Transcriptomic analysis of the immune microenvironment of non-hematopoietic human tumors

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Thèse de doctorat

Discipline : Immunologie

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Soutenue le 21 septembre 2015 devant le jury composé de :

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Remerciements

A mon directeur de thèse **Hervé Fridman**. Pour la confiance que vous avez témoigné à mon égard, d'abord en acceptant d'encadrer ma thèse en sachant que je ne venais pas d'une formation d'immunologie, puis en me laissant la liberté de proposer et d'explorer des pistes de recherche. Pour votre disponibilité, largement motivée par l'envie de découvrir et discuter des résultats nouveaux. Pour vos connaissances, manifestées aussi bien dans celles que vous m'avez transmises que dans la manière d'en aborder de nouvelles avec un regard à la fois ouvert et critique. Et enfin, pour votre sympathie et votre conception chaleureuse d'une relation professeur-élève, qui nous a permis d'échanger sur tant de sujets enrichissants.

A mon co-directeur **Aurélien de Reyniès**. Tout d'abord pour avoir incarné ce qu'est une collaboration scientifique, motivée par un intérêt mutuel pour une thématique, et qui t'a amené à accepter d'encadrer ma thèse en cours de route et de façon altruiste. Pour m'avoir fait partager tes valeurs d'une science visant à améliorer concrètement la connaissance globale plus que l'égo du chercheur. Pour ton souci du détail et ta rigueur, et surtout pour ton amicale bienveillance.

A **Catherine Sautès-Fridman**. Pour votre humour et votre bienveillante autorité, cette si fine capacité à organiser, faciliter et harmoniser les relations de travail de vos collaborateurs. Pour votre humanité et l'oreille attentive que vous portez à nos petits et grands soucis autant qu'à nos humbles ou démesurées fiertés ou nos humeurs quotidiennes.

A **Caroline**, pour ton immense soutien, non seulement pour ces trois années de recherche où tu as écouté autant mes doutes que mes joies, partagé tes conseils avisés, embrassé mes ambitions, mais surtout d'avoir partagé et égayé ma vie en dehors du laboratoire. Pour nos jours de bonheurs passés, présents et à venir, merci.

A ma mère **Isabelle**, pour ton immense et perpétuel soutien affectif et logistique, pour m'avoir éveillé aux goûts de la connaissance, de la science, de la culture et m'avoir transmis ta philanthropie.

A mon père **Dominique**, qui n'aura pas pu connaître ni le début ni la fin de ce travail, mais dont je ne doute pas qu'il aurait été très fier.

A mon frère **Quentin** et ma belle-sœur **Neila**, ma sœur tant aimée **Mathilde**, pour votre disponibilité et votre bienveillance perpétuelle à mon égard, pour tout ce qui nous unit par nos vies si longtemps partagées et qui bien souvent nous permet de nous comprendre sans avoir besoin de nous parler.

A ma belle-mère **Martine**, pour ton côté direct, pour l'affection et le souci que tu as pour ceux qui t'entourent, pour la liberté que tu manifestes au quotidien, et pour avoir la première révélé qu'un conflit peut parfois s'avérer nécessaire et bénéfique.

A mes **grands-parents** et en particulier **Grandmi**, mon nouvellement père **Eric**, toute les **familles Becht** et **Cholley**, ma belle-mère **Florence** et toute **mes belles-familles**, pour votre amour et votre sympathie, et pour l'intérêt que nous éprouvons pour nos vies respectives.

A **Véro** et **Xavier**, **Lyliane** et **Selçuk**, qui m'avez fait grandir depuis le berceau et dans l'âge adulte par votre affection et votre bienveillance.

A **Nicolas Giraldo-Castillo**, pour avoir tant échangé sur nos vies personnelles et professionnelles, pour ta joie de vivre et ton enthousiasme, ton humour et ton espièglerie. A **Laetitia Lacroix**, pour ton éternelle motivation, ton constant souci de donner le meilleur de toi-même, de la force et de la joie dont tu témoignes au quotidien. A **Ivo Natario** pour ta fougue. A **Bénédicte Buttard** pour ta force de caractère. A **Bénédicte le Clec'h** pour ta franchise. A **Yann Vano** pour ta curiosité. A **Aude Versavel** pour ta sympathie.

A **Isabelle Cremer**, pour nos enthousiasmantes collaborations et ta cordialité. A **Marie Caroline Dieu-Nosjean** pour nos discussions scientifiques passionnées et ton amabilité. A **Diane Damotte** pour m'avoir fait partager un autre regard sur des problématiques communes. A **Jean-Luc Teillaud** pour ta sympathie et ta joie de vivre. A **Lubka Roumenina** pour ta motivation et ton enthousiasme. A **Véronique Frémeaux-Bacchi** pour ton humour et ta franchise. A **Sophie Sibéril** pour sa sympathie.

A **Tessa Fredriksen** pour ta générosité et ta timide exubérance. A **Amélie Bilocq** pour ta sympathie. A **Claire Deligne** pour ta franchise, tes conseils et ton humour. A **Claire Germain** pour ton sérieux et ton aplomb. A **Jérôme Biton** pour ta sympathie. A **Jérémy Goc** pour ton dévouement. A **Romain Remark** pour m'avoir fait découvrir le laboratoire. A **Audrey Lupo** pour son humour et son opiniâtreté. A **Sophie Chauvet** pour ta sympathie. To **Priyanka Devi** for your kindness. To **Shambu Prasad**, for your curiosity. A **Nicolas Merle** et **Anne Grunenwald** pour leur amabilité. A **Benoit Milcent** pour m'avoir fait partager ses points de vue. A **Mikael Perez**, **Samantha Knockaert, Nathalie Josseaume** et **Claudia Gutierrez** pour votre joie de vivre. A **Samuel Mouyal** pour ton intelligence et ta curiosité. A **Rémy Nicole**, **Sylvie Job**, **Laetitia Marisa**, **Yuna Blum**, **Noemie Robil**, **Aurélie Kamoun**, **Nabila Elarouci**, **Fabien Petel**, **Mira Ayadi, Lucile Armenoult** et **Eric Letouzé** pour m'avoir intégré à votre équipe. A **Estelle Devevre** et **Hélène Fohrer-Ting** pour votre expertise et votre sympathie. A **Pierre Validire**, **Catherine Julié** et **Janick Selves** pour l'intérêt que vous portez à nos travaux. To **Jitka Fučíková** for your kindness, your humor, your dedication and our collaborations.

A **Pierre Laurent-Puig** pour votre sympathie, votre franchise, votre curiosité, votre humour et votre ingéniosité. A **Jessica Zucman-Rossi** pour votre sagacité et votre sympathie.

A **Johanna Anglio**, **Jasmina Rogozarski**, **Nathalie Jupiter**, **Lamia Froment**, **Eliane Montlouis** pour votre l'aide que vous nous apportez au quotidien.

Mes amis **Matthias** et **Sylviane**, **Julien**, **Audren**, **Nicola** et **Marie-Albane**, **Franck** et **Anne Sophie**, **Alexandre Millot**, **Jules**, **Alexandre Bury**, **Jérémy**, **Nathanaël**, **Maxime Chammas, Aude**, et **Yu**, pour votre soutien, votre sympathie, votre humour, votre grandeur d'âme et le bonheur que vous m'offrez.

A tous **les collègues**, **amis**, **membres de la famille** et **collaborateurs** que je ne peux remercier spécifiquement faute de place, merci de votre affection et de votre intérêt.

Abstract

Transcriptomic analysis of the immune microenvironment of non-hematopoietic human tumors

Abstract

Tumors grow within a complex microenvironment composed of immune cells, fibroblasts, endothelial cells and other non-malignant cells. The study of the composition of tumor microenvironments has led to classifications with prognostic and theranostic values, as well as the discovery of treatments modulating the composition and the functional orientation of the microenvironment. Concurrently, molecular classifications of tumors have proposed taxonomies within cancers that define groups of patients with different prognoses and are associated with response to treatments.

Recent evidence suggest that the phenotype of the malignant cell is a critical determinant in the shaping of its microenvironment, suggesting potential correlations between immune and molecular classifications. The goal of this PhD project was therefore to analyze the microenvironment of molecularly-classified human tumors.

Colorectal cancer represents a paradigm for tumor immunology, as it is the human cancer in which it was exemplified that an adaptive immune response can control tumor growth and metastasis. Conversely, clear-cell renal cell carcinoma represents an exception in tumor immunology, as an extensive adaptive immune response is associated with more aggressive diseases.

Molecular transcriptomic classifications were recently proposed for both of these apparently immunologically contrasted cancers. In this work, I propose a methodology that enables the characterization of the tumor microenvironment using transcriptomic data, and apply it to describe the immune contexture of molecular subgroups of colorectal and clear-cell renal cell carcinomas.

These analyses argue in favor of the unification of molecular and immune classifications of human cancers, challenge our current views of the relationship between the composition of the tumor microenvironment and patient's prognosis, and suggest immunotherapeutic approaches that could benefit subgroups of patients in these two cancers.

Analyse transcriptomique du microenvironnement immunitaire des tumeurs humaines non-hématopoïetiques

Résumé

Le microenvironnement des tumeurs est composé de cellules immunitaires, de fibroblastes et de cellules endothéliales, ainsi que d'autres cellules non-malignes. Son étude a permis d'établir des classifications qui ont une valeur pronostique et théranostique, ainsi que de développer des traitements modulant la composition et l'orientation fonctionnelle du microenvironnement. En parallèle, des classifications moléculaires des tumeurs ont proposé des taxonomies stratifiant les cancers humains en sous-groupes associés à des différences de survie des patients et leur réponse aux traitements.

Des études récentes suggèrent que le phénotype de la cellule cancéreuse est un facteur critique dans le façonnement du microenvironnement tumoral, suggérant un possible consensus entre les classifications immunitaires et moléculaires. Le but de cette thèse était donc de caractériser le microenvironnement des sous-groupes moléculaires de tumeurs humaines.

Le cancer colorectal a été le premier cancer humain dans lequel il a été mis en évidence qu'une réponse immunitaire adaptative était associée à un contrôle de la croissance tumorale, et représente ainsi un exemple type pour l'immunologie des tumeurs. A l'inverse, le carcinome du rein à cellules claires est une exception vis-à-vis de l'immunologie des tumeurs, puisqu'une forte réponse immunitaire adaptative y est associée à des tumeurs plus agressives.

Des classifications transcriptomiques ont été récemment établies pour ces deux cancers, qu'à première vue tout oppose sur le plan immunitaire. Dans ce travail, je propose une méthode permettant l'étude du microenvironnement tumoral à partir de données transcriptomiques, et décris son application à l'étude du contexte immunitaire des cancers colorectaux et du rein à cellules claires.

Ces analyses suggèrent qu'une unification des classifications moléculaires et immunitaires des tumeurs humaines est possible, remettent en cause notre conceptualisation des liens entre la composition du microenvironnement tumoral et le pronostic du patient, et évoque des pistes immunothérapeutiques potentiellement adaptées à certains sous-groupes de patients dans ces cancers.

Contents

Notations

APC	Antigen-presenting cell
CAF	Cancer-associated fibroblasts
ccRCC	Clear-cell renal cell carcinoma
CIMP	CpG-island methylator phenotype
CIN	Chromosomal instability
CMP	Common myeloid progenitor
CMS	Consensus molecular subtype
CRC	Colorectal cancer
CRCSC	Colorectal cancer subtyping consortium
DAMP	Danger-associated molecular patterns
DLBCL	Diffuse large B-cell lymphoma
ER	Estrogen-receptor
FAP	Familial adenomatous polyposis
GMP	Granulocytic-monocytic progenitor
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
MDSC	Myeloid-derived suppressor cell
MMR	Mismatch-repair machinery
mRNA	messenger RNA
MSI	Microsatellite-instability
NK	Natural killer
NSCLC	Non-small cell lung cancer
OS	Overall-survival
PAMP	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor
PFS	Progression-free survival
ROS	Reactive oxygen species
T_{h1}	T helper 1
T_{h17}	T helper 17
T_{h2}	T helper 2
T_{reg}	Regulatory T cell
TAM	Tumor-associated macrophages
TCR	T-cell receptor
TKI	Tyrosine-kinase inhibitor
VEGF	Vascular-endothelial growth factor

Chapter 1

Introduction

In this body of work I describe a comprehensive analysis of the relationship between tumor infiltration by several immune cell subsets and both molecular characteristics of the tumor cells and clinical outcome of the patients. I first introduce previously-published relevant results.

1.1 Introduction to immunology

The immune system comprises a set of organs and circulating cell populations. Its best known function is to defend the organism against pathogens. It is also implicated in other processes such as maintenance of tissues' homeostasis through the clearance of apoptotic bodies. Its role in preventing the occurrence of cancer has been debated for a long time, but is now widely accepted. We first present general immunology results that help describe and interpret the spectrum of immune responses to cancer.

1.1.1 Effectors and modulators of immune responses

Before discussing in more details the relationship between the immune system and cancer, I first detail the various currently described populations that constitute the immune system, as well as the molecules that regulate their functions. The main role of the immune system is to recognize the *self* from the *non-self* and eliminate the latter.

1.1.1.1 Regulation of immune cells functions

1.1.1.1.1 Chemokines guide immune cells trafficking

Most immune cells exert their functions locally, either through contact-dependent mechanisms or by secreting molecules with short ranges of action. In any case, precise trafficking of immune cells to sites of inflammation or to lymphoid organs is required. This trafficking is mostly regulated by chemokines. These molecules are specific for one or several receptors expressed on mobile cells. Sensing of a chemokine gradient by receptor-expressing cells will lead the movement of the cell towards the direction of highest chemokine concentration.

1.1.1.1.2 Interleukins orientate the functionality of immune responses

More than thirty types of interleukins have been described in humans. Interleukins orientate the development of immune cells in primary lymphoid organs. In the periphery, interleukins mediate the functional orientation of immune cells, triggering proliferative,

activating, inhibiting or phenotype-polarizing events in cells expressing the corresponding receptors.

1.1.1.2 Classifications of immune cell subsets

Immune cells can be classified according to their function or to their developmental origin. These classifications are strongly related, although not entirely redundant. I first present the functional classification of immune cells, as it eases the conceptualization of immune responses. I then detail the process of hematopoiesis, as it is a theoretical framework that heavily influence the methodology we introduce later.

1.1.1.2.1 Functional classification of immune cells

Immune cells have historically been separated functionally into innate and immune compartments. I present in this section the main characteristics of these two compartments.

1.1.1.2.1.1 Innate immunity

Innate immunity refers to the process of defending the organism against pathogens (viruses, bacteria, fungi and parasites) in generic ways. Thus, its action begins with anatomical barriers (such as the skins, gut or respiratory mucosa) which prevent pathogens from entering the body. If this first line of defense fails, mechanisms aimed at eliminating pathogens will take place.

1.1.1.2.1.1.A Danger signals

The first role of the innate immune system is to identify situations threatening the organism. Cells of the innate immune system will recognize intruding pathogens. To do so, these cells express receptors to molecules known as Pathogen Associated Molecular Patterns (PAMPs). Well-known PAMP-receptor families include *Toll-like receptors* and *nucleotide olgomerization domain-like receptors*. These PAMPs-receptors recognize *non-self* molecules commonly expressed by pathogens. For instance, TLR4 recognizes, among other ligands, Lipopolysaccharide[1], a constituent of the outer membrane of Gram-negative bacteria. TLR3 recognizes double-stranded RNA[1], which can be found in some viruses but not in eukaryotic cells. NOD2 recognizes peptidoglycan, a sugar found in Gram-positive bacteria[1]. Apart from pathogens, cells of the innate immune system can also sense *Danger Associated Molecular Patterns* (DAMPs), such as molecules of the *self* that are released in extracellular compartements by damaged cells (for instance nuclear DNA)[2]. The innate immune system also comprises soluble molecules able to recognize danger signals, such as the complement system. The patterns recognized by the innate immune system are limited to a few tens, but have been selected through the co-evolution of organisms and pathogens during millions of years, and are therefore able to sense dangers in many non-physiological situations.

1.1.1.2.1.1.B Effector functions

When danger signals are detected by the innate immune system, a reaction known as acute inflammation is triggered. Cells sensing danger signals release pro-inflammatory molecules that lead to the recruitment and the activation of immune cells at the site of inflammation. These lead to the elimination of pathogens or damaged cells through phagocytosis, infected cells killing, creation of physical barriers to trap pathogens and antigen presentation to cells of the adaptive immune system.

1.1.1.2.1.2 Adaptive immunity

The fundamental diffence between the adaptive and the innate immune system is the way the pool of recognized molecules is generated. While the innate immune system is based on a limited and fixed set of patterns, the adaptive immune system is based on the selection of randomly generated receptors to discriminate between *self* or *non-self* molecules. Molecules that trigger adaptive immune responses are termed *antigens*.

1.1.1.2.1.2.A Repertoire

During their development, receptors of the cells of the adaptive immune system express undergo a process known as *V(D)J recombination*. Briefly, receptors of adaptive immune cells are stochastichally chosen through random selection of several DNA loci. If these random rearrangements lead to out-of-frame DNA sequences, the cell will undergo apoptosis. Otherwise, it will continue its development. At this point, the cell is equipped with a randomly-generated receptor, which could *a priori* bind to either *self, non-self* or both. To prevent auto-immunity, these cells are presented with peptides derived from the *self*, and those whose receptors recognize *self antigens* with high affinity are eliminated. Those passing these selection criteria are therefore able to express a receptor that will not react with *self antigens*, and will be allowed to circulate within the organism. In the event of a binding of the receptor carried by this cell to an *antigen*, the cell bearing this *antigen* should therefore be *non-self* and eliminated.

1.1.1.2.1.2.B Effector functions

Cells of the adaptive immune system become activated when they detect *non-self antigens*. This activation can lead to the activation of other immune cell subsets, elimination of infected host cells, inactivation of circulating molecules such as toxins and designation of targets to other immune cells. A distinctive functional feature of adaptive immunity is its ability to memorize *non-self* antigens. Upon antigen-specific activation, a subset of the effector cell differentiate into long-live Central Memory cells, able to patrol the body for years and rapidly expand upon antigen re-encounter.

1.1.1.2.2 Immune cells phylogeny

Immune cells all originate from hematopoietic stem cells (HSC), a common progenitor that is located in the bone marrow. A succession of lineage commitments, a process known as **hematopoiesis**, orientates the differentiating immune cell towards its terminal differentiation stage. The developping immune cell first commit toward the myeloid lineage or the lymphoid lineage.

Most myeloid cells belong to innate immunity, while most lymphoid cells are related to adaptive immunity. Myeloid lineage commitment of the HSC gives rise to a Common Myeloid Progenitor cell (CMP), that can further differentiate into a Megakaryocyte-erythroid progenitor which give rise to non-immune blood cells (erythrocytes and megakaryocytes), or to a Granulocyte/Macrophage Progenitor (GMP) which can develop into myeloid immune cells. The GMP can differentiate into cells of the granulocytic lineage (Neutrophils, Basophils, Eosinophils), Mast cells, or into monocytes. Monocytes can then give rise to macrophages or myeloid dendritic cells.

The lymphoid lineage begins with the commitment of HSC into a Common Lymphoid Progenitor (CMP). CMPs can then commit to the B or the T lineage. The origin of Natural Killer (NK) cells is not fully established but studies support the idea that they

originate from a common T/NK lineage[3–5]. Innate lymphoid cells, a recently identified subtype of lymphoid cells that are tied to innate immunity and whose main function is cytokine production, are believed to originate from the T/NK lineage. The developmental origin of plasmacytoid Dendritic Cells (pDC) is still controversial, as these cells express lymphoid specific markers and are reported able to arise from both myeloid[6] or lymphoid[7] progenitors.

1.1.1.2.3 Functions of myeloid cells

We detail in this section the function of myeloid cell subsets during immune responses. The function of lymphoid cells is developped in the next section. Figure 1.1 graphically illustrates the main functions of immune and other stromal cells during inflammatory responses.

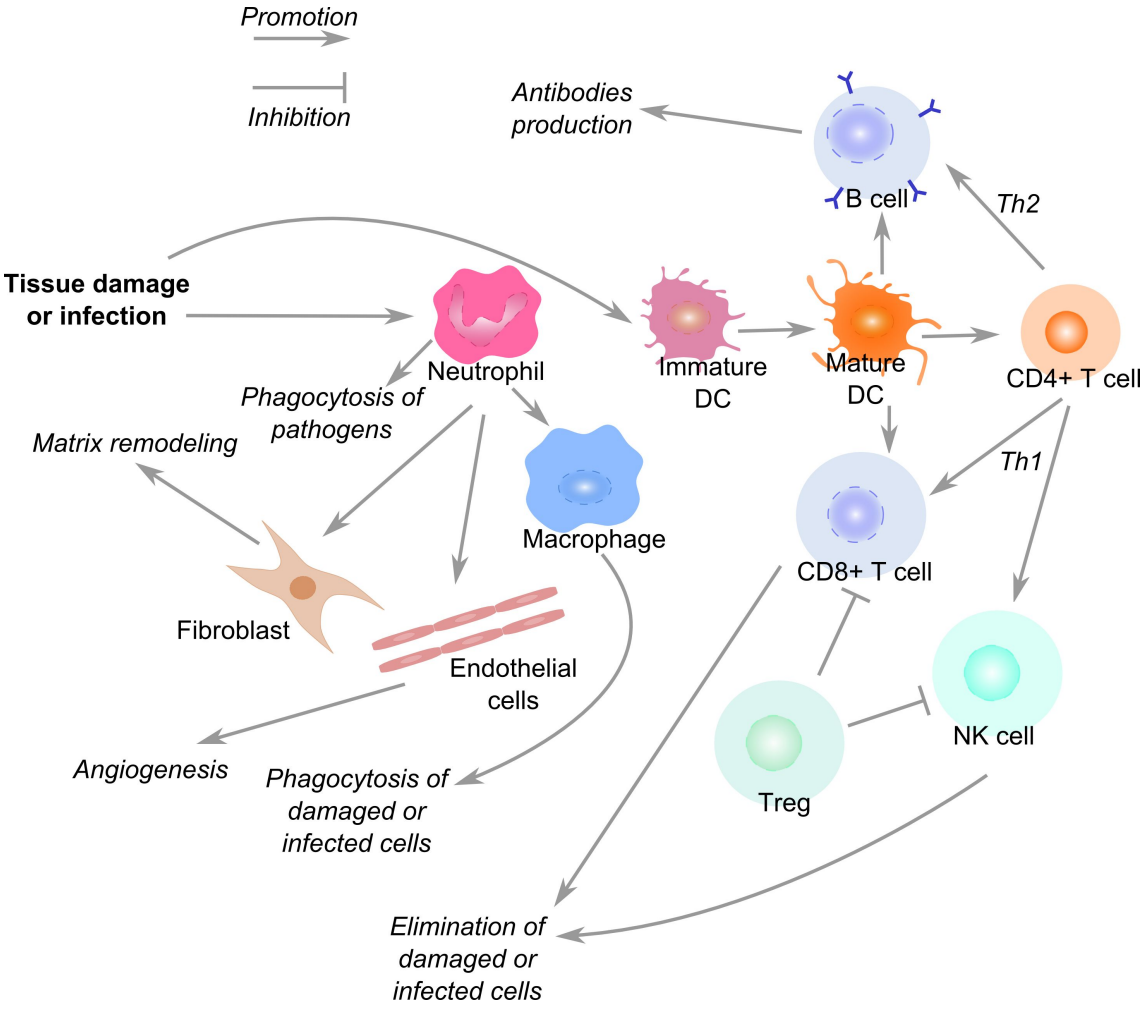

Figure 1.1: The crosstalk and effector functions of immune and other stromal cells during inflammatory responses.

1.1.1.2.3.1 Monocytes

Monocytes are immature myeloid cells which are mostly found in the blood. Upon stimulation by danger signals and/or cytokines, they differentiate into macrophages or

myeloid dendritic cells.

1.1.1.2.3.2 Macrophages

Macrophages are effector cells arising from monocytes upon entrance in tissues from the peripheral blood. Their major function is phagocytosis, which is the internalization of cells, bacteria or other small bodies, which are then destroyed by acidification, action of proteases and reactive oxygen species. Macrophages are capable of low levels of antigen presentation to $CD4^+$ T lymphocytes.

1.1.1.2.3.3 Dendritic cells

Dendritic cells are specialized antigen-presenting cells (APC). Their names come from the long dendrites that extend from their bodies, which they use to sense surrounding molecular patterns. Dendritic cells are capable of phagocytosis, which enable them to present antigens originating from the extracellular milieu to $CD4^+$ T lymphocytes, as well as to $CD8^+$ T cells through a process known as *antigen cross-presentation*. Apart from phagocytosis, dendritic cells are also capable of macropynocytosis, which is the internalization of small volumes of extracellular fluids, whose antigenic content is subsequently processed and presented. Finally, dendritic cells can express co-stimulatory or co-inhibitory ligands, as well as a wide spectrum of cytokines, which depends on their state of maturation and the stimuli they received during maturation. These secondary signals will orientate the functionality of the dendritic cells-stimulated immune cells.

1.1.1.2.3.4 Neutrophils

Neutrophils are large immune cells featuring cytoplasmic granules. These granules contain cytotoxic molecules which are released upon encountering bacteria. Neutrophils are capable of phagocytosis, but mostly target bacteria rather than large cellular bodies. Neutrophils are highly abundant in the peripheral blood, and can rapidly mobilize upon early inflammatory signals.

1.1.1.2.3.5 Other myeloid cell subsets

Other granular myeloid cells include basophils and eosinophils, whose mains functions are to defend the organism against parasites. Mast cells are found in tissues and are characterized by histamine-containing granules. Upon binding of allergens to mast cells-bound IgE immunoglobulins, they release pro-inflammatory mediators such as histamine, which is notably associated with allergic reactions.

1.1.1.2.4 Functions of lymphoid cells

1.1.1.2.4.1 T lymphocyte

T lymphocytes are lymphoid cells that differentiate from CMP cells in the bone marrow and then migrate to the thymus. Inside the thymus, the immature T cells (Thymocytes) randomly rearrange DNA fragments encoding the T cell receptor molecules leading to a selection of a randomly generated sequence. This sequence is then translated into proteins forming the TCR. The TCR is able to sense peptides presented by Major Histocompatibility Complex (MHC) molecules. Thymocytes are selected for their capacity to positively interact with MHC molecules, and negatively selected for reactivity against *self antigens*. T cells able to fulfill both of these criteria will leave the thymus and circulate within

the body. T cells are however functionally heterogeneous, presenting cytotoxic, helper or suppressive phenotypes.

1.1.1.2.4.1.A Naive and Memory T cells

T lymphocytes leave the thymus in a naive state, marked by expression of the CD45RA membranous epitope. Upon antigen recognition, naive T cells mature into memory T cells, marked by a switch of CD45 expression to the CD45RO isoform. CD45 is a tyrosine-phosphatase which regulates signal transduction in immune cells, although the functions of each of its six different isoforms is poorly understood[8]. Most of the memory T lymphocytes will feature an effector-memory (T_{EM}) $CD45RO^+CCR7^-$ phenotype and home to peripheral organs to exert effector function. A subset will feature a central-memory (T_{CM}) $CD45RO^+CCR7^+$ phenotype and home towards lymphoid organs following chemoattraction by the ligands of CCR7, CCL19 and CCL21, to watch for the re-apparition of their target antigen[9]. Memory T cells can exert different functions according to their phenotypes.

1.1.1.2.4.1.B $CD4^+$ Helper T cells

$CD4^+$ T cells are T cells expressing the membrane-bound CD4 co-receptor. This co-receptor enable them to interact with **class II** MHC molecules. Class II MHC molecules are mostly expressed by antigen-presenting cells, and present peptides from the extracellular environment. Upon recognition of class II MHC-bound peptides and in the presence of co-stimulatory molecules, $CD4^+$ T cells secrete molecules that modulate the function of surrounding immune cells, hence their 'helper' denomination. Helper $CD4^+$ T cells come in a wide variety of functional orientations that we briefly recapitulate.

1.1.1.2.4.1.B.a T helper 1 cells

T helper 1 (T_{h1}) cells foster cellular immune responses. The main cytokines produced by T_{h1} cells are IFNγ and IL2, which activate $CD8^+$ T cells and NK cells, as well as macrophages, and promotes $CD4^+$ T cells differentiation to the T_{h1} subtype. IFNγ stimulates IL12 production by dendritic cells which in turn enhances IFNγ production. T_{h1} cells also produce IL2 which foster survival and proliferation of T lymphocytes.

1.1.1.2.4.1.B.b T helper 2 cells

T helper 2 (T_{h2}) cells foster humoral immune responses. They produce the IL4 and IL13 cytokines which foster the T_{h2} differentiation of $CD4^+$ T cells, and favors IgE antibody class-switching of B cells which in turns activate basophils and mast cells. Other Th2-associated cytokines are IL5 which activate eosinophils and IL9 which activates mast cells and lymphocytes.

1.1.1.2.4.1.B.c Regulatory T cells

Uncontrolled immune reactions can disrupt host's tissues homeostasis through inflammatory reactions, allergy or auto-immunity. In physiological conditions, suppressive signals are able to weaken immune reactions and avoid immunity-related collateral damage. Regulatory T cells are $CD4^+$ T cells which produce IL10 and TGFβ and therefore suppress the activity of other lymphocytes and dampen inflammation.

1.1.1.2.4.1.B.d T helper 17 cells

T helper 17 (T_{h17}) cells are induced by IL6, IL21 or IL23 signaling in the presence

of TGFβ. They release IL17A, IL17F, IL22 which have pro-inflammatory properties, and IL21 to stimulate T_{h17} differentiation in an autocrine manner.

1.1.1.2.4.1.C $CD8^+$ Cytotoxic T cells

Cytotoxic T cells are T cells expressing the membrane-bound CD8 co-receptor. This co-receptor enable them to interact with the **class I** family of MHC molecules. Class I MHC molecules are expressed in all nucleated cells of the body[10], and apart from specialized antigen-presenting cells, exclusively present peptides from proteins synthesized by the cell. Notably, when an infecting virus hijacks the host's cellular machinery to translate viral-encoded proteins, viral-derived peptides are presented on the cell membrane by class I MHC molecules. $CD8^+$ T cells are able to sense class 1 MHC-bound peptides and therefore recognise intracellular *non-self antigens*. Upon recognition, $CD8^+$ T cells secrete cytotoxic molecules (notably granzymes and perforin), leading to the elimination of the target cell and the release of IFNγ which further promotes a T_{h1} orientation of the immune response.

1.1.1.2.4.1.D Natural Killer cells

Natural Killer cells (NK cells) are cytotoxic lymphocytes that do not express a variable receptor and thus are classified as innate immune cells. They are able to exert their function against antibody-marked targets, as well as on host cells that lost class I MHC expression. Loss of class I MHC expression can be induced by some viruses to evade $CD8^+$ T cell-mediated elimination, in which case NK cells can intervene. Like $CD8^+$ T cells, they secrete granzymes and perforin and release IFNγ upon activation.

1.1.1.2.4.1.E Other cell subsets of the T/NK lineage

T cells can differentiate into other recently described subsets, such as T helper 9, which release the pro-inflammatory IL9 cytokine. T follicular helper (T_{fh}) cells specialize into supporting B cell follicules in lymphoid organs. Other recently identified lymphoid subpopulations originate from the T/NK lineage. Tγδ cells (T cells with a more limited TCR repertoire that originates from the γ and δ loci instead of the α and β loci) which are thought to be involved in the recognition of lipids presented by the CD1d molecule in an MHC-independent manner[11]. NKT cells are closely related to Tγδ cells in that they also recognise CD1d-bound lipids, but differ in that they express a TCR originating from a limited repertoire generated by the αβ loci[12]. Innate Lymphoid Cells are cells that resemble NK cells but instead of exerting cytotoxic activity release cytokines upon activation.

1.1.1.2.4.2 B lymphocytes

B lymphocytes are the main effectors of the adaptive humoral immunity, through the production of antibodies. Like T cells, they express a randomly generated receptor by V(D)J recombination, the B-cell receptor (BCR). Unlike the TCR, the BCR does not require the presentation of peptides by MHC molecules to exert its function. The BCR resembles a membrane-bound antibody. B cells are selected against auto-reactivity within the bone marrow. Upon *non-self* antigen recognition, B cells release antibodies that can bind to the recognized antigen. This process can directly neutralize the antigen or mark its carrier for elimination by other immune cells, such as macrophages or NK cells. Like T cells, they leave the bone marrow in a naive state. B cells can bind to their target antigens either directly or through T cell-mediated presentation. Upon recognition, most B cells differentiate into Plasma cells, terminally differentiated B cells which lose their capacity to

proliferate but which greatly enhances their capacity to produce and release antibodies. A fraction of activated B cells differentiate into memory B cells, long-lived cells that rapidly activate and proliferate upon antigen re-encounter.

1.2 Cancer immunology

Now that I have briefly introduced the mechanisms of actions and cooperation involved in immune responses, mostly in the context of pathogens encounters, I present how these mechanisms interact with tumors. I provide evidence for tumor-induced immune reactions and present the cellular and molecular mediators at play in each situation.

1.2.1 Control of cancer growth through adaptive immunity

A fundamental aspect of cancer immunology is that the immune system, mainly through its adaptive arm, is able to identify and eliminate host cells undergoing malignant transformations and control tumor growth. Although the idea of a cancer-protecting function of the immune system was proposed more than a century ago[13], it remained controversial during the 20^{th} century[14, 15]. This idea is now widely accepted and considered a hallmark of cancer[16]. In the following section, I report the results in favor of the acceptation of an anti-tumor role of the immune system.

In 2004, R. Schreiber and colleagues proposed the **3E** theory[17], standing for **E**limination, **E**quilibrium and **E**scape. The idea behind this theory is that most cells undergoing malignant transformation are recognized by immune cells and *eliminated*. Sometimes, a tumor cell manage to proliferate fast enough to compensate for immune-mediated elimination, the overall tumor size remaining at *equilibrium*. In the third stage, tumor cells manage to *escape* elimination from the immune system and grow uncontrolled. The challenge in studying how immune cells are able to eliminate cancer cells is that clinically detectable tumors have, by definition, managed to grow from a single mutated cell to a mass of derived malignant cells, and are therefore at the *escape* stage. Nevertheless, it was reported that in colorectal cancer, tumor stage inversely correlated with infiltration by cytotoxic T cells, suggesting that advanced lesions are less controlled by the immune system[18].

1.2.1.1 Indirect and direct evidence of adaptive immunity-mediated cancer control

Epidemiological observations in immunodeficient patients indirectly hinted at a protective role of the adaptive immune system against cancer occurrence. In particular, it was observed in the 1980-1990 decade that patients suffering from HIV/AIDS have higher chances of developing Kaposi's sarcoma[19–21], a transforming virus-associated cancer, caused by *HHV8*. Independantly from the host's immunocompetence, a pool of *HHV8* persists in endothelial cells, whose malignant proliferation cannot be controlled by immunodeficient patients[22]. Transplant recipients, who receive immunosuppressive therapies to avoid transplant rejection, have a consistently higher risk of developing this malignancy[23]. Strikingly, transplant recipients are also at higher risk of developing other solid tumors, such as brain[24] and other central nervous system malignancies[24], thyroid[24], bone[24], colon[25], lung[25], prostate[25], stomach[25], esophagus[25], pancreas[25], ovary[25], breast[25], melanoma[24, 25], leukemia[25], hepatobiliary tumors[24, 25], cervical and vulvovaginal cancers[25], testicular[25], bladder[25], kidney[25], nonmelanoma skin cancers[25], oral cavity cancers[24], as well as non hodgkin-lymphoma[25].

Animal models were then used to study whether immunodeficient animals were at

higher risk of developing cancer. Several studies showed an increase in carcinogen-induced tumors numbers in immunodeficient mice compared to wild-type mice[21, 26]. Importantly, it was observed that a knock-out of the Rag2 gene, which encodes the Recombination activating gene 2, is sufficient to induce this effect. Rag2 is necessary for the *V(D)J* recombination and therefore for the survival of B and T cells in the periphery, supporting the idea that the adaptive arm of the immune system is responsible for the control of tumor occurrence. Direct evidence was later obtained using *in-vivo* two-photon imaging: C57BL/6 mice were injected with the syngenic EL4 cell line expressing the exogenous OVA protein and latter injected with OT-I T cells specific for OVA antigens. In-situ and *in-vivo* imaging of the CD8 protein and the activity of CASP3, an apoptosis marker, showed that the cytotoxic lymphocytes $CD8^+$ T cells were actively killing tumor cells[27]. The authors also noted that the elimination was a slow process, taking on average 6 hours, and proposed that the amount of tumor-targeting $CD8^+$ T cells could be a limiting factor in the control of tumor growth.

1.2.1.2 Identification of tumors as *non-self*

The control of tumor growth by the adaptive immune system is unintuitive. As I presented earlier, the term 'adaptive' refers to the fact that this system is able to dynamically discriminate between *self* and *non self*. The distinction is clear in the case of invasive pathogens, but cancer cells are host's transformed cells and could therefore be thought as belonging to the *self*. The malignant transformation of normal cells involves mutations[28] in pathways controlling cell proliferation, resistance to apoptosis and other fundamental characteristics of cancers[16]. Mutated genes encode proteins that differ from those expressed by the host's normal cells and could therefore be considered as *non-self*, marking the malignant cell as a target for the adaptive immune system. Peptides capable of eliciting an adaptive immune response are known as *neoantigens* or *tumor antigens*. $CD8^+$ T cells clones specific for tumor antigens were found against the cyclin-dependant kinase 4 gene CDK4[29] or mutated β-catenin[30] in melanoma, and the tumor-expressed MHC class II HLA-A2 gene in renal cell carcinoma[31].

Interestingly, host's non-mutated proteins were also found to be targeted by the adaptive immune system. In particular, proteins that are expressed in MHC class I-negative cells, such as sperm cells or trophoblasts, and aberrantly expressed by cancer cells, are able to elicit immune responses. The first example identified in human is an epitope of the MAGEA1[32] testis-restricted protein aberrantly expressed in melanoma. Antigenic properties were later found in proteins encoded by the MAGE family of antigens and other germline-specific genes, such as BAGE1, GAGE1, XAGE1B, CTAG2, CTAG1 and SSX2, in melanoma, but also lung, colorectal, breast and prostate carcinomas[33].

More surprisingly, some proteins constitutively expressed by non-malignant cells were also found to elicit immune responses. These proteins are usually overexpressed in tumor cells, leading to a TCR-mediated activation of the corresponding specific lymphocytes[33], while their expression is too low in normal cells to reach the threshold leading to T cells activation. Examples of such antigens include the 'prostate-specific antigen' (PSA) protein in prostate cancer[34, 35], the HER2/neu antigen[36] encoded by the amplified ERBB2 gene in breast and ovarian cancers, as well as the Melan-A protein in melanoma[37–39].

A more straightforward class of tumor associated antigen are the peptides associated with carcinogenic viruses. These peptides remain expressed in the transformed cells and can elicit immune responses, as shown with Human Papilloma Virus (HPV) infection in head and neck squamous cell carcinoma[40], Epstein-Barr virus in Hodgkin's lymphoma, nasopharyngeal carcinoma, NKT lymphoma and Burkitt's lymphoma[41]. Altogether,

these examples show that although tumor cells are derived from normal cells, they can express antigens that can be recognized as *non-self*.

The recognition of *non-self* antigens is not sufficient to induce immune responses, as the sensing of DAMPs or PAMPs is necessary to activate inflammatory cells which can in turn co-stimulate adaptive immune cells. Examples of tumor-expressed DAMPs include IL6, which can be expressed by tumor cells, and necrosis-associated molecules such as proteins of the S100 family[42].

1.2.1.3 Clinical impact of tumor infiltration by adaptive immune cells

Infiltration by adaptive lymphocytes is heterogeneous across tumors of a same malignancy. Since these cells are able to recognize neoantigens expressed by tumor cells[33] and that the $CD8^+$ T cell-mediated elimination is slow[27] the amount of $CD8^+$ T cells infiltrating a tumor can reflect a patient's immune system's ability to control tumor growth. Consistently, presence of intratumor T cells were shown to be associated with a good prognosis in human cancers, including ovarian cancer[43] and glioma[44, 45]. In the last decade, it was shown that a precise quantification of the density of tumor-infiltrating T cells was associated with favorable outcome in colorectal cancer[46]. Further analyses first identified $CD8^+$ cytotoxic T cells[47], then cytotoxic T cells that encountered an antigen ($CD8^+$ T_{EM} cells), as a strong prognostic factor for relapse-free survival in colorectal cancer. It was also shown that the prognostic value associated with a high density of $CD8^+$ and T_{EM} cells is independent from the UICC/TNM classification, the current gold-standard for prognosis prediction in colorectal cancer. These results enabled the development of the *Immunoscore*, an immunohistochemistry-based quantification of two immune markers (CD3 and CD8, or CD8 and CD45RO) in two areas of the tumor (the center as well as the invasive margin), resulting in a score ranging from 0 (poor prognosis) to 4 (good prognosis). Patients with a score of 0 have a low infiltration for each marker in all areas, while patients with a score of 4 have a high infiltration in all areas. The immunoscore is a tool proposed for the prediction of patient's prognosis[48–51] and its response to treatments[52, 53].

Consistently with their predictive power in colorectal cancer, infiltrating $CD8^+$ T_{EM} cells or a corresponding T_{h1} functional orientation of the tumor microenvironment were shown to be associated with favorable outcome in most human malignancies[54], including melanoma, head and neck, breast, bladder, ovarian, oesophageal, prostate, lung, pancreatic, cervical, liver, gastric, urothelial and merk cell cancers. The consensus emerging from these analyses is that the infiltration by adaptive cytotoxic lymphocytes is associated with favorable outcome.

1.2.1.4 Amplifying immune responses as a therapeutic strategy

Cancer regressions, especially sarcoma regressions, following the injection of immunostimulating agents, have been reported more than a century ago[55]. Specifically, intentionally causing Erysipelas, an acute infection of the upper dermis by a *streptococcus* bacteria suspension, now referenced as Coley's toxin, was reported to yield durable responses in about 20% of sarcoma patients. At that time, the mechanism of action was far from being understood, and Coley proposed that the efficacy of his toxin was due either to a direct toxicity of the toxin on sarcoma cells, or due to the induced fever, or even that the sarcoma was composed of bacteria which were overcome by the ones in the toxin[55]. The efficacy of Coley's toxin is nowadays believed to rely on multiple immune-related mechanisms, including lipopolysaccharide-mediated TLR activation of macrophages, which in turn release TNFα that induce direct tumor cells death and IL12 which stimulate previously-activated

T cells[56].

More recent immunostimulatory treatment modalities include administration of human interferon α (IFNα) and/or interleukin-2 (IL2). IFNα is a cytokine part of the type I family of interferons. It can trigger cell cycle arrest, differentiation or apoptosis in cancer cells[57]. In addition to these direct effects, IFNα increases the expression of class I MHC and chemokine production in tumor cells, resulting the attraction of lymphocytes to the tumor nest. It enhances NK cells abilities to kill target cells and produce IFNγ which locally activate $CD8^+$ T cells and polarize $CD4^+$ T cells towards a T_{h1} phenotype[58]. IFNα also enhances APCs abilities to present antigens[59, 60] and to secrete the T_{h1} cytokine IL12[61]. Most importantly, IFNα directly activates $CD8^+$ T cells by increasing their production of IFNγ[62], their proliferative capacities[63] and their differentiation into memory T cells[64] and favor their clonal expansion[65, 66]. Clinical indications for IFNα include melanoma, renal cell carcinoma, hairy cell leukemia, multiple myeloma, chronic myelo-proliferative syndrome, chronic myeloid leukemia, hemangioma and AIDS-related Kaposi's sarcoma[67].

IL2 is a cytokine used in the treatment of metastatic renal cell carcinoma and melanoma[68]. The doses employed in a clinical setting have to be carefully adjusted, as low dose have an immunosuppressive effect, while high dose can lead to irreversible toxicities[69]. Its mechanism of action involve activation of $CD8^+$ T cells and NK cells[70, 71] which in turn reduce tumor vascularization[71].

Some strategies involve the expansion, activation, optionally *ex-vivo* modifications, and re-injection of patient's autologous immune cells. Such strategies are termed adoptive cell transfers. Dendritic cells have been widely used in this context. Monocytes are purified from patient's peripheral blood, differentiated *in-vitro* into dendritic cells, which are then pulsed by patient's tumor lysates or exogenous cell lines. Dendritic cells are afterwards able to migrate to patient's lymph nodes to generate systemic responses leading to tumor regressions[72]. Such a treatment protocol, Sipuleucel-T, has been FDA and EMA-approved for the treatment of metastatic prostate cancer[73, 74].

1.2.2 Tumor outgrowth through pro-inflammatory signals

In addition to "avoiding immnune destruction"[16], enabling "tumor-promoting inflammation", emerged in the second edition of the "hallmarks of cancer" review[16, 75] as the other immunity-related item. Inflammation is associated with many carcinogenic events. For instance, mutation of the RET oncogene in thyrocytes is sufficient to induce papillary thyroid carcinoma and is accompanied with up-regulation of pro-inflammatory genes[76]. Mutation of RAS oncogenes in an ovarian cancer cell line xenografted in athymic mice is associated with the production of interleukin-8 (CXCL8) by the tumor cells, resulting in increased angiogenesis[77]. Activating mutation of the IL6-receptor signal transducer gp130 (IL6ST) triggers an inflammatory program in hepatocytes, favoring adenoma formation which can later develop into hepatocarcinoma[78]. Exogenous conditions can also trigger inflammatory signals. Infection by *Helicobacter pylori* is associated with gastric cancer[79–81] and mucosa-associated lymphoid tissue (MALT) lymphoma[82, 83], viral infection with Hepatitis-B or Hepatitis-C viruses is associated with the development of hepatocellular carcinoma[84] and tobacco smoke exposure triggers chronic lung inflammation[85]. It has been proposed that 15% of all diagnosed cancers are caused by infection[86].

Inflammation is a very broad concept that refers to immune-response promoting conditions and can designate almost every immune cell populations. In the context of tumor immunology, it usually refers to immune cell populations or cytokines that promote tumor growth. It is paradoxical as inflammation is associated with the promotion of immune re-

sponses, including adaptive immune responses. Some authors described adaptive immune responses as 'good inflammation' and the pro-tumor signals as 'bad inflammation'[87]. In this manuscript we use 'inflammation' to refer to the latter. In the following section, we detail some of tumor-promoting effects associated with inflammation.

1.2.2.1 Inflammation triggers genetic instability

Inflammation, while being associated with clinically-apparent tumors, can occur either in early or late stages of carcinogenesis. Pre-existing inflammation can directly promote carcinogenesis through mutational effects. In particular, reactive oxygen species (ROS), which are macrophage effector molecules which purpose is to destroy pathogens, have been shown to directly modify DNA sequences[88]. Inflammatory reactions can also lead to epigenetic modifications of the DNA, albeit no causal effect on carcinogenesis was observed[89].

1.2.2.2 Anti-apoptotic signals and proliferative signals

Inflammatory factors, such as IL6, IL1B or IL22, induce inflammatory responses leading to the activation of transcription factors NF-κB and STAT3 in cancer cells. These pathways can lead to the expression of anti-apoptotic molecules[90, 91], such as BCL2[87] and BCL-X (BCL2L1)[92] which promotes survival of malignant B cells in follicular lymphoma[93]. Mutation of STAT3 in non-tumorigenic immortalized fibroblasts is able to enable their successful xenografting in nude mice[94], and activation of the IL6-STAT3 pathway has been shown to have pro-carcinogenesis activities in many malignancies[92], including pancreatic ductal adenocarcinoma[95] and intraepithelial carcinoma[96], lung[97] and gastric adenocarcinoma[98]. NF-κB activation inhibits TNF-mediated apoptotis[99]. Inflammation also triggers proliferation in malignant cells[100], notably by increasing the expression of the cyclins B, D1 and D2[92, 101, 102].

1.2.2.3 Angiogenic signals

When the tumor reaches a certain size, oxygen supply becomes too limited for it to diffuse in all the areas of the tumor and hypoxic conditions arise. Inflammation is one of the mechanisms subverted by tumors to sustain neo-angiogenesis and increase blood supply. Inflammatory mediators released either by malignant, hematopoietic or other stromal cells can increase local angiogenesis. For instance, IL1β released by cancer cells simultaneously triggers angiogenesis and the recruitment of inflammatory cells to the tumor bed in a MYC-dependent pancreatic β-cell mice cancer model[103], where mast cells in turn can promote angiogenesis[87, 104]. While tumor cells have been known for a long time to induce local angiogenesis, the contribution of stromal cells, and in particular innate immune cells, to neo-angiogenesis is now established[105–110]. Immune-induced angiogenesis is notably mediated by the release of vascular-endothelial growth factors (VEGF), epidermal growth factor, fibroblast growth factor 2, TNFα, TGF-β platelet-derived growth factors (PDGF), placental growth factor, neuropilin-1 and IL8 (CXCL8)[110]. Among innate immune cells, macrophages are the most abundant in tumors and were shown to control angiogenesis in a mouse model of breast cancer[105] through their ability to secrete VEGF-A[111]. Inhibition of the colony stimulating factor 1 receptor (CSF1R), which is required for macrophages differentiation and survival, was shown to inhibit neo-vascularization in a glioma model[112].

1.2.2.4 Inflammatory biomarkers as therapeutic targets

Until recently, the prevailing model for macrophage polarization described two types of macrophage subtypes infiltrating tumors. M1 macrophages differentiate from monocytes under the influence of T_{h1} cytokines such as IFNγ, have higher phagocytic capacities than M2 macrophages and stimulate $CD8^+$ T cells and NK cells through cytokine production and antigen presentation. On the other hand, M2 macrophages are described as pro-angiogenic and as suppressors of adaptive immune cells. However, the diversity and plasticity of macrophage populations is too large to be accurately reflected by this simple model[113], and authors have since begun to use empirical classifications governed by the function of the analyzed myeloid cells[114], using the generic name tumor-associated macrophage (TAM) denomination to indicate tumor-infiltrating macrophages, independently of their orientation[115].

In the absence of a consensus on macrophage nomenclature, most studies examining the relationship between TAMs and cancer-related outcome have been carried out with the CD68 and CD163 markers, which respectively label macrophages and M2-polarized macrophages, using enzymatic immunohistochemistry[115, 116]. The majority (about 80%[115, 116]) of studies concluded in an association between high TAMs infiltration and poor prognosis. Yet subtleties appear, even within a given malignancy. For instance, in lung non small cell lung cancer (NSCLC), $CD68^+$ $IL10^+$ macrophages were found to be associated with poor prognosis[117] and half of the $CD68^+$ population was found positive for IL10 expression in late stage patients[117], while another study in the same tumor type showed that macrophages infiltrating early stage NSCLC were mostly M1-polarized and found in higher numbers in long patients with long survival[118].

Another difficulty is that inflammation leads to chemoattraction of many different immune cell types, and macrophage infiltration can be partly correlated with infiltration by $CD8^+$ T cells, for instance in colorectal cancer[119]. Thus, some authors proposed multi-marker classifications based on the quantification of multiple infiltrating immune cells populations. Examples include $CD3_+$ T cells / $CD68_+$ macrophages, associated with favorable outcome in bladder cancer[120], or classifications based on CD8 (cytotoxic T cells), CD4 (T helper cells) and CD68 (macrophages) for the stratification of breast cancer survival[121].

Numerous studies suggest that modulating inflammation is a potential treatment modality for the treatment of some cancers. Firstly, prophylactic use of anti-inflammatory agents such as aspirin has been shown to be associated with a reduction in colorectal cancer[122–124] incidence, a finding that has then been extended to breast[125], oesophageal, gastric, prostate and lung cancers[126]. Secondly, suppressing inflammatory signals in highly inflammatory clinically detectable cancers can lead to a halt of tumor growth[127], potentially synergizing with cytotoxic agents[2].

1.2.3 Tumor escape of the adaptive immune control

DNA instability in tumors, associated with selective pressure from immune-mediated elimination of tumor cells, lead to the emergence of escape mechanisms in tumor cells. These can either be directly induced by the tumor cells, or through other cell populations of the microenvironment.

1.2.3.1 Tumor-cell mediated escape

1.2.3.1.1 Reduction of the immunogenicity of malignant cells

$CD8^+$ T cells are the main effector of anti-tumor immune responses, but their activity requires the presentation of peptides by target cells by class I MHC molecules. These molecules are heterodimers consisting of two chains. The α chain is encoded by six different genes (HLA-A, HLA-B, HLA-C, HLA-D, HLA-E, HLA-F). The product of any of these genes associates non-covalently with the β2-microglobulin unit, encoded by a single gene, B2M. Some tumor cells harbour inactivating mutations in the B2M gene, abrogating expression of any functional class I MHC molecule and inhibiting the activity of $CD8^+$ T cells[128]. In the absence of functional class I MHC molecules, NK cells can sense the loss of class I MHC expression and exert contact-dependent cytotoxicity. However, tumor-infiltrating NK cells have been reported to display inhibited phenotypes compared to NK cells populating non-malignant tissues distant from the tumor. NK cells infiltrating Non-Small Cell Lung Cancer (NSCLC) tumors were shown to down-regulate the expression of the activating receptors NKp30, NKp80, CD16, NKG2D and DNAM-1 and consistently to have lower degranulation and cytotoxic capacities *ex-vivo*[129], possibly caused by TGF-β signaling[130]. Similar results have been observed in melanoma[131].

Loss of class I MHC expression is a striking illustration[132] of tumor adaption to immune pressure. However, in most cases, immune escape occurs in a more subtle and slow way through the selection of peptides with low immunogenicity. Immunogenecity refers to the capacity of a given peptide to elicit an immune response from the host. It includes its capacity to be presented by highly allelic-variable class I HLA molecules and to not chemically resemble non-mutated tolerated *self* peptides. Mouse models have successfully illustrated this phenomenon, as 3-methylcholanthrene (MCA)-induced tumors from immunocompetent mice have higher xenograft success rates in syngenic fully immunocompetent mice compared to those grown in $Rag^{-/-}$ mice lacking adaptive lymphocytes[26]. In human melanoma, vaccination based on the tumor-expressed gp100 peptide induced a reduction in tumor gp100 expression compared to pre-vaccination samples[133].

1.2.3.1.2 Expression of immunosuppressive molecules

The immune system is a double-edged sword that is efficient in preventing pathogens from colonizing the body, while autoimmune reactions can lead to damage to host's tissues. As other immune cells, $CD8^+$ T cells express, either constitutively or after activation, inhibitory receptors (also known as *immune checkpoints*) that regulate their activity. Tumors are able to subvert this mechanism and avoid $CD8^+$ T cell-mediated elimination. A notable example include the expression of PD1 (PDCD1) ligands by tumor cells. PD1 is an inhibitory receptor expressed by a variety of immune cells, including T cells. It can bind to either PD-L1 (CD274) or PD-L2 (PDCD1LG2), which results in reduced cytotoxic capacities[134], proliferative capacities[135, 136] and response to TCR-stimulation[136]. In physiological conditions, IFNγ the major cytokine of the T_{h1} axis, produced by activated T_{h1} and $CD8^+$ cells, has been shown to induce PD-L1 and PD-L2 expression in surrounding cells[137, 138]. Immune checkpoints include other molecules such as LAG-3, CTLA4 and TIM-3. Contact-dependent mechanisms can also mediate T cell elimination, such as Fas-ligand expressed by tumor cells which can bind to Fas expressed by surrounding lymphocytes, inducing their apoptosis[139, 140], although the importance of this effect is still debated[141, 142].

Tumor cells can also release soluble factors that result in the suppression of T cell responses in the microenvironment. TGF-β and VEGF-A orientate the functionality of surrounding hematopoietic cells towards a suppressive phenotype[143]. Other factors, such as the anti-inflammatory interleukin IL10, Galectin-1 (LGALS1)[144], gangliosides[145] and prostaglandin E2 (PGE_2)[146, 147] are implicated in the direct inhibition or elimination of infiltrating T cells.

1.2.3.2 Microenvironment-mediated suppression

Many suppressive pathways involve multiple cell populations from the tumor microenvironment. A critical step in the TCR-mediated actiation of T lymphocytes is its interaction with an antigen-presenting cell (APC), which usually are dendritic cells, but also macrophages and some B cell subsets. In addition to presenting class I and class II MHC-bound peptides to $CD4^+$ and $CD8^+$ T cells respectively (primary activation signal), APCs deliver so-called *secondary activation signals*. The secondary signal is antigen aspecific, but depends on which ligand/receptor couples are engaged between the T cell and the APC. Various activating or inhibiting receptor families have been described. The type of signal transduced depends mostly on the activation status of the APC. Notably, dendritic cells which sensed danger signals through DAMPs will mature and consequently transduce co-stimulatory signals to the T cell. On the other hand, immature dendritic cells presenting a specific antigen to a T cell will transduce tolerogenic signals, by repressing the T cell's ability to respond to future TCR stimulation, apoptosis or differentiation to a T_{reg} phenotype.

Several studies reported a defective presentation by dendritic cells infiltrating tumors. Firstly, the maturation of monocytes to dendritic cells is dampened in favor of a macrophage differentiation through the action of IL6 and Macrophage Colony Stimulating Factor 1 (CSF1)[148]. Secondly, the maturation process of dendritic cells is inhibited[149–154] by several mechanisms. Molecular mediators of the response to hypoxia pathway such as VEGF-A have been implicated in impairment of DC maturation through the inhibition of the inflammatory transcription factor NF-κ B[155]. The theory of *immunogenic cell death* proposes that activation of DC mostly depends on the type of cell death tumor cells underwent before their uptake by phagocytes[156]. Markers of immunogenic cell death, notably translocation of the chaperone calreticulin from the cytosol to the plasma membrane and the release of adenosine triphosphate (ATP) and of high-mobility group box 1 (HMGB1) protein in the extracellular milieu. Mice models have shown that after cancer cell line injection and clearance due to cytotoxic chemotherapy, and depending on the type of cell death, subsequent re-challenge using the same cell line will lead to rejection only in the case of immunogenic cell death in an immunocompetent host[157]. The fact that myeloid cells, in the tumor microenvironment, exert tolerogenic roles led to the functional definition of *myeloid-derived suppressor cells* (MDSCs)[143]. Lack of consensual markers in humans hamper comprehensive analyses of the MDSC populations in human tumors, and a wide variety of factors have been implicated in their expansion and polarization, including VEGF, GM-CSF, G-CSF, M-CSF, gangliosides, prostaglandins, IFNγ, complement C5a, TGF-β, interleukins IL1β, IL6, IL10, IL12, IL13 and chemokines CCL2, CXCL5 and CXCL12[143]. However, the mechanisms by which they exert their suppressive functions have been well studied, and notably include the depletion of L-arginine by the enzyme Arginase 1 which leads to impaired T cell proliferation[158, 159].

Lymphoid cells, and notably regulatory T cells (T_{reg}) are involved in antigen-specific suppressive function. Upon TCR activation, T_{reg} release the immunosuppressive cytokine IL10 which downregulates T_{h1} cytokines and co-stimulatory molecules on APCs. T_{reg} are produced under the influence of TGF-β and IL2 stimulation through the IL2 high-affinity receptor CD122-CD25 heterodimer. Other cytokines, notably VEGF-A, have been shown to induce regulatory polarization of $CD4^+$ T cells[160].

Hypoxia is in general linked to increased immunosuppression. It directly inhibits T cell responses, as hypoxic conditions inhibit IL2 and IFNγ release after TCR-mediated T cell activation[161]. In ovarian cancer, tumor cells response to hypoxia was shown to induce the expression of the chemokine CCL28 which attracts T_{reg}[162]. In mice, hypoxic area have been shown to favor the M2 polarization of macrophages[163], and VEGF-A signaling was shown to directly induce T cells expression of PD1 and other immune checkpoints, notably Tim-3 and CTLA-4[164].

Fibroblasts can also modify T cell responses, through several mechanisms. As major producers of the extracellular matrix, they control the trafficking of T cells from the invasive margin to the tumor stroma[165]. Fibroblasts can directly inhibit TNF and IFNγmediated anti-tumor immunity[166], hamper dendritic cells maturation[167] and inhibit T cell proliferation[168–170]. They have been shown to constitutively express the immune checkpoint ligand PD-L1[171], and this expression is upregulated upon IFNγ stimulation[138].

1.2.3.3 Immunosuppression as a therapeutic target

Since $CD8^+$ T cells are able to eliminate tumor cells and are limited in this function by tumor and microenvironment factors, the modulation of inhibitory signals has emerged as a therapeutic target during the last years. Above all, immune checkpoint molecules have been successfully targeted by blocking agents. The first inhibitory receptor that was successfully targeted is CTLA4, an inhibitory co-receptor expressed by T cells and which binds to CD80 and CD86 on APCs. Ipilimumab[172] and tremelimumab[173] are two fully humanized monoclonal antibodies specific for CTLA4. Clinical trials began in the early 21^{th} century and clinical responses were observed in around 10% of advanced melanoma patients, despite reports of high grade autoimmune adverse events in approximately 30% of them[172, 173]. Further investigations showed that enterocolitis, an auto-immune manifestation occuring in the intestines, was associated with higher response rates in treated melanoma and renal cell carcinoma patients and clinically manageable by administering TNFα inhibitors[174]. Tremelimumab showed no survival benefit compared to chemotherapy in a phase III trial[175], and has not currently received approval. Ipilimumab proved to confer a survival benefit compared to the melanoma peptide vaccine gp100 in a phase III trial[176] and is now FDA and EMA-approved.

The second checkpoint pathway that was successfully targeted is the PD1 - PD-L1/L2 axis. Nivolumab, an antibody targeting the receptor PD1 have shown clinical activity, first in advanced melanoma, renal cell carcinoma , lung and advanced prostate cancer[177], hodgkin lymphoma[178] and other malignancies[179]. Other antibodies targeting PD1 yielded clinical responses in some hematologic malignancies[180–182]. Compared to ipilimumab, it appears to be associated with less toxicity[183, 184]. Blockade of the ligand PD-L1 also led to clinical benefit in advanced melanoma, lung, ovarian, renal cell carcinoma[185, 186] and metastatic bladder[187] cancers.

Other immune checkpoints, such as LAG3 (T cells-expressed), B7-H3 (CD276, inhibitory ligand of unknown receptor) are currently evaluated as therapeutic targets in clinical trials[184], while others are investigated in preclinical models. The promising re-

sults of these new therapeutic approaches are likely to result in new therapeutic targets in the upcoming years. MDSC-mediated suppression is also a candidate for stimulation of anti-tumor immune responses[188–190]. The relationship between hypoxia and immunosuppression led some authors to propose combination of anti-angiogenic and anti-checkpoint treatments to evaluate potential synergy[191].

Adoptive transfer strategies that address inhibitory secondary signals include chimeric antigen receptors (CAR) T cells[192]. These are genetically-engineered T cells, designed to recognize tumor cells epitopes (for instance CD19 in the case of B cell malignancies), whose TCR is fused to the endodomain of the T cell activating receptor CD28 and therefore bypass inhibitory secondary signals, and whose epitope recognition is independent of MHC presentation[193]. CAR T cells are able to exert cytotoxicity to cells expressing their specific antigen, and appear as a successful treatment modality in the context of B cell malignancies[193].

1.3 Classification of tumors in the era of omic techniques

Tumor classifications have been established and continuously refined by clinicians and researchers. Their goals are to integrate our knowledge of the biology of each malignancies to ameliorate patient's management. Accurately predicting the evolution of a cancer has been of crucial importance since the advent of cytotoxic chemotherapies, to avoid potentially-damaging unnecessary treatments. More recently, the development of targeted therapies, drugs that interfere with a particular feature of the tumor biology, has opened new therapeutic options and simultaneously pushed for a better characterization of potential responders. In the following section, we recapitulate the evolution of tumor classifications, with a particular attention on the recent molecular classifications.

1.3.1 Anatomopathological classifications of tumors

1.3.1.1 The origin of the tumor cell: the main determinant of the tumor's biology

The most straightforward and currently almost subconsciously accepted way to classify cancers is according to the organ in which the primary tumor originated. As an illustration, the World Health Organization/International Agency for Research on Cancer classification is made of seven books, each covering one or several anatomical compartment ([digestive system], [breast and female genital organs], [soft tissue and bone], [skin], [urinary and male genital organs], [head and neck], and [lung, pleura, thymus and heart])[194–200]. Chapters within these books cover cancers of a given organ. This organ-based classification still stands after centuries of use. We now understand that cancer is a disease caused by molecular modifications of normal host cells which disrupt the mechanisms controlling cell proliferation, apoptosis, motility, among other features[16]. Depending on the cellular identity of the precursor cells, different mutations will lead to either malignant transformation or no phenotypic modification. The phenotype of the transforming cell is therefore the most critical factor in our current understanding of cancers biologies.

This classification has strong implications for the therapeutic management and the prognosis of the patient. Some cancers are highly aggressive and difficult to treat, while for others the proportion of long term survivors is much higher. For instance, in 2009, the United-States 5 year survival rates were of 6.5% for pancreatic cancer, 17.5% for liver cancers, but 87.6% for Hodgkin lymhpoma and 95.6% for testis cancer[201].

Within most malignancies, the current gold-standard for prognosis prediction is the

Union Internationale Contre le Cancer / TNM (TNM) staging classification, which is based on the assessment of the size or the depth of the primary tumor (T stage), its spread to local or distant lymph nodes (N stage) and its metastatic dissemination to distant organs (M stage)[202]. The TNM classification is able to accurately depict cancer progression and is therefore strongly associated with prognosis. It is however inapplicable to diffuse cancers such as leukaemia.

Other anatomopathological examinations of tumor tissues help clinicians predict the patient's outcome associated with a given tumor. Notably, the tumor grade is a classification based on the appearance of tumor cells. Its definition depends on the malignancy but recapitulates the agressivity of tumor lesions. One possible grading system is to assess the extent of the differentiation of malignant cells, with low grade tumors resembling non-malignant well-differentiated surrounding cells, and high grade tumors looking more anaplastic. In some cancers, such as brain malignancies, the grading system is used instead of the TNM staging system to assess patient's prognosis.

1.3.1.2 Molecular classifications of tumors

Anatomopathological classifications of tumors are useful for the evaluation of prognosis, and can also reflect different tumor biologies within malignancies[203–205]. They are based on the visual examination of tumor samples either at the macroscopic or the microscopic scale, but rely on other techniques to describe the mechanisms sustaining carcinogenesis. During the last couple of decade, the emergence of high-throughput molecular biology techniques, the so-called *omic* techniques, enabled the simultaneous quantitative assessment of thousands of molecular entities in tumor samples. Transcriptomics refers to the quantification of messenger RNAs (mRNA) in a sample, reflecting the activation levels of genes. Genomics concerns the characterization of DNA sequences, and can be used to assess allelic variability and most importantly tumor-specific mutations or inherited genetic susceptibility allelic variants. Methylomics measures the amount of methylation per DNA locus, which reflects epigenetical modifications of the genome. Proteomics simultaneously measure the amount of thousands of proteins. All of these techniques enable wide spectrum analyses, with at least several thousands of characterized targets, and can integrate prior biological knowledge of context-relevant targets. Their utilization in creating molecular classification of tumors enabled unsupervised taxonomies to emerge, along with their phenotypical and clinical characteristics.

1.3.1.2.1 Genomic classifications

Observations of chromosomal aberrations in cancer cells first hinted at genetic abnormalities as a possible cause for cancer[206]. In the middle of the 20^{th} century, the role of DNA as the main support of traits heritability was established[207], along with observations that DNA-damaging agents caused cancer. Shortly after, it was shown that the transfer of cancer genome in non malignant cells enabled their malignant transformation[208, 209]. The characterization of these modifications enabled researchers to better understand the causal events underlying carcinogenesis. Genomic translocations between chromosomes 9 and 22 were reported in chronic myeloid leukaemia[210], and identification of single-nucleotide mutations in oncogenes were identified in bladder epithelial cells[211, 212].

Mutations stochastically affect cancer cells[213] and can be either advantageous or deleterious, in the context of the cells' microenvironment. A Darwinian selection process is believed to filter out the cells unable to proliferate or survive. Understanding the mutations associated with clinically apparent or benign tumors help understanding which

disrupted pathways are favoring cancer's onset. Such genomic events are termed *driver mutations*. It can encompass point mutations but also base deletions and insertions, large chromosomic rearrangements and change in allelic ploidy (amplifications or deletions of chromosome fragments)[214]. Other genomic events, such as the integration of exogenous viral sequences, can also lead to cancer. Genomic classifications seek to identify genomic modifications of the cancer genome compared to the host's non malignant genome, and characterize their effect on the tumor cell's biology.

The number of known driver mutations ranges in the hundreds[28, 214], but the true number of possible driver events is likely to range in the thousands[214]. Some driver mutations are highly prevalent in many cancers, such as inactivating mutations in the TP53 tumor suppressor gene which occurs in approximately 50% of tumors[215]. Others are rare and specific to one or a few malignancies, such as the APC gene which is frequently mutated in colorectal cancer, and occasionally mutated in papillary thyroid and adrenocortical cancers[216].

The knowledge of mutated genes in a cancer can help establish molecular classification, in particular to develop targeted therapies or predict responders to treatments. For instance, imatinib is a tyrosine-kinase inhibitor specifically targeting the translocation of the ABL oncogene in chronic myeloid leukaemia[217]. Trastuzumab, a monoclonal antibody targetting the HER2/neu antigen, targets the product of the amplified ERRB2 gene, a genomic event which occurs in 20 to 30% of breast cancers[218]. Cetuximab, a monoclonal antibody targeting the EGFR receptor, is unable to induce clinical responses in KRAS-mutated tumors[219]. Recently-identified mutations provided by genome-wide association studies could therefore develop into drug targets or biomarkers predictive of response to treatments.

Such classifications are useful in a clinical setting, but it is difficult to integrate the whole-genome mutation spectrum into unified taxonomies. Driver mutations occur in particular pathway which sustain survival, growth and other cancer hallmarks, such as resistance to immune elimination. Driver mutations can sometimes be exclusive, but this fact usually indicate dependency of the encoded proteins[220, 221] and therefore biological proximity. Creating biologically-relevant classification of tumors requires to be able to discriminate between passenger and driver mutations, which is difficult as most somatic mutations in cancers are believed to be passenger[28]. Driver mutations then need to be sorted according to the pathway they affect, and their effect on the corresponding protein (inactivation, activation or no effect) characterized. The identification of mutated genes in cancer nonetheless provides important insights in which pathways are dysregulated during carcinogenesis as well as potential drug targets.

1.3.1.2.2 Transcriptomic classifications

High-throughput transcriptome characterization became possible with the development of DNA microarrays, followed by massively parallel RNA-sequencing methods. Although cancer is above all a genetic disease, the study of the transcriptome has proven more useful to establish cancer-specific classifications which rely on different biological processes and can be studied independently. The transcriptome is more straightforward in terms of interpretation that genomic mutations, as it is difficult to predict the effect of a mutation through DNA sequence alone. Since mRNAs indicate gene activation, the simultaneous quantification of tens of thousands of gene products in large human cohorts and their classification through unsupervised statistical learning techniques enabled researchers to discover classifications strongly associated with genomic events, but which also gave insights into the biological processes at play. The first classifications were estab-

lished respectively in breast cancer and B cell lymphoma, in the early 21th century.

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma. In 1994, a classification of non-Hodgkin's lymphomas based on morphological and molecular parameters was published, but suggested that DLBCL covered distinct diseases, although no useful subclassification could be established[222]. Using cluster analyses of DNA microarrays on various lymphoma samples and purified normal hematopoietic cells, A. Alizadeh and colleagues showed that DLBCL was molecularly different from other lymphomas (chronic lymphoid leukaemia, follicular lymphoma), but also separated into two molecular subgroups[223]. Comparison of the gene expression profiles to those of non-malignant samples showed that the first subgroup expressed markers of germinal center B cells (GC-like), and the second subgroup of activated B cells (activated B). Further classifications using these two sets of markers separated DLBCL patients into two subgroups with clinically different behaviors: GC-like lymphomas are associated with better prognosis than activated B lymphomas, independently of their International Prognostic Index, a clinical tool for prognosis prediction based on patient's age, performance status and the extent and location of the malignancy[224].

In breast cancer, the study of 65 surgical specimens from 42 different individuals established the first molecular transcriptomic classification of a non-hematopoietic cancer[225]. This seminal study, based on the quantification of 8102 genes showed that the molecular profile of two samples from a single individual were closer to each other than to unrelated samples, supporting the idea that transcriptomic profiles could faithfully describe a patient's tumor. Clustering analyses of tumor samples revealed four tumor classes. Clustering of the genes according to their co-expression profiles across patients identified gene sets that were differentially expressed across clusters, including ones related to previously genomic events such as ERBB2 amplification, or clinical features such as estrogen receptor (ER) loss of expression. This two-ways clustering enabled the denomination of the four tumor classes :

basal-like Basal epithelial cells markers, ER negative

ERBB2+ ERBB2 overexpression, low ER expression

Normal-breast-like Markers of adypocytes

epithelial/ER+ Luminal epithelial cells markers, ER positive

This molecular classification, which partially corresponds to previously-identified molecular alterations of breast tumors, later proved useful in a clinical setting, as variations based on this classification were able to predict patient's outcome[226] and response to chemotherapy[227]. Interestingly, the gene sets identified by this analysis identified both genomic features (for instance ERBB2 overexpression) and microenvironment features (expression of adypocytes markers, B cells-related genes). This approach, although it is focused on tumor portraits, still identifies microenvironment-related patterns.

These transcriptomic classifications were later confirmed, refined[228–234], used in preclinical models and to stratify clinical trials[235], and inspired the discovery of clinically and biologically heterogeneous subgroups in many other malignancies, including colorectal cancer[236–241], renal cell carcinoma[242, 243], glioma[244, 244, 245], liver[246–249], bladder[250] and prostate[251], acute myeloid leukaemia[252–256], and other cancers[257–259].

1.3.1.2.3 Multi-omics classifications

In addition to genomic and transcriptomic characterization of tumors, other high-dimensional techniques are able to identify tumor molecular subtypes. Notably, the methylome, proteome and miRNome quantify molecular features that result in modifications of biological pathways in tumor cells and their microenvironments. Analog of the techniques, such as clustering algorithms, used for transcriptome-based tumor classifications have been applied to data obtained from these multiple omic techniques. Notably, The Cancer Genome Atlas (TCGA) consortium aims at a multi-omic characterization of major human cancers. Their studies, as well as others, show that other omic classifications are significantly correlated with those established using transcriptomics[239, 260–273].

1.3.1.3 Molecular classifications of colorectal cancer

In this section, I present genomic and transcriptomic characterizations of colorectal cancer (CRC).

1.3.1.3.1 The adenoma carcinoma sequence

The prevailing model for CRC carcinogenesis was proposed in 1988 by B. Vogelstein and colleagues[274], based on the genetic characterization of four genomic events (mutations in genes of the Ras family, deletions in chromosomes 5, 17 and 18) in early adenomas, advanced adenomas and colorectal carcinomas samples. This model describes the accumulation of genomic events implicated in the progression from benign colorectal adenomas to malignant carcinomas. The first event is a loss of the APC tumor suppressor gene function, which controls the β-catenin pathway. *Familial adenomatous polyposis* (FAP) is a hereditary condition, most frequently caused by germline inactivation of the APC gene, leading to the development of many adenomatous polyps in the large intestine. Loss of the APC-containing 5p21 genetic locus occured only in patients without FAP, indicating that either germline or somatic loss of FAP was the main factor associated with the development of adenomas. Secondly, the authors observed an increase in the incidence of RAS mutations in both advanced (large) adenomas and in carcinomas compared to early (small) adenomas, indicating that mutation in the RAS pathway is associated with the size of adenomas. Thirdly, loss of the 18q chromosome arm, which carries the SMAD4 oncogene and the still debated tumor suppressor DCC, was most prevalent in carcinomas, followed by advanced adenomas. Finally, loss of the 17p chromosome arm, which notably hosts the TP53 tumor suppressor gene, was almost exclusively associated with carcinomas.

1.3.1.3.2 Pathways associated with carcinogenesis

Nearly all colorectal tumors feature disregulation of the Wnt/β-catenin pathway[275]. This pathway is important for the migration of colonic stem-cell from the bottom to the top of the colon crypt. Inactivation of the Wnt/β-catenin pathway lead to a proliferation of stem cells which remain in an undifferentiated state in the bottom part of the crypt, leading to the formation of adenomas. The transition from adenomas to carcinoma necessitates the acquisition of other malignant hallmarks.

Inactivation of the APC pathway is not sufficient to permit the growth of the adenoma. Advanced adenoma frequently harbor mutations activating cell-cycle pathways. Notably, mutations in Ras oncogenes (KRAS, NRAS, HRAS) , BRAF, or mutations affecting the PI3K pathway (such as activating mutations of PIK3CA or inactivating mutation

of PTEN), leading to increased cell proliferation within the pre-malignant lesion[275].

Other frequently disregulated pathways include the TP53 pathway, responsible for cell-cycle control and sensing of DNA integrity, the TGFβ pathway which promotes cell survival, invasion, metastasis and immunosuppression[275].

1.3.1.3.3 Genomic and epigenetic events associated with carcinogenesis

In colorectal cancer, high genomic instability is reported as the main factor contributing to the acquisition of these traits. Three major mechanisms have been shown to enable it.

1.3.1.3.3.1 Chromosomal instability

Chromosomal instability (CIN) is observed in up to 85% of colorectal cancers. It is characterized by frequent loss or gains of whole of chromosomes during cell replications, which can lead to aneuploidy (inconsistent number of copies of chromosomes), or chromosomal fragments amplifications or deletions and loss of heterozygosity. The mechanisms underlying this phenomenon are still poorly understood, most likely because of its complexity: hundreds of genes have been shown to induce CIN in yeast, while only around ten have been identified in humans[276]. Most of these include genes related to chromosome segregation during replication, such as BUB1, BUB1B and AURKA[276, 277], and genes involved response to DNA damage such as TP53, BRCA1 and BRCA2[276].

1.3.1.3.3.2 CpG-island methylator phenotype

CpG-island methylator phenotype (CIMP) is defined by hypermethylation of the symetrical dinucleotide CpG, and a global DNA hypomethylation. CpG-islands are DNA sequences where CpG dinucleotides are found in high frequencies, although there is no consensus the definition of CpG islands[278]. Many human genes harbor a CpG-island in their promoter region, and methylation of the cysteines of the CpG-island have been associated with transcriptional silencing of the gene[278]. CIMP is believed to contribute to carcinogenesis through the methylation of promoters of tumor suppressor genes[279], and/or by promoting microsatellite instability, another mechanism involved in genomic instability.

1.3.1.3.3.3 Microsatellite instability

Microsatellite instability (MSI) is caused by a defect in the DNA mismatch repair (MMR) machinery. MMR is involved in the correction of DNA replication errors. Microsatellites are short repetitive sequences of DNA, which increase the chance for the DNA-polymerase to 'stutter', leading to an increase in replication errors[275]. MSI is therefore the marker of a dysfunctional MMR system. MSI can be due to germline mutations of MMR enzymes, such as MLH1, MSH2, MSH6 and PMS2, which causes a condition known as Lynch syndrome[275, 280], associated with an increased risk of developping colorectal, gastric, ovarian and other cancers[280]. MSI can also occur in the absence of germline mutations, notably through epigenetic silencing of MLH1 in $CIMP^+$ tumors[275, 278].

As a result of deficient MMR, MSI^+ tumors are hypermutated, with almost 10 times more non-synonymous mutations per tumor than mutagens-induced lung cancers and melanoma[28]. MSI occurs in approximately 15% of CRC cases[275].

1.3.1.4 Transcriptomic subtypes of colorectal cancer

Unsupervised transcriptomic classification of colorectal cancer has been actively studied during the recent years. Six teams have independently reported molecular subtyping studies between 2012 and 2014[236–241]. These classifications seem to be mostly consistent with each others: while the reported number of subtypes vary from 3 to 6, some subgroups, identified by their respective association with molecular and clinical characteristics, appear coherent. I will now briefly introduce the commonly identified subgroups.

1.3.1.4.1 MSI-enriched subgroup

A subgroup highly enriched for microsatellite instable tumors has been identified in all proposed classifications[236–241], and is also highlighted in the TCGA publication[281]. It is reported to have high CIMP phenotype[239–241, 281], consistent the majority of MSI tumors being sporadic MSI and associated with CIMP. It also highly expresses cell proliferation-related genes[238, 239, 241], and is enriched in BRAF mutated tumors[238–241, 281].

It is reported to have high immune infiltration in five studies[236, 237, 239, 241], including the TCGA study[281], but the precise characterization of this infiltration is ambiguous: this subgroup was reported to overexpress genes related to antigen processing, HLA class I and II[236, 239], TLR-signaling, NK cells cytotoxicity and TCR signaling[239], interferons and interferon-induced transcripts of the IFI family[237, 241], chemokines[237], immune-system or inflammatory-response related pathways[236, 281].

This subgroup is also associated with favorable clinical outcome in five publications[237–241] and forms a poor prognosis higher-order group when merged with another subgroup in one publication[236].

1.3.1.4.2 Mesenchymal subgroup

All six publications identified a subgroup with overexpression of mesenchymal markers[236–241], colon bottom-crypt signature[237, 239], stem-cell phenotype[237, 239, 241], higher TGF-β pathway signaling[238–241], serrated-adenoma signature[239, 240], desmoplastic histology[241], and low cell proliferation[238, 239, 241].

It is associted with poor-prognosis[236–241] and resistance to therapies, both *in-vivo* to 5-fluouracil (5FU) chemotherapy[238, 240], Cetuximab (a monoclonal antibody targeting the VEGF receptor)[240], and *in-vitro* to a panel of targeted therapies[236]. It could however respond to FOLFIRI therapy (a tri-chemotherapy regimen combining folinic acid, 5FU and irinotecan)[237].

Immune infiltration may also be associated with this subgroup, as it has overexpression of genes related to TLR-signaling and hematopoietic cell lineage[239] or an immune signature[241] in two publications. Other publications did not however report differentially expressed genes related to immunity in this subgroup[236, 237].

1.3.1.4.3 Other subgroups

The other reported subgroups are less consensual based on the reported results. Most studies however report epithelial subgroups with a CIN phenotype[239, 240] and low immune infiltration[239, 241].

The Colorectal Cancer Subtyping Consortium is currently investigating the primary data of each reported studies, as well as other datasets, to prove the consistency of the various approaches and summarize it by proposing a consensual classification[282]. In addition to the three above-mentioned subtype, it proposes a fourth subtype, enriched for KRAS and IGFBP2 mutations[282].

1.3.2 Molecular classifications of clear cell Renal Cell Carcinoma

Compared with colorectal cancer, the known sequence of genomic events associated with the carcinogenesis clear cell Renal Cell Carcinoma (ccRCC) are less understood. It is dominated by the disruption of the 'cellular response to hypoxia' pathway, while secondary events begin to emerge. In parallel, transcriptomic classifications are being proposed, and may increase our understanding of the heterogeneity of ccRCC in terms of clinical behavior[283].

1.3.2.1 Genomic events associated with ccRCC carcinogenesis

1.3.2.1.1 Response to hypoxia pathway

The first identified germline mutation predisposing to ccRCC is a loss-of-function mutation of the von Hippel-Lindau (VHL) tumor-suppressor gene[284]. VHL encodes a protein which associates with products of the TCEB1 (elongin B), TCEB2 (elongin C) and CUL2 (cullin-2) genes to form a protein complex with ubiquitin-ligase activity[285]. In normoxic conditions, this complex ubiquitinates the hypoxia-inducible factor 1a (HIF1a), leading to its degradation. HIF1a is a transcription factor mediating the cellular response to hypoxia pathway. In hypoxic conditions or when the ubiquitin-ligase complex is inactivated, HIF1a escapes proteasome-mediated degradation and triggers a transcriptomic program which fosters glycolysis and production of VEGF which promotes angiogenesis.

VHL inactivation have been reported in around 90% of ccRCC tumors[264, 285]. Most of these events are recessive loss-of-function mutations, but are associated with a loss of heterozygosity of the 3p21-3p25 chromosomal loci which contains the VHL gene[264, 285]. Driver genomic events in VHL wild-type ccRCC tumors are still poorly characterized[264], but a study notably reported recurrent mutations in the elongin B gene TCEB1, mutually exclusive with VHL mutations[285].

1.3.2.1.2 Chromatin remodeling

Epigenetics appear to also play a role in the carcinogenesis of ccRCC. VHL can be inactivated through hypermethylation of its promoter instead of a mutation, an event seen in around 7-15% of ccRCC tumors[264, 284, 285]. A less understood epigenomic event is the disruption of the chromatin remodeling complex SWI/SNF. Several studies observed a significant rate of somatic mutations affecting this complex, in genes such as the PBRM1[264, 285, 286], ARID1A, SMARCA4[264]. It has been proposed that the SWI/SNF complex could be implicated in heterochromatin conversion of chromosomal regions, leading to their epigenetic silencing.

SETD2 mutation appears to be selected as a secondary carcinogenic event among VHL-mutated tumors: SETD2 is located in the same 3p21-3p25 region as VHL[264, 285] and

therefore affected by frequent LOH, and mutated SETD2 are detected at lower allelic frequencies that VHL mutations, suggesting a subclonal selection of SETD2 among mutated VHL tumors[285]. SETD2 seems implicated in widespread DNA hypomethylation[264] and associated with faster relapse[285].

1.3.2.1.3 PI3K-AKT-mTOR pathway

The PI3K-AKT-mTOR pathway, which regulates cell cycle, ranks third in terms of mutation frequency in ccRCC, with mutations affecting PTEN, PIK3CA, AKT and mTOR in around one-quarter of tumors[264, 285].

1.3.2.1.4 NF-κB pathway

Some authors suggested that NF-κB, the main transcriptomic factor implicated in inflammatory transcriptomic programs, but that also regulates cell survival, was overactivated in VHL-mutated tumors[283]. NF-κB activation was associated with resistance to TNF-α-mediated apoptosis. NF-κB activation also leads to the production of inflammatory cytokines, such as IL6 and CXCL8 (IL8)[283].

1.3.2.2 Transcriptomic classifications of ccRCC

Two studies, other than the one I was involved in and which is presented in the 'Results' chapter, identified transcriptomic subgroups of ccRCC. The first used a consensus clustering approach on a discovery cohort of 51 ccRCC samples to identify two subgroups, termed ccA and ccB. ccA corresponded to 24 samples, ccB to 15 samples, while 12 samples were unclassified. ccA tumors overexpressed genes related to angiogenesis and metabolism, and ccB tumors genes related to epithelial-to-mesenchymal transition (EMT), TGFβ and Wnt pathways. No association between subtypes and the rate of VHL mutations or promoter hypermethylation was found, and no other mutational events were investigated. This classification was reproduced on an independent set of 177 ccRCC tumors, in which the relationship with patient survival was investigated. ccA tumors were shown to have a better survival rate than ccB tumors, although ccA tumors were enriched for early stage and early grade patients. After correcting for stage, grade and performance status, the survival advantage of ccA over ccB patients was not anymore significant, although a trend towards better prognosis for ccA patients was still present (p=0.089). The authors noted that a continuous score based on the expression of 177 probesets and used to classify tumors in the validation set was significantly associated with survival after taking into account the confounding factors[243]. This first study therefore showed that two molecular subtypes of ccRCC could be identified and were associated with different prognostic values. It was latter confirmed on a meta-analysis of 480 publically available ccRCC transcriptomic profiles. ccA and ccB clusters were again identified, with ccA being associated with favorable outcome, and a third minor cluster correlated with VHL wild-type tumors or tumors with variant histologies. When variant histologies were removed, the analysis was dominated by signatures related to patient's sex and batch effects, preventing a deeper classification[242]. In both studies, there is no mention of association between the molecular subtypes and immune responses.

The multi-omic classification provided by the TCGA study identified four mRNA subgroups of ccRCC, termed m1, m2, m3 and m4. Very little details about the pathways

specifically associated with each clusters are reported, and no mention of immune-related information is given. m1 is characterized by chromatin remodeling, with higher frequencies of PBRM1 mutations. m3 tumors featured an increased rate of PTEN mutations and CDKN2A deletions, both genes being related to the regulation of cell cycle. m4 showed an enrichment in BAP1 mutations, a gene involved in DNA repair, and in the mTOR gene. Importantly, this study confirmed the clinical impact of the classification established by Brannon and colleagues[243], by showing that the ccA cluster corresponded to the m1 cluster, while ccB was splitted into m2 and m3 clusters, and with the m1 cluster being associated with better outcome. The authors of the TCGA study proposed that the m4 cluster correspond to the previously unclassified samples.

1.4 Shaping of the immune contexture by cancer cells

The study of the immune contexture of tumors, which greatly accelerated in the early 21^{th} century, has led to a better understanding of the interactions between tumor cells and their microenvironments, which opened promising, albeit recent, therapeutic avenues. In the meantime, the advent of high throughput molecular biology approaches, has greatly sped up our understanding of the molecular mechanisms that underlie the carcinogenesis and the heterogeneity of tumors within and across malignancies. In contrast, our understanding of the genesis of the variety of immune responses is limited. Within a single malignancy, tumors from different patients feature highly diverse immune responses, as shown by heterogeneity in the densities and the phenotypes of the immune and non-immune stromal cells of the microenvironment. Recent results suggest that the phenotype of the tumor cells is one of the major determinant of the tumor's immune contexture. In this section, I review the current knowledge of tumor immunology in relationship with tumor cell's phenotypes in colorectal cancer (CRC) and clear-cell renal cell carcinoma (ccRCC), and illustrate how these examples invite us to integrate the characterization of the immune contexture with the knowledge of tumor molecular characteristics.

1.4.1 Colorectal cancer: a canonical example for tumor immunology

Tumor immunology has been extensively studied in colorectal cancer. It is the cancer where it was established that an immune response characterized with extensive infiltration by $CD8^+$ T cells and a T_{h1} immune orientation are associated with favorable outcome. In this section, I will expand on these findings, comment on the relationship between inflammation and colorectal cancer's carcinogenesis, and on the state of immunotherapeutic approaches this malignancy.

1.4.1.1 Colorectal cancer arises in an inflammatory background

Inflammation is involved in the early stages of colorectal cancer's carcinogenesis. This statement is widely accepted for patients suffering from inflammatory-bowel diseases (IBD), a group of inflammatory conditions which include Crohn's disease or ulcerative colitis. Patients suffering from IBD are at increased risk of developping colorectal cancer, in which inflammation drives carcinogenesis, notably through the activation of the IL6/STAT3 pathway. In this case, cancer arises from flat dysplasic lesions instead of adenomas[287], with mutation of TP53 occuring early[288] and dysregulation of the Wnt pathway occuring late in the carcinogenesis[287].

Non IBD-associated colorectal cancer's carcinogenesis is, on the other hand, mainly

thought to be a purely genomic and epigenetic event, and the role of inflammation in the adenoma-carcinoma sequence was at first downplayed[289]. The fact that prophylactic use of anti-inflammatory drugs such as aspirin[122–124] is associated with a reduced risk of developing colorectal cancer, and that COX2 inhibitors were shown to reduce the number of polyps in patients affected by FAP[290] instead suggest that inflammation plays a role in sustaining carcinogenesis even in early non IBD-associated colorectal cancer.

In the case of adenoma-arising colorectal cancer, the prevailing model is that an inflammatory response with a T_{h1} functional orientation prevails in the adenoma stage, with production of IFNγ and the presence of M1-polarized macrophages as well as mature dendritic cells. These elements are still present in the carcinoma stage, along with T_{h17} cells and M2-polarized macrophages that together favor cell survival and proliferation as well as extracellular-matrix remodelling. Infiltration by inflammatory myeloperoxidase-positive cells (Neutrophils) increases from normal epithelial colonic tissue to dysplasic crypt foci and adenoma, and is highest in carcinoma samples[291], reflecting an inflammatory gradient.

1.4.1.2 T_{h1} functional orientation and extensive infiltration by $CD8^+$ T cells are associated with favorable prognosis in colorectal cancer

Histochemical quantifications of $CD45RO^+$ memory T cells first showed that anatomopathologically-classified 'high-risk' tumors, positive for lymphatic invasion, vascular emboli or perineural invasion, and tumors from patients with lymph node or distant metastases, also featured lower infiltration by memory T cells[46]. It was also shown that tumors from patients who relapsed had a comparatively lower expression of genes related to a T_{h1} functional orientation[46]. Expanding on these results, Galon and colleagues showed that the density of infiltrating $CD3^+$ adaptive T cells, $CD45RO+^+$ memory T cells, potentially-cytotoxic $CD8^+$ T cells or cytotoxic granzyme-B^+ T cells were all predictive of the overall-survival and relapse-free survival of patients in non-metastatic colorectal cancer, both in the center of the tumor and its invasive margin[47]. This seminal study showed that the simultaneous analysis of the type (cytotoxic and memory cells), the density and the location of tumor infiltrating cells could be combined into a single measure able to predict non-metastatic colorectal cancer patients' prognoses independently of their tumors' stages[48–51].

Other T lymphocytes subsets have been associated with prognosis in colorectal cancer, such as regulatory T (T_{reg}) and IL17-producing cells. Unlike other tumor types[54, 292], T_{reg} cells appear to be associated with a favorable prognosis in colorectal cancer[293–295]. This might be due to the fact that T_{reg} cells tamper the inflammation induced by IL17, which are associated with poor prognosis[296, 297] and tumor growth[298] in colorectal cancer.

1.4.1.3 Microsatellite instability is associated with immune response

MSI is associated with favorable outcome in colorectal cancer. MSI tumors accumulate mutations that lead to the presentation of *non-self* antigens by tumor cells, and indeed have a marked increase in tumor-infiltrating immune cells[18, 293, 299, 300]. Yet, it appears that the prognostic-value of tumor-infiltrating T lymphocytes densities is independent of the MSI-status of the tumor[18, 293]. The high rate of mutations in MSI

tumors could both hint at a better intrinsic control of the tumor growth by the adaptive immune system and represent a target for immunotherapy[301–303].

1.4.1.4 Until recently, immunotherapy was unsuccessful in colorectal cancer

The only immunotherapeutic drugs currently approved in the treatment of colorectal cancer are monoclonal antibodies. Although some immunostimulatory effects have been reported[304], these drugs are mostly believed to antagonize tumor growth through the inhibition of angiogenesis and survival-signals mediated by VEGF-signaling. Cetuximab, a monoclonal antibody targeting the EGF receptor, is FDA and EMA-approved for the treatment of metastatic $KRAS^{WT}$, but most of its therapeutic effects is unlikely to stem from immuno-stimulatory functions of the antibody. Indeed, patients whose tumors are mutated for KRAS, a downstream signal-transducer of the EGFR receptor, fail to respond to Cetuximab[219]. Bevacizumab, a monoclonal antibody targeting VEGF-A and approved for the treatment of metastatic colorectal cancer, is also believed to exert its efficacy through the inhibition of neo-angiogenesis[305].

Other strategies, including peptide or tumor cells vaccines, *ex-vivo* dendritic cells activation and transfer, or cytotoxic lymphocytes adoptive transfers, have been experimented in humans, but no phase III clinical trial using these strategies demonstrated a clinical benefit[306]. Currently approved recombinant cytokines IL2 and IFNα, are not indicated for the treatment of colorectal cancer[306].

Immune checkpoint inhibitors have also been tested for the treatment of CRC, and initially yielded a poor response rate: only one CRC patient ouf of nineteen enrolled responded to the anti-PD1 monoclonal antibody Nivolumab in a phase I trial[307, 308], and none out of eighteen responded to an anti PD-L1 monoclonal antibody in another phase I trial[185]. The facts that the only colorectal cancer patient who responded had an MSI tumor phenotype, and that MSI tumors are associated with an increased expression of checkpoint molecules[302, 303], prompted a clinical trial for Pembrolizumab, another anti-PD1 antibody, stratified by MSI phenotype. This phase I clinical trial showed a strong association between the response to Pembrolizumab and the MSI status of the tumors, with proficient MMR tumors having higher response rates. Consistently, patients with MSI tumors had an increase progression-free survival and overall-survival following treatment compared to those harboring microsatellite stable tumors[179]. This finding appears to translate to other tumors where MSI phenotypes exist, such as endometrial, gastric and small intestine cancers[179].

1.4.2 ccRCC: a counter-example for the paradigms of tumor immunology

Clear-cell renal cell carcinoma has been reported as an immunogenic tumor, based on the fact that like melanoma but unlike colorectal cancer, cytokine-based immunotherapies (IL2 and IFNα) have been approved for its treatment[309]. Yet, unlike most malignancies[54], an extensive infiltration by $CD8^+$ T cells is associated with poor prognosis in ccRCC. In this section, I will present results underlying the singularity of ccRCC for tumor immunology.

1.4.2.1 The immunoscore does not apply to ccRCC

Tumor infiltration by $CD8^+$ T cells is associated with a favorable outcome in most malignancies[54]. Surprisingly, this rule does not hold true for ccRCC. Several independent studies have reported that infiltration by $CD8^+$ T cells was associated with a poor prognosis[149, 310, 311] or with a non-significant trend towards poor prognosis[312] Consistent correlations with survival were obtained for $CD4^+$ T cells and $CD45RO^+$ T cells[312]. High proportion of $CD3^+$ T cells among tumor-infiltrating lymphocytes is also associated with a poor prognosis in ccRCC[313], as well a high expression of lymphoid markers[314] or the expression of IFNγ[149] using transcriptomics. In parallel, while most tumors feature down-regulation of class I MHC, which is believed to mediate escape from $CD8^+$ T cells-mediated elimination, ccRCC do not feature such a down-regulation, but rather an up-regulation[315].

On the other hand, it appears that the quantification of proliferating $CD8^+$ T lymphocytes[310] is associated with favorable prognosis, suggesting that actively proliferating $CD8^+$ T cells, which are likely antigen-activated, associated with favorable outcome. Consistently, an analysis of the repertoire of $CD8^+$ T cells infiltrating ccRCC tumors highlighted the polyclonality of these cells, indicating little *in-situ* clonal activation and expansion[316]. The same study also reported an upregulation of $PD1^+$ and $LAG3^+$ T cells in ccRCC compared to peripheral blood lymphocytes from the same patients[316], while tumor infiltration by $CD8^+$ T cells was shown to correlate with infiltration by $PD1^+$ and $LAG3^+$ cells. Altogether, these result suggest that tumor-infiltrating $CD8^+$ T cells are specific for tumor antigens but functionally inhibited.

As I presented earlier, ccRCC carcinogenesis is tightly linked to disruption of the response to hypoxia pathway, through the loss-of-function mutation or promoter hypermethylation of the VHL gene[264, 285], or mutations of TCEB1[285], leading to a high expression of the pro-angiogenic factor VEGF-A by tumor cells. It has been shown that VEGF-A modulates the expression of immune checkpoint molecules on tumor-infiltrating T cells[164] and that anti-angiogenic treatments were able to reduce the percentage of T_{reg} cells in ccRCC tumors in patient's peripheral blood[317, 318], which correlates with an increase in T_{h1}[318] cytokines and with response to treatment[317]. It has therefore been proposed that the high angiogenesis associated with ccRCC carcinogenesis could be responsible for the apparent lack of efficiency of tumor-infiltrating $CD8^+$ T cells.

1.4.2.2 Immunotherapy has been a successful modality for the treatment of ccRCC

Spontaneous regressions of ccRCC tumors have been observed and first suggested that the immune system was able to mediate cancer regressions in ccRCC[319]. At the same time, clinical trials to evaluate the efficacy of IL2 or IFNα cytokines were ongoing[320–323], and yielded clinical responses in up to 30% of patients, and durable responses in up to 7%[324]. However, high dose IL2 therapy induces severe toxicities and its use is therefore restricted to patients with high performance status[325]. Moreover, a comprehensive meta-analysis did not conclude that there was a survival benefit in the overall survival of patients treated with high dose IL2 monotherapy compared to IFNα monotherapy[326], nor for low dose IL2 plus IFNα therapy compared to IFNα monotherapy, while IL2 was associated with higher toxicity[326]. Use of IFNα is also limited by high treatment-related toxicity, but has been shown to increase patient's overall survival compared with conven-

tional treatments such as chemotherapy[327].

IL2 and IFNα have been the main treatments for advanced renal cell carcinoma for almost two decades, but have recently been outshadowed by targeted therapies, specifically those targeting angiogenesis or the mTOR pathway[324]. Although these therapies provide a progression-free survival benefit over cytokine-based immunotherapies, patients ultimately relapse and die of their diseases[325]. However, the new generation of immunotherapies, based on immune checkpoint blocking antibodies, has resulted in that a subset of ccRCC patients responded, and that these responses could be durable[185, 307, 328]. A recent phase II clinical trial with the anti-PD1 antibody Nivolumab showed a clinical activity and acceptable safety profile in patients with metastatic ccRCC refractory to anti-angiogenic treatments[329]. Ongoing phase III clinical trials comparing checkpoint blocking antibodies to targeted therapies will tell whether these immunotherapies will yield similar or better short-term clinical benefits than other targeted therapies, and similar or better long-term responses as cytokine-based therapies.

1.4.3 The shaping of immune responses by cancer cells

I presented results underlying the fact that the immune system was able to control tumor growth, and that it could be therapeutically leveraged. I highlighted the critical role of the phenotype of the cancer's progenitor cell in conditioning carcinogenesis, and presented genomic classifications of tumors which help deciphering the critical molecular events associated with carcinogenesis. Results from tumor immunology in ccRCC and CRC show that immune contextures vary from one malignancy to the other. I will now discuss whether these different immune contextures are shaped by tumor cells or by the surrounding organ's tissues.

1.4.3.1 Immune responses are conserved during metastasis

Studying the tumor metastasis microenvironment is useful to delineate the impacts of the surrounding organ and of the malignant cells in shaping their immune contextures. Remark and colleagues reported immunohistochemical quantifications of tumor infiltrating $CD8^+$ T cells, DC-$Lamp^+$ mature dendritic cells and $NKp46^+$ NK cells in lung metastases stemming from colorectal cancers or renal cell carcinoma[311]. Despite being resected from the same organs, the densities of lung metastasis-infiltrating immune cells was different between the two malignancies: the density of DC-$Lamp^+$ cells was higher in CRC lung metastases compared to ccRCC lung metastases, and the density of $NKp46^+$ cells lower[311].

For both cancers, immune infiltration was heterogeneous across patients in both lung metastases and corresponding primary tumors. However, the density of all the quantified immune cells were highly correlated between primary tumors and their corresponding metastases, in both cancers[311]. These two results reveal that tumors of different molecular phenotype that evolve within the same surrounding tissue display different immune contextures, suggesting that the tumor cells influence the density of the infiltrating immune cells.

1.4.3.2 The prognostic associated with a given immune contexture in the primary tumor is recapitulated in metastases

I presented evidence of a prognostic association between a high density of tumor infiltrating $CD8^+$ T cells in primary CRC and primary ccRCC. However, it is a positive association in CRC[47] and a negative one in ccRCC[149]. Strikingly, these results were reproduced in study of Remark and colleagues: infiltration by $CD8^+$ T cells was associated with favorable outcome in CRC lung metastases and poor outcome in ccRCC lung metastases[311]. Of note, in lung cancer, like in CRC, $CD8^+$ T cells infiltration is associated with favorable outcome[330]. Therefore, malignant cells rather than the surrounding organ condition the prognostic value associated with a high density of a given immune cell phenotype.

Altogether, these results suggest that there is a strong influence of the malignant cell's phenotype on their immune contexture[311, 314, 331–333].

1.5 Correlating immune contextures and tumor's molecular phenotypes

I have presented results from both tumor immunology and omic analyses, that led to classifications of tumors, which are useful to predict prognosis and response to therapies. I also detailed the similarity of the immune contexture of primary and matched pulmonary metastases of CRC and ccRCC tumors. In this setting, the density of infiltrating immune cells was correlated between matched malignant lesions, suggesting that tumor cells influence the recruitment of adaptive and inflammatory immune cells, possibly through the release of cytokines or inflammatory factors, as well as the antigenicity and number of presented neoantigens. The prognostic impact associated with an extensive infiltration by adaptive immune cells was also consistent between primary and metastatic tumors (positive for CRC and negative for ccRCC), suggesting that the effect of the immune contexture on tumor control or promotion depends on the phenotype of the cancer cell rather than on the surrounding non-malignant tissue. These results suggest that molecular characteristics of tumors (activation of pro-inflammatory or immunosuppressive transcriptomic programs, expression of highly or poorly immunogenic antigens) influence the densities of immune cells in the tumor microenvironment, as well as their effect on tumor evolution. Since transcriptomic classifications aim at identifying 'intrinsic' tumor phenotypes that may correspond to different molecular phenotypes of the cancer cells, these classifications could therefore also identify groups of tumors with different immune contextures. In order to challenge this hypothesis, I will thus introduce possible approaches to investigate the relationship between omic and immunological classifications of tumors.

1.5.1 Canonical techniques to study the immune microenvironment

Most researches aiming at quantifying cell populations within the immune microenvironment are based on immunohistochemistry. This technique can be performed on fixed samples which are then sectioned in thin slices and mounted on glass slides. Antibodies specifically targeting proteins of interest (for instance CD8) are then incubated on the surface of the tissue. Antibodies are usually conjugated to an enzyme which catalyzes a color-producing reaction, usually a peroxidase. Once antibodies are bound to their epitopes, the enzyme's substrate is added on the tissue slide and subsequently processed by

the enzyme, producing a color co-localizing with the epitope of interest. The number of positive cells, extent of positive surface or color intensity can then be quantified, and their spatial localization observed. Quantification of positive cells can be achieved through eye-counting using a microscope, or through image-processing softwares. In both cases, the process is time consuming, requires expertise and access to tissue samples. Moreover, it is difficult to simultaneously quantify multiple cell populations, as the number of distinct colors that can be used simultaneously is limited. Given the wide number of cell populations interacting within the tumor microenvironment, a quantitative analysis of multiple cell populations is difficult. The number of simultaneously-targeted epitopes can be increased with the use of antibody-bound fluorescent dyes, which is useful to assess patterns of co-expression of the corresponding proteins within a tissue, but makes the quantification of positive cells difficult.

Flow-cytometry uses fluorochromes-bound antibodies to target cells suspended in a liquid media, which is then ran through a cytometer equipped with lasers, each emitting light at wavelengths matching the distinct fluorochromes' excitation windows. Fluorescence is then measured by detectors and mapped to epitope-specific antibodies based on the emission wavelength. Other signals, such as the forward light scatter and the side light scatter are measured and enable cells stratifications depending on their sizes and granularity. This technique enables the quantification of refined cell populations based on the combination of epitopes expressed, at the cost of the loss of spatial localization. A number of confounding events (cellular debris, dead or bound cells) restricts this quantification to be relative to a reference cell populations. One can for instance measure $CD8^+$ T cells among $CD45^+$ hematopoietic cells, or among $CD3^+$ cells, but this technique is not used measure absolute numbers of immune cells within a tumor sample. Moreover, it requires access to fresh tissue samples and therefore cannot be used in retrospective analyses of tumor cohorts.

1.5.2 Studying the immune microenvironment through gene expression

As transcriptomic classifications reflect differences in carcinogenesis pathways and mutational events, and are able to capture immune-related information[225], it is tempting to study the microenvironment through transcriptomic data, and directly assess the correlation between the amount of infiltrating immune cells' densities and cancer molecular subgroups. In this section, I will present why such a study is conceptually different from those performed using immunohistochemistry or immunofluorescence, and review the methods that have been proposed to tackle this issue.

1.5.2.1 Enrichment analyses

Features (genes) clustering analyses can identify sets of co-expressed genes, and supervised analyses identify gene sets differentially expressed between two or more conditions. These two types of approaches can then be used to identify overrepresented gene sets (for instance genes that belong to a particular pathway) among a gene cluster or a set of differentially expressed genes. This can be achieved through the use of a hypergeometric test, or with other statistical methods such as Gene Set Enrichment Analysis (GSEA)[334], or by ad-hoc analyses that rely on the expertise of researchers to infer the biological signification of a gene set. Such analyses have been performed to highlight the enrichment of immune-related genes in molecular classifications[239], or to analyze immune signatures in tumor cohorts[335]. Although the approach is in theory valid, it requires either

a suitable database for the definition of gene sets or knowledge based on empirically defined pathways. These are, however, highly context-dependent: for instance, the gene set *GO:0050863 regulation of T cell activation* is suitable within an experiment that compares two T cell samples and should reflect a differential activation of T cells within one sample. In the context of analyzing the tumor microenvironment, it could reflect activation of T cells, the presence of T cells, or even an upregulation of genes involved in T cell activation but which are in fact expressed by other cell types. Gene sets from public databases are highly overlapping, and such analyzes usually return many related pathways as overrepresented, enabling analysts to highlight differential expression of immune-related genes, but not to perform a deeper characterization of the type of immune response at play.

1.5.2.2 The tumor transcriptome: a convoluted measure of gene expressions

Unlike immunohistochemistry and immunofluorescence, where events are acquired at a cellular level, transcriptomic measures is performed at the sample level. A tumor sample is a heterogeneous mixture of malignant and normal populations, including immune and other stromal cells. A measure of the expression of a gene in a tumor sample is therefore the sum of the expressions of the gene by all individual cells in the sample. The gene expression measure is therefore the convolution of each cell populations' gene expression profiles and their corresponding proportions in the sample. If we consider a 'perfect' measure (with no noise and perfect linearity), then the following model holds :

Let a sample's transcriptome e be a measure of n features. These can be represented by a *n-dimensional* vector $e = e_{1...n} = (e_1, \dots, e_n)$. We can consider that the sample is composed of a finite number of m distinct cell populations. Let $p = p_{1...m}$ represent each population's proportions. p verifies equations (1.1) and (1.2).

$$\sum_{k=1}^{m} p_k = 1 \tag{1.1}$$

$$\forall k \in 1 \dots m, p_k \geq 0 \tag{1.2}$$

For a given feature $1 \leq j \leq n$, and a cell population $1 \leq k \leq m$, let $g_{j,k}$ be the measure of the expression of the feature j in population k. Then the sample's expression of the feature e_j verifies equation (1.3), which states that the measured expression e_j is the weighted average of the expression levels of j in each cell populations $g_{j,k}$, weighted by their respective proportions p_k.

$$e_j = \sum_{k=1}^{m} g_{j,k} \times p_k \tag{1.3}$$

Since equation (1.3) holds for any feature j, the sample's transcriptome e follows equation (1.4).

$$e_{1...n} = \begin{pmatrix} e_1 \\ e_2 \\ e_3 \\ \dots \\ e_{n-1} \\ e_n \end{pmatrix} = \begin{pmatrix} g_{1,1} & g_{1,2} & \dots & g_{1,m} \\ g_{2,1} & g_{2,2} & \dots & g_{2,m} \\ g_{3,1} & g_{3,2} & \dots & g_{3,m} \\ \vdots & \vdots & \ddots & \vdots \\ g_{n-1,1} & g_{n-1,2} & \dots & g_{n-1,m} \\ g_{n,1} & g_{n,2} & \dots & g_{n,m} \end{pmatrix} \times \begin{pmatrix} p_1 \\ p_2 \\ p_3 \\ \vdots \\ p_{m-1} \\ p_m \end{pmatrix} \tag{1.4}$$

Finally, let s be the number of samples measured (for instance s different tumor samples). The transcriptomic measure then becomes a $n \times s$ matrix (equation (1.5)), whose i^{th} column ($1 \leq i \leq s$) is the transcriptome of sample i. Each sample verifies equation (1.4), so the matrix $e_{1...n,1...s}$ verifies equation (1.6)

$$e_{1...n,1...s} = \begin{pmatrix} e_{1,1} & e_{1,2} & \dots & e_{1,s} \\ e_{2,1} & e_{2,2} & \dots & e_{2,s} \\ e_{3,1} & e_{3,2} & \dots & e_{3,s} \\ \vdots & \vdots & \ddots & \vdots \\ e_{n-1,1} & e_{n-1,2} & \dots & e_{n-1,s} \\ e_{n,1} & e_{n,2} & \dots & e_{n,s} \end{pmatrix} \tag{1.5}$$

$$e_{1...n,1...s} = \begin{pmatrix} g_{1,1} & g_{1,2} & \dots & g_{1,m} \\ g_{2,1} & g_{2,2} & \dots & g_{2,m} \\ g_{3,1} & g_{3,2} & \dots & g_{3,m} \\ \vdots & \vdots & \ddots & \vdots \\ g_{n-1,1} & g_{n-1,2} & \dots & g_{n-1,m} \\ g_{n,1} & g_{n,2} & \dots & g_{n,m} \end{pmatrix} \times \begin{pmatrix} p_{1,1} & p_{1,2} & \dots & p_{1,s} \\ p_{2,1} & p_{2,2} & \dots & p_{2,s} \\ p_{3,1} & p_{3,2} & \dots & p_{3,s} \\ \vdots & \vdots & \ddots & \vdots \\ p_{m-1,1} & p_{m-1,2} & \dots & p_{m-1,s} \\ p_{m,1} & p_{m,2} & \dots & p_{m,s} \end{pmatrix} \tag{1.6}$$

where $p_{j,k}$ is the proportion of the cell population j in sample k.

1.5.2.3 Deconvolution approaches

The goal of deconvolution approaches is to estimate the unknown matrix of proportions $p = p_{1...m,1...s}$. The matrix $e = e_{1...n,1...s}$ is measured (known). Various approaches have been proposed to estimate the proportions p, that I will review in this section, with a particular focus on their scopes (cell populations surveyed and types of analyzable samples)

1.5.2.3.1 Complete deconvolution

The matrix $g = g_{1...n,1...m}$, whose columns are the transcriptome of each cell populations, is also unknown. Complete deconvolution algorithms attempt to simtultaneously estimate both g and p, given e[336]. The first study proposing an algorithm to solve this problem was published in 2001 by Venet and colleagues[337]. It is the first stating the above-stated mixture model (equation (1.6)). In their paper, the authors proposed a method that iteratively estimates e then g to minimize the error $|e - g \times p|$, and applied it to a measure of 1988 genes across 62 colon cancer samples. Interestingly, their method was able to identify four cell populations, two of which could be identified as hematopoietic cells and fibroblasts[337]. The authors however discuss the limitations of their algorithm, notably the absence of experimental validation and the difficulty in estimating the number of cell populations k.

Repsilber and colleagues built on the method proposed by Venet et al., notably by using a least-square non-negative matrix factorization algorithm in the iterative steps, which enable them to alleviate one of the hypotheses necessary for the applicability of the previously-proposed model[338]. The authors also tested the predicted proportions against cytometry-measured proportions in blood samples, and the cell populations-specific expression profiles against those of FACS-sorted cells from the corresponding blood samples. They were able to reach a Pearson's correlation of 0.86 between the predicted and measured expression profile, and a significant correlation between the proportions of cytometry-measured and computationally-predicted cell populations.

No other complete deconvolution method that did not use external information was otherwise published[336]. Erkkiläe and colleagues implemented a bayesian statistical framework that requires only the number of distinct cell populations k to perform a complete deconvolution[339]. In addition, tools have been proposed that relied either on the external measurement of cellular proportions p to infer the cell populations' transcriptomic profiles g, or on the measure of the transcriptomic profiles g to estimate the proportions p. For instance, Kuhn and colleagues, as well as Zhong and colleagues, implemented methods which estimate the proportions based on the expression of user-provided populations-specific markers, assumed to be expressed in only one cell population, and then use these proportions to estimate their corresponding gene expression profiles[340, 341]. Ahn et al. as well as Quon et al. used a two-populations model, and a corresponding set of genes externally-measured for both populations, to first estimate the proportions of the two populations and then their corresponding transcriptomes[342, 343], with the goal of ameliorating the measurement of the malignant cell's transcriptomes.

1.5.2.3.2 Partial deconvolution

Instead of trying to estimate simultaneously both g and p, most published methods rely on the measurement of cell proportions to estimate population specific expression profiles[344–346], or measurement of specific expression profiles to estimate proportions[343, 347–353]. I will focus on the latter, as our purpose is to quantify immune cell populations within a tumor's microenvironment.

Lu and colleagues[347] were the first to deconvolve cell proportions in the transcriptome of yeast cultures in different phases of the cell cycle, by using gene expression measurements in yeast cultures synchronized at a given phase of the cell cycle. Shortly after, the first study applying deconvolution to tumor samples was published[348], aiming at estimating the proportion of malignant cells and contaminating stroma in a tumor sample. Wang and colleagues increased the number of cell populations whose proportions are estimated, in murine mammary gland samples. They notably included immune samples ($CD4^+$ and $CD8^+$ T cells, B and plasma cells and macrophages) and fibroblasts in their reference transcriptomic prolifes, along with mammary epithelial cells, as well as brown and white adipose tissues[349]. Gong et al. showed that such approaches could apply to next-generation RNAseq data[352].

Studies focusing on estimating immune cells' proportions mostly focused on blood samples[350, 351], which is arguably of lower complexity in terms of cellular composition than solid tissues. Using transcriptomic profiles of purified cell populations, these studies were able to estimate either the proportions of lymphocytes, neutrophils and monocytes[351], or 18 different immune cell populations[350].

Finally, a recent study tackled the issue of the estimation of immune cell proportions within tumor tissues[353]. It relies on the expression from the gene signatures of Abbas et al.[350], or user-provided signatures, then perform a feature selection step followed by support vector regression to estimate the proportions of the populations covered by the signatures[353]. Applied to tumor samples and using user-provided signatures, it quantifies the relative proportions of immune cells within the hematopoietic contingent of cells in the tumor microenvironment.

1.5.2.4 Marker-based approaches

The definition of a transcriptomic marker for a cell population is unclear and varies from one study to the other. Within partial deconvolution approaches, we presented studies that used markers of cell populations[350, 353], which in this context are genes differentially expressed between the populations surveyed. Deconvolution approaches try to minimize $|e - g \times p|$ given e and g, but part of the transcriptome e is sometimes irrelevant, as some genes are expressed in none of the cell types of interest or at equal levels by all of them. Partial deconvolution approaches therefore use a subset of the features $n' \in [1, n] \cap \mathbb{N}$ where $\forall j \in n'$, j is a gene differentially expressed among the cell populations surveyed.

In another type of approaches, markers are thought as genes entirely specific to one population and not expressed at all in the others, and could therefore directly correlate with tumor infiltration by their corresponding immune population. The idea behind marker-based approaches is, formally, to identify the set of genes $n_{spec} \in [1, n] \cap \mathbb{N}$, so that for all cell populations, the expression of the marker is null except in one, ie $\forall j \in n_{spec}, \exists! k(j) \in [1, m] \; such \; as \; g_{j,k(j)} \neq 0$.

Equation (1.5) is then equivalent to (1.7).

$$e_{1...n_{spec},1...s} = \begin{pmatrix} g_{1,1} & 0 & \dots & 0 \\ g_{2,1} & 0 & \dots & 0 \\ 0 & g_{3,2} & \dots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & \dots & g_{n_{spec}-1,m} \\ 0 & 0 & \dots & g_{n_{spec},m} \end{pmatrix} \times \begin{pmatrix} p_{1,1} & p_{1,2} & \dots & p_{1,s} \\ p_{2,1} & p_{2,2} & \dots & p_{2,s} \\ p_{3,1} & p_{3,2} & \dots & p_{3,s} \\ \vdots & \vdots & \ddots & \vdots \\ p_{m-1,1} & p_{m-1,2} & \dots & p_{m-1,s} \\ p_{m,1} & p_{m,2} & \dots & p_{m,s} \end{pmatrix} \tag{1.7}$$

For a given cell population k_0, whose markers are n_{k0}, we have

$$\forall j \in n_{k_0}, e_j = \sum_{k=1}^{m} g_{j,k} \times p_k = g_{j,k_0} \times p_{k_0} \tag{1.8}$$

ie $p_{k_0} \propto e_j$, there is a direct proportionality between the proportion of the cell population k_0 and e_j, the sample's expression of j.

The system of equations given in (1.8) is overdetermined, as there are more equations than variables, and it is therefore unsure whether a solution mathematically exist. To estimate the proportion of interest, it is however possible to use estimators such as the mean $\overline{\left(\frac{e_j}{g_{j,k_0}}\right)}_{j \in n_{k_0}}$.

The markers investigated are sometimes proposed on the basis of the knowledge of immunology[119, 354, 355], but are most often based on empirical data measuring the level of expression in transcriptomes of purified (FACS-sorted, *in-vitro* cultured, or microdissected tissue sections) cell populations[119, 355–360]. Interestingly, in the pioneering work of Venet et al. on complete deconvolution[337], the authors define a transcriptomic marker as described above, and show that the existence of markers for each population surveyed is one of the conditions for their algorithm to be deterministic. However, in the implementation of their method, they do not use such markers but instead relax their

definition to genes with a high differential expression[337]. Only one study attempted to identify 'strong' markers, with a non-null expression in only one cell population as shown in equation (1.7)[356]. The other studies based on identification of markers only characterized 'weak' markers, overexpressed in a given cell population but with potential non-null expression levels in the others[119, 357–360]. Finally, Abbas et al. and Palmer et al. focused on blood samples, with little or no control for solid tissues, respectively[356, 358]. Shoemaker et al. propose a web-based tool performing enrichment analyses of user-provided gene sets, which could be applied to the study of tumor molecular subtypes. Yoshihara et al. use two sets of markers to estimate stromal and immune contaminations in tumors, making their approach inapplicable for the precise characterization of immune responses in tumor subtypes[360]. Finally, Bindea et al. proposed list of overexpressed markers for twenty-six cell populations and two types of colorectal controls[119].

1.5.3 Transcriptomic studies of the tumor microenvironment

In the previous section, I presented methods related to the estimation of immune-cell infiltration in heterogeneous samples. I this section, I will present the results obtained with these methods, with unsupervised analyses, or a combination of both, on transcriptomic cohorts of human colorectal, ccRCC, or in multiple cancers at once.

1.5.3.1 Transcriptomic analyses of the microenvironment in colorectal cancer

1.5.3.1.1 Signatures associated with immune infiltration

Transcriptomic analyses have been performed in the seminal study by Galon and colleagues which associates extensive infiltration by $CD3^+$, $CD8^+$ or $CD45RO^+$ T cells in colorectal tumors' microenvironments with favorable outcome for the patient[47], using a quantitative rt-PCR approach on 18 genes related to immunity. Clustering analyses on this small gene set enabled the identification of a cluster of co-expressed genes associated with T cells cytotoxicity and a T_{h1} functional orientation (GZMB, GNLY, IFNG, IRF1, CD3Z, CD8A, TBX21), suggesting that this signature represents the infiltration by $CD8^+$ T cells. Expanding on these results, Mlecnik et al. later identified, using *in-silico* gene networks reconstructions, CX3CL1, CXCL9 and CXCL10 as the main cytokines associated with infiltration by these cells in colorectal cancer[361]. Finally, Coppola et al. identified a set of twelve cytokines (CCL4, CCL5, CXCL9, CXCL10, CXCL11, CXCL13, CCL2, CCL3, CCL8, CCL18, CCL19, CCL21) which identifies the presence of tertiary lymphoid structures in colorectal tumors stroma[361]. Tertiary lymphoid structures are lymph-node like follicles that emerge at sites of chronic inflammation and their presence have been associated with favorable outcome in a wide variety of tumors[333]. The signature proposed by Coppola and colleagues overlaps with the one identified by laser capture microdissection in lung cancer (which includes CCL19, CCL21, CXCL13)[362].

1.5.3.1.2 Cellular quantifications using transcriptomics

The first study analyzing the microenvironment in colorectal cancer tumors using transcriptomics was performed by Bindea and colleagues[119]. In this paper, the authors proposed a list of markers for twenty-six cell populations of the microenvironment. These markers were then used to perform clustering analyzes, independently of the markers' associated cell populations, which stratified patients into two clusters (a third set of patient was left unclassified). Samples of the cluster 1, highly expressing the proposed markers of $CD8^+$ T cells and of T_{h1} lymphocytes, were associated with favorable outcome. Samples of the cluster 2 highly expressed the proposed markers of NK cells, eosinophils,

central-memory T cells, T_{H2}, T_{h17}, and regulatory T cells, and were associated with poorer outcome. The same approach was used by Angelova et al., who added markers for myeloid-derived suppressor cells based on published immunological knowledge[355]. It identified genomic features associated with immune infiltration, and notably that microsatellite instability is associated with infiltration by adaptive lymphocytes.

Two studies recently analyzed the microenvironments of colorectal cancer molecular subgroups, with a particular focus on the poor-prognosis mesenchymal subgroup. Both studies used gene set enrichment analyses and identified that this subgroup was highly infiltrated by leukocytes (without further precisions about their phenotypes), endothelial cells and fibroblasts[363, 364]. Calon et al. proposed that tumor-associated fibroblasts were actively promoting the metastatic capacities of cancer cells in this subgroup, which could explain the poor prognosis associated with this subgroup[363]. Isella et al. proposed that expression of fibroblasts markers could identify this subtype and predict poor response to chemotherapy[364].

1.6 Transcriptomic analyses of the microenvironment in ccRCC

Most transcriptomic studies focusing on kidney cancer attempted to characterize pathways differentially expressed between normal kidney and either renal cell carcinoma[365] or ccRCC[366–369]. All of these agreed on the fact that ccRCC tissues overexpress genes related to immunity compared with adjacent kidney tissue. Pathways identified included both lymphocytes-related and inflammation-related pathways[365], antigen-processing and presentation, NK-cell mediated cytotoxicity and cytokine-cytokine receptor interactions[369], IFNγ-mediated immune response and innate immune response[368], cytokines, TLRs and T-cells related genes[367]. Tan et al. measured the expression of 681 immune-related genes and found virtually all of them overexpressed in ccRCC samples compared to adjacent kidney tissues[366]. These pathways suggest an over-infiltration by many different immune cell types.

One study focused on one particular gene, CD1d, which they report as a marker common to myeloid cells of the monocytic lineage and B cells. They show that a higher expression of this gene is associated with higher tumor stage and grade, and predicts a poor outcome[370]. I presented studies suggesting that high infiltration by $CD8^+$ T cells is associated with poor outcome in ccRCC. This study suggests that a high infiltration by other immune cell types could also identify high-risk patients in ccRCC.

1.7 Pan-cancer analyses of the tumor microenvironment

Several studies have recently been published on pan-cancer analyses, two of them focusing on the tumor microenvironment. Hoadley and colleagues performed a clustering analyses spanning twelve different malignancies using TCGA data, and highlighted pathways related to 'T and B lymphocytes', 'PD1 signaling', 'CTLA4' and 'interferon signaling'[371]. The first three were highly correlated, and the highest expression was found in ccRCC (KIRC TCGA project), followed by lung adenocarcinoma (LUAD TCGA project). Colorectal cancer was among the cancers with the lowest expression for these three pathways[371]. Their result suggest that ccRCC is one of the highest lymphocytes-infiltrated cancers.

The second study is also based on TCGA data and includes 18 TCGA projects. It

analyzes the expression of a two-genes score (the mean of $\log_2$(GZMA) and $\log_2$(PRF1)), which is proposed to reflect simultaneously the infiltration and the activation of cytotoxic lymphocytes within tumor samples[354]. Their results also suggest that ccRCC is one of the most infiltrated malignancies and that colorectal cancer rather poorly infiltrated. Interestingly, the authors suggest that the number of non-synonymous mutations, as well as the number of mutations potentially generating epitopes able to bind to the patient's HLA molecules, are correlated with their two-genes score. They also propose that the expression of endogenous retroviruses transcripts, as well as some particular gene mutations, are associated with the two-genes score.

Finally, Gentles and colleagues applied the CIBERSORT algorithm[353] on 18,000 tumors spanning 39 malignancies to study the prognosis associated with various immune subsets[372]. Since CIBERSORT only outputs the proportion of immune cells subpopulations relative to a sample's level of immune infiltration, they could not compare the level of infiltration across malignancies. They however show a high proportion of myeloid cells among immune cells in brain cancers compared to other solid tumors. The relative proportions of the surveyed immune cell subpopulations is shown to be similar across other solid tumors[372]. Their results also support that relative infiltration by T$\gamma\delta$ cells is the best immune marker for favorable outcome, and relative infiltration by neutrophils the best immune marker for adverse outcome[372]. The association of the relative frequency of immune cell subpopulations with survival is however cancer-dependent[372].

Chapter 2

Hypotheses, objectives and strategies

In the introduction, I presented results from both tumor immunology and molecular studies of tumors. Researches in tumor immunology have shown that in most cancers, adaptive immune cells can control tumor growth, and consistently that their extensive infiltration in tumors correlate with favorable outcome. They also showed that inflammation, on the other hand, fuels tumor growth, notably by inhibiting the activity of the adaptive immune response and by promoting angiogenesis and tumor cells proliferation. These results led to a large number of proposed immunological biomarkers to predict patient's prognosis or response to therapies. In parallel, omic classifications have delineated tumor molecular subgroups in many different malignancies, which are able to identify different carcinogenic mechanisms within a given cancer. These subgroups are associated with different genomic and epigenetic alterations, and are often informative about patient's prognosis and response to treatments.

Little is known about the overlap between immunological and molecular classifications. Studies that propose molecular stratifications of cancers often report dysregulation of immunity-related genes in some subgroups, suggesting a consistency between immune and molecular classifications, although most often only little precision about the type of inflammatory or adaptive immune mechanisms at play is given. On the other hand, the analysis of matched primary and metastatic tumors performed by Remark et al.[311], where the molecular phenotype of the tumor cell is likely to be mostly maintained between matched samples while the surrounding tissue is different, reveals that the phenotype of tumor cells critically influence the density of infiltrating immune cells and the prognostic value associated with a given immune infiltrate.

Thus, we hypothesized that there is a correlation between the molecular phenotypes of tumors and their immune contextures. To test this hypothesis, we proposed to analyze the immune contexture of large cohorts of molecularly-classified human tumors. Several mechanisms could underly this correlation, notably the type of cytokines released by cancer cells, their antigenicity or the production of molecules regulating their stroma.

The main objective of my PhD project is therefore to correlate immune contextures and the molecular subtypes of tumors. It serves several translational purposes:

- Firstly, both genomic and immune classifications were shown to predict patient's prognosis and response to treatments. A unified genomic and immune classification could accelerate their conjoint use in the clinic.
- Secondly, several treatments modulate features of the tumor microenvironment,

notably anti-angiogenic treatments which modulate the tumor vasculature, anti-checkpoint and cytokine-based immunotherapies which enhance anti-tumor immune responses, and anti-inflammatory treatments which tame tumor-promoting inflammation. Several studies suggest that cancer-associated fibroblasts also represent potential therapeutic targets in the tumor microenvironment. Analyzing the tumor microenvironment of tumor molecular subgroups could therefore hint at the groups of patients most likely to respond to these microenvironment-targeting therapies.

A secondary objective is to confirm, and investigate why, in clear-cell renal cell carcinoma, unlike most other cancers, an extensive infiltration by $CD8^+$ T cells correlate with poor prognosis. This objective is important for tumor immunology, in order to ameliorate our understanding of the interactions between malignant cells and their immune microenvironment.

2.1 Methodological objective: transcriptomic quantification of cells populations of the tumor microenvironment

Investigating multiple cell populations and functional mediators of the tumor microenvironment through classical techniques, such as immunohistochemistry and immunofluorescence, in relationship with tumor molecular phenotypes, is extensively time and money consuming, and requires access to both fresh or frozen tumor tissue to collect nucleic acids material for molecular analyses, and flash-frozen paraffin-embedded tissues for *in-situ* analyses. On the other hand, since the tumor transcriptome holds immune-related information, and is readily available from public repositories when molecular classifications are performed and published, I proposed to develop a transcriptomic approach to quantify immune cells infiltration.

2.2 Methodological objective: transcriptomic analyzis of the functional orientation of the tumor microenvironment

Since the functional orientation of cells within the microenvironment is mostly modulated by cytokines and other soluble mediators, I proposed a litterature-based approach to select genes important in the functional orientation of the tumor microenvironment.

2.3 Main objective: unifying immune and molecular classifications

We set-up collaborations with researchers proposing molecular classifications of ccRCC and CRC and re-analyzed their data to describe the immune contextures of the identified tumor subtypes, using the methodology we designed. *In-situ* analyzes were realized to confirm the computational predictions.

2.4 Secondary objective: investigate and explain the prognosis of ccRCC tumors highly infiltrated by CD8$^+$ T cells

I focused on ccRCC tumors highly infiltrated by cytotoxic lymphocytes and analyzed other features of their microenvironment. I notably focused on the other cell populations present in these tumors and on the functional orientation of immune cells in their microenvironments.

Chapter 3

Results

3.1 Article 1 : the immune contextures of ccCRC molecular subtypes

3.1.1 Summary of the article

3.1.1.1 Motivation of the study

This article is a collaborative work in which we proposed a transcriptomic classification of ccRCC tumors along with the description of the respective immune contextures of the tumor subtypes. The study was motivated by the fact that tyrosine-kinase inhibitors (TKI), which are drugs that interfere with intracellular signaling pathways and which are proposed as first-line therapy to advanced ccRCC patients, yield inconsistent responses across patients, while no biomarkers for the response to these treatments is available. Additionally, since immune checkpoint-blockade therapies have been recently reported to induce clinical responses in metastatic ccRCC patients who did not respond to TKI, a description of the immune contexture of ccRCC molecular subtypes could help identifying patients who could benefit from checkpoint-blockade immunotherapies.

The study is based on a multi-omic analyses of primary ccRCC tumors of patients at the metastatic stage, who received at least one cycle of Sunitinib, a multi-targeted tyrosine-kinase inhibitor, whose targets include VEGF receptors and PDGF receptors. These receptors transduce angiogenic and proliferative signals. In the absence of an untreated control group, the endpoints analyzed were the response to treatment according to RECIST 1.0 criteria, overall-survival (OS) and progression-free survival (PFS).

Supervised analyses of genomic data failed to identify genomic markers associated with the primary endpoints, which could be due to the molecular heterogeneity of ccRCC tumors. A transcriptomic classification of these tumors was therefore performed and analyzed the distribution of Sunitinib-responders across subgroups, as well as the OS and PFS associated with each subgroup. Since the number of tumors analyzed in this discovery cohort was modest (53 ccRCC samples), a rt-qPCR classifier was designed and tested on the discovery cohort and then applied to an independent validation serie of 47 samples.

3.1.1.2 Results: identification and genomic characterization of four molecular subgroups of ccRCC

Three clustering techniques based on the 1% most variable probesets of the transcriptomic arrays each identified four tumor clusters, termed ccrcc1-4, with a strong consistency of the classifications established with each method. These findings confirmed and extended

previously published classifications that stratified ccRCC into two[243] or three molecular subgroups[242]. The ccrcc1 subgroup is characterized by lower mutation rates of the VHL and PBRM1 genes, hypermethylation of CpG-islands, hypermethylation of genes encoding proteins of the polycomb-group which control cell differentiation and a consistent higher Fuhrman grade, and overexpression of genes downstream of the MYC transcription factor which controls cell proliferation. ccrcc2 has the highest VHL mutation rate of the four subgroups, an intermediate expression of the 'cellular response to hypoxia' pathway and lower Fuhrman grade compared with the other subgroups, but otherwise showed little specific genomic events or disrupted pathways. ccrcc3 is a 'normal-like' group, whose transcriptome and methylome resemble the one of tumor-distant normal kidney samples, and has a low activation of hypoxia pathways. Finally, the ccrcc4 subgroup resembles ccrcc1 in terms of genomic amplification events and disrupted pathways, but is characterized by lower rate of PBRM1 and VHL mutations as well as an overexpression of immunity-related genes. It also showed recurrent copy-number alterations of the 2p12, 2p22.3 and 8q21.13 genomic loci.

3.1.1.3 Results: association of the molecular subgroups with response to sunitinib and prognosis

The classification into four molecular subgroups showed a significant association with response rates to Sunitinib treatment, with 70% of responders (complete or partial responses as evaluated by RECIST 1.0) for ccrcc3 tumors, 53% for cccrcc2, 41% for ccrcc1 and only 20% for ccrcc4 tumors. Consistently, patient survival after treatment was significantly different across the four subgroups, with patients of the ccrcc2 and ccrcc3 groups having the best prognosis for both OS and PFS, followed by ccrcc1, while patients in the ccrcc4 subgroup had the worst outcome.

3.1.1.4 Results: high immune infiltration in the ccrcc4 poor-prognosis subgroup

Pathological examination of tumor tissue sections identified that ccrcc4 tumors were more inflammatory. Consistently, pathway analyses relying on public gene sets databases identified an upregulation and hypomethylation of genes involved in 'T-cell activation', 'Regulation of immune response' and 'Chemotaxis' in ccrcc4 tumors. As these results suggested a high infiltration by immune cells, I used previously-established transcriptomic signatures of immune cells[119] to identify whether and which immune subpopulations were infiltrating ccrcc4 tumors. These cell signatures had been designed for colorectal cancer, and I first validated that ccRCC cell lines did not express the genes covered by these signatures. Genes in the cell signatures of T cells, cytotoxic lymphocytes, B cells, NK cells and macrophages were found to have a good specificity, and their expression were therefore analyzed in the transcriptomic profiles of the four groups of tumors. I found that the expression of these immune signatures were all associated with the molecular classification of tumors, except for the genes specific for NK cells, suggesting a high infiltration by B, T, $CD8^+$ T cells and macrophages in ccrcc4 tumors. I then analyzed a list of manually-curated genes related to immune modulation, and showed that ccrcc4 tumors overexpressed cytokines involved in the attraction of memory T cells (CXCL9, CXCL10, CXCL11) and a T_{h1} polarization of the immune response (IFNG, IL12A, IL12B, TBX21). Tumors of the ccrcc4 subgroup also overexpressed genes related to inflammation (CCL2, TNF, CSF1). Strikingly, genes encoding molecules inhibiting adaptive immune responses were overexpressed in ccrcc4 tumors, including PDCD1 (PD1) and its two ligands, TIM3 (HAVCR2), LAG3, as well

as soluble immunosuppressant such as TGFB1 and IL10. Immunohistochemical analyses confirmed that this poor-prognosis subgroup was highly infiltrated by $CD8^+$ T cells.

3.1.1.5 Discussion

This article confirms and extends previous transcriptomic classifications of ccRCC. It has translational relevance, as this stratification of ccRCC is associated with response to Sunitinib treatment. In particular, patients of the ccrcc4 subgroup poorly responded to Sunitinib and had the worst prognosis, in terms of both OS and PFS, despite being extensively infiltrated by $CD8^+$ T cells. This subgroup was characterized by a canonical T_{h1} immune response, which could be counterbalanced by the presence of inflammatory cells (macrophages) and expression of pro-inflammatory molecules, as well as a high expression of checkpoint molecules and immunosuppressive soluble factors, which could explain why $CD8^+$ T cells are not associated with favorable prognosis in ccRCC. The fact that patients in this subgroup are refractory to Sunitinib but express molecules of the PD1 pathway suggest that they could respond to antibodies interfering with the PD1 pathway.

3.1.2 Article

This published article is available at `http://clincancerres.aacrjournals.org/content/early/2015/01/10/1078-0432.CCR-14-1128`.

3.2 Article 2 : the immune contextures of CRC molecular subtypes

3.2.1 Summary of the article

This *in-preparation* article addresses two objectives of my project: the development of a methodology to analyze the microenvironment of human tumors, and its application to study the immune microenvironment of CRC molecular subtypes.

3.2.1.1 Objectives and methodology

A large number of independent studies have proposed transcriptomic classifications of CRC in the past few years[236–241]. The Colorectal Cancer Subtyping Consortium (CRCSC) is currently proposing a consensus classification based on these independent studies[282], and their results are currently under evaluation. We retrieved the Consensus Molecular Subtype (CMS) annotations from the CRCSC and investigated the immune contextures of each CMS using a transcriptomic approach. The main goal of this study is the investigation of the correlation between molecular and immune classifications, which provide insights into potential immunotherapeutic approaches for each patient subgroups.

3.2.1.2 Methodology: Transcriptomic quantification of cell populations in the tumor microenvironment

To achieve it, I developped and introduce a marker-based transcriptomic quantification method of tumor infiltrating immune and stromal populations, and a knowledge-based approach to study their functional orientations. The marker-based approach is based on the curation of transcriptomes from 1114 immune, 36 endothelial and 50 fibroblastic pure cell samples, and as control the transcriptome of 745 non-hematopoietic cancer cell lines,

from public repositories. I consistently annotated these samples and normalized them using fRMA[373, 374], an algorithm which enables the integration of multiple transcriptomic studies into a single dataset. I curated profiles from samples obtained with as many culture conditions (for instance with or without stimulations) and purification methods (FACS-sorted or *in-vitro* differentiated, purified from peripheral blood or from tumors...) as possible, in order to account for possible phenotypic alterations of the cells in the tumor microenvironment that could affect markers' expression.

I first performed unsupervised analyses to investigate whether similarly-labeled transcriptomes displayed consistent gene expression profiles. These analyses showed that the samples clustered together according to their phenotypic annotation rather than batch effects. It also showed that non-immune samples (cell lines, fibroblasts and vessels) segregated from hematopoietic samples. Interestingly, hematopoietic samples further segragated into myeloid and lymphoid lineages, which then separated into monocytic and granulocytic clusters, and T/NK and B clusters, respectively, as previously observed by Abbas and colleagues[356]. Unsupervised analyses failed in identifying more refined phenotypes, for instance $CD4^+$ T cells from $CD8^+$ T cells, as observed by Palmer et al.[358]. The consistency of these unsupervised classification analyses led me to classify samples according to their position in the hematopoiesis tree, and then perform a supervised screen on the complete transcriptome to identify each populations' universally-expressed markers.

Unlike previous marker-based studies, I pursued the characterization of 'strong' markers, as first defined by Lu et al.[347], ie markers expressed in one and only one cell population and not in the others (see equation (1.8)). The transcriptome we curated are produced using Affymetrix Human Genome U133 Plus 2.0 Arrays, which are single-color cDNA arrays. There is no straightforward method to assess the non-expression of a gene using this technique, as non-expressed genes are attributed non-null values which correspond to background noise signals. In my supervised analysis, I chose a set of three statistical criteria, which together ensure the specific expression of the target probeset by one and only one cell population.

These markers were experimentally validated using two different approaches. First, mRNA were extracted from pure immune populations and a colorectal cancer cell line, and mixed together in varying proportions. These mixtures were hybridized on Affymetrix Human Genome U133 Plus 2.0 Arrays, and the markers' expression levels were summarized and compared with the known proportions of the corresponding cell populations in each mixture. This *in-vitro* analysis showed a very high correlation between the predicted and known proportions.

Immunohistochemical quantifications were performed on tissue sections from transcriptomically-characterized tumors for three cell populations (cytotoxic lymphocytes, macrophages and fibroblasts), which revealed a strong correlation between the two techniques.

3.2.1.3 Methodology: Functional orientation of cell populations in the tumor microenvironment

Unlike cell-populations' markers, cytokines and immunomodulatory factors do not necessitate specific expression to infer the functional orientation of tumor-infiltrating immune cells: indeed, these factors are either soluble or membrane-bound, and it is therefore their overall quantity in the microenvironment that is relevant. I therefore applied a knowledge-based approach to study the function of cells in the tumor microenvironment, and curated gene sets related to inflammation, angiogenesis, immunosuppression and T_{helper} cells ori-

entations.

3.2.1.4 Results: immune contextures of each CMS

Four CMS are proposed by the CRCSC:

MSI-like: enrichment in MSI, CIMP and BRAF-mutated tumors, and favorable outcome

Canonical: enrichment in CIN tumors and activation of the Wnt pathway, and intermediate outcome

Metabolic: enrichment in CIN and KRAS-mutated tumors and dysregulation of metabolic pathways, and intermediate outcome

Mesenchymal: overexpression of mesenchymal genes, and poor outcome

The cell signatures revealed that the MSI-like and Mesenchymal subgroups were both highly infiltrated by immune cells, whereas the Canonical and Metabolic subgroups had low infiltration by immune cells. MSI-like and Mesenchymal tumors were both highly infiltrated by cells of the monocytic lineage, which were shown to be macrophages by immunohistochemistry. MSI-like tumors had the highest expression of genes specific for cytotoxic lymphocytes, which were shown to be of $CD8^+$ phenotype by immunohistochemical analyses. Mesenchymal tumors had intermediate expression of genes specific for cytotoxic lymphocytes ($CD8^+$ T cells), and very high expression of genes specific for endothelial cells and fibroblasts. Immunohistochemical analyses revealed prominent stromal infiltration in Mesenchymal tumors, with a high density of cancer-associated fibroblasts.

Genes encoding functional molecules revealed that the MSI-like subgroup had the highest expression of genes associated with T cell infiltration[361] and activation[375], T_{h1} functional orientation[47], that were previously reported as associated with favorable outcome in CRC, and immune checkpoints, whose expression were previously shown to be increased in MSI CRC tumors[302, 303]. The Mesenchymal subtype was associated with highest expression of pro-angiogenic, non-checkpoint immunosuppressive factors (TGFβ, LGALS1), and complement components, as well as intermediate expression of PD1 ligands. The two poorly-infiltrated Canonical and Metabolic subgroups had low expression of class I MHC molecules, which present antigens to surrounding $CD8^+$ T cells.

Analyses of the transcriptomic profiles of pure cell populations revealed that the genes found specifically overexpressed in Mesenchymal tumors are mostly overexpressed in fibroblasts, endothelial cells and macrophages. Fibroblasts overexpress pro-angiogenic factors, which can favor the proliferation of surrounding endothelial cells. Endothelial cells were shown to overexpress inflammatory molecules, which can recruit cells of monocytic origin. Both fibroblasts and endothelial cells overexpress immunosuppressive factors. Finally, fibroblasts, endothelial cells and macrophages all produce complement molecules which together may locally activate the complement cascade and further fuel inflammation.

3.2.1.5 Discussion

This study revealed, like in ccRCC, a strong association between transcriptomic subgroups and immune contextures in CRC. Two subgroups were found to be highly infiltrated by immune cells. The MSI-like subgroup was expected, as it corresponds to a genomic subgroup previously reported to be highly infiltrated by adaptive immune cells. The functional

orientation and cellular infiltration found in this subgroup perfectly corresponds to the immune contexture described by the team of J. Galon[18, 46–49, 51, 53, 119, 361], which is associated with favorable outcome in CRC, consistently with the favorable outcome of patients harboring MSI-like tumors. The high expression of checkpoint molecules in this subgroup suggests that it could benefit from checkpoint-blockade immunotherapies, as previously proposed using *in-situ* or *ex-vivo* approaches[302, 303].

The second highly-infiltrated subgroup was unexpected, and despite a higher infiltration by $CD8^+$ T cells than the Canonical and Metabolic subgroups, is associated with the worst prognosis. Along with this infiltration by $CD8^+$ T cells, this subgroup features extensive infiltration by fibroblastic and endothelial cells. Fibroblasts were already shown to support metastasis in this subgroup[363, 364], but our work suggest that they also directly favor angiogenesis which in turn promote recruitment of monocytic cells. These three cellular contingents express different complement molecules which together may permit the local activation of the complement cascade, further promoting inflammation and angiogenesis. The immune contexture found in this subgroup suggest that these patients may benefit from anti-inflammatory agents or CAR T cells adoptive transfers, the latter being specifically designed to overcome the type of immunosuppressive signals found in Mesenchymal tumors.

3.2.2 Article

Colorectal cancer of mesenchymal type shape an inflammatory, angiogenic and immunosuppressive microenvironment

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ABSTRACT

The tumor microenvironment contains many distinct and complexly-interacting cell populations, whose composition may predict prognosis and response to therapies. In the present work, using 1194 samples of purified cell populations, we defined specific and robust transcriptomic markers of the immune and stromal cell populations of the tumor microenvironment, and we quantitatively validated them in an *in-vitro* RNA mixture model. Colorectal cancer (CRC) is a heterogeneous disease in which distinct molecular subgroups have been described. We report that in three independent CRC cohorts (n=1388), CRC molecular subgroups and microenvironmental signatures are highly correlated. Out of the four molecular subgroups, two highly expressed immune-specific genes. The good-prognosis microsatellite-instable-enriched subgroup (CMS1) is characterized by overexpression of genes specific to cytotoxic lymphocytes. In contrast, the poor-prognosis Mesenchymal subgroup (CMS4) expresses markers of lymphocytes and of cells of monocytic origin. The Mesenchymal subgroup also displays an angiogenic, inflammatory and immunosuppressive signature, a coordinated pattern also observed in breast (n=254), ovarian (n=97), lung (n=80) and kidney (n=143) cancers. Pathological examination revealed that the Mesenchymal subtype was characterized by a high density of fibroblasts that likely produce the chemokines and cytokines which favor tumor-associated inflammation and support angiogenesis, resulting in a poor prognosis.

INTRODUCTION

The acquisition of genetic modifications during carcinogenesis results in altered cellular transcriptomic programs which lead to a variety of tumor cell phenotypes[(1)]. Transcriptomic analysis of large cohorts of human cancers enabled to propose classifications which partly correspond to genomic or clinically-established parameters and delineate previously unknown heterogeneity[(2)]. These stratifications allowed to identify molecular subgroups associated with distinct risks of disease progression[(3)].

Transcriptomic classifications of Colorectal Cancer (CRC) have recently been reported independently by six different laboratories[(4-9)]. An international consortium is proposing a Consensus Molecular Subtype (CMS) classification in four subgroups. CMS1 or MSI-like contains most Microsatellite Instable (MSI) tumors, with mutations in genes encoding DNA mismatch-repair proteins, resulting in high mutational burden. MSI-like is also enriched for tumors with a CpG-Island Methylator Phenotype (CIMP) and mutations in the BRAF oncogene. CMS2 or Canonical is a subtype with high Chromosomal Instability (CIN) as well as activation of the Wnt and MYC pathways. CMS3 or Metabolic is enriched in tumors with KRAS mutations shows a disruption of metabolic pathways. Finally, CMS4 or Mesenchymal has a mesenchymal phenotype and frequent CIMP phenotype.

This classification stratifies CRC into intrinsic subtypes with different prognosis and therapeutic responses. It has been well established that the composition of the microenvironment in which the malignant cells grow and expand is essential for predicting patient's prognosis[(10, 11)] and can be a target for cancer therapies[(12)]. Indeed, in most cancers, strong tumor infiltration of memory T cells with a Th1 orientation and potentially cytotoxic CD8+ T cells, in primary and in metastatic sites, correlates with longer patient's survival[(11)]. CRC represents a paradigm in this respect. Indeed, our

laboratory has demonstrated that patients whose tumors are highly infiltrated by memory T cells, particularly cytotoxic CD8+ T lymphocytes, had a better Progression-Free (PFS) and Overall Survival (OS)[10, 13-16]. We have hypothesized that tumor-associated antigens could locally induce anti-tumor adaptive immune responses and have characterized Tertiary Lymphoid Structures (TLS), adjacent to the tumor nests, that could be sites where anti-tumor immunity is generated[17]. Indeed, we found that high T and B cell infiltration and a high expression of genes coding for lymphocyte-attracting chemokines, i.e. CX3CL1, CXCL9, CXCL10 for T cells[15] and CXCL13 for B cells[16], as well as genes involved in a T_{h1} orientation (IFNG, TBX21) and cytotoxicity (GZMB, GNLY)[10], are associated with favorable prognosis[10, 14, 16]. MSI tumors, with their high mutational load and high leukocyte infiltration, fall perfectly in this category. It has recently been reported that metastatic CRC tumors with this phenotype responded to treatments with PD-1 immune checkpoint-blocking antibodies which increases the local immune reaction, potentially against tumor associated antigens[18, 19]. The Metabolic, KRAS-mutated subtype is known to be resistant to anti-EGFR antibodies[20]. Anti-angiogenic treatments yield inconsistent therapeutic responses, probably due to the lack of predictive markers.

In the era of targeted therapies, particularly immunotherapies which are dependent on the composition of the tumor microenvironment, it is essential to establish the immune landscape of all CRC tumors. Indeed, since a high immune infiltration is not restricted to MSI tumors, we undertook to precisely analyze and quantify the immune, inflammatory, angiogenic and fibroblastic elements in the different molecular subtypes, as well as the expression of functional chemokines, cytokines and inflammatory mediators in the tumors. For this purpose, we developed robust molecular signatures for the corresponding cells, based on 1114 transcriptomic profiles of pure immune cell populations, 36 profiles of endothelial cells and 50 profiles of fibroblasts. We validated these signatures both in *in-vitro* experiments, and by immunohistochemistry on a subset of molecularly classified tumors. In these two settings, we found a highly significant correlation between the expression of our gene signatures and the presence of the corresponding cell population. We applied these transcriptomic signatures to quantify immune and stromal infiltration of the four CMS subtypes of CRC, and discuss potential immunotherapeutic approaches which could benefit each subtype.

MATERIAL AND METHODS

Public transcriptomic datasets

The complete lists of selected Gene Expression Profiles (GEP), related type and experimental conditions are given in Tables S1, S2 and S3.

Microenvironment purified cells

We screened the GEO database[21] for GEP of purified samples of human immune cells, fibroblasts and endothelial cells hybridized on Affymetrix HG-U133Plus2.0 microarrays. We collected 1194 GEP from 80 series, including 1114 immune, 36 endothelial and 50 fibroblast samples.

Pan-cancers tumor cell lines

The Affymetrix HG-U133Plus2.0 GEP from the 917 (including 745 non-hematopoietic) tumor cell lines from the Cancer Cell Lines Encyclopedia[22] series were selected as tumor controls.

Colorectal tumors samples and subtypes annotations

The GEP from 1750 colorectal tumor samples were collected. The GSE39582 dataset(fresh frozen samples, Affymetrix HG-U133Plus2.0, n=566) was used as a discovery cohort (herein termed CIT discovery). Samples from series GSE13067 (n=74), GSE13294 (n=155), GSE17536 (n=177) and GSE33113 (n=90) were aggregated as a validation meta-serie (herein termed CIT validation) (fresh frozen samples, Affymetrix HG-U133Plus2.0, n=496). Samples from the PETACC3 (ArrayExpress:E-MTAB-990) serie (n=688, formalin-fixed, paraffin-embedded samples, custom Affymetrix microarrays) were used to validate the non-dependency of the results on microarray technology and sample processing. The CMS subtype annotation of all tumors analyzed was provided by the Colorectal Cancer Subtyping Consortium (CRCSC). CMS-unclassified samples reduced the numbers of samples analyzed to 458 for the CIT discovery cohort (81% classified), 404 for the CIT validation cohort (81% classified) and 526 for the PETACC3 cohort (76% classified). The total number of CRC tumors analyzed was therefore 1388.

Multi-cancers dataset

The GEP of breast (n=254), colorectal (n=173), kidney (n=144), ovarian (n=97), lung (n=80) and endometrial (n=69) were retrieved from expO dataset (GEO:GSE2109).

RNA mixture models

Peripheral immune cells sorting

Peripheral venous blood was extracted for 3 healthy donor using heparin vacuntainer tubes (BD Bioscience). Peripheral blood mononuclear (PBMC) or polymorphonuclear cells (PMN) were isolated using Ficoll-Paque PLUS (GE Healthcare Life Sience) or Polymorph Prep (Axis-Shield) density gradient, respectively. PBMCs were stained with anti-CD3 FITC (Clone UCHT1), anti-CD14 APC (MΦP9), anti-CD19 ECD (J3-119) and anti-CD56 PE (B159); and PMNs with anti-CD66b FITC (G10F5), anti-CD19 ECD (J3-119), anti-CD3 PE (UCHT1), anti-CD56 PE (B159) and anti-CD14 APC (MΦP9). Cell sorting was done in a FACS Aria cytometer (BD Bioscience), and cell purity higher than 97% was always achieved. We sorted the following populations: T cells (DAPI-/CD3+/CD14-/CD19-/CD56-), monocytes (DAPI-/CD3-/CD14+/CD19-/CD56-), B cells (DAPI-/CD3-/CD14-/CD19+/CD56-) and NK cells (DAPI-/CD3-/CD14-/CD19-/CD56+) on PBMCs, and neutrophils (DAPI-/CD66b+/CD19-/CD3-/CD56-/CD14-) on PMNs.

Cell culture

HCT116 were purchased from ATCC and cultured according to vendor's instructions.

RNA extraction

Cells were lysed in RLT (QIAGEN)-1% mercaptoethanol buffer, and RNA was purified with a Maxwell 16 simplyRNA Kit (Promega) according to manufacturer's instructions. Genetic material purity and quantity was determined with a 2100 Bioanalyzer Instrument (Agilent Technologies).

Immunohistochemistry

Serial 5 µm formalin-fixed paraffin-embedded (FFPE) tissue sections from colorectal cancer were stained using autostainerPlus Link 48 (Dako). Antigen retrieval and deparaffinization were carried out on a PT-Link (Dako) using the EnVision FLEX Target Retrieval Solutions (Dako). The antibodies used are listed in Table S4. Peroxidase activity was detected using diaminobenzidine substrate (Dako). Slides stained with anti-CD8 and anti-CD68 were digitalized with a NanoZoomer scanner (Hamamatsu) and analyzed with Calopix software (Tribvn, France). The degree of Smooth Muscle Actin expression in the tumor stroma was quantified following the next grading system: (1) scarce fibroblasts; (2) 1-3 layers of fibroblast; (3) >3 layers of fibroblasts and fibroblast area <50% of tumor area; (4) >3 layers of fibroblasts and fibroblast area >50% of tumor area.

Microarrays hybridization

Biotinylated double strand cDNA targets were prepared from 10 ng of total RNA using the NuGEN Ovation Pico WTA System V2 Kit (Cat # 3302) followed by the NuGEN Encore Biotin Module Kit (Cat # 4200) according to manufacturer recommendations. Following fragmentation and labeling, 4.55 µg of cDNAs were hybridized for 16 hours at 45°C, 60 rpm on Human GeneChip HG-U133 plus 2.0 arrays (Affymetrix). The chips were washed and stained in the GeneChip Fluidics Station 450 (Affymetrix) using the FS450_0004 script and scanned with the GeneChip Scanner 3000 7G (Affymetrix) at a resolution of 1.56 µm. Raw data (.CEL Intensity files) were extracted from the scanned images using the Affymetrix GeneChip Command Console (AGCC) version 4.0.

Data deposition

The data produced for validation of the immune signatures have been deposited in NCBI's Gene Expression Omnibus[21] and are accessible through GEO Series accession number GSE64385.

Microarrays analysis

GEP normalization

The GEP from microenvironment purified cells and pan-cancers cell lines were normalized independently for each series, using the frozen RMA method on each independent series (fRMA R package). The RMA normalized GEP from the CIT CRC discovery series were downloaded directly from GEO. The GEP from PETACC3 CRC series were normalized in batch using RMA method (affy R package). Each GEP series from the CIT CRC validation meta-series was normalized independently using frozen RMA method; then the corresponding matrices were combined into one matrix, further

normalized with Combat method[23], using series' identifiers as batch variables and no covariates. The GEP from RNA mixture models were normalized using the RMA method. When mapping probesets to HUGO Gene Symbols, the mean across probesets was chosen to represent the gene's expression level.

Supervised screening of microenvironment populations specific markers

Samples of microenvironment purified cells were labeled according to their reported immune or stromal populations, resulting in 43 distinct classes. These populations can be organized in a pyramidal graph (Fig S1) with nodes representing populations (classes), and directed edges representing relations of inclusion. For instance, the categories "CD8+ T cells", "NK cells" and "Tγδ cells" form the "Cytotoxic lymphocytes" category, which itself is included in the "T/NK lineage" category. Of these 43 classes of samples, some correspond to terminal leaves of this pyramid (ex. "regulatory T cells"), while some others do correspond to higher level nodes (ex. Peripheral-Blood Mononuclear Cells, "PBMC"). We designed 36 meta-categories from these 43 categories (Table 1, Table S5). Some of these 43 classes were either too general (ex. "Hematopoietic cells"), or represented by too few samples/series (ex. "Eosinophils"), to establish a robust and specific signature. Thus, in this pyramid, we selected 9 immunologically and 2 stromal relevant categories (Table 1, Fig S1), with enough representative samples available, and retained specific markers only for these 11 categories.

When screening markers of a category, denoted C, (ex. CD8+ T cells), it was compared to a "negative" category, denoted $\bar{C}$, containing all other samples, excepted those with a content overlapping that of category C (Table S5) (ex. samples of CD3+ T cells were excluded when screening for the Cytotoxic-lymphocytes class as they contain CD8+ CD3+ T cells mixed with CD4+ CD3+ T cells). To select probeset markers, the selection criteria were based on a triplet of probeset-level statistics, the positive Area Under the Curve (AUC), the fold-change (FC) and a specific fold-change (sFC), with the following definitions:

(1) $FC = X - \overline{X}$

(2) $sFC = (X - \overline{X}_{min})/(\overline{X}_{max} - \overline{X}_{min})$

where we denote by X the centroid (i.e. average across all samples) of category C , $\overline{X}$ the centroid of $\bar{C}$, $\overline{X}_j$ the centroid of any class j composing $\bar{C}$ (j=1..k) , $\overline{X}_{min}$ the min value across centroids of classes composing $\bar{C}$ ($\overline{X}_{min} = \min_{j \in 1..k} \{\overline{X}_j\}$), $\overline{X}_{max}$ the max value across centroids of classes composing $\bar{C}$ ($\overline{X}_{max} = \max_{j \in 1..k} \{\overline{X}_j\}$). The specific fold-change both accounts for a high expression in C compared to $\bar{C}$ and a low variability within $\bar{C}$.

For each of the 11 categories of interest, probesets with AUC > 0.97, Fold-Change >2 and FCspec >1.5 were retained (Table S6). In the rare cases of probesets selected in multiple categories, they were removed from both categories. When aggregating probesets to gene symbols, symbols selected in multiple categories were removed from both categories.

Computation of single sample metagenes scores

Given a gene signature (i.e. set of specific markers) of a given category C, we computed a corresponding per-sample score, called hereafter a *metagene score*, using the mean expression of the genes from that signature, after row-centering each gene across all samples.

Supervised tests of differential expression

ANOVA tests were used to assess the dependency of genes or metagenes scores to the molecular subgroups classification. Student's t-tests were used to investigate differential expression of genes between subgroups or cell line phenotypes. To test for differential level of the metagene scores in a given molecular subgroup, Student's t-tests against the cohort's median metagene score were used. To test for differential level of metagenes between molecular subgroups, pairwise one-tailed t-tests with Bonferroni correction were used (Table S7).

List of immune-related genes

We curated a list of genes known to encode proteins with immunomodulatory functions (Table S8). Representatives of the chemokines, chemokine-receptors, interleukins, interleukins-receptors, TNF and TNF-receptors, growth factors, interferons and interferon-receptors, inhibitory receptors and their ligands, TLR and class I MHC gene families were included.

Results

1-Robust transcriptomic signatures of the tumor-microenvironment cell populations predict the proportions of related populations in controlled mixtures

We focused on three microenvironment cellular contingents: immune cells, endothelial cells and fibroblasts. Redundant and overlapping transcriptomic signatures of these populations and of their subdivisions have been published based on various statistical frames[24]. To define a set of non-redundant, non-overlapping signatures, based on a unified statistical frame, we collected publicly available transcriptomic profiles of tumor cells, immune cells, fibroblasts and endothelial cells from 80 independent series, leading to a set of 1194 samples, including 1114 immune samples (Table S1). The experimental design (including cell purification methods, culture conditions, tissue sources, hybridization batches) can introduce biases in the identification procedure of specific gene markers. Thus several independent series based on distinct experimental designs were analyzed for each cell population (Table 1), in order to select specific gene markers, robustly expressed independently of the experimental design.

Firstly we performed a Principal Component Analysis (PCA) of the genome-wide normalized transcriptomic profiles of the immune samples (n=1114), colorectal cancer cell lines (n=55), and stromal (fibroblasts and endothelial cells) samples (n=86), in order to assess in a non-supervised way whether or not these profiles would show distinct patterns across distinct populations of cells. PCA showed a clear separation between non-hematopoietic (cancer cell lines, fibroblasts and endothelial cells) and hematopoietic cells (Fig 1A). Among the latter, PCA showed distinct clusters for lymphoid cells, cells of monocytic origin, and granulocytes (Fig 1A). To further study the immune populations, PCA was applied separately to lymphoid, monocytic and granulocytic subsets. Among lymphocytes, the analysis allowed to discriminate between T and NK cells on the one hand and B cells on the other hand (Fig 1B). PCA also separated monocytes from macrophages and myeloid dendritic cells (DC) within the monocyte-derived lineage (Fig 1C). Finally, PCA did not clearly discriminate between granulocytic populations (Fig 1D). PCA could not separate cytotoxic from non-cytotoxic lymphocytes (Fig S2A), nor CD4+ T cells from CD8+ T cells and NK cells (Fig S2B).

Since these distinct patterns were reminiscent of splits of the hematopoietic tree, we created 36 biologically relevant sets of samples corresponding to immune or stromal cell populations (Table 1, Table S5). A supervised analysis using stringent statistical criteria (Material and Methods) was conducted to retain only genes with high specificity for the corresponding cell populations (Table 1). Since some signatures were highly redundant ("B cells lineage" and "B cells"), while others featured a suspiciously high number of markers ("Eosinophils", "Th17") or simply no markers, we retained 11 signatures out of the 36 for further analysis. It resulted in the selection of 779 probesets mapping to 456 unique gene symbols. A heatmap representing the level of expression of the selected markers across the different cell populations is illustrated in Fig 1E and the precise signatures are reported in Table S6. To validate our approach, we designed an artificial mixture model composed of RNA extracted from five circulating immune cell populations (CD3+ T cells, CD56+ NK cells, CD19+ B cells, CD66b+ Neutrophils, CD14+ Monocytes) mixed with RNA extracted from the HCT116 colorectal cancer cell line, and hybridized on Affymetrix HG-U133Plus2.0 microarrays. Mixture ratios were ordered in two transposed Latin squares (Table S9) to assess the specificity of the tested signatures to one and only one cell population. The metagene scores derived from these signatures showed a linear relation to the mixture log-proportions, meaning that they were able to almost perfectly predict the mixture proportions (Fig 1F).

For these 11 gene signatures, we analyzed the corresponding GEP in three CRC cohorts, the "CIT cohort"[7], the "CIT validation cohort"[7] and PETACC3[9], and the expO pan-cancer cohort (Table S2). Within all signatures, except the Granulocytes, a reproducible cluster of highly correlated markers was found, supporting that their expression is representative of the corresponding population proportion (Fig S3). The markers of granulocytes showed poor pairwise correlations (Fig S3), indicating that they may infiltrate tumors in too low numbers to be accurately measured by microarray technologies. The high correlation of the Monocytic-lineage and the Myeloid-lineage metagenes' scores (r=0.89, 0.86, 0.80 and 0.71 respectively on CIT, CIT validation, expO and PETACC3 cohorts) compared to the lower correlation of the Myeloid and Granulocyte metagenes' scores (r=0.38, 0.45, 0.34 and 0.12), suggested that infiltrating granulocytes had a low impact on the overall Myeloid signature expression. These results are consistent with a previous report showing that granulocytes are rarer than cells of monocytic origin in CRC[16].

2-CRC molecular subgroups show distinct expression patterns of immune and stromal signatures

In the CIT and CIT validation cohorts, the molecular subgroups showed consistent and distinct patterns for the 11 signatures. Tumors of the MSI-like and Mesenchymal subtypes had a high expression of lymphoid (Fig 2A,B) as well as myeloid cells-specific genes (Fig 2A,C), thus exhibiting a strong immune and inflammatory contexture, whereas tumors of the Canonical and Metabolic subtypes had low expression of the lymphocytic and myeloid signatures. Tumors of the MSI-like and Mesenchymal subtypes differed in that MSI-like samples exhibited a higher cytotoxic-cells signature expression, reflecting high infiltration by activated CD8+ and NK cells. Granulocyte-specific transcripts were poorly discriminative (Fig 2A,C). In addition, Mesenchymal samples exhibited a high expression of the fibroblastic and endothelial cell-signatures, compatible with highly vascularized and inflammatory tumors that have a strong presence of cancer-associated fibroblasts (CAF) in their microenvironments (Fig 2A,D).

The immune infiltrations in the four subtypes predicted by our gene signatures were confirmed using immunohistochemical analyses in a subset of 38 randomly selected tumors from the CIT discovery cohort. CD8+ T cells and CD68+ macrophages were quantified within the tumor center. These analyses showed a significant correlation between the density of CD8+ cells in the tumor and the cytotoxic metagene from transcriptomic analyses ($p=2.10^{-5}$, r=0.67) and between CD68+ macrophages and the monocytic-lineage metagene ($p=1.10^{-5}$, r=0.68) (Fig S4). We confirmed that the MSI-like and the Mesenchymal-like subgroups had higher densities of CD8 T cells and CD68 macrophages than the Canonical and Metabolic subtypes, validating the transcriptomic predictions (Fig 3A, Fig 3B). In addition, we performed smooth-muscle actin (SMA) immunohistochemical labeling, which marks the tumor stroma. The Mesenchymal subtype had the highest SMA grading, supporting the fact that the transcriptomic fibroblastic signature was reflecting a high presence of CAF (Fig 3C, 3D).

Having analyzed patterns related to microenvironment cell populations, we focused on features related to immune cells function and migration. We thus analyzed the expression of genes encoding molecules involved in T cell chemotaxis, activation and inhibition, inflammation and complement components, angiogenesis as well as major histocompatibility complex 1 (MHC1) molecules (Fig 4, Table S8). The 4 consensus molecular subgroups again showed strikingly reproducible data across the 2 independent cohorts. The MSI-like subtype exhibited a high expression of genes coding for chemokines T cells (CXCL9[15], CXCL10[15], CXCL16) or involved in the formation of tumor-adjacent Tertiary Lymphoïd Structures CXCL13[25, 26]), as well as the Th1 cytokine IFNG and IL15, all of which have been shown to correlate with good prognosis in CRC[10, 14-16]. In contrast, the Mesenchymal subtype exhibited a high expression of the myeloid chemokine CCL2, complement components (C1QA, C1QB, C1QC, C1R, C1S, C3, C3AR1, C5AR1, C7, CFD, CFH, CFI), angiogenic factors (VEGFB, VEGFC and PDGFC) and immunosuppressive molecules (TGFB1, TGFB3, LGALS1[27], CXCL12). CD274 and PDCD1LG2, the genes encoding the PD-1 ligands, were highly expressed in MSI-like tumors but also in some tumors of the Mesenchymal group. Strikingly, MHC1 genes, whose products present peptides to CD8+ T cells, were poorly expressed in the poorly-infiltrated Canonical subtype.

We were able to reproduce these results on an independent cohort of 688 CRC samples, whose RNA was extracted from paraffin-embedded tissues and hybridized on another microarray platform, indicating strong reproducibility (Fig S5).

3-Mesenchymal tumor cells induce an inflammatory and angiogenic tumor microenvironment

The poor-prognostic C4 CRC subgroup is characterized by a fibroblastic signature, as well as a high expression of the myeloid and endothelial-cells metagenes. We found that the fibroblastic signature highly correlated with the endothelial one ($p<10^{-15}$ on the two cohorts, Pearson's r = 0.84, 0.84 for CIT and CIT validation respectively) and myeloid cells metagene ($p<10^{-15}$ on the two cohorts, pearson's r=0.6, 0.46 for CIT and CIT validation respectively) (Fig S6A). In contrast, there was no correlation between the fibroblastic and cytotoxic-cells signatures (Fig S6A). Correlations between the fibroblastic signature and both the endothelial and myeloid cell-signatures were also observed in breast, lung and ovary cancers, and confirmed in CRC (Fig S6B), suggesting that the immune contexture found in Mesenchymal CRC tumors also exist in these cancers. In kidney cancer the correlation between the fibroblast and the myeloid metagenes' scores was weaker, and it was absent in endometrium cancer (Fig S6B).

The coordination of these signatures led us to hypothesize that fibroblasts promote angiogenesis and inflammatory-cells recruitment in the Mesenchymal CRC tumors' microenvironment. Since tumor samples correspond to a mixture of tumor cells and microenvironment cells, transcriptomic samples of pure cell populations were used to investigate the cellular origin of the inflammatory and angiogenic signatures of the Mesenchymal molecular subgroup. We first identified the genes upregulated in the Mesenchymal subtype compared to each of the other subtypes (Student's T tests against each of the other three subtypes, all $p<0.05$, Table S8). We then investigated the expression of these genes by immune, stromal and malignant cells. B, T and NK lymphocytes, as well as colorectal cancer cell lines, each overexpressed only a small subset of these genes. Fibroblasts had the highest expression for the pro-angiogenic factors VEGFB, VEGFC and PDGFC, the immunosuppressive factors LGALS1, CXCL12, PTGS1, TGFB3 and the complement components C1S, C1R, CFH, C7, CFHR2 and can thus promote angiogenesis and immunosuppression. Endothelial cells had the highest expression of the myeloid chemoattractant CCL2, the angiogenic factor PDGFB and immunosuppressive molecules TGFB1 and TGFB2. Finally, monocytic cells expressed complement components (C1QA, C1QC, C3, C3AR1, C5AR1) and chemokines attracting macrophages (CCL19, CCL23). Altogether, these results show that fibroblasts can promote angiogenesis, which can promote the recruitment of cells of the monocytic lineage, which further promote the recruitment of macrophages. Endothelial cells and fibroblasts express immunosuppressive molecules specific to the Mesenchymal subtype. Finally, all three populations express complement components which, if activated, can locally fuel inflammation.

Discussion

In the last decade, the interplay between tumors and the immune system has emerged as a critical aspect of tumor biology and is strongly associated with the host ability to control tumor growth and to respond to therapies. Incorporating precise immune-related information in descriptive cancer-classification studies or in prospective clinical trials is therefore critical. However, no suitable tool was available to interpret transcriptomic immune signatures. In the present work, we define robust gene signatures to embrace the heterogeneity of the immune, inflammatory, angiogenic and fibroblastic tumor microenvironment and we apply this tool on a previously published molecular classification of colorectal cancer.

Transcriptomic immune signatures were previously published, but did not control for background expression by cancer cells[28], or did not address the question of the specificity to one and only one cell population of the selected markers[16], nor were they quantitatively validated. In an attempt to propose robust and quantitative immune gene signatures, data from 80 published datasets of stromal and hematopoietic cells comprising 1194 samples, controlled against 745 solid-cancer cell lines from 23 primary sites, were used to select robust markers, expressed in several conditions, and to reduce the selection of false positives arising from batch effects or high dimensionality. We collected samples representing the main cellular contingents of the microenvironment (tumor, immune and endothelial cells and fibroblasts) and are thus the major contributors of a tumor-sample's RNA. The expression of the specific markers almost perfectly recapitulated the proportion of their corresponding population in a mixture model.

The expression of the immune cell-specific signatures, enriched by the analysis of a large array of functionally relevant genes, in three CRC cohorts stratified using a previously published molecular classification revealed a strong association between the tumor cell phenotype and both the composition and the functional orientation of its immune microenvironment. Notably, we demonstrate that Mesenchymal tumors are associated with a pro-inflammatory, pro-angiogenic and immunosuppressive microenvironment.

In the three cohorts, two subgroups were characterized by high expression of immune signatures: the expected MSI-rich CMS1 group and the unexpected Mesenchymal CMS4 group. Strikingly, while the MSI-like group correlated with favorable patient's prognostic in terms of RFS[7], the Mesenchymal subgroup of patients had the worst prognosis in the CIT publication[7]. Consistently, the mesenchymal subgroup had the worst prognosis in other CRC[4-6, 8, 9] classification studies. We describe for the first time a group of CRC tumors with high lymphoid gene expression associated with poor prognosis for the patients. This subgroup is characterized by an extensive tumor-infiltration by Cancer Associated Fibroblats (CAF) (Fig 2, Fig 3), correlating with high angiogenesis and myeloid-cells infiltration (Fig 2, Fig S6A). We hypothesize that this strong inflammatory component hampers the positive value of the Th1/CD8+ T cells in these tumors, by repressing the anti-tumor activity of cytotoxic T cells while fueling tumor growth, angiogenesis and stroma remodeling.

Even more importantly, the fibroblastic signature, found in the Mesenchymal tumors extends to other cancers than CRC (Fig S6B). It is thus tempting to postulate that similar immune, inflammatory and immunosuppressive microenvironments might also be found in these tumors, indicating that similar therapies aimed at modifying the tumor-microenvironment could be applied to cohorts of cancers of different origins and locations exhibiting a Mesenchymal phenotype. In particular, anti-angiogenic treatments and/or inhibitors of LGALS1-encoded protein[29] should be tested in Mesenchymal CRC and the Mesenchymal-like tumors. The Mesenchymal subgroup also exhibit an angiogenic and inflammatory signature which is probably the consequence of their high fibroblastic infiltration. Angiogenesis and inflammation are intertwined pathways, which both fuel tumor growth through the production of survival and proliferative signals and by favoring blood supply[30]. Yet, since the Mesenchymal subtype is highly infiltrated by CD8+ T cells, one could expect it to be associated with favorable outcome[11]. However, an extensive number of studies have shown that inflammatory and angiogenic microenvironments were associated to the inhibition of anti-tumor cytotoxic T cell immune responses, notably through the inhibition of the maturation of dendritic cells[30]. Immature dendritic cells deliver inhibitory secondary signals to T cells upon antigen presentation, inhibiting their activation. Some immunotherapeutic strategies, such as T cells with a Chimeric Antigen Receptor (CAR), are specifically designed to bypass the need of a co-stimulatory signal upon antigen recognition[31], and could therefore mediate tumor elimination in this subgroup. Moreover, antibodies blocking the pro-inflammatory receptor IL6R were shown to reduce fibroblast-mediated angiogenesis in a mouse model of CRC[32], supporting the use of anti-inflammatory CAF-targeting agents in the treatment of Mesenchymal CRC.

MSI-like is the other "immune-high subgroup" of CRC. This group contains the patients harboring MSI tumors, and is known to be associated with a good prognosis, and to feature a strong CD8+ T cell infiltration. Strikingly, MSI-like is the group featuring the highest expression of class I MHC genes, as well as genes specific for cytotoxic lymphocytes (Fig 2, Table S7) or attracting memory T cells (CXCL9, CXCL10), activating T cells (IFNG), supporting proliferation of T and NK cells (IL15) and helping in the

formation of Tertiary Lymphoid Structures - TLS - (CXCL13) where anti-tumor adaptive immune responses are likely shaped[33](Fig 4, Table S8). High expression of these genes have been reported to be associated with good prognosis in CRC[10, 11, 15, 16]. CXCL13 and IL15 have been shown to be produced by the tumor cells, whereas IFNG is clearly produced by the infiltrating cells. MSI-like is also characterized by a lower expression of the myeloid and endothelial cells signatures (Fig 2) as well as angiogenesis inducing genes (Fig 4). It is therefore likely that MSI-like contains highly immunogenic tumors, in the context of mild inflammation and angiogenesis, which results in the generation of anti-tumor adaptive immune responses educated in tumor-adjacent TLS[34]. Effector memory CD8 T cells[34] and B cells[35] would then control the growth and metastasis in this subgroup[36], as exemplified in NSCLC[37]. IFNG produced by infiltrating T cells is known to induce a phenomenon called "adaptive resistance" by increasing the expression of the inhibitory checkpoint molecule PD-1 on T cells[38] and of its ligands CD274 (PD-L1)[38] and PDCD1LG2 (PD-L2)[39] on the tumor cells, which may result in inefficient anti-tumor T cell reaction[40]. It is striking that MSI-like tumors also shows the highest expression of PD-L1 and PD-L2 genes, followed by Mesenchymal tumors (Fig 3, Table S8). These results prompt to treat CRC MSI-like patients with agents blocking the PD-1/PD-L1 pathway, such as Nivolumab and anti-PDL1. Recent evidence using in-situ immunohistochemical staining of immune checkpoints molecules support the use of anti-checkpoint immunotherapies in MSI patients[41]. Since MSI-like is highly enriched for MSI patients but also includes a group of MicroSatellite Stable (MSS) patients[7], the use of molecular classifications might help identify responders among MSS patients, and non-responders among MSI patients.

Tumors of the Canonical and Metabolic subgroups were characterized by poor infiltration by immune cells and low class I MHC expression, and are thus most likely poorly immunogenic. They could represent targets for bi-specific antibodies targeting a tumor-associated antigens[42], which promote tumor-targeting adaptive immune responses. Mutated KRAS, which is highly prevalent in the Metabolic subgroup, can represent such a target.

Since the transcriptomic classification of CRC is strongly associated to different immune contextures, the present work paves the way of novel classifications of cancers, based on the relationships between the phenotype of the cancer cell and the corresponding immune and stromal profile of its microenvironment, potentially identifying the most appropriate treatments, including anti-angiogenic drugs and immunotherapies.

Our results have important implications in the understanding of tumor immunology, as they demonstrate how the phenotype of the cancer cell is associated with immune and stromal patterns. Furthermore, they provide potential clinical implications since they allow the identification of immunological, angiogenic and inflammatory targets that may be modulated by appropriate immunotherapies in each CRC molecular subgroup, such as anti-checkpoint antibodies or anti-inflammatory agents.

References

1. Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, L.A.J. & Kinzler, K.W. Cancer genome landscapes. Science 339, 1546-58(2013).

2. Rhodes, D.R. & Chinnaiyan, A.M. Integrative analysis of the cancer transcriptome. Nat. Genet. 37 Suppl, S31-7(2005).

3. Yuan, Y., Van Allen, E.M., Omberg, L., Wagle, N., Amin-Mansour, A., Sokolov, A. et al. Assessing the clinical utility of cancer genomic and proteomic data across tumor types. Nat. Biotechnol. 32, 644-52(2014).

4. Schlicker, A., Beran, G., Chresta, C.M., McWalter, G., Pritchard, A., Weston, S. et al. Subtypes of primary colorectal tumors correlate with response to targeted treatment in colorectal cell lines. BMC Med Genomics 5, 66(2012).

5. Sadanandam, A., Lyssiotis, C.A., Homicsko, K., Collisson, E.A., Gibb, W.J., Wullschleger, S. et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nat. Med. 19, 619-25(2013).

6. Roepman, P., Schlicker, A., Tabernero, J., Majewski, I., Tian, S., Moreno, V. et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. Int. J. Cancer 134, 552-62(2014).

7. Marisa, L., de Reyniès, A., Duval, A., Selves, J., Gaub, M.P., Vescovo, L. et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. PLoS Med. 10, e1001453(2013).

8. De Sousa E Melo, F., Wang, X., Jansen, M., Fessler, E., Trinh, A., de Rooij, L.P.M.H. et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat. Med. 19, 614-8(2013).

9. Budinska, E., Popovici, V., Tejpar, S., D'Ario, G., Lapique, N., Sikora, K.O. et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. J. Pathol. 231, 63-76(2013).

10. Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pagès, C. et al. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. Science 313, 1960-1964(2006).

11. Fridman, W., Pagès, F., Sautès-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer 12, 298-306(2012).

12. Chen, D.S. & Mellman, I. Oncology meets immunology: the cancer-immunity cycle. Immunity 39, 1-10(2013).

13. Pagès, F., Berger, A., Camus, M., Sanchez-Cabo, F., Costes, A., Molidor, R. et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N. Engl. J. Med. 353, 2654-66(2005).

14. Mlecnik, B., Bindea, G., Angell, H.K., Sasso, M.S., Obenauf, A.C., Fredriksen, T. et al. Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. Sci Transl Med 6, 228ra37(2014).

15. Mlecnik, B., Tosolini, M., Charoentong, P., Kirilovsky, A., Bindea, G., Berger, A. et al. Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. Gastroenterology 138, 1429-40(2010).

16. Bindea, G., Mlecnik, B., Tosolini, M., Kirilovsky, A., Waldner, M., Obenauf, A.C. et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity 39, 782-95(2013).

17. Dieu-Nosjean, M., Goc, J., Giraldo, N.A., Sautès-Fridman, C. & Fridman, W.H. Tertiary lymphoid structures in cancer and beyond. Trends Immunol. 35, 571-80(2014).

18. Xiao, Y. & Freeman, G.J. The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy. Cancer Discov 5, 16-8(2015).

19. Llosa, N.J., Cruise, M., Tam, A., Wicks, E.C., Hechenbleikner, E.M., Taube, J.M. et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov 5, 43-51(2015).

20. Lièvre, A., Bachet, J., Le Corre, D., Boige, V., Landi, B., Emile, J. et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 66, 3992-5(2006).

21. Edgar, R., Domrachev, M. & Lash, A.E. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30, 207-10(2002).

22. Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A.A., Kim, S. et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483, 603-7(2012).

23. Johnson, W.E., Li, C. & Rabinovic, A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8, 118-27(2007).

24. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U.S.A. 102, 15545-50(2005).

25. de Chaisemartin, L., Goc, J., Damotte, D., Validire, P., Magdeleinat, P., Alifano, M. et al. Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer. Cancer Res. 71, 6391-9(2011).

26. Gu-Trantien, C., Loi, S., Garaud, S., Equeter, C., Libin, M., de Wind, A. et al. $CD4^+$ follicular helper T cell infiltration predicts breast cancer survival. J. Clin. Invest. 123, 2873-92(2013).

27. Soldati, R., Berger, E., Zenclussen, A.C., Jorch, G., Lode, H.N., Salatino, M. et al. Neuroblastoma triggers an immunoevasive program involving galectin-1-dependent modulation of T cell and dendritic cell compartments. Int. J. Cancer 131, 1131-41(2012).

28. Abbas, A.R., Baldwin, D., Ma, Y., Ouyang, W., Gurney, A., Martin, F. et al. Immune response in silico (IRIS): immune-specific genes identified from a compendium of microarray expression data. Genes Immun. 6, 319-31(2005).

29. Astorgues-Xerri, L., Riveiro, M.E., Tijeras-Raballand, A., Serova, M., Neuzillet, C., Albert, S. et al. Unraveling galectin-1 as a novel therapeutic target for cancer. Cancer Treat. Rev. 40, 307-19(2014).

30. Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation. Nature 454, 436-444(2008).

31. Maher, J., Brentjens, R.J., Gunset, G., Rivière, I. & Sadelain, M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. Nat. Biotechnol. 20, 70-5(2002).

32. Nagasaki, T., Hara, M., Nakanishi, H., Takahashi, H., Sato, M. & Takeyama, H. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. Br. J. Cancer 110, 469-78(2014).

33. Dieu-Nosjean, M., Goc, J., Giraldo, N.A., SautÃ¨s-Fridman, C. & Fridman, W.H. Tertiary lymphoid structures in cancer and beyond. Trends in Immunology 35, 571-580(2014).

34. Camus, M., Tosolini, M., Mlecnik, B., Pagès, F., Kirilovsky, A., Berger, A. et al. Coordination of intratumoral immune reaction and human colorectal cancer recurrence. Cancer Res. 69, 2685-93(2009).

35. Germain, C., Gnjatic, S., Tamzalit, F., Knockaert, S., Remark, R., Goc, J. et al. Presence of B cells in tertiary lymphoid structures is associated with a protective immunity in patients with lung cancer. Am. J. Respir. Crit. Care Med. 189, 832-44(2014).

36. Becht, E., Goc, J., Germain, C., Giraldo, N.A., Dieu-Nosjean, M., Sautès-Fridman, C. et al. Shaping of an effective immune microenvironment to and by cancer cells. Cancer Immunol. Immunother. 63, 991-7(2014).

37. Goc, J., Germain, C., Vo-Bourgais, T.K.D., Lupo, A., Klein, C., Knockaert, S. et al. Dendritic cells in tumor-associated tertiary lymphoid structures signal a Th1 cytotoxic immune contexture and license the positive prognostic value of infiltrating CD8+ T cells. Cancer Res. 74, 705-15(2014).

38. Taube, J.M., Anders, R.A., Young, G.D., Xu, H., Sharma, R., McMiller, T.L. et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 4, 127ra37(2012).

39. Latchman, Y., Wood, C.R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I. et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat. Immunol. 2, 261-8(2001).

40. Francisco, L.M., Sage, P.T. & Sharpe, A.H. The PD-1 pathway in tolerance and autoimmunity. Immunol. Rev. 236, 219-42(2010).

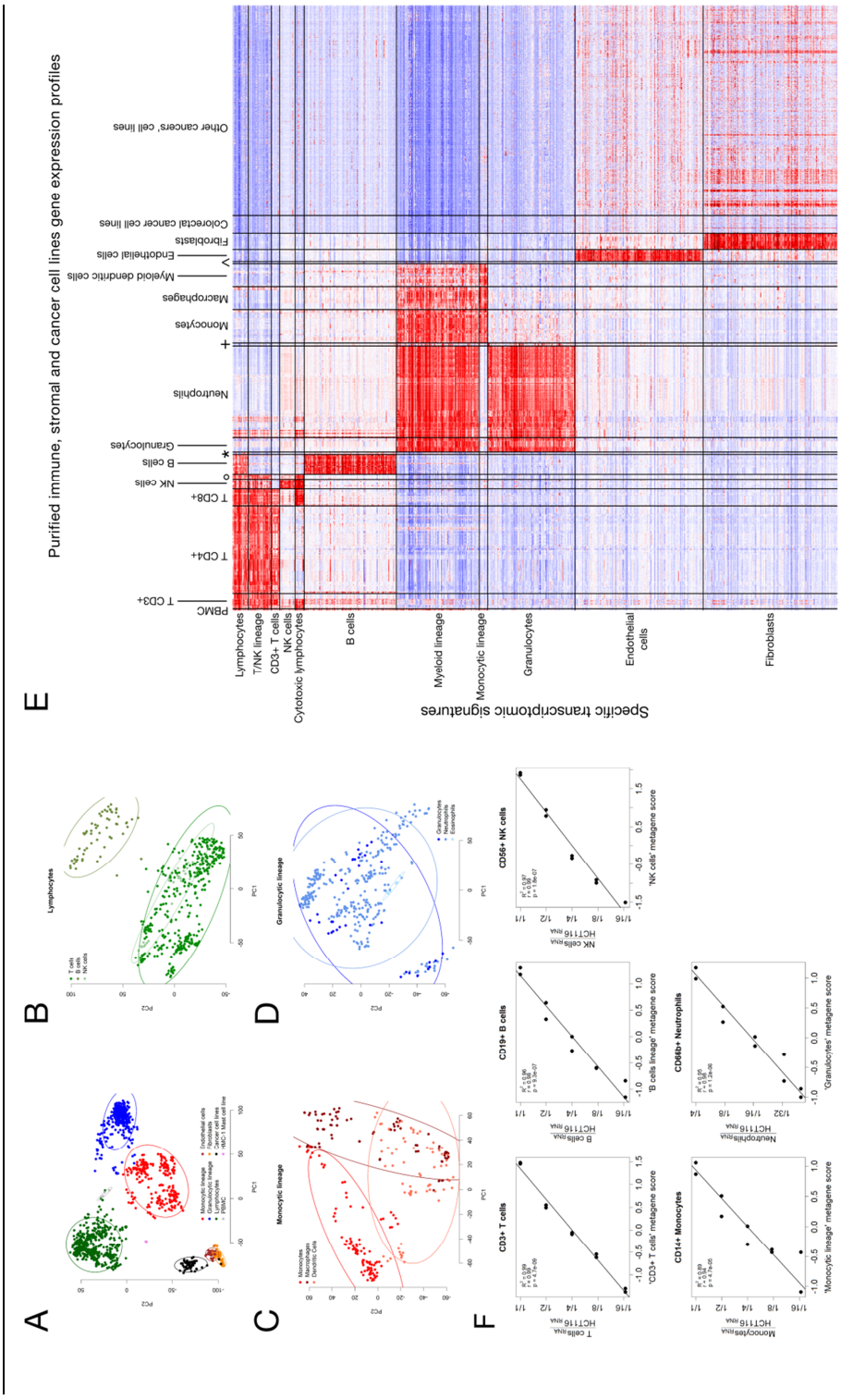
41. Llosa, N.J., Cruise, M., Tam, A., Wick, E.C., Hechenbleikner, E.M., Taube, J.M. et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov , (2014).

42. Bargou, R., Leo, E., Zugmaier, G., Klinger, M., Goebeler, M., Knop, S. et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science 321, 974-7(2008).

Financial support: This work was supported by the 'Institut National de la Santé et de la Recherche Médicale', the University Paris-Descartes, the University Pierre et Marie Curie, the Institut National du Cancer (2011-1-PLBIO-06-INSERM 6-1), CARPEM (CAncer Research for PErsonalized Medicine), Labex Immuno-Oncology (LAXE62_9UMS872 FRIDMAN), the Fondation ARC pour la recherche sur le cancer, the Cancéropôle Ile-de-France, Institut National du Cancer (2011-1-PLBIO-06-INSERM 6-1,PLBIO09-088-IDF-KROEMER), the Universidad de los Andes School of Medicine (N.A.G.), Colciencias (NAG). EB is supported by B3MI doctorate fellowship and NAG by PPATH doctorate fellowship.

Acknowledgments: Authors wish to acknowledge members of the Centre d'Imagerie Cellulaire et de Cytométrie "CICC" plateform of the Cordeliers Research Center, and the "Plateforme Biopuces et Séquençage" of the IGBMC for their respective technical expertises. The efforts of Gene Expression Omnibus, arrayExpress, the expression project for Oncology and the International Genomics Consortium, and all the teams that shared their GEP results are greatly acknowledged. We thank Ivo Natario for his help with data collection. We thank Lubka Roumenina, Gabriela Bindea, Jerome Galon, Bernhard Mlecnik, Estelle Devevre, Audrey Lupo and Marie-Caroline Dieu-Nosjean for their fruitful discussions.

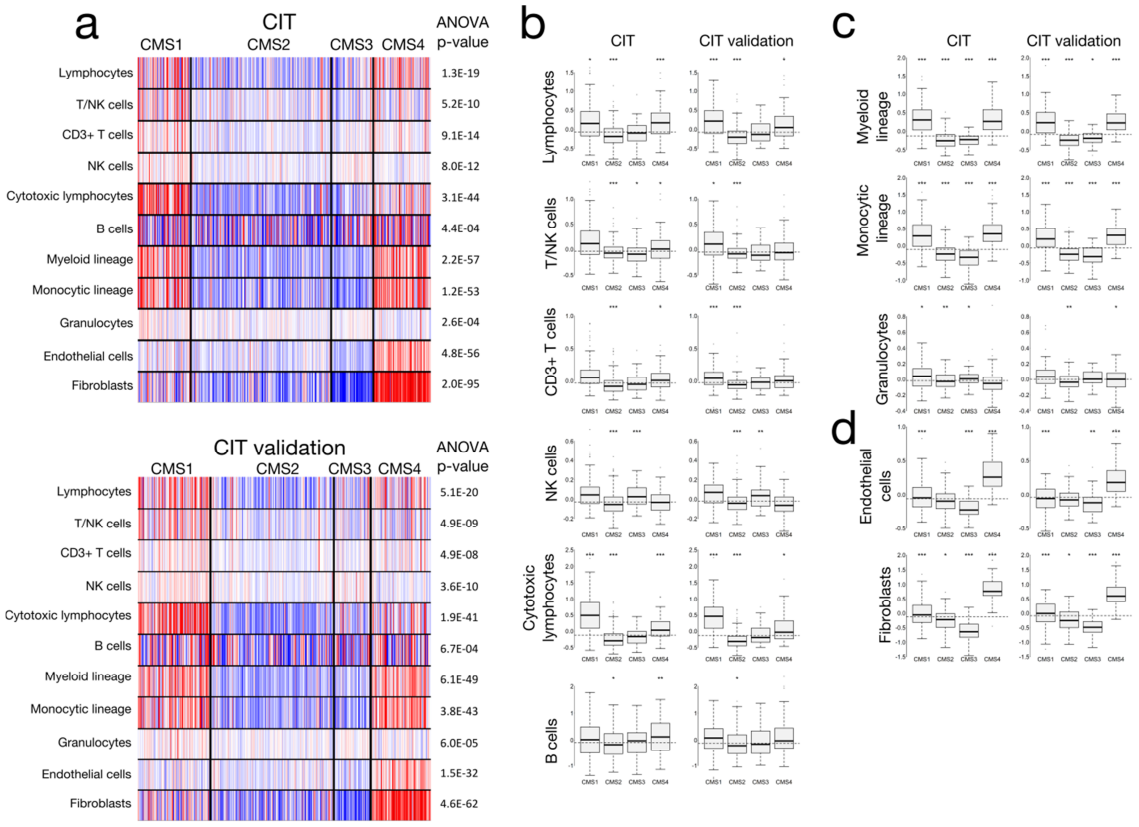
Fig 1


A
B
C
D
E
F
Purified immune, stromal and cancer cell lines gene expression profiles
Specific transcriptomic signatures
PBMC
T CD3+
T CD4+
T CD8+
NK cells
B cells
Granulocytes
Neutrophils
Monocytes
Macrophages
Myeloid dendritic cells
Endothelial cells
Fibroblasts
Colorectal cancer cell lines
'Other cancers' cell lines
Lymphocytes
T/NK lineage
CD3+ T cells
NK cells
Cytotoxic lymphocytes
B cells
Myeloid lineage
Monocytic lineage
Granulocytes
Endothelial cells
Fibroblasts
Lymphocytes
Monocytic lineage
Granulocytic lineage
CD3+ T cells
CD19+ B cells
CD56+ NK cells
CD14+ Monocytes
CD66b+ Neutrophils
'CD3+ T cells' metagene score
'B cells lineage' metagene score
'NK cells' metagene score
'Monocytic lineage' metagene score
'Granulocytes' metagene score

Definition and validation of specific transcriptomic signatures of immune and stromal cells

Principal Components Analysis (PCA) based on the 5% most variable probesets applied to **A)** 1114 immune, 50 fibroblast, 36 endothelial and 55 colon cancer cell lines samples **B)** the subset of lymphocytic samples, **C)** the subset of granulocytic samples, **D)** the subset of samples of monocytic origin. **E)** Heatmap showing the level of expression of the supervised specific signatures among immune cell subpopulations and non-hematopoietic samples. ° : γδ T cells. * : Plasmacytoid dendritic cells. + : Eosinophils. ^ : HMC-1 mast cell line. Rows were centered. Red denotes a higher expression and blue a lower expression. White denotes an average expression. **F)** Correlations between dilutions of mRNA extracted from purified immune cell populations mixed with mRNA extracted from cancer cell lines and the metagene score of the corresponding signature. The solid black line represents a least-square linear regression fit.

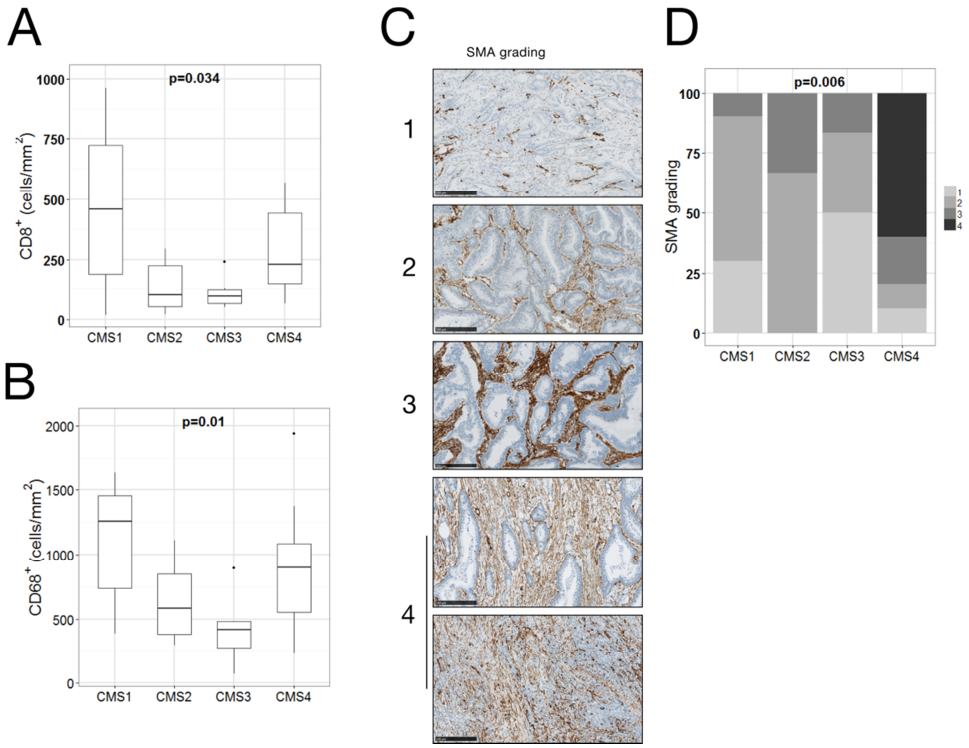
Fig 2



Immune and stromal signatures of the four molecular subgroups of CRC

A) Heatmap showing the level of the metagene scores of the immune and stromal signatures among 2 transcriptomic cohorts of CRC patients, that were classified in 4 molecularly-defined CRC subgroups. Distributions of the **B)** lymphocytic, **C)** myeloid, **D)** stromal metagene scores across subgroups in the 2 cohorts. * : p <0.05. ** : p<0.001. *** : p<0.0001 compared to the cohort's median using a Student's t-test.

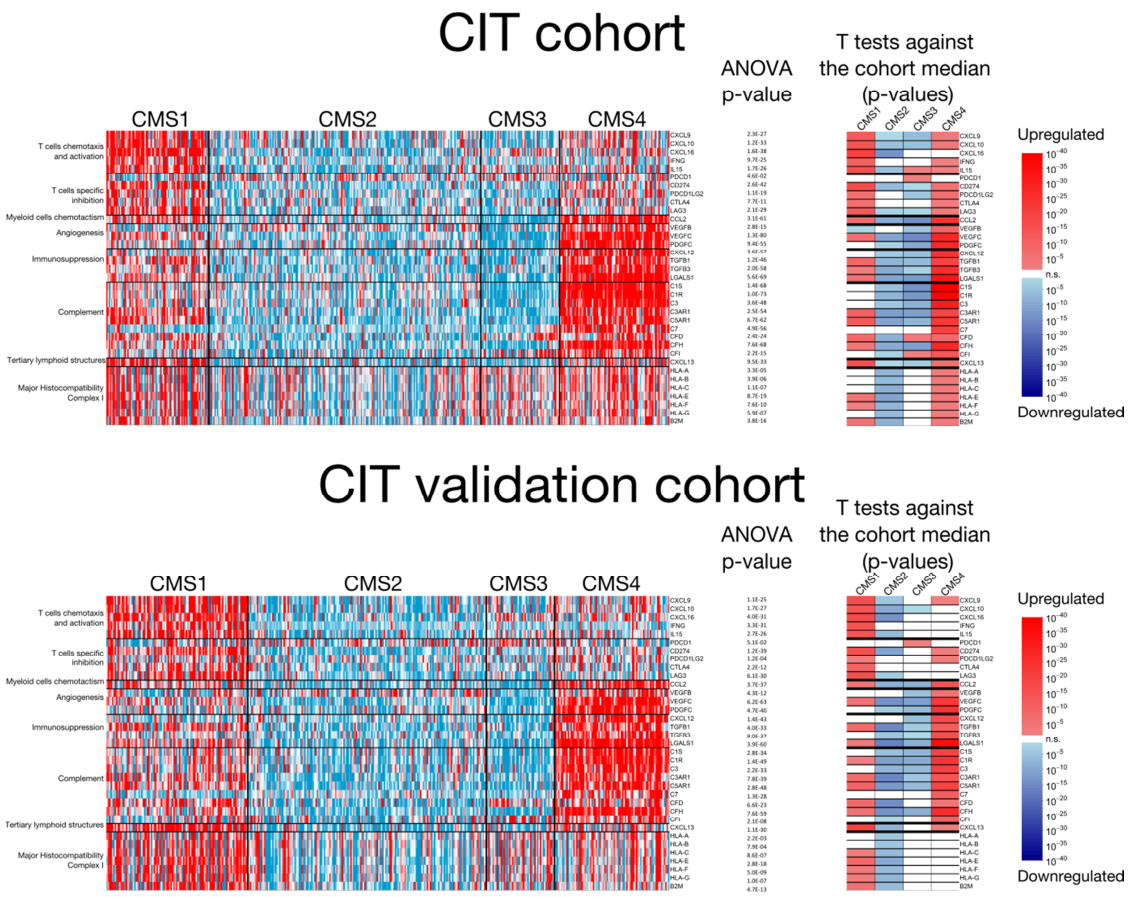
Fig 3



Immunohistochemical characterization of the four CRC subgroups

A) Distributions of the densities of tumor-infiltrating $CD8^+$ T cells in the four subgroups. **B)** Distributions of the densities of tumor-infiltrating $CD68^+$ macrophages in the four subgroups. P-values were assessed using the Kruskall-Wallis test. **C)** Representative tumor areas of each SMA grades. SMA-positive areas are labeled in brown. **D)** Distributions of each SMA grades in the four subgroup. P-value were assessed using Fisher's exact test.

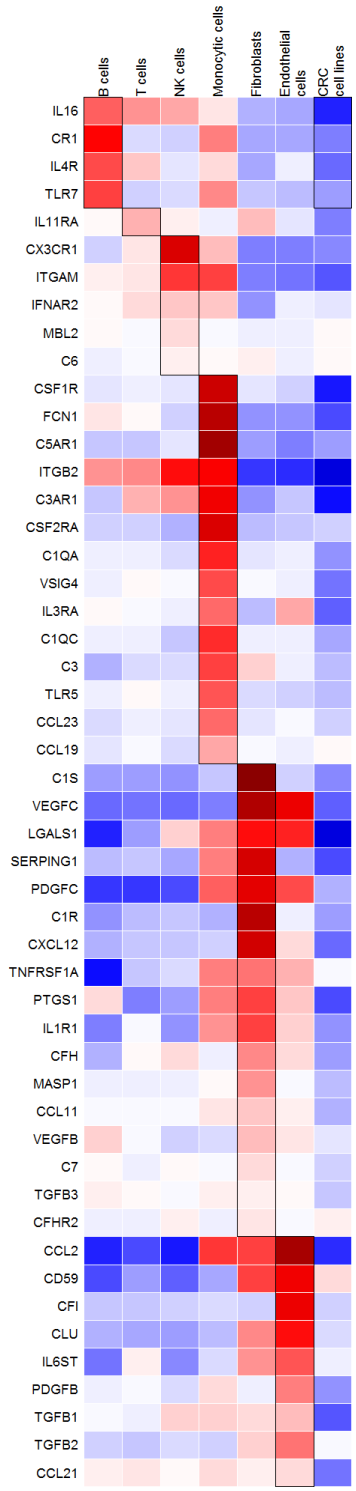
Fig 4



Expression of functionally relevant immune genes among the 4 subgroups in the two cohorts.

The heatmaps on the left represents the level of expression of the genes. Rows were centered and scaled. Red denotes a higher expression and blue a lower expression. The heatmaps on the right represents the p-value of a Student's t-test against the cohort median, for each gene.

Fig 5


B cells
T cells
NK cells
Monocytic cells
Fibroblasts
Endothelial cells
CRC cell lines
IL16
CR1
IL4R
TLR7
IL11RA
CX3CR1
ITGAM
IFNAR2
MBL2
C6
CSF1R
FCN1
C5AR1
ITGB2
C3AR1
CSF2RA
C1QA
VSIG4
IL3RA
C1QC
C3
TLR5
CCL23
CCL19
C1S
VEGFC
LGALS1
SERPING1
PDGFC
C1R
CXCL12
TNFRSF1A
PTGS1
IL1R1
CFH
MASP1
CCL11
VEGFB
C7
TGFB3
CFHR2
CCL2
CD59
CFI
CLU
IL6ST
PDGFB
TGFB1
TGFB2
CCL21

Inflammatory, angiogenic and suppressive molecules overexpressed in Mesenchymal tumors are highly expressed by fibroblastic, endothelial and monocytic cells

Expression of the genes specifically upregulated in Mesenchymal tumors and related to inflammation, angiogenesis, immunosuppression and immune cell functional orientations, in homogeneous samples of immune, stromal or colorectal cancer cell lines. Black frames indicate that the corresponding cell population has the highest expression of the gene.

Table 1

Signature	Number of independent datasets	Number of samples			Number of markers		
		positive class	negative class	ignored	Probesets	Mapping genes	Unmapped probesets
Non Hematopoietic	13	831	1104	4	119	83	6
Endothelial cells	7	36	1903	0	166	108	6
Lymphatics	3	18	1921	0	38	27	6
HUVECs	4	18	1921	0	20	14	0
Fibroblasts	5	50	1889	0	175	108	5
Cancer Cell Lines	1	745	1190	4	30	25	4
Hematopoietic cells	67	1104	831	4	358	232	29
Lymphoid lineage	37	497	1438	4	24	17	3
CD3+ T cells	29	400	1535	4	27	17	3
CD4+ T cells	22	349	1549	41	11	5	0
CD8+ T cells	20	282	1602	55	3	1	0
Tγδ	7	53	1831	55	2	1	0
Memory T cells	3	31	1560	348	1	1	0
Regulatory T cells	6	65	1667	207	4	3	1
Th1 cells	2	15	1713	211	0	0	0
Th17 cells	1	18	1710	211	121	93	19
Th2 cells	1	12	1720	207	2	2	0
B cells lineage	9	62	1873	4	119	38	55
B cells (excluding Plasma cells)	9	57	1878	4	49	20	12
Memory B cells	2	8	1886	45	11	5	1
Naive B cells	2	8	1886	45	10	4	6
Plasma cells	2	5	1930	4	75	30	36
NK cells	4	30	1905	4	20	11	5
T/NK lineage	31	430	1505	4	34	23	1
Myeloid lineage	33	603	1328	8	111	75	12
Monocytic lineage	24	253	1678	8	11	8	0
Granulocytes	14	350	1585	4	116	71	20
Neutrophils	10	299	1592	48	93	54	13
Eosinophils	2	7	1884	48	245	131	107
Myeloid dendritic cells	11	72	1863	4	2	2	0
Dendritic cells	11	77	1858	4	1	0	1
Monocytes	11	106	1829	4	6	5	1
Macrophages	6	75	1860	4	4	3	0
Cytotoxic lymphocytes	11	97	1787	55	12	7	3
B lineage or plasmacytoid dendritic cell	9	67	1868	4	123	41	58
Plasma cell or plasmacytoid dendritic cells	3	10	1925	4	12	7	3

The 36 meta-categories that were used in the supervised screen for marker selection.

Bold font and gray background highlight the 11 signatures selected for further analysis.

Supplementary Materials:

Fig S1

Pyramidal representation of the inclusion relationships between samples

Fig S2

Subsets of the T/NK lineage are not separable using Principal Component Analysis

Fig S3

Each signature, except the Granulocytic, includes a reproducible cluster of highly correlated genes

Fig S4

Results are reproducible on the independent PETACC3 CRC cohort (n=688)

Fig S5

Cytotoxic-lymphocytes and monocytic-lineage metagenes predict tumor infiltration by the corresponding cell populations

Fig S6

The fibroblast metagene score correlates with endothelial and myeloid cells metagene scores in CRC and other cancers

Table S1

Immune, and stromal samples used to define the immune metagenes

Table S2

Colorectal cancer samples constituting the 3 cohorts analyzed, along with their molecular subgroups

Table S3

Samples from the Cancer Cell Line Encyclopedia

Table S4

Antibodies used for immunohistochemical analyses

Table S5

Definition of the purified immune and stromal meta-categories according to the reported phenotype of the samples used for microarray analyzes

Table S6

Probesets selected to belong to the immune and stromal metagenes

Table S7

RNA ratios used in the RNA mixture models

Table S8

Molecular subgroup-level statistics on each signature's metagene score

Table S8

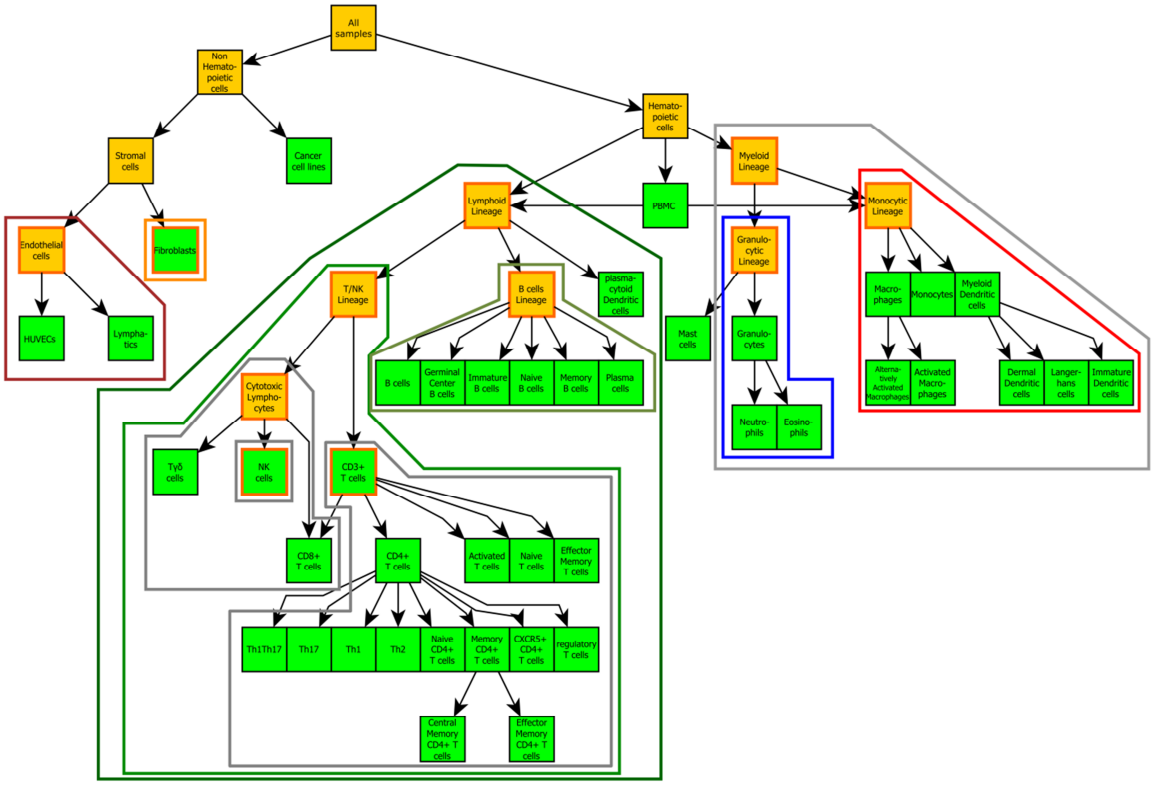
Levels of expression of genes related to inflammation, angiogenesis and immunomodulation in CRC cohorts

Supplementary Information

Colorectal cancer cells of mesenchymal type shape an inflammatory, angiogenic and immunosuppressive microenvironment

Authors: Etienne Becht, Aurélien de Reyniès, Nicolas A Giraldo, Camilla Pilati, Bénédicte Buttard, Laetitia Lacroix, Janick Selves, Catherine Sautès-Fridman, Pierre Laurent-Puig, and Wolf-Herman Fridman

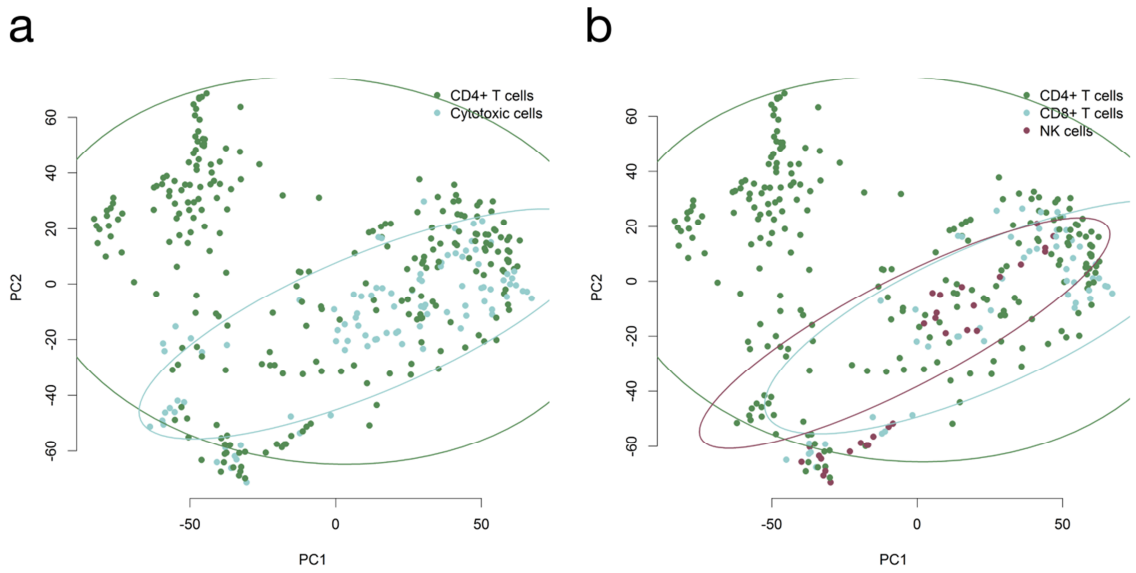
Fig S1



Pyramidal representation of the inclusion relationships between samples

A green background indicates that the class was collected as a transcriptomic sample. Yellow nodes enable organizing the samples into a pyramidal graph. Nodes or leaves with orange frames indicate that the signatures were selected in the set of 11 specific signatures. Frames encompassing several nodes and leaves indicate selected meta-categories, and the color corresponds to the one use in Fig1ABCD if applicable, and is gray otherwise.

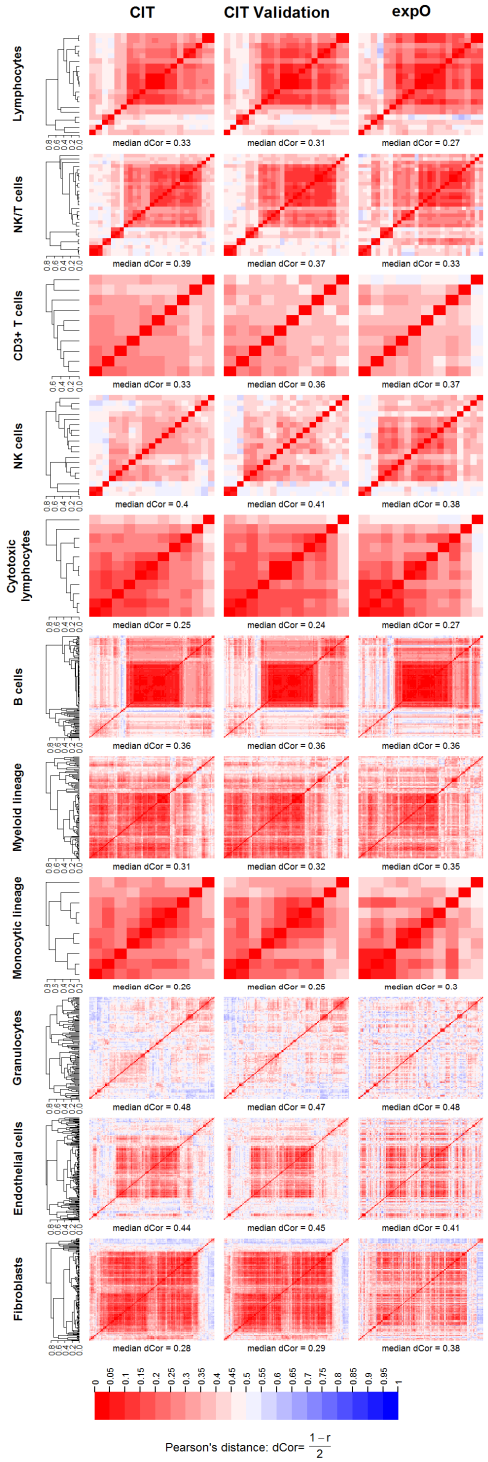
Fig S2



Subsets of the T/NK lineage are not separable using Principal Component Analysis

Principal Components Analysis (PCA) based on the 5% most variable probesets applied on **A)** CD4+ T cells and cytotoxic-lymphocytes **B)** CD4+ T cells, CD8+ T cells, NK cells.

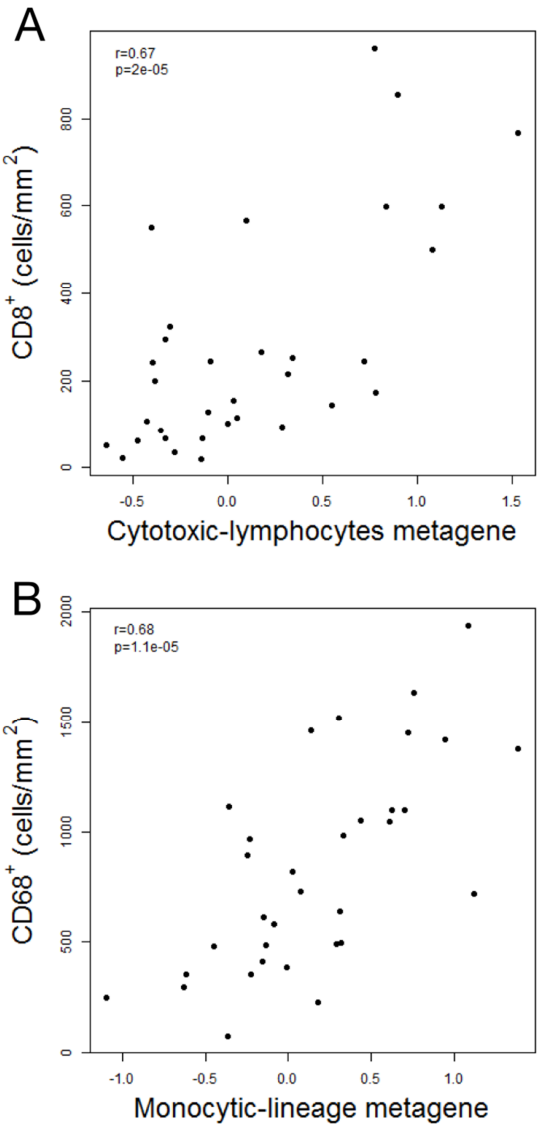
Fig S3


CIT
CIT Validation
expO
Lymphocytes
median dCor = 0.33
median dCor = 0.31
median dCor = 0.27
NK/T cells
median dCor = 0.39
median dCor = 0.37
median dCor = 0.33
CD3+ T cells
median dCor = 0.33
median dCor = 0.36
median dCor = 0.37
NK cells
median dCor = 0.4
median dCor = 0.41
median dCor = 0.38
Cytotoxic lymphocytes
median dCor = 0.25
median dCor = 0.24
median dCor = 0.27
B cells
median dCor = 0.36
median dCor = 0.36
median dCor = 0.36
Myeloid lineage
median dCor = 0.31
median dCor = 0.32
median dCor = 0.35
Monocytic lineage
median dCor = 0.26
median dCor = 0.25
median dCor = 0.3
Granulocytes
median dCor = 0.48
median dCor = 0.47
median dCor = 0.48
Endothelial cells
median dCor = 0.44
median dCor = 0.45
median dCor = 0.41
Fibroblasts
median dCor = 0.28
median dCor = 0.29
median dCor = 0.38
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5 0.55 0.6 0.65 0.7 0.75 0.8 0.85 0.9 0.95 1
Pearson's distance: dCor= 1 − r / 2

Each signature, except the Granulocytic, includes a reproducible cluster of highly correlated genes

For each signature, the Pearson's correlation distance matrix between probesets is shown in the CIT, CIT validation and expO cohorts. Probesets were clustered using hierarchical clustering with complete linkage and Pearson's distance on the CIT cohort (left panels). The same clustering order is displayed for the three cohorts. The color code is given at the bottom.

Fig S4

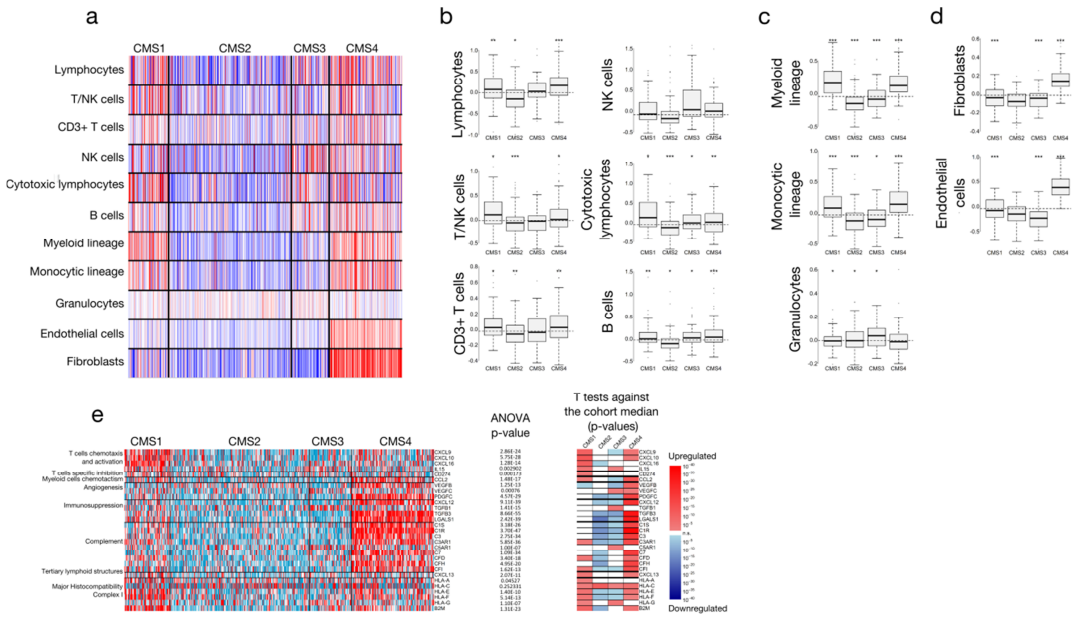


Cytotoxic-lymphocytes and monocytic-lineage metagenes predict tumor infiltration by the corresponding cell populations

A) Scatterplot representing the Cytotoxic-lymphocytes metagene expression and the corresponding quantification of $CD8^+$ T cells in 38 CRC tumors. **B)** Scatterplot representing the

Monocytic-lineage metagene expression and the corresponding quantification of $CD68^{+}$ Macrophages cells in 38 CRC tumors. P-values were assessed by Student's T tests; r corresponds to the Pearson's correlation coefficient.

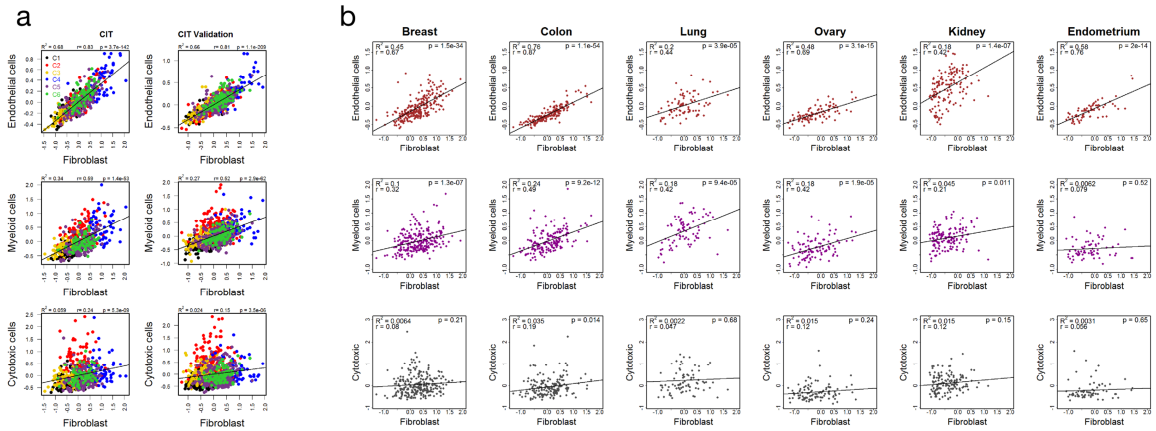
Fig S5



Results are reproducible on the independent PETACC3 CRC cohort (n=688).

A) Heatmap showing the level of the metagene scores of the immune and stromal signatures in the PETACC3 CRC transcriptomic cohort, that was classified according to the 6 molecularly-defined CRC subgroups. Distributions of the **B)** lymphocytic, **C)** myeloid **D)** and stromal metagenes' scores across subgroups in the 2 cohorts. * : p <0.05. ** : p<0.001. *** : p<0.0001 compared the the cohort's median using a Student's t-test. **E)** The heatmap on the left represents the level of expression of the genes. Rows were centered and scaled. Red denotes a higher expression and blue a lower expression. The heatmap on the right represent the p-value of a Student's t-test against the cohort median, for each gene.

Fig S6



The fibroblast metagene score correlates with endothelial and myeloid cells metagene scores in CRC and other cancers

Scatterplots representing the relationships between the endothelial cells, myeloid cells and cytotoxic cells metagenes scores compared to the fibroblast metagene score **A)** in the 2 CRC cohorts **B)** across 6 cancers, including CRC, in the expO dataset.

3.3 Article 3 : the immune contextures of CRC and ccRCC molecular subtypes

3.3.1 Summary of the results

This *in press* article draws a parallel between the analyzes of the immune contextures of ccRCC and CRC molecular subgroups. It updates our findings in ccRCC presented in **Article 1** by using the gene signatures presented in **Article 2**, which confirms our previously-published results.

This paper also reports immune contextures in the six transcriptomic subgroups reported by Marisa et al[239]. The correspondence between the six subgroups of Marisa and colleagues and the CMS is as follow:

CMS	MSI-like	Canonical	Metabolic	Mesenchymal
Marisa	C2	C1 C5 C6	C3	C4

The results obtained on this classification are consistent with the ones proposed in **Article 2**. In addition, it shows that the C6 'normal-like' subgroup proposed by Marisa et al.[239] but which has no direct equivalent in the CMS classification[282] is highly infiltrated by B cells, but not by myeloid cells, which suggests that this subgroup has a different immune contexture than C1 and C5, although these subgroups are merged in the CMS classification.

This short paper illustrates the strong association between ccRCC and CRC molecular subtypes, which prompts to perform similar studies in other malignancies in order to unify genomic and immune classifications.

3.3.2 Article

Title : Prognostic and theranostic impact of molecular subtypes and immune classifications in Renal Cell Cancer (RCC) and Colorectal Cancer (CRC)

Etienne Becht[1,2,3], Nicolas A Giraldo[1,2,3], Benoit Beuselinck[4,5], Sylvie Job[6], Laetitia Marisa[6], Yann Vano[1,2,3,7], Stéphane Oudard[2,7], Jessica Zucman-Rossi[4,7], Pierre Laurent-Puig[8,9], Catherine Sautès-Fridman[1,2,3], Aurélien de Reyniès[6], Wolf Herman Fridman[1,2,3,9,]*

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[4]INSERM, UMR-1162, Génomique fonctionnelle des tumeurs solides, Paris, France
[5]University Hospitals Leuven, KULeuven, Leuven, Belgium
[6]Programme Cartes d'Identité des Tumeurs, Ligue Nationale Contre le Cancer, Paris, France
[7]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Department of Oncology, Paris, France
[8]Université Paris Descartes, Sorbonne Paris Cite; INSERM, UMR-S1147, Paris, France
[9]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Department of Biology, Paris, France
[*]Corresponding author

Keywords : colorectal cancer, renal cell cancer, cancer molecular subtypes, immune classification, tumor microenvironment, prognostic and theranostic markers

Abbreviations :

ccRCC : clear cell renal-cell carcinoma
CRC : colorectal cancer
MSI : microsatellite instable
MSS : microsatellite stable
CIN: chromosomal instability

Abstract : Molecular and immune classifications powerfully predict cancer patient's survival and response to therapies. We hereby describe the immune tumor microenvironment of molecular subgroups of colorectal and renal cell cancers revealing a strong correlation between tumor subtypes and distinct immune profiles.

Manuscript : During the last decade, two major prognostic classifications of human cancers have emerged, respectively based on the phenotype of tumor cells and on the composition of the immune infiltrate. The first, molecular classification of cancer, stratifies patients according to genetic mutations, translocations, amplifications or deletions of chromosome fragments in malignant cells[(1)]. The second, immune classification, stratifies patients according to the location, quality and quantity of the tumoral immune infiltrate[(2)]. So far, no correlation between these two classifications had been performed.

The molecular classification has proven to be useful, in many cancer types, to identify groups of patients with distinct prognosis and responses to therapies. Thus, patients presenting mutations in driver oncogenes can be treated by specific inhibitors such as vemurafenib that targets mutated BRAF in melanoma[(3)] or gefitinib and erlotinib that targets EGFR mutations in lung tumors[(4)(5)]. Acute lymphocytic leukemia and chronic lymphoid leukemia patients with a translocation of BCR-ABL genes are sensitive to imatinib[(6)]. Amplification of the HER2/neu gene in breast cancer cells results in an overexpression of the encoded HER2 protein which is a therapeutic target for the monoclonal antibodies trastuzumab and pertuzumab in HER2-positive patients[(7)]. Conversely, patients whose colorectal cancer (CRC) are mutated for the KRAS oncogene are resistant to treatment with cetuximab, a monoclonal antibody targeting the EGF-Receptor[(8)].

Whole-transcriptome analyses of tumor cohorts also define molecular subgroups with prognostic and theranostic values. It has been exemplified in a recent publication by our group who analyzed a cohort of patients with clear cell Renal Cell Carcinoma (ccRCC)[(9)] who had developed a metastatic disease and were treated with sunitinib, a tyrosine-kinase inhibitor (TKI) targeting VEGFR1, VEGFR2,

RET, CKIT, FLT3 and PDGF-RB[10]. Transcriptomic analyses were performed on resected primary ccRCC tumors from these patients. An unsupervised consensus clustering approach identified 4 robust ccRCC subtypes (ccrcc1 to ccrcc4) that were associated with different responses to sunitinib treatment[9]. ccrcc4 had the lowest response rate to sunitinib and the shortest progression-free survival (PFS) and overall survival (OS) than ccrcc2 and ccrcc3 (Figure 1A). ccrcc4, exhibited a stem-cell polycomb signature and a sarcomatoid differentiation[9].

Six independent laboratories have reported transcriptomic molecular classifications of CRC[11](12)(12)(12)(13)(13)(13)(14)(14)(14)(15)(15)(15)[16]. They all agree on the identification of a subgroup with Microsatellite Instability (MSI) associated with longer PFS and OS, as well as on the identification of a mesenchymal subgroup, characterized by TGFß activation, stromal cells invasion and angiogenesis, and that is associated with the worst prognosis. This classification could also have a theranostic value since patients with tumors of the mesenchymal subgroup are more resistant to targeted therapies[12], including cetuximab[13] and more recently it has been reported that the CRC patient who responded to anti-checkpoint PD-1-targeting antibodies (nivolumab) belonged to the MSI subgroup[17]. One of these CRC classifications have been reported by some of us, dividing CRC in 6 subgroups (C1 to C6)[16]. C1 displayed chromosomal instability (CIN) with a significant down regulation of immune pathways, C2 comprises the MSI tumors which are known to be highly infiltrated by T lymphocytes, C3 was enriched for tumors with KRAS mutations, C4 has an upregulation of cancer stem cell like phenotype signatures, C5 features CIN with activation of the Wnt pathway and C6 was also CIN but have an expression profile similar to normal tissues[16]. As expected, patients of the C2 subgroup had the best clinical outcome, in terms of PFS and OS, whereas patients from the C4 subgroup had the worst prognosis[16] (Figure 2A).

In addition, these two molecular classifications of RCC and CRC were shown to correlate with immunological and inflammatory signatures[9][16]. For instance, pathway analyses revealed an overexpression and hypomethylation of genes involved in immune response and chemotaxis in the

ccRCC4 group of tumors. In CRC, the “Hematopoietic cell lineage” pathway was overrepresented in C2 and C4, suggesting increased infiltration by immune cells.

However, in-depth analyses of the composition of the immune microenvironment in relation with the molecular subgroups are still lacking. Such analyses appear mandatory since the immune classification of cancers is the other major prognostic factor that emerged during the last decade. Initiated by the pioneering work of Zhang et al in ovarian cancer[18] and Galon et al in CRC[19] who showed that the density of intratumoral T cells, particularly memory CD8+ T cells and a Th1 orientation was the strongest prognostic factor for PFS and OS, it was extended and confirmed for most cancer types and led to the concept of immune contexture which proposes that the density, location, functional orientation and local education of memory T cells strongly impacts patients' clinical outcome[2]. It has allowed the establishment of a standardized, robust and reproducible immunoscore which value as a routine laboratory test is being validated in a worldwide consortium[20]. The immune classification of human tumors also has theranostic value. For instance, the presence of CD8+ T cells is necessary, although not always sufficient[21], for response to therapy with anti-PD-1 antibodies in melanoma tumors[22]. It also represents a theranostic marker for other immunotherapies, since high T cell infiltration, in association with the presence of a high number of Tertiary Lymphoid Structures[23], accompanies the potential efficacy of therapeutic vaccines[24] or anti-checkpoint antibodies[25]. There are, however, exceptions to the beneficial effect of a high infiltration by CD8+ T cells, as observed in Head and Neck cancer[26], Hodgkin lymphoma[27], diffuse large B-cell lymphoma[28] and ccRCC[2][29].

We have revisited the ccRCC case by studying the immune contexture of 135 primary ccRCC[30] and 51 lung metastases of ccRCC[30][31]. We first reported that a high density of CD8+ T cells in primary as well as in metastatic sites was associated with shorter patient's survival[31]. Analysis of The Cancer Genome Atlas[32] expression data revealed that the expression of most of the genes, associated with a CD8+T cell-oriented immune response and including most notably INFg, correlated with a poor

prognosis. A more detailed analysis of the immune infiltrates revealed that many CD8+ T cells co-expressed immune checkpoint inhibitors such as PD-1 and LAG-3, and showed that high densities of PD-1 and/or LAG-3 expressing T cells correlated with poor prognosis[30]. In some patients, tumor cells expressed PD-L1 and PD-L2, while tumor-infiltrating T cells expressed PD-1. Strikingly, this co-expression was associated with a higher risk of relapse and death[30].

In contrast, high densities of CD8 T cells correlated with longer patient's survival in primary sites[19] as well as in liver[33] or lung[31] metastatic sites in CRC. The clear opposite prognostic impacts of the CD8+T cell infiltrates between ccRCC and CRC patients were observed both in the primary and the metastatic sites, suggesting that the clinical impact of the immune contexture depends on the tumor type rather than the tumor site[34]. These results prompted us to investigate the correlations between the molecular subgroups and the immune infiltrate. To study large cohorts of patients and sets of transcriptomic data, we established a robust and selective immunome, defining metagenes for all lymphocyte subsets (CD3+, CD4+, CD8+, Th1, Th2, Th17, Treg, NK, Tgd, B cells...) monocyte-derived cells, mast cells, granulocytes[35] but also endothelial cells and fibroblasts (Becht, submitted). The immunome was applied to the ccRCC and CRC molecular subgroup classifications presented above[9][16].

In the ccRCC cohort, the immunome identified the ccrcc4 subgroup as exhibiting the highest expression of genes expressed in T, B cells, cytotoxic cells and myeloid cells whereas the ccrcc1 subgroup had the lowest expression of immune metagenes (Figure 1B), confirming our previous observations[9]. Among the genes overexpressed in ccrcc4, in addition to genes involved in Th1 polarization (IFNg, TBX21), T cell activation (IL12R) and chemotaxis (CXCL9, CXCL10), were genes governing T cell inhibition (including PD-1 (PDCD1), LAG3, and TGFß), as well as genes attracting (CXCL12) and activating (CSF1) myeloid cells[9](Figure 1C). Indeed, the ccrcc4 subgroup also exhibited an hypomethylation of genes involved in the regulation of T cell activation, regulation of the immune response, chemotaxis and caspase cascades involved in apoptosis[9]. Finally, immunohistochemical

analyses revealed that tumors of the ccrcc4 subgroup had the strongest CD8+ T cells infiltration, together with PD-1 expression on their membrane and PD-L1 expression on tumor cells[9]. The combined analyses of molecular subgroups of ccRCC and immune classifications therefore allowed to identify an "immune high" and inflammatory subgroup, likely shaped by the sarcomatoid differentiated malignant cells producing chemokines and cytokines regulating the immune contexture, and inducing T cell exhaustion (PD-1 expression) and immunosuppression (TGFß). It identifies a poor-prognostic cohort, in which tumor-infiltrating lymphocytes express immune checkpoint inhibitors (PD-1, LAG-3) whose ligands are expressed by tumor cells. We consequently proposed that the ccrcc4 subgroup identifies patients responding to immune checkpoint modulators [9].

Application of the immunome to the CRC classification published by Marisa et al.[16] identified two "immune high" subgroups, as shown in Figure 2B. The expected MSI-enriched "C2" subgroup highly expressed T and NK cell metagenes and to a lesser extent the myeloid-cells metagene. It was the subgroup with the highest expression of genes involved in Th1 orientation (IFNg), PD-1 and chemokines attracting T cells (CXCL9, CXCL10), of IL15, which activates cytotoxic lymphocytes and promotes survival of memory CD8+T cells[36], and of genes implicated in the formation of Tertiary Lymphoid Structures (CXCL13), confirming previous observation of Bindea et al[35]. Surprisingly, C2 was not the only subgroup characterized by high immune metagenes expression (Figure 2C). The C4 subgroup, with a stem cell-like transcriptomic profile and expressing markers of epithelial-to-mesenchymal transition, comprised tumors with high T and NK metagenes expression but in the context of high myeloid cells metagene signature, and of endothelial and fibroblastic cells markers expression. Some tumors of this subgroup also expressed the PD-1 ligands, CD274 and PDCD1LG2. In accordance with high expression of the myeloid cells metagenes, the C4 subgroup also exhibited a high expression of genes encoding myeloid cells attracting chemokines (CCL2), angiogenic factors (VEGFA, VEGFC, PDGF), and TGFB1 (Figure 2C). These observations are reminiscent of the fact that high VEGF gene expression impaired the beneficial clinical impact of high granulysin gene expression

in CRC tumors[37]. The C1 and C5 subgroups were characterized by low immune and inflammatory metagenes expression. It was associated with a low expression of MHC class I genes which may explain the low CD8 T lymphocyte infiltration of these subgroups (Figure 2B and 2C). Altogether, the combined analysis of cancer molecular subgroups and immune classifications of CRC revealed unexpected immune and inflammatory associated heterogeneity in CRC tumors. Whereas the C2/MSI subgroup presents a canonical Th1/Memory CD8+ T cells cytotoxic infiltration correlating with good prognosis, the C4 subgroup exhibits a strong lymphocyte infiltration associated with myeloid cell infiltration, along with angiogenesis and high density of tumor-associated fibroblasts. These last three components most likely impair the immune reaction and are partly responsible for the poor prognosis of patients from this subgroup. Despite these deleterious elements in the microenvironment of C4 tumors, the presence of PD-1 and LAG-3 positive lymphocytes and PD-L1 expressing cells opens the possibility of targeted immunotherapies for the corresponding group of patients.

These results show similarities at the subgroup level between distinct tumor types such as ccRCC and CRC and allow to define new groups of immune high patients associated with distinct prognosis. They illustrate the high potential of combining the analyses of cancer molecular subgroups with immune classifications to define new groups of patients with similar tumoral and microenvironmental signatures, independently of tumor types. By associating the mutational, differentiation or methylation status of the tumor cells together with the tumor microenvironments that they shape, these molecular and immune based classifications has a high prognostic value and will provide targets and markers for targeted therapies.

Acknowledgments

This work was supported by the 'Institut National de la Santé et de la Recherche Médicale', the University Paris-Descartes, the University Pierre et Marie Curie, the Institut National du Cancer (2011-1-PLBIO-06-INSERM 6-1), CARPEM (CAncer Research for PErsonalized Medicine), Labex Immuno-Oncology (LAXE62_9UMS872 FRIDMAN), the Universidad de los Andes School of Medicine (NAG), Colciencias (NAG). EB is supported by B3MI doctorate fellowship, NAG by PPATH doctorate fellowship and YV by CARPEM doctorate fellowship.

References

1. Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, L.A.J. & Kinzler, K.W. Cancer genome landscapes. Science 339, 1546-58(2013).

2. Fridman, W., Pagès, F., Sautès-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer 12, 298-306(2012).

3. Chapman, P.B., Hauschild, A., Robert, C., Haanen, J.B., Ascierto, P., Larkin, J. et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N. Engl. J. Med. 364, 2507-16(2011).

4. Rosell, R., Carcereny, E., Gervais, R., Vergnenegre, A., Massuti, B., Felip, E. et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 13, 239-46(2012).

5. Maemondo, M., Inoue, A., Kobayashi, K., Sugawara, S., Oizumi, S., Isobe, H. et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N. Engl. J. Med. 362, 2380-8(2010).

6. Weisberg, E., Manley, P.W., Cowan-Jacob, S.W., Hochhaus, A. & Griffin, J.D. Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. Nat. Rev. Cancer 7, 345-56(2007).

7. Piccart-Gebhart, M.J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I. et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N. Engl. J. Med. 353, 1659-72(2005).

8. Karapetis, C.S., Khambata-Ford, S., Jonker, D.J., O'Callaghan, C.J., Tu, D., Tebbutt, N.C. et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N. Engl. J. Med. 359, 1757-65(2008).

9. Beuselinck, B., Job, S., Becht, E., Karadimou, A., Verkarre, V., Couchy, G. et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin. Cancer Res. 21, 1329-39(2015).

10. Papaetis, G.S. & Syrigos, K.N. Sunitinib: a multitargeted receptor tyrosine kinase inhibitor in the era of molecular cancer therapies. BioDrugs 23, 377-89(2009).

11. The Cancer Genome Atlas Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330-7(2012).

12. Schlicker, A., Beran, G., Chresta, C.M., McWalter, G., Pritchard, A., Weston, S. et al. Subtypes of primary colorectal tumors correlate with response to targeted treatment in colorectal cell lines. BMC Med Genomics 5, 66(2012).

13. Sadanandam, A., Lyssiotis, C.A., Homicsko, K., Collisson, E.A., Gibb, W.J., Wullschleger, S. et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nat. Med. 19, 619-25(2013).

14. De Sousa E Melo, F., Wang, X., Jansen, M., Fessler, E., Trinh, A., de Rooij, L.P.M.H. et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat. Med. 19, 614-8(2013).

15. Budinska, E., Popovici, V., Tejpar, S., D'Ario, G., Lapique, N., Sikora, K.O. et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. J. Pathol. 231, 63-76(2013).

16. Marisa, L., de Reyniès, A., Duval, A., Selves, J., Gaub, M.P., Vescovo, L. et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. PLoS Med. 10, e1001453(2013).

17. Lipson, E.J., Sharfman, W.H., Drake, C.G., Wollner, I., Taube, J.M., Anders, R.A. et al. Durable cancer regression off-treatment and effective reinduction therapy with an anti-PD-1 antibody. Clin. Cancer Res. 19, 462-8(2013).

18. Zhang, L., Conejo-Garcia, J.R., Katsaros, D., Gimotty, P.A., Massobrio, M., Regnani, G. et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N. Engl. J. Med. 348, 203-13(2003).

19. Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pagès, C. et al. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. Science 313, 1960-1964(2006).

20. Galon, J., Pagès, F., Marincola, F.M., Angell, H.K., Thurin, M., Lugli, A. et al. Cancer classification using the Immunoscore: a worldwide task force. J Transl Med 10, 205(2012).

21. Herbst, R.S., Soria, J., Kowanetz, M., Fine, G.D., Hamid, O., Gordon, M.S. et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 515, 563-7(2014).

22. Taube, J.M., Klein, A., Brahmer, J.R., Xu, H., Pan, X., Kim, J.H. et al. Association of PD-1, PD-1 Ligands, and Other Features of the Tumor Immune Microenvironment with Response to Anti-PD-1 Therapy. Clin. Cancer Res. 20, 5064-74(2014).

23. Dieu-Nosjean, M., Goc, J., Giraldo, N.A., SautÃ¨s-Fridman, C. & Fridman, W.H. Tertiary lymphoid structures in cancer and beyond. Trends in Immunology 35, 571-580(2014).

24. Maldonado, L., Teague, J.E., Morrow, M.P., Jotova, I., Wu, T.C., Wang, C. et al. Intramuscular therapeutic vaccination targeting HPV16 induces T cell responses that localize in mucosal lesions. Sci Transl Med 6, 221ra13(2014).

25. Lutz, E.R., Wu, A.A., Bigelow, E., Sharma, R., Mo, G., Soares, K. et al. Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation. Cancer Immunol Res 2, 616-31(2014).

26. Badoual, C., Hans, S., Merillon, N., Van Ryswick, C., Ravel, P., Benhamouda, N. et al. PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer Res. 73, 128-38(2013).

27. Scott, D.W., Chan, F.C., Hong, F., Rogic, S., Tan, K.L., Meissner, B. et al. Gene expression-based model using formalin-fixed paraffin-embedded biopsies predicts overall survival in advanced-stage classical Hodgkin lymphoma. J. Clin. Oncol. 31, 692-700(2013).

28. Muris, J.J.F., Meijer, C.J.L.M., Cillessen, S.A.G.M., Vos, W., Kummer, J.A., Bladergroen, B.A. et al. Prognostic significance of activated cytotoxic T-lymphocytes in primary nodal diffuse large B-cell lymphomas. Leukemia 18, 589-96(2004).

29. Nakano, O., Sato, M., Naito, Y., Suzuki, K., Orikasa, S., Aizawa, M. et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res. 61, 5132-6(2001).

30. Giraldo, N., Becht, E., Pages, F., Skliris, G.P., Verkarre, V., Vano, Y. et al. Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer. Clin. Cancer Res. , (2015).

31. Remark, R., Alifano, M., Cremer, I., Lupo, A., Dieu-Nosjean, M., Riquet, M. et al. Characteristics and Clinical Impacts of the Immune Environments in Colorectal and Renal Cell Carcinoma Lung Metastases: Influence of Tumor Origin. Clin. Cancer Res. 19, 4079-4091(2013).

32. Cancer Genome Atlas Research Network Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499, 43-9(2013).

33. Halama, N., Michel, S., Kloor, M., Zoernig, I., Benner, A., Spille, A. et al. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. Cancer Res. 71, 5670-7(2011).

34. Becht, E., Goc, J., Germain, C., Giraldo, N.A., Dieu-Nosjean, M., Sautès-Fridman, C. et al. Shaping of an effective immune microenvironment to and by cancer cells. Cancer Immunol. Immunother. 63, 991-7(2014).

35. Bindea, G., Mlecnik, B., Tosolini, M., Kirilovsky, A., Waldner, M., Obenauf, A.C. et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity 39, 782-95(2013).

36. Mlecnik, B., Bindea, G., Angell, H.K., Sasso, M.S., Obenauf, A.C., Fredriksen, T. et al. Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. Sci Transl Med 6, 228ra37(2014).

37. Camus, M., Tosolini, M., Mlecnik, B., Pagès, F., Kirilovsky, A., Berger, A. et al. Coordination of intratumoral immune reaction and human colorectal cancer recurrence. Cancer Res. 69, 2685-93(2009).

Figure legends:

Figure 1: correlation between molecular subgroups and immune and inflammatory gene expression in ccRCC

A) Kaplan-Meier curves representing the progression-free survival (left) and overall-survival (right) of ccRCC4 patients compared to non-ccRCC4 patients **B)** Relative expression of immune cell-specific markers in the 4 ccRCC subgroups (red : high expression, blue : low expression). Percentages indicate the frequency of each subgroups within the cohort. **C)** Relative expression of functionally-relevant immune genes in the 4 ccRCC subgroups (red : high expression, blue : low expression). Dataset : ArrayExpress E-MTAB-3269

Figure 2: correlation between molecular subgroups and immune and inflammatory gene expression in CRC

A) Kaplan-Meier curves representing the relapse-free survival (left) and overall-survival (right) of C2, C4 and non-C2/C4 patients **B)** Relative expression of immune cell-specific markers in the 6 CRC subgroups (red : high expression, blue : low expression). Percentages indicate the frequency of each subgroups within the cohort. **C)** Relative expression of functionally-relevant immune genes in 6 CRC subgroups (red : high expression, blue : low expression). Dataset : Gene Expression Omnibus GSE39582

Figure 1:

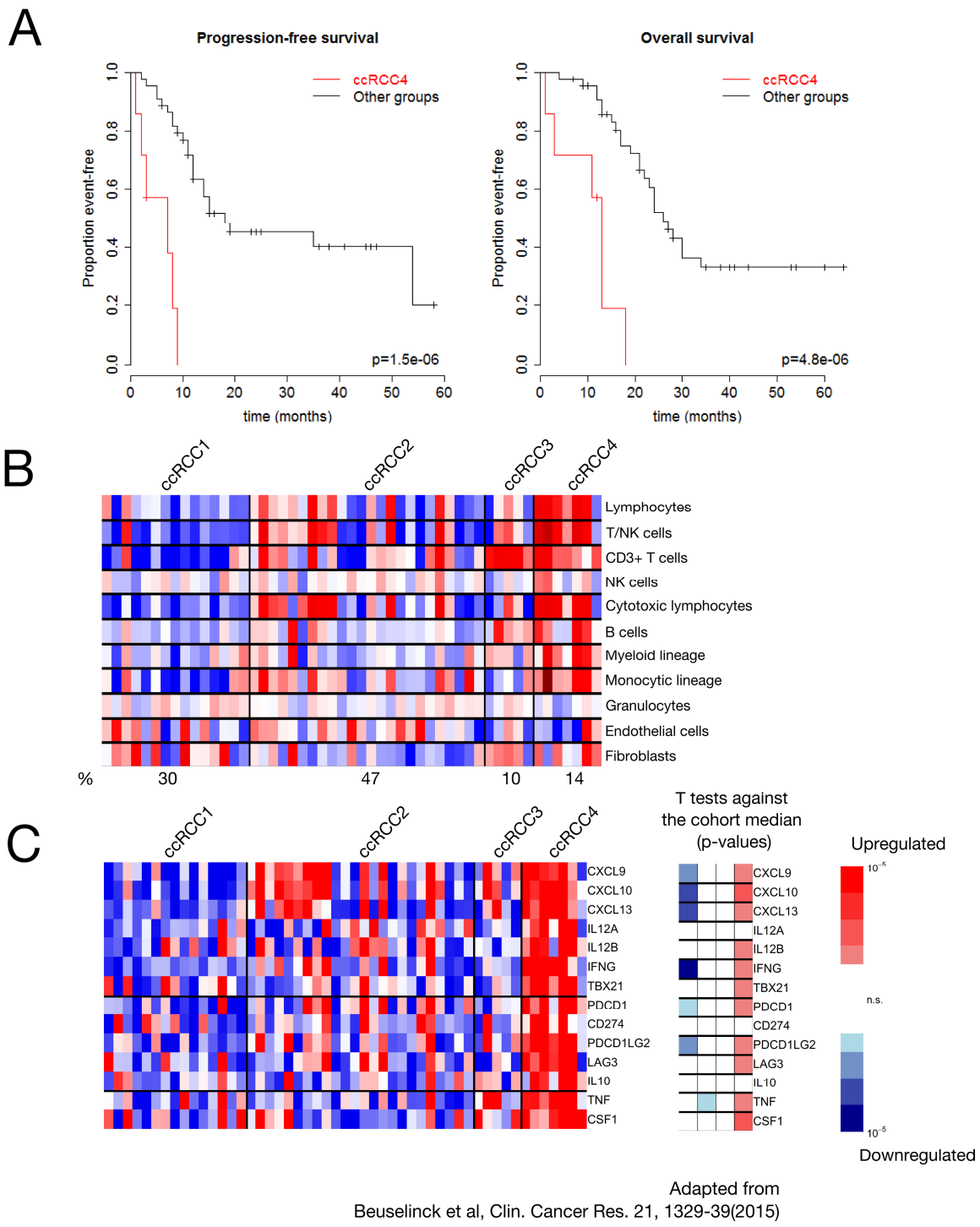
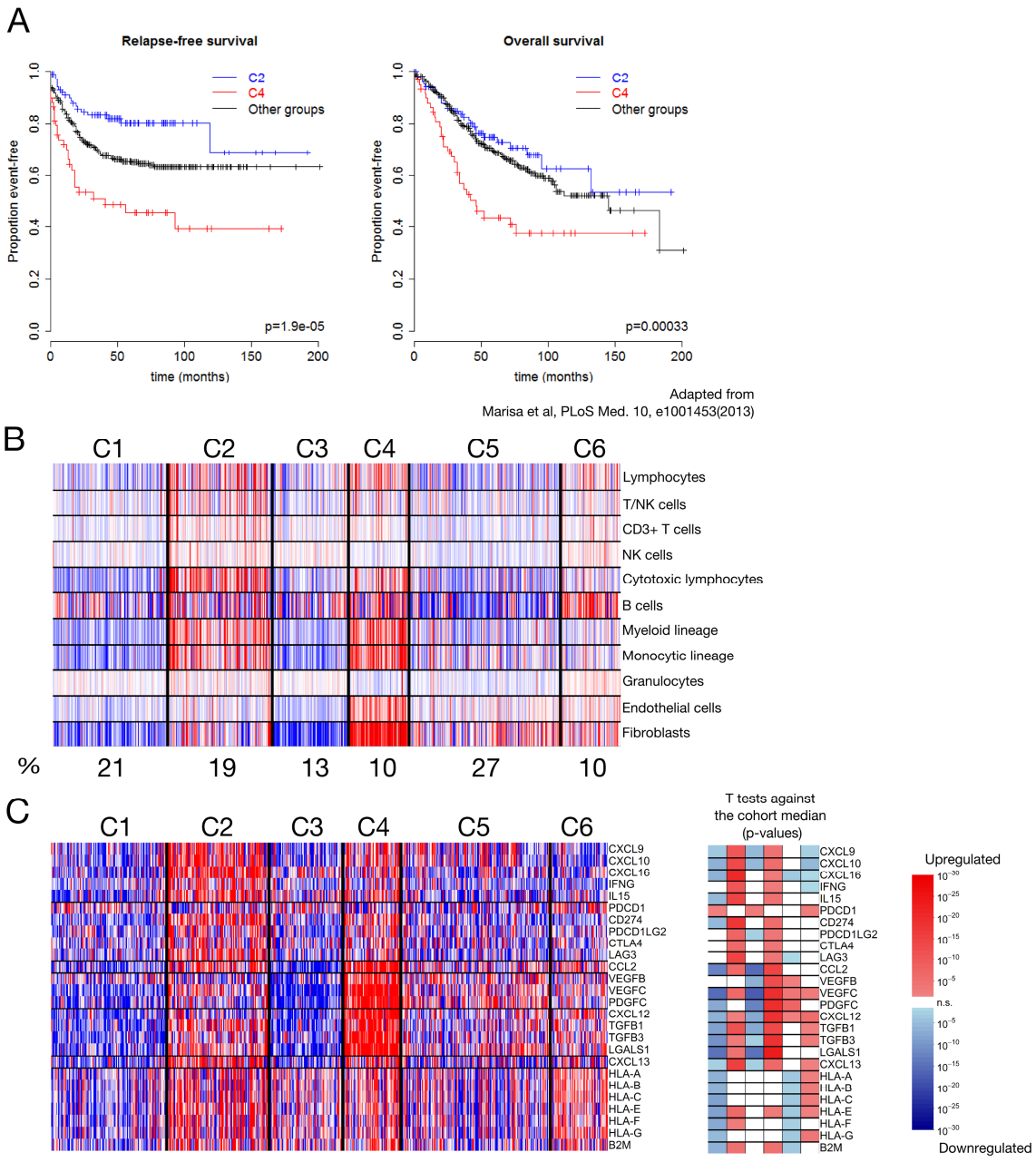

A
Progression-free survival
Overall survival
ccRCC4
Other groups
Proportion event-free
time (months)
p=1.5e-06
p=4.8e-06
B
ccRCC1
ccRCC2
ccRCC3
ccRCC4
Lymphocytes
T/NK cells
CD3+ T cells
NK cells
Cytotoxic lymphocytes
B cells
Myeloid lineage
Monocytic lineage
Granulocytes
Endothelial cells
Fibroblasts
%
30
47
10
14
C
CXCL9
CXCL10
CXCL13
IL12A
IL12B
IFNG
TBX21
PDCD1
CD274
PDCD1LG2
LAG3
IL10
TNF
CSF1
T tests against the cohort median (p-values)
Upregulated
n.s.
Downregulated
Adapted from
Beuselinck et al, Clin. Cancer Res. 21, 1329-39(2015)

Figure 2:


A
Relapse-free survival
Overall survival
C2
C4
Other groups
Proportion event-free
time (months)
p=1.9e-05
p=0.00033
Adapted from
Marisa et al, PLoS Med. 10, e1001453(2013)
B
C1
C2
C3
C4
C5
C6
Lymphocytes
T/NK cells
CD3+ T cells
NK cells
Cytotoxic lymphocytes
B cells
Myeloid lineage
Monocytic lineage
Granulocytes
Endothelial cells
Fibroblasts
%
21
19
13
10
27
10
C
T tests against
the cohort median
(p-values)
CXCL9
CXCL10
CXCL16
IFNG
IL15
PDCD1
CD274
PDCD1LG2
CTLA4
LAG3
CCL2
VEGFB
VEGFC
PDGFC
CXCL12
TGFB1
TGFB3
LGALS1
CXCL13
HLA-A
HLA-B
HLA-C
HLA-E
HLA-F
HLA-G
B2M
Upregulated
n.s.
Downregulated

Chapter 4

Discussion

In this chapter, I first discuss advantages and drawbacks of the method enabling transcriptomic quantifications of cellular proportions of tumor microenvironments that I proposed. I subsequently discuss the immune contexture of ccRCC and CRC molecular subgroups, their relationship to tumor immunology theories and their translational relevance. Finally, I propose perspectives that could enrich the analyses presented in this manuscript.

4.1 Transcriptomic quantification of cell populations in tumor microenvironments

I introduced several studies dealing with the issue of cellular heterogeneity in transcriptomic samples. I will now discuss the methodology I developed in the context of previously published methods.

4.1.1 Depth of characterized cell phenotypes

The method I developed enables the quantification of nine immune and two stromal cell populations of the tumor microenvironment. It includes both broad cell categories such as 'Myeloid' and 'Lymphoid' cells, and more precise phenotypes such as 'T cells' or 'Granulocytes'. The number of phenotypes characterized is intermediate when compared to previously-published marker-based approaches[356, 358, 360]. Abbas et al.[356] and Palmer et al.[358] respectively characterized 6 and 3 cell populations, and their results only consider blood cells and are thus inapplicable in tumor samples. Yoshiara et al.[360] proposed a method suitable for the analysis of tumors, but proposes only two signatures, one for immune cells and one for the tumor stroma, and therefore cannot discriminate between immune cell subpopulations. Bindea et al.[119] proposed signatures for 28 populations, but the number of pure samples analyzed and the statistical criteria used are not stringent enough to allow specificity of the proposed markers to one and only one cell population. Also, since microarray technologies can lack sensitivity for lowly expressed transcripts, it may be difficult to measure infiltration by rare cell populations such as dendritic cells using this approach. I discussed in **Article 2** the depth of characterized phenotypes, based on the number of identified markers, the number of independent studies including a given cell population and the relevance of the population characterized by the gene signature. Unsupervised analyses were also used to identify unsupervised separability of homogeneous samples, and revealed that even intermediate-depth populations such as $CD4^+$ T cells and $CD8^+$ T cells were indistinguishable through these approaches. It is possible that deconvolution algorithms, that do not necessarily rely on genes with

binary expression patterns, might be able to quantify more subtle phenotypes than the approached I proposed. I did however introduce a set of functional molecules that enable the analysis of the functional orientation of infiltrating immune cells to compensate the relatively modest depth of the cell phenotypes quantified.

4.1.2 Choice of the approach

Deconvolution approaches use features whose expression levels vary across cell populations, while marker-based approaches rely on features specifically expressed by one population. These approaches rely on strong theoretical frameworks, as described in equation (1.4), that come at the price of hypotheses on the statistical distribution of the variables and relationship between specific mRNA concentrations and the corresponding gene expression measures. Different authors have argued in favor of the use of either linear expression values[338, 342, 343, 350, 351, 353] or logarithmic expression[346] values in these models. It is unclear whether the object of this debate actually affects the potency of these methods, as recently published algorithms did not report major output differences using both scales[353]. A more questionable hypothesis relies in the assumption of linearity between mRNA concentration and expression measures. Microarray technologies overestimate lowly-expressed genes due to background noise which overcomes the specific signal, and underestimate highly-expressed genes due to probe saturation (see figure 4.1). These non-linear effects therefore handicap deconvolution methods based on linear models. The authors of the CIBERSORT methods for instance reported systematic overestimation of rare cell subsets[372], which could be due to these phenomenons.

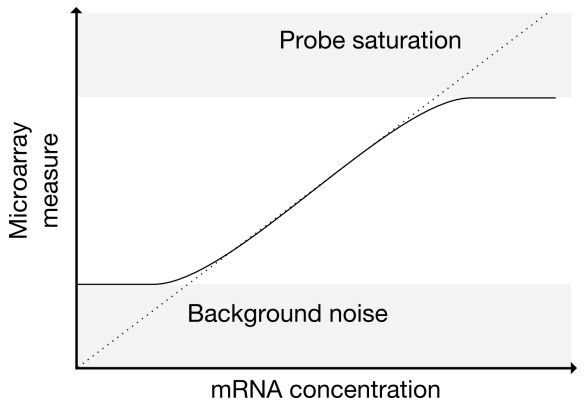


Figure 4.1: Illustration of non-linear effects in DNA microarray measurements

I attempted to implement linear models to deconvolve the proportions of known cell proportions in the RNA mixture model used in **Article 2, Fig 1 and Table S9**, but achieved lower accuracy compared to marker-based quantification, and therefore pursued with the marker-based approach.

4.1.3 Control samples

No other published studies used controls suitable for pan-cancer analyses. In the approach I introduced, I used 745 cancer cell lines spanning 24 anatomic locations, therefore controlling for the non-expression of selected markers by a wide range of cancer cell lines. Although endothelial cells were included in the populations screened by Bindea et al.[119], no other marker-based study characterized the transcriptomic markers of fibroblasts.

Despite the large number of cell populations characterized, some cell populations were

not profiled on Affymetrix Human Genome U133 Plus 2.0 Arrays and therefore could not be included in our analysis. Such populations include NKT cells and basophils. Mast cells were represented only by the HMC-1 cell line, a human cell line derived from a patient with mast cell leukaemia[376]. HMC-1 samples were therefore only used as controls and could not be used to reliably identify markers specific for mast cells.

Many non-immune cell populations present in tumor microenvironments could also not be included. Such cells include non-malignant enterocytes, goblet cells and colonic stem cell that are physiologically present in the colonic crypt, and could therefore contaminate CRC samples. Transcriptomic profiles of such non-malignant cells are not available for most organs and were therefore not included in our analysis. These cells can in theory express some markers that we selected and could potentially disturb the estimated proportions. I however presented *ex-vivo* validations that argue in favor of reliable quantifications provided by the proposed gene-signatures (**Article 2, Fig 3, Fig S3, Fig S4**), that I develop in the following section.

4.1.4 Experimental validations

Three validation settings were presented and support that the gene signatures identified accurately measure the proportions of infiltrating cells. We first performed *in-vitro* mixtures of mRNA from five immune populations further diluted in a CRC cell line mRNA. The proportions introduced in the mixtures were organized in two transposed latin squares (**Article 2, Table S9**) to ensure the strict specificity of the selected markers to one and only one cell population, as this setting reduced the collinearity of the mRNA proportions between the different cell populations. The amount of mRNA introduced in each mixture greatly varied, with mRNA proportions spanning five $\log_2$ units. In this challenging setting, the summarized gene signatures were very highly correlated to the known proportions in the mixtures, with Pearson's correlation coefficients ranging from 0.94 to 0.99.

I then reported the correlation between markers of specific cell populations (**Article 2, Fig S3**). This analysis is based on the theoretical considerations developed in equation (1.8), which states that the expression of a feature specific for a given cell population is proportional to this population's proportion in a sample. By transitivity, we therefore expect a high correlation of the markers of a given population with each others. This analysis also suggest a consistency between unsupervised clustering of features and our supervised marker-based approach. A high correlation between markers was observed within each signature, except for the one specific to granulocytes. This result suggests that granulocytes either infiltrate CRC tumors in too low numbers to be accurately quantified, or that their mRNA content is damaged before sample fixation, these cells being notoriously instable as they die upon activation[377]. However, the co-expression patterns of non-granulocytic cells' markers are consistent with a high enrichment in marker genes. Selected probesets that show an overall poor correlation to the other genes of the signature could reflect a downregulation of the corresponding transcript by the target cell population, an expression value in tumors below the lower limit of detection of the measurement technique, or falsely selected probesets.

Finally, we immunohistochemically-characterized the immune microenvironment of transcriptomically profiled CRC tumors for three markers. For each marker, we observed a high consistency between the transcriptomic predictions and the corresponding cell infiltrations (**Article 2, Fig 3, Fig S4**).

4.1.5 Translational applications

In addition to their use for the study of tumor microenvironments, these gene signatures could be applied in the clinic to quantify immune and stromal cell infiltrations in resected tumors or tumor biopsies. Since the composition of the immune microenvironment yields prognostic information[54], and since multiple markers are necessary to accurately predict prognosis (**Article 2**) but immunohistochemical quantifications are time-consuming, our transcriptomic approach could enable a fast and harmonized quantitative characterization of the microenvironment of clinical samples.

4.2 Immune contextures of CRC and ccRCC molecular subtypes

In both cancers, we found a high correlation between the expression of immune and stromal signatures and molecular subgroups. In this section, I discuss the implications of these findings for tumor immunology and for the clinical management of the patients in these subgroups.

4.2.1 Tumor immunology and the immune contexture of molecular subgroups

I introduced tumor immunology through two major axes, the adaptive anti-tumor immune response associated with favorable outcome and the pro-tumor inflammation associated with poor outcome. I will now present how these two axes stratify tumor molecular subgroups.

4.2.1.1 Immune-low subgroups

In both cancers, poorly infiltrated tumors corresponded to specific transcriptomic subgroups. In ccRCC, the ccrcc1, ccrcc2 and ccrcc3 subgroups were poorly infiltrated compared to ccrcc4 (**Article 1**). In CRC, the Canonical and Metabolic subgroups in the CMS classification (**Article 2**), or the C1, C3 and C5 subgroups in the CIT classification (**Article 3**) had the lowest infiltration by immune cells. These tumors therefore appear as immune-ignored, unable to elicit both inflammatory and adaptive immune responses. The reasons for this immune ignorance or escape are unclear.

A recent study suggested that tumors with a high mutational load were extensively infiltrated by cytotoxic lymphocytes[354]. The number of mutations is indeed much higher in MSI tumors compared to non-MSI tumors in CRC, but Mesenchymal CRC tumors have mutation rates comparable with the ones found in the Canonical and Metabolic tumors, suggesting that the low number of mutations in immune-low CRC tumors is not sufficient to explain their low immune infiltration. I reported that class I HLA molecules were downregulated in immune-low CRC subgroups (**Article 2**). Lack of expression of class I MHC molecules could lead to a low activation of $CD8^+$ T cells, preventing their local proliferation and thus lowering their overall proportion within the tumor microenvironment. A third possibility is that malignant cells in these subgroups do not display danger signals upon apoptosis, resulting in non-immunogenic cell death and therefore a lack of local inflammation, resulting in low infiltration by both lymphocytes and myeloid cells.

In ccRCC, the average number of mutations across the four subgroups has not been investigated, and therefore could account for higher infiltration in ccrcc4. Tumor immune infiltration was found lower in ccrcc1-3 when compared to ccrcc4. In these subgroups, meta-

genes representing T cells and Macrophages infiltrations had an higher expression than in distant non-malignant kidney tissues, suggesting that even poorly-infiltrated ccRCC tumors are more infiltrated than normal kidney tissues, as previously reported[365–369]. It is unclear whether the intermediate immune infiltration found in these subgroups is able to control tumor growth. ccrcc1 and ccrcc2 feature the lowest expression of metagenes specific for macrophages, as well as a trend for higher NK cells infiltration than the poorly-immunogenic ccrcc3 (**Article 1, Fig S2**). NK cells have been previously associated with favorable outcome in ccRCC[311], and could mediate anti-tumor functions in these subgroups , although NK cells infiltrating ccRCC tumors have been reported to display a dysfunctional phenotype[378–380]. ccrcc1 had the lowest expression of genes regulating immune cells functions (**Article 1, Fig S1**), suggesting that these tumors are the least immunogenic of the four subgroups.

Immunotherapeutic approaches in these subgroups should aim at eliciting an adaptive immune response in the context of poor tumor cells immunogenicity. Bi-specific antibodies could represent such a treatment modality. Bi-specific are artificial antibodies engineered to simultaneously target two distincts epitopes. One epitope is usually a tumor-associated antigen, and the second is usually a T cell receptor specific epitope on the molecules forming the CD3-signaling complex[381]. These antibodies take advantage of the high affinity of antibodies which can bind to native, non-processed antigens, and then recruit and activate effector T cells regardless of their intrinsic specificity. CAR T cells offer an alternative, notably as they possess the capacity to bind to mutated antigens independently of MHC presentation[193]. MHC molecules are downregulated in the intermediate prognosis and poorly immunogenic ccrcc1 subgroup (**Article 1, Fig S1**), and in the poorly immunogenic CRC subgroups (**Article 2, Fig 4** and **Article 3, Fig 2C**), and these tumors may therefore lack the antigen-presentation machinery. These cells may therefore be able to mediate tumor elimination despite low class I MHC expression.

4.2.1.2 MSI-like, $CD8^{+}{}_{high}$, T_{h1}-oriented CRC subgroup

I have cited a large corpus of publications supporting that extensive infiltration by $CD8^{+}$ T cells or a consistent T_{h1} functional orientation is associated with favorable prognosis in most malignancies[54], and most notably in CRC[18, 46, 47, 119, 361, 375, 382]. It is striking that the immune contexture described in these studies perfectly fits with the one I reported in CRC tumors of the MSI-like transcriptomic subgroup. MSI-like, or the corresponding C2 subgroup, have the highest tumor infiltration by cytotoxic lymphocytes (**Article 2, Fig 2** and **Article 3, Fig 2B**), which we showed to correspond to infiltration by $CD8^{+}$ T cells (**Article 2, Fig 3A, Fig S4A**). This subgroup also have the highest expression of chemokines attracting memory T lymphocytes (CXCL9, CXCL10, CXCL11)[18], of T_{h1} molecules such as IFNγ[47], of the IL15 cytokine which activates T and NK cells[375], and CXCL13 which is associated with formation of Tertiary Lymphoid Structures which locally prime adaptive immune responses[119, 333, 383]. The high consistency between the good-prognosis MSI-like subgroup described in transcriptomic classifications of CRC[236–241, 282] and the tummor-immunology good-prognosis $CD8^{high}/T_{h1}$ subgroup is a striking illustration of the relationship between immune and molecular classifications.

This subgroup has the highest expression of molecules of the PD-1 pathway, consistently with recent publications supporting the use of checkpoint blockade antibodies for the treatment of these patients[179, 302, 303]. The high number of mutations found in MSI tumors is consistent with their high infiltration by cytotoxic lymphocytes[354], and supports the idea that the long relapse-free survival that these patients experience is in

part due to the control of tumor growth and dissemination by tumor antigens-targeting cytotoxic T cells. It is therefore likely that MSI tumors of relapsing CRC patients feature higher expression of molecules implicated in immune-escape mechanisms, and checkpoint-blockade therapies could therefore mostly benefit relapsing MSI patients.

4.2.1.3 Inflammatory subgroups in CRC and ccRCC

In both cancers, poor-prognosis highly-infiltrated subgroups were identified. In both cases, *in-situ* analyses showed a high infiltration by $CD8^+$ T cells (**Article 1, Fig S2, Fig S3** and **Article 2, Fig 2, Fig 3**) contradicting the idea that tumors highly infiltrated by $CD8^+$ T cells have an inherently better prognosis. It is striking that even in CRC, a prototypical illustration of this idea, a poor-prognosis subgroup with high $CD8^+$ T cells infiltration exists.

In RCC, ccRCC4 displayed the highest infiltration by B and T lymphocytes, as well as macrophages (**Article 1, Fig S2** and **Article 3, Fig 2B**), with a concomitant T_{h1} functional orientation (expression of TBX21 and IFNγ), T and B cells-attracting chemokines (CXCL9, CXCL10, CXCL13) (**Article 1, Fig S1** and **Article 3, Fig 2C**) and should therefore theoretically be associated with favorable outcome. Yet, both soluble (TGFβ, IL10) and contact-dependent (PDCD1 [PD1], CD274 [PD-L1], PDCD1LG2 [PD-L2], LAG3, HAVCR2 [TIM3]) inhibitory molecules are highly expressed in this subgroup and suggest that immunosuppressive mechanisms counteract the cytotoxic/T_{h1} functional orientation of tumor-infiltrating lymphocytes. TGFβ and IL10 are notable T_{reg} related cytokines, and suggest an additional extensive infiltration by regulatory T cells in these tumors. Immune checkpoint can further promote immunosuppression in this subgroup. These tumors are resistant to Sunitinib treatment (**Article 1, Fig 3A**), but express markers that were previously associated with response to PD1-blocking antibodies[185, 307, 308], suggesting that the molecular classification proposed in **Article 1** might be used as a theranostic tool to predict patient's response to these treatments. Pathways related to angiogenesis were found overexpressed in this subgroup (**Article 1, Table 2**), although no increase in markers specific for endothelial cells were observed (**Article 3, Fig 1B**). ccRCC is however a highly vascularized malignancy[264], and it is therefore likely that even moderately vascularized ccRCC tumors feature high levels of angiogenesis. Anti-angiogenic treatments have been shown to synergize with checkpoint-blockade therapies[164] and to inhibit the proliferation of T_{reg} cells in ccRCC, and it is thus tempting to experiment treatments regimen combining anti-angiogenic and anti-checkpoint drugs that could synergistically alleviate both immunosuppressive mechanisms at play in ccrcc4 tumors and leverage their extensive infiltration by $^+$ lymphocytes.

The Mesenchymal subgroup of CRC resembles ccrcc4 in that it is highly infiltrated by inflammatory cells and highly vascularized (**Article 2, Fig 2, Fig 3** and **Article 3, Fig 2B**). A notable difference is that this immune contexture appears to be mainly initiated by cancer-associated fibroblasts (CAF) (**Article 2, Fig 5**), which were not specifically associated with ccrcc4 in ccRCC (**Article 3, Fig 1B**). It has been suggested that ccRCC tumor cells release inflammatory mediators[87, 283] that could shape their microenvironments in the way CAF shape the immune contexture of Mesenchymal CRC tumors. Like ccrcc4, these tumors highly expressed PD1 ligands and other checkpoint molecules such as LAG3 and CTLA4 (**Article 2, Fig 4** and **Article 3, Fig 2C**) and T_{h1} molecules, albeit at lower levels than MSI-like CRC tumors. They however specifically expressed suppressive molecules such as TGFβ and LGALS1 and pro-inflammatory molecules such as the CCL2 chemokine or complement molecules. It therefore appears that compared to the MSI-like subgroup, immunosuppression is mediated, in addition to checkpoint molecules, by high

inflammation and angiogenesis. Both of these axes should be targeted simultaneously in order to restore anti-tumor adaptive immune responses in this subgroup. Treatment strategies could involve combination therapies using anti-angiogenic and anti-checkpoint agents. CAR T cells, in addition to their ability to sense antigens in an MHC-independent manner, are also designed to constitutively receive co-stimulatory signals upon antigen-specific activation[193]. Since antigen-presenting cells feature immature phenotypes in angiogenic or hypoxic environments, it is likely that immature antigen presenting myeloid cells participate in immunosuppression by delevering co-inhibitory signals to T cells in this subgroup. CAR T cells could potentially bypass these co-inhibitory signals to exert direct anti-tumor functions.

4.2.1.4 Immune classification of tumor subtypes

In light of these analyses, I propose that tumor subtypes distribute along the two axes of inflammation and adaptive immunity as shown in figure 4.2. This diagram suggests that multiple markers are necessary to interpret the immune contexture of tumors and accurately predict prognosis and response to treatments. Analyzing how tumor subtypes in other cancers distribute in this diagram might enrich our knowledge of the interactions between the immune system and tumors, and help targeting immunotherapeutic treatments.

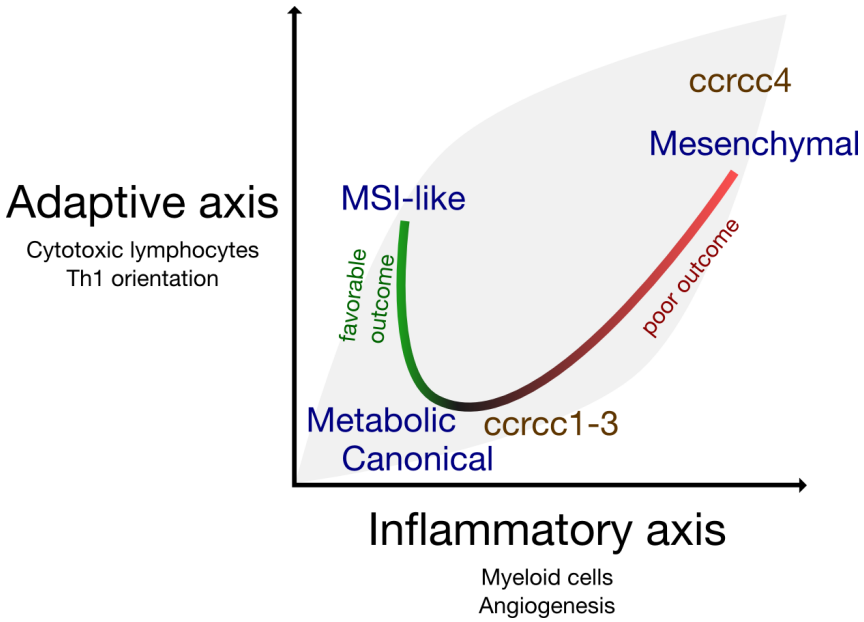


Figure 4.2: Transcriptomic subgroups differ in inflammatory and adaptive immune components, which correlate with prognosis.

4.3 Perspectives

To conclude this discussion, I propose several approaches to tackle issues raised by our results.

4.3.1 Improving the transcriptomic quantification of tumor-infiltrating immune cells

Microarray technologies are strongly affected by non-linear effects. However, recent gene-expression measurement techniques, such as Nanostring assays or RNA-sequencing technologies may possess wider linear dynamic ranges and could therefore benefit from more refined statistical models, which could improve measurement accuracy and permit the quantification of more precise cell phenotypes. RNA-sequencing also provides non-ambiguous characterization of non-expressed genes, and reproducing our marker-based approach on this technology might identify more accurate sets of specific genes with binary expression patterns. Such analyses are however dependent on sample availability. RNA-sequencing allows the estimation of splice variants, and some of these could be specific for currently unadressed cell populations.

4.3.2 Phenotypical characterization of tumor-infiltrating immune cells

Our analyses also advocate for finer characterization of the immune contextures of the transcriptomic subgroups. As molecular subgroups of CRC and ccRCC feature different infiltration by immune populations, and functional mediators were also differentially expressed across these subgroups, it is likely that the phenotype of tumor-infiltrating immune cells differ across the subgroups. Cytometry-based phenotypic analyses on molecularly-classified fresh tumor samples might provide insights in these regards. It would be particularly relevant to study the repertoire of T cells in the MSI-like and Mesenchymal CRC subgroups, to study whether there are differences in the clonality of the tumor-infiltrating T cells between these subgroups. Lower T cells clonality in Mesenchymal CRC might indicate a lack of tumor-specific adaptive immune reaction. In addition, characterizing the myeloid compartment would address our hypotheses that macrophages feature a M1 phenotype in MSI-like and a M2 phenotype in Menenchymal CRC tumors, and that dendritic cells feature a mature phenotype in MSI-like and immature phenotype in Mesenchymal CRC tumors.

4.4 Conclusion

The results reported in this manuscript show that immune classifications correlate with molecular subtypes in both CRC and ccRCC, and suggest immunotherapeutic approaches for the various tumor subtypes in these malignancies. Transcriptomic classifications have been established for a large numbers of cancers. Comprehensively characterizing the immune contexture of tumor subtypes in these other malignancies should result in unified immune and molecular classifications, and guide the development and the prescription of both tumor-targeted therapies and microenvironment-targeted therapies.

Bibliography

[1] Masayuki Fukata, Arunan S. Vamadevan, and Maria T. Abreu. Toll-like receptors (tlrs) and nod-like receptors (nlrs) in inflammatory disorders. *Semin Immunol*, 21(4):242–253, Aug 2009.

[2] Douglas R. Green, Thomas Ferguson, Laurence Zitvogel, and Guido Kroemer. Immunogenic and tolerogenic cell death. *Nat Rev Immunol*, 9(5):353–363, May 2009.

[3] H. R. Rodewald, P. Moingeon, J. L. Lucich, C. Dosiou, P. Lopez, and E. L. Reinherz. A population of early fetal thymocytes expressing fc gamma rii/iii contains precursors of t lymphocytes and natural killer cells. *Cell*, 69(1):139–150, Apr 1992.

[4] G. Leclercq, V. Debacker, M. de Smedt, and J. Plum. Differential effects of interleukin-15 and interleukin-2 on differentiation of bipotential t/natural killer progenitor cells. *J Exp Med*, 184(2):325–336, Aug 1996.

[5] J. R. Carlyle, A. M. Michie, C. Furlonger, T. Nakano, M. J. Lenardo, C. J. Paige, and J. C. Zúñiga-Pflücker. Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J Exp Med*, 186(2):173–182, Jul 1997.

[6] Boris Reizis. Regulation of plasmacytoid dendritic cell development. *Curr Opin Immunol*, 22(2):206–211, Apr 2010.

[7] Boris Reizis, Anna Bunin, Hiyaa S. Ghosh, Kanako L. Lewis, and Vanja Sisirak. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol*, 29:163–183, 2011.

[8] Nick Holmes. Cd45: all is not yet crystal clear. *Immunology*, 117(2):145–155, Feb 2006.

[9] Yolanda D. Mahnke, Tess M. Brodie, Federica Sallusto, Mario Roederer, and Enrico Lugli. The who's who of t-cell differentiation: human memory t-cell subsets. *Eur J Immunol*, 43(11):2797–2809, Nov 2013.

[10] Eric W. Hewitt. The mhc class i antigen presentation pathway: strategies for viral immune evasion. *Immunology*, 110(2):163–169, Oct 2003.

[11] Erin J. Adams, Siyi Gu, and Adrienne M. Luoma. Human gamma delta t cells: Evolution and ligand recognition. *Cell Immunol*, 296(1):31–40, Jul 2015.

[12] Patrick J. Brennan, Manfred Brigl, and Michael B. Brenner. Invariant natural killer t cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol*, 13(2):101–117, Feb 2013.

[13] P. Ehrlich. *Über den jetzigen Stand der Karzinomforschung.* 1908.

[14] O. Stutman. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science*, 183(4124):534–536, Feb 1974.

[15] O. Stutman. Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose. *J Natl Cancer Inst*, 62(2):353–358, Feb 1979.

[16] Douglas Hanahan and Robert A. Weinberg. Hallmarks of cancer: the next generation. *Cell*, 144(5):646–674, Mar 2011.

[17] Gavin P. Dunn, Lloyd J. Old, and Robert D. Schreiber. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*, 21(2):137–148, Aug 2004.

[18] Bernhard Mlecnik, Marie Tosolini, Amos Kirilovsky, Anne Berger, Gabriela Bindea, Tchao Meatchi, Patrick Bruneval, Zlatko Trajanoski, Wolf-Herman Fridman, Franck Pagès, and Jérôme Galon. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol*, 29(6):610–618, Feb 2011.

[19] J. W. Pape, B. Liautaud, F. Thomas, J. R. Mathurin, M. M. St Amand, M. Boncy, V. Pean, M. Pamphile, A. C. Laroche, and WD Johnson, Jr. Characteristics of the acquired immunodeficiency syndrome (aids) in haiti. *N Engl J Med*, 309(16):945–950, Oct 1983.

[20] H. W. Haverkos and D. P. Drotman. Prevalence of kaposi's sarcoma among patients with aids. *N Engl J Med*, 312(23):1518, Jun 1985.

[21] T. H. Schreiber and E. R. Podack. A critical analysis of the tumour immunosurveillance controversy for 3-mca-induced sarcomas. *Br J Cancer*, 101(3):381–386, Aug 2009.

[22] Louise Giffin and Blossom Damania. Kshv: pathways to tumorigenesis and persistent infection. *Adv Virus Res*, 88:111–159, 2014.

[23] D. Farge. Kaposi's sarcoma in organ transplant recipients. the collaborative transplantation research group of ile de france. *Eur J Med*, 2(6):339–343, 1993.

[24] R. E. Curtis, P. A. Rowlings, H. J. Deeg, D. A. Shriner, G. Socíe, L. B. Travis, M. M. Horowitz, R. P. Witherspoon, R. N. Hoover, K. A. Sobocinski, JF Fraumeni, Jr, and JD Boice, Jr. Solid cancers after bone marrow transplantation. *N Engl J Med*, 336(13):897–904, Mar 1997.

[25] Bertram L. Kasiske, Jon J. Snyder, David T. Gilbertson, and Changchun Wang. Cancer after kidney transplantation in the united states. *Am J Transplant*, 4(6):905–913, Jun 2004.

[26] V. Shankaran, H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber. Ifngamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*, 410(6832):1107–1111, Apr 2001.

[27] Béatrice Breart, Fabrice Lemaître, Susanna Celli, and Philippe Bousso. Two-photon imaging of intratumoral cd8+ t cell cytotoxic activity during adoptive t cell therapy in mice. *J Clin Invest*, 118(4):1390–1397, Apr 2008.

[28] Bert Vogelstein, Nickolas Papadopoulos, Victor E. Velculescu, Shibin Zhou, Luis A Diaz, Jr, and Kenneth W. Kinzler. Cancer genome landscapes. *Science*, 339(6127):1546–1558, Mar 2013.

[29] T. Wölfel, M. Hauer, J. Schneider, M. Serrano, C. Wölfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K. H. Meyer zum Büschenfelde, and D. Beach. A p16ink4a-insensitive cdk4 mutant targeted by cytolytic t lymphocytes in a human melanoma. *Science*, 269(5228):1281–1284, Sep 1995.

[30] P. F. Robbins, M. El-Gamil, Y. F. Li, Y. Kawakami, D. Loftus, E. Appella, and S. A. Rosenberg. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med*, 183(3):1185–1192, Mar 1996.

[31] D. Brändle, F. Brasseur, P. Weynants, T. Boon, and B. Van den Eynde. A mutated hla-a2 molecule recognized by autologous cytotoxic t lymphocytes on a human renal cell carcinoma. *J Exp Med*, 183(6):2501–2508, Jun 1996.

[32] P. van der Bruggen, C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. A gene encoding an antigen recognized by cytolytic t lymphocytes on a human melanoma. *Science*, 254(5038):1643–1647, Dec 1991.

[33] Pierre G. Coulie, Benoît J. Van den Eynde, Pierre van der Bruggen, and Thierry Boon. Tumour antigens recognized by t lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer*, 14(2):135–146, Feb 2014.

[34] P. Correale, K. Walmsley, C. Nieroda, S. Zaremba, M. Zhu, J. Schlom, and K. Y. Tsang. In vitro generation of human cytotoxic t lymphocytes specific for peptides derived from prostate-specific antigen. *J Natl Cancer Inst*, 89(4):293–300, Feb 1997.

[35] Brian M. Olson, Thomas P. Frye, Laura E. Johnson, Lawrence Fong, Keith L. Knutson, Mary L. Disis, and Douglas G. McNeel. Hla-a2-restricted t-cell epitopes specific for prostatic acid phosphatase. *Cancer Immunol Immunother*, 59(6):943–953, Jun 2010.

[36] B. Fisk, T. L. Blevins, J. T. Wharton, and C. G. Ioannides. Identification of an immunodominant peptide of her-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic t lymphocyte lines. *J Exp Med*, 181(6):2109–2117, Jun 1995.

[37] P. G. Coulie, V. Brichard, A. Van Pel, T. Wölfel, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, J. P. Szikora, J. C. Renauld, and T. Boon. A new gene coding for a differentiation antigen recognized by autologous cytolytic t lymphocytes on hla-a2 melanomas. *J Exp Med*, 180(1):35–42, Jul 1994.

[38] Y. Kawakami, S. Eliyahu, K. Sakaguchi, P. F. Robbins, L. Rivoltini, J. R. Yannelli, E. Appella, and S. A. Rosenberg. Identification of the immunodominant peptides of the mart-1 human melanoma antigen recognized by the majority of hla-a2-restricted tumor infiltrating lymphocytes. *J Exp Med*, 180(1):347–352, Jul 1994.

[39] Y. Kawakami, S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki, and S. A. Rosenberg. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous t cells infiltrating into tumor. *Proc Natl Acad Sci U S A*, 91(9):3515–3519, Apr 1994.

[40] Sjoerd H. van der Burg and Cornelis J M. Melief. Therapeutic vaccination against human papilloma virus induced malignancies. *Curr Opin Immunol*, 23(2):252–257, Apr 2011.

[41] Heather M. Long, Gregory Parsonage, Christopher P. Fox, and Steven P. Lee. Immunotherapy for epstein-barr virus-associated malignancies. *Drug News Perspect*, 23(4):221–228, May 2010.

[42] Abhishek D. Garg, Aleksandra M. Dudek, and Patrizia Agostinis. Cancer immunogenicity, danger signals, and damps: what, when, and how? *Biofactors*, 39(4):355–367, 2013.

[43] Lin Zhang, Jose R. Conejo-Garcia, Dionyssios Katsaros, Phyllis A. Gimotty, Marco Massobrio, Giorgia Regnani, Antonis Makrigiannakis, Heidi Gray, Katia Schlienger, Michael N. Liebman, Stephen C. Rubin, and George Coukos. Intratumoral t cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med*, 348(3):203–213, Jan 2003.

[44] W. H. Brooks, W. R. Markesbery, G. D. Gupta, and T. L. Roszman. Relationship of lymphocyte invasion and survival of brain tumor patients. *Ann Neurol*, 4(3):219–224, Sep 1978.

[45] L. Palma, N. Di Lorenzo, and B. Guidetti. Lymphocytic infiltrates in primary glioblastomas and recidivous gliomas. incidence, fate, and relevance to prognosis in 228 operated cases. *J Neurosurg*, 49(6):854–861, Dec 1978.

[46] Franck Pagès, Anne Berger, Matthieu Camus, Fatima Sanchez-Cabo, Anne Costes, Robert Molidor, Bernhard Mlecnik, Amos Kirilovsky, Malin Nilsson, Diane Damotte, Tchao Meatchi, Patrick Bruneval, Paul-Henri Cugnenc, Zlatko Trajanoski, Wolf-Herman Fridman, and Jérôme Galon. Effector memory t cells, early metastasis, and survival in colorectal cancer. *N Engl J Med*, 353(25):2654–2666, Dec 2005.

[47] Jérôme Galon, Anne Costes, Fatima Sanchez-Cabo, Amos Kirilovsky, Bernhard Mlecnik, Christine Lagorce-Pagès, Marie Tosolini, Matthieu Camus, Anne Berger, Philippe Wind, Franck Zinzindohoué, Patrick Bruneval, Paul-Henri Cugnenc, Zlatko Trajanoski, Wolf-Herman Fridman, and Franck Pagès. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*, 313(5795):1960–1964, Sep 2006.

[48] Jérôme Galon, Franck Pagès, Francesco M. Marincola, Magdalena Thurin, Giorgio Trinchieri, Bernard A. Fox, Thomas F. Gajewski, and Paolo A. Ascierto. The immune score as a new possible approach for the classification of cancer. *J Transl Med*, 10:1, 2012.

[49] Jérôme Galon, Franck Pagès, Francesco M. Marincola, Helen K. Angell, Magdalena Thurin, Alessandro Lugli, Inti Zlobec, Anne Berger, Carlo Bifulco, Gerardo Botti, Fabiana Tatangelo, Cedrik M. Britten, Sebastian Kreiter, Lotfi Chouchane, Paolo Delrio, Hartmann Arndt, Martin Asslaber, Michele Maio, Giuseppe V. Masucci, Martin Mihm, Fernando Vidal-Vanaclocha, James P. Allison, Sacha Gnjatic, Leif Hakansson, Christoph Huber, Harpreet Singh-Jasuja, Christian Ottensmeier, Heinz Zwierzina, Luigi Laghi, Fabio Grizzi, Pamela S. Ohashi, Patricia A. Shaw, Blaise A. Clarke, Bradly G. Wouters, Yutaka Kawakami, Shoichi Hazama, Kiyotaka Okuno, Ena Wang, Jill O'Donnell-Tormey, Christine Lagorce, Graham Pawelec, Michael I.

Nishimura, Robert Hawkins, Réjean Lapointe, Andreas Lundqvist, Samir N. Khleif, Shuji Ogino, Peter Gibbs, Paul Waring, Noriyuki Sato, Toshihiko Torigoe, Kyogo Itoh, Prabhu S. Patel, Shilin N. Shukla, Richard Palmqvist, Iris D. Nagtegaal, Yili Wang, Corrado D'Arrigo, Scott Kopetz, Frank A. Sinicrope, Giorgio Trinchieri, Thomas F. Gajewski, Paolo A. Ascierto, and Bernard A. Fox. Cancer classification using the immunoscore: a worldwide task force. *J Transl Med*, 10:205, 2012.

[50] Maria-Gabriela Anitei, Guy Zeitoun, Bernhard Mlecnik, Florence Marliot, Nacilla Haicheur, Ana-Maria Todosi, Amos Kirilovsky, Christine Lagorce, Gabriela Bindea, Dan Ferariu, Mihai Danciu, Patrick Bruneval, Viorel Scripcariu, Jean-Marc Chevallier, Franck Zinzindohoué, Anne Berger, Jérôme Galon, and Franck Pagès. Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clin Cancer Res*, 20(7):1891–1899, Apr 2014.

[51] Jérôme Galon, Bernhard Mlecnik, Gabriela Bindea, Helen K. Angell, Anne Berger, Christine Lagorce, Alessandro Lugli, Inti Zlobec, Arndt Hartmann, Carlo Bifulco, Iris D. Nagtegaal, Richard Palmqvist, Giuseppe V. Masucci, Gerardo Botti, Fabiana Tatangelo, Paolo Delrio, Michele Maio, Luigi Laghi, Fabio Grizzi, Martin Asslaber, Corrado D'Arrigo, Fernando Vidal-Vanaclocha, Eva Zavadova, Lotfi Chouchane, Pamela S. Ohashi, Sara Hafezi-Bakhtiari, Bradly G. Wouters, Michael Roehrl, Linh Nguyen, Yutaka Kawakami, Shoichi Hazama, Kiyotaka Okuno, Shuji Ogino, Peter Gibbs, Paul Waring, Noriyuki Sato, Toshihiko Torigoe, Kyogo Itoh, Prabhu S. Patel, Shilin N. Shukla, Yili Wang, Scott Kopetz, Frank A. Sinicrope, Viorel Scripcariu, Paolo A. Ascierto, Francesco M. Marincola, Bernard A. Fox, and Franck Pagès. Towards the introduction of the 'immunoscore' in the classification of malignant tumours. *J Pathol*, 232(2):199–209, Jan 2014.

[52] Paolo A. Ascierto, Mariaelena Capone, Walter J. Urba, Carlo B. Bifulco, Gerardo Botti, Alessandro Lugli, Francesco M. Marincola, Gennaro Ciliberto, Jérôme Galon, and Bernard A. Fox. The additional facet of immunoscore: immunoprofiling as a possible predictive tool for cancer treatment. *J Transl Med*, 11:54, 2013.

[53] Gabriela Bindea, Bernhard Mlecnik, Helen K. Angell, and Jérôme Galon. The immune landscape of human tumors: Implications for cancer immunotherapy. *Oncoimmunology*, 3(1):e27456, Jan 2014.

[54] Wolf Herman Fridman, Franck Pagès, Catherine Sautès-Fridman, and Jérôme Galon. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*, 12(4):298–306, Apr 2012.

[55] W. B. Coley. Ii. contribution to the knowledge of sarcoma. *Ann Surg*, 14(3):199–220, Sep 1891.

[56] Kangla Tsung and Jeffrey A. Norton. Lessons from coley's toxin. *Surg Oncol*, 15(1):25–28, Jul 2006.

[57] Ratih Asmana Ningrum. Human interferon alpha-2b: a therapeutic protein for cancer treatment. *Scientifica (Cairo)*, 2014:970315, 2014.

[58] V. Brinkmann, T. Geiger, S. Alkan, and C. H. Heusser. Interferon alpha increases the frequency of interferon gamma-producing human cd4+ t cells. *J Exp Med*, 178(5):1655–1663, Nov 1993.

[59] S. M. Santini, C. Lapenta, M. Logozzi, S. Parlato, M. Spada, T. Di Pucchio, and F. Belardelli. Type i interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in hu-pbl-scid mice. *J Exp Med*, 191(10):1777–1788, May 2000.

[60] Laura Santodonato, Giuseppina D'Agostino, Roberto Nisini, Sabrina Mariotti, Domenica M. Monque, Massimo Spada, Laura Lattanzi, Maria Paola Perrone, Mauro Andreotti, Filippo Belardelli, and Maria Ferrantini. Monocyte-derived dendritic cells generated after a short-term culture with ifn-alpha and granulocyte-macrophage colony-stimulating factor stimulate a potent epstein-barr virus-specific cd8+ t cell response. *J Immunol*, 170(10):5195–5202, May 2003.

[61] Grégory Gautier, Martine Humbert, Florence Deauvieau, Mathieu Scuiller, John Hiscott, Elizabeth E M. Bates, Giorgio Trinchieri, Christophe Caux, and Pierre Garrone. A type i interferon autocrine-paracrine loop is involved in toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J Exp Med*, 201(9):1435–1446, May 2005.

[62] Khuong B. Nguyen, Wendy T. Watford, Rachelle Salomon, Sigrun R. Hofmann, Gary C. Pien, Akio Morinobu, Massimo Gadina, John J. O'Shea, and Christine A. Biron. Critical role for stat4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science*, 297(5589):2063–2066, Sep 2002.

[63] P. Marrack, J. Kappler, and T. Mitchell. Type i interferons keep activated t cells alive. *J Exp Med*, 189(3):521–530, Feb 1999.

[64] Agnes Le Bon, Vanessa Durand, Elisabeth Kamphuis, Clare Thompson, Silvia Bulfone-Paus, Cornelia Rossmann, Ulrich Kalinke, and David F. Tough. Direct stimulation of t cells by type i ifn enhances the cd8+ t cell response during cross-priming. *J Immunol*, 176(8):4682–4689, Apr 2006.

[65] Ganesh A. Kolumam, Sunil Thomas, Lucas J. Thompson, Jonathan Sprent, and Kaja Murali-Krishna. Type i interferons act directly on cd8 t cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*, 202(5):637–650, Sep 2005.

[66] Peter Aichele, Heike Unsoeld, Marie Koschella, Oliver Schweier, Ulrich Kalinke, and Smiljka Vucikuja. Cd8 t cells specific for lymphocytic choriomeningitis virus require type i ifn receptor for clonal expansion. *J Immunol*, 176(8):4525–4529, Apr 2006.

[67] Sandra Hervas-Stubbs, Jose Luis Perez-Gracia, Ana Rouzaut, Miguel F. Sanmamed, Agnes Le Bon, and Ignacio Melero. Direct effects of type i interferons on cells of the immune system. *Clin Cancer Res*, 17(9):2619–2627, May 2011.

[68] JaniceP Dutcher, DouglasJ Schwartzentruber, HowardL Kaufman, SanjivS Agarwala, AhmadA Tarhini, JamesN Lowder, and MichaelB Atkins. High dose interleukin-2 (aldesleukin) - expert consensus on best management practices-2014. *Journal for Immunotherapy of Cancer*, 2(1), 2014.

[69] A delicate balance: tweaking il-2 immunotherapy. *Nat Med*, 18(2):208–209, Feb 2012.

[70] K. A. Margolin. Interleukin-2 in the treatment of renal cancer. *Semin Oncol*, 27(2):194–203, Apr 2000.

[71] Connie Jackaman, Christine S. Bundell, Beverley F. Kinnear, Alison M. Smith, Pierre Filion, Deborah van Hagen, Bruce W S. Robinson, and Delia J. Nelson. Il-2 intratumoral immunotherapy enhances cd8+ t cells that mediate destruction of tumor cells and tumor-associated vasculature: a novel mechanism for il-2. *J Immunol*, 171(10):5051–5063, Nov 2003.

[72] Michal Podrazil, Rudolf Horvath, Etienne Becht, Daniela Rozkova, Pavla Bilkova, Klara Sochorova, Hana Hromadkova, Jana Kayserova, Katerina Vavrova, Jan Lastovicka, Petra Vrabcova, Katerina Kubackova, Zdenka Gasova, Ladislav Jarolim, Marek Babjuk, Radek Spisek, Jirina Bartunkova, and Jitka Fucikova. Phase i/ii clinical trial of dendritic-cell based immunotherapy (dcvac/pca) combined with chemotherapy in patients with metastatic, castration-resistant prostate cancer. *Oncotarget*, May 2015.

[73] Celestia S. Higano, Paul F. Schellhammer, Eric J. Small, Patrick A. Burch, John Nemunaitis, Lianng Yuh, Nicole Provost, and Mark W. Frohlich. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-t in advanced prostate cancer. *Cancer*, 115(16):3670–3679, Aug 2009.

[74] Philip W. Kantoff, Celestia S. Higano, Neal D. Shore, E Roy Berger, Eric J. Small, David F. Penson, Charles H. Redfern, Anna C. Ferrari, Robert Dreicer, Robert B. Sims, Yi Xu, Mark W. Frohlich, Paul F. Schellhammer, and I. M. P. A. C. T Study Investigators . Sipuleucel-t immunotherapy for castration-resistant prostate cancer. *N Engl J Med*, 363(5):411–422, Jul 2010.

[75] D. Hanahan and R. A. Weinberg. The hallmarks of cancer. *Cell*, 100(1):57–70, Jan 2000.

[76] Maria Grazia Borrello, Luisella Alberti, Andrew Fischer, Debora Degl'innocenti, Cristina Ferrario, Manuela Gariboldi, Federica Marchesi, Paola Allavena, Angela Greco, Paola Collini, Silvana Pilotti, Giuliana Cassinelli, Paola Bressan, Laura Fugazzola, Alberto Mantovani, and Marco A. Pierotti. Induction of a proinflammatory program in normal human thyrocytes by the ret/ptc1 oncogene. *Proc Natl Acad Sci U S A*, 102(41):14825–14830, Oct 2005.

[77] Anke Sparmann and Dafna Bar-Sagi. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell*, 6(5):447–458, Nov 2004.

[78] Sandra Rebouissou, Mohamed Amessou, Gabrielle Couchy, Karine Poussin, Sandrine Imbeaud, Camilla Pilati, Tina Izard, Charles Balabaud, Paulette Bioulac-Sage, and Jessica Zucman-Rossi. Frequent in-frame somatic deletions activate gp130 in inflammatory hepatocellular tumours. *Nature*, 457(7226):200–204, Jan 2009.

[79] J. Parsonnet, G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med*, 325(16):1127–1131, Oct 1991.

[80] A. Nomura, G. N. Stemmermann, P. H. Chyou, I. Kato, G. I. Perez-Perez, and M. J. Blaser. Helicobacter pylori infection and gastric carcinoma among japanese americans in hawaii. *N Engl J Med*, 325(16):1132–1136, Oct 1991.

[81] D. Forman, D. G. Newell, F. Fullerton, J. W. Yarnell, A. R. Stacey, N. Wald, and F. Sitas. Association between infection with helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. *BMJ*, 302(6788):1302–1305, Jun 1991.

[82] A. C. Wotherspoon, C. Ortiz-Hidalgo, M. R. Falzon, and P. G. Isaacson. Helicobacter pylori-associated gastritis and primary b-cell gastric lymphoma. *Lancet*, 338(8776):1175–1176, Nov 1991.

[83] A. C. Wotherspoon, C. Doglioni, T. C. Diss, L. Pan, A. Moschini, M. de Boni, and P. G. Isaacson. Regression of primary low-grade b-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of helicobacter pylori. *Lancet*, 342(8871):575–577, Sep 1993.

[84] Sergei I. Grivennikov, Florian R. Greten, and Michael Karin. Immunity, inflammation, and cancer. *Cell*, 140(6):883–899, Mar 2010.

[85] Hiroyuki Takahashi, Hisanobu Ogata, Reiko Nishigaki, David H. Broide, and Michael Karin. Tobacco smoke promotes lung tumorigenesis by triggering ikkbeta- and jnk1-dependent inflammation. *Cancer Cell*, 17(1):89–97, Jan 2010.

[86] Lisa M. Coussens and Zena Werb. Inflammation and cancer. *Nature*, 420(6917):860–867, 2002.

[87] Alberto Mantovani, Paola Allavena, Antonio Sica, and Frances Balkwill. Cancer-related inflammation. *Nature*, 454(7203):436–444, Jul 2008.

[88] Francesco Colotta, Paola Allavena, Antonio Sica, Cecilia Garlanda, and Alberto Mantovani. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*, 30(7):1073–1081, Jul 2009.

[89] Maria A. Hahn, Torsten Hahn, Dong-Hyun Lee, R Steven Esworthy, Byung-Wook Kim, Arthur D. Riggs, Fong-Fong Chu, and Gerd P. Pfeifer. Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. *Cancer Res*, 68(24):10280–10289, Dec 2008.

[90] Chenguang Fan, Jusan Yang, and John F. Engelhardt. Temporal pattern of nfkappab activation influences apoptotic cell fate in a stimuli-dependent fashion. *J Cell Sci*, 115(Pt 24):4843–4853, Dec 2002.

[91] Khandaker Al Zaid Siddiquee and James Turkson. Stat3 as a target for inducing apoptosis in solid and hematological tumors. *Cell Res*, 18(2):254–267, Feb 2008.

[92] Eran Elinav, Roni Nowarski, Christoph A. Thaiss, Bo Hu, Chengcheng Jin, and Richard A. Flavell. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat Rev Cancer*, 13(11):759–771, Nov 2013.

[93] Y. Tsujimoto, L. R. Finger, J. Yunis, P. C. Nowell, and C. M. Croce. Cloning of the chromosome breakpoint of neoplastic b cells with the t(14;18) chromosome translocation. *Science*, 226(4678):1097–1099, Nov 1984.

[94] J. F. Bromberg, M. H. Wrzeszczynska, G. Devgan, Y. Zhao, R. G. Pestell, C. Albanese, and JE Darnell, Jr. Stat3 as an oncogene. *Cell*, 98(3):295–303, Aug 1999.

[95] Akihisa Fukuda, Sam C. Wang, John P Morris, 4th, Alexandra E. Folias, Angela Liou, Grace E. Kim, Shizuo Akira, Kenneth M. Boucher, Matthew A. Firpo, Sean J. Mulvihill, and Matthias Hebrok. Stat3 and mmp7 contribute to pancreatic ductal adenocarcinoma initiation and progression. *Cancer Cell*, 19(4):441–455, Apr 2011.

[96] Marina Lesina, Magdalena U. Kurkowski, Katharina Ludes, Stefan Rose-John, Matthias Treiber, Günter Klöppel, Akihiko Yoshimura, Wolfgang Reindl, Bence Sipos, Shizuo Akira, Roland M. Schmid, and Hana Algül. Stat3/socs3 activation by il-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer Cell*, 19(4):456–469, Apr 2011.

[97] Sizhi Paul Gao, Kevin G. Mark, Kenneth Leslie, William Pao, Noriko Motoi, William L. Gerald, William D. Travis, William Bornmann, Darren Veach, Bayard Clarkson, and Jacqueline F. Bromberg. Mutations in the egfr kinase domain mediate stat3 activation via il-6 production in human lung adenocarcinomas. *J Clin Invest*, 117(12):3846–3856, Dec 2007.

[98] Dana M. Bronte-Tinkew, Mauricio Terebiznik, Aime Franco, Michelle Ang, Diane Ahn, Hitomi Mimuro, Chihiro Sasakawa, Mark J. Ropeleski, Richard M Peek, Jr, and Nicola L. Jones. Helicobacter pylori cytotoxin-associated gene a activates the signal transducer and activator of transcription 3 pathway in vitro and in vivo. *Cancer Res*, 69(2):632–639, Jan 2009.

[99] Olivier Micheau and Jürg Tschopp. Induction of tnf receptor i-mediated apoptosis via two sequential signaling complexes. *Cell*, 114(2):181–190, Jul 2003.

[100] Yasuo Adachi, Chieko Aoki, Naoko Yoshio-Hoshino, Koichi Takayama, David T. Curiel, and Norihiro Nishimoto. Interleukin-6 induces both cell growth and vegf production in malignant mesotheliomas. *Int J Cancer*, 119(6):1303–1311, Sep 2006.

[101] Julia Bollrath, Toby J. Phesse, Vivian A. von Burstin, Tracy Putoczki, Moritz Bennecke, Trudie Bateman, Tim Nebelsiek, Therese Lundgren-May, Ozge Canli, Sarah Schwitalla, Vance Matthews, Roland M. Schmid, Thomas Kirchner, Melek C. Arkan, Matthias Ernst, and Florian R. Greten. gp130-mediated stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell*, 15(2):91–102, Feb 2009.

[102] Sergei Grivennikov, Eliad Karin, Janos Terzic, Daniel Mucida, Guann-Yi Yu, Sivakumar Vallabhapurapu, Jürgen Scheller, Stefan Rose-John, Hilde Cheroutre, Lars Eckmann, and Michael Karin. Il-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell*, 15(2):103–113, Feb 2009.

[103] Ksenya Shchors, Elena Shchors, Fanya Rostker, Elizabeth R. Lawlor, Lamorna Brown-Swigart, and Gerard I. Evan. The myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. *Genes Dev*, 20(18):2527–2538, Sep 2006.

[104] Laura Soucek, Elizabeth R. Lawlor, Darya Soto, Ksenya Shchors, Lamorna Brown Swigart, and Gerard I. Evan. Mast cells are required for angiogenesis and macroscopic expansion of myc-induced pancreatic islet tumors. *Nat Med*, 13(10):1211–1218, Oct 2007.

[105] Elaine Y. Lin, Jiu-Feng Li, Leoid Gnatovskiy, Yan Deng, Liyin Zhu, Dustin A. Grzesik, Hong Qian, Xiao-nan Xue, and Jeffrey W. Pollard. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res*, 66(23):11238–11246, Dec 2006.

[106] Enrico Giraudo, Masahiro Inoue, and Douglas Hanahan. An amino-bisphosphonate targets mmp-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J Clin Invest*, 114(5):623–633, Sep 2004.

[107] Rose Du, Kan V. Lu, Claudia Petritsch, Patty Liu, Ruth Ganss, Emmanuelle Passegué, Hanqiu Song, Scott Vandenberg, Randall S. Johnson, Zena Werb, and Gabriele Bergers. Hif1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell*, 13(3):206–220, Mar 2008.

[108] L. Bingle, C. E. Lewis, K. P. Corke, M W R. Reed, and N. J. Brown. Macrophages promote angiogenesis in human breast tumour spheroids in vivo. *Br J Cancer*, 94(1):101–107, Jan 2006.

[109] Farbod Shojaei, Xiumin Wu, Cuiling Zhong, Lanlan Yu, Xiao-Huan Liang, Jenny Yao, Dominique Blanchard, Carlos Bais, Franklin V. Peale, Nicholas van Bruggen, Calvin Ho, Jed Ross, Martha Tan, Richard A D. Carano, Y Gloria Meng, and Napoleone Ferrara. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature*, 450(7171):825–831, Dec 2007.

[110] Lee B. Rivera and Gabriele Bergers. Intertwined regulation of angiogenesis and immunity by myeloid cells. *Trends Immunol*, 36(4):240–249, Apr 2015.

[111] Elaine Y. Lin, Jiu-feng Li, Gabriel Bricard, Weigang Wang, Yan Deng, Rani Sellers, Steven A. Porcelli, and Jeffrey W. Pollard. Vascular endothelial growth factor restores delayed tumor progression in tumors depleted of macrophages. *Mol Oncol*, 1(3):288–302, Dec 2007.

[112] Stephanie M. Pyonteck, Leila Akkari, Alberto J. Schuhmacher, Robert L. Bowman, Lisa Sevenich, Daniela F. Quail, Oakley C. Olson, Marsha L. Quick, Jason T. Huse, Virginia Teijeiro, Manu Setty, Christina S. Leslie, Yoko Oei, Alicia Pedraza, Jianan Zhang, Cameron W. Brennan, James C. Sutton, Eric C. Holland, Dylan Daniel, and Johanna A. Joyce. Csf-1r inhibition alters macrophage polarization and blocks glioma progression. *Nat Med*, 19(10):1264–1272, Oct 2013.

[113] Luke C. Davies, Stephen J. Jenkins, Judith E. Allen, and Philip R. Taylor. Tissue-resident macrophages. *Nat Immunol*, 14(10):986–995, Oct 2013.

[114] Peter J. Murray, Judith E. Allen, Subhra K. Biswas, Edward A. Fisher, Derek W. Gilroy, Sergij Goerdt, Siamon Gordon, John A. Hamilton, Lionel B. Ivashkiv, Toby Lawrence, Massimo Locati, Alberto Mantovani, Fernando O. Martinez, Jean-Louis Mege, David M. Mosser, Gioacchino Natoli, Jeroen P. Saeij, Joachim L. Schultze, Kari Ann Shirey, Antonio Sica, Jill Suttles, Irina Udalova, Jo A. van Ginderachter, Stefanie N. Vogel, and Thomas A. Wynn. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, 41(1):14–20, Jul 2014.

[115] Yoshihiro Komohara, Masahisa Jinushi, and Motohiro Takeya. Clinical significance of macrophage heterogeneity in human malignant tumors. *Cancer Sci*, 105(1):1–8, Jan 2014.

[116] Moniek Heusinkveld and Sjoerd H. van der Burg. Identification and manipulation of tumor associated macrophages in human cancers. *J Transl Med*, 9:216, 2011.

[117] E. Zeni, L. Mazzetti, D. Miotto, N. Lo Cascio, P. Maestrelli, P. Querzoli, M. Pedriali, E. De Rosa, L. M. Fabbri, C. E. Mapp, and P. Boschetto. Macrophage expression of interleukin-10 is a prognostic factor in nonsmall cell lung cancer. *Eur Respir J*, 30(4):627–632, Oct 2007.

[118] C. M. Ohri, A. Shikotra, R. H. Green, D. A. Waller, and P. Bradding. Macrophages within nsclc tumour islets are predominantly of a cytotoxic m1 phenotype associated with extended survival. *Eur Respir J*, 33(1):118–126, Jan 2009.

[119] Gabriela Bindea, Bernhard Mlecnik, Marie Tosolini, Amos Kirilovsky, Maximilian Waldner, Anna C. Obenauf, Helen Angell, Tessa Fredriksen, Lucie Lafontaine, Anne Berger, Patrick Bruneval, Wolf Herman Fridman, Christoph Becker, Franck Pagès, Michael R. Speicher, Zlatko Trajanoski, and Jérôme Galon. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity*, 39(4):782–795, Oct 2013.

[120] Gottfrid Sjödahl, Kristina Lövgren, Martin Lauss, Gunilla Chebil, Oliver Patschan, Sigurdur Gudjonsson, Wiking Månsson, Mårten Fernö, Karin Leandersson, David Lindgren, Fredrik Liedberg, and Mattias Höglund. Infiltration of cd3+ and cd68+ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. *Urol Oncol*, 32(6):791–797, Aug 2014.

[121] David G. DeNardo, Donal J. Brennan, Elton Rexhepaj, Brian Ruffell, Stephen L. Shiao, Stephen F. Madden, William M. Gallagher, Nikhil Wadhwani, Scott D. Keil, Sharfaa A. Junaid, Hope S. Rugo, E Shelley Hwang, Karin Jirström, Brian L. West, and Lisa M. Coussens. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*, 1(1):54–67, Jun 2011.

[122] Enrico Flossmann, Peter M. Rothwell, British Doctors Aspirin Trial , and the UK-TIA Aspirin Trial. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet*, 369(9573):1603–1613, May 2007.

[123] Annemijn M. Algra and Peter M. Rothwell. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol*, 13(5):518–527, May 2012.

[124] Andrew T. Chan, Shuji Ogino, and Charles S. Fuchs. Aspirin and the risk of colorectal cancer in relation to the expression of cox-2. *N Engl J Med*, 356(21):2131–2142, May 2007.

[125] Gretchen L. Gierach, James V Lacey, Jr, Arthur Schatzkin, Michael F. Leitzmann, Douglas Richesson, Albert R. Hollenbeck, and Louise A. Brinton. Nonsteroidal anti-inflammatory drugs and breast cancer risk in the national institutes of health-aarp diet and health study. *Breast Cancer Res*, 10(2):R38, 2008.

[126] Mangesh A. Thorat and Jack Cuzick. Role of aspirin in cancer prevention. *Curr Oncol Rep*, 15(6):533–540, Dec 2013.

[127] Frances Balkwill. Tumour necrosis factor and cancer. *Nat Rev Cancer*, 9(5):361–371, May 2009.

[128] Monica Bernal, Francisco Ruiz-Cabello, Angel Concha, Annette Paschen, and Federico Garrido. Implication of the β2-microglobulin gene in the generation of tumor escape phenotypes. *Cancer Immunol Immunother*, 61(9):1359–1371, Sep 2012.

[129] Sophia Platonova, Julien Cherfils-Vicini, Diane Damotte, Lucile Crozet, Vincent Vieillard, Pierre Validire, Pascale André, Marie-Caroline Dieu-Nosjean, Marco Alifano, Jean-François Régnard, Wolf-Herman Fridman, Catherine Sautès-Fridman, and Isabelle Cremer. Profound coordinated alterations of intratumoral nk cell phenotype and function in lung carcinoma. *Cancer Res*, 71(16):5412–5422, Aug 2011.

[130] Sarah S. Donatelli, Jun-Min Zhou, Danielle L. Gilvary, Erika A. Eksioglu, Xianghong Chen, W Douglas Cress, Eric B. Haura, Matthew B. Schabath, Domenico Coppola, Sheng Wei, and Julie Y. Djeu. Tgf-β-inducible microrna-183 silences tumor-associated natural killer cells. *Proc Natl Acad Sci U S A*, 111(11):4203–4208, Mar 2014.

[131] Gabriella Pietra, Claudia Manzini, Silvia Rivara, Massimo Vitale, Claudia Cantoni, Andrea Petretto, Mirna Balsamo, Romana Conte, Roberto Benelli, Simona Minghelli, Nicola Solari, Marina Gualco, Paola Queirolo, Lorenzo Moretta, and Maria Cristina Mingari. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res*, 72(6):1407–1415, Mar 2012.

[132] Trevor E. Angell, Melissa G. Lechner, Julie K. Jang, Jonathan S. LoPresti, and Alan L. Epstein. Mhc class i loss is a frequent mechanism of immune escape in papillary thyroid cancer that is reversed by interferon and selumetinib treatment in vitro. *Clin Cancer Res*, 20(23):6034–6044, Dec 2014.

[133] A. Riker, J. Cormier, M. Panelli, U. Kammula, E. Wang, A. Abati, P. Fetsch, K. H. Lee, S. Steinberg, S. Rosenberg, and F. Marincola. Immune selection after antigen-specific immunotherapy of melanoma. *Surgery*, 126(2):112–120, Aug 1999.

[134] Christian Blank, Ian Brown, Amy C. Peterson, Mike Spiotto, Yoshiko Iwai, Tasuku Honjo, and Thomas F. Gajewski. Pd-l1/b7h-1 inhibits the effector phase of tumor rejection by t cell receptor (tcr) transgenic cd8+ t cells. *Cancer Res*, 64(3):1140–1145, Feb 2004.

[135] LauraL. Carter, Lynette A. Fouser, Jason Jussif, Lori Fitz, Bija Deng, Clive R. Wood, Mary Collins, Tasuku Honjo, Gordon J. Freeman, and Beatriz M. Carreno. Pd-1:pd-l inhibitory pathway affects both cd4(+) and cd8(+) t cells and is overcome by il-2. *Eur J Immunol*, 32(3):634–643, Mar 2002.

[136] G. J. Freeman, A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, and T. Honjo. Engagement of the pd-1 immunoinhibitory receptor by a novel b7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*, 192(7):1027–1034, Oct 2000.

[137] Melissa M. Mazanet and Christopher C W. Hughes. B7-h1 is expressed by human endothelial cells and suppresses t cell cytokine synthesis. *J Immunol*, 169(7):3581–3588, Oct 2002.

[138] Sang-Keun Lee, Sam-Hwa Seo, Byoung-Soo Kim, Chang-Deok Kim, Jeung-Hoon Lee, Jung-Soo Kang, Pil Jae Maeng, and Jong-Soon Lim. Ifn-gamma regulates the expression of b7-h1 in dermal fibroblast cells. *J Dermatol Sci*, 40(2):95–103, Nov 2005.

[139] D. B. Chappell and N. P. Restifo. T cell-tumor cell: a fatal interaction? *Cancer Immunol Immunother*, 47(2):65–71, Oct 1998.

[140] J. O'Connell, G. C. O'Sullivan, J. K. Collins, and F. Shanahan. The fas counterattack: Fas-mediated t cell killing by colon cancer cells expressing fas ligand. *J Exp Med*, 184(3):1075–1082, Sep 1996.

[141] F. H. Igney, C. K. Behrens, and P. H. Krammer. Tumor counterattack–concept and reality. *Eur J Immunol*, 30(3):725–731, Mar 2000.

[142] Frederik H. Igney and Peter H. Krammer. Tumor counterattack: fact or fiction? *Cancer Immunol Immunother*, 54(11):1127–1136, Nov 2005.

[143] Dmitry I. Gabrilovich and Srinivas Nagaraj. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*, 9(3):162–174, Mar 2009.

[144] Gabriel A. Rabinovich, Dmitry Gabrilovich, and Eduardo M. Sotomayor. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol*, 25:267–296, 2007.

[145] Ali Chahlavi, Patricia Rayman, Amy L. Richmond, Kaushik Biswas, Renliang Zhang, Michael Vogelbaum, Charles Tannenbaum, Gene Barnett, and James H. Finke. Glioblastomas induce t-lymphocyte death by two distinct pathways involving gangliosides and cd70. *Cancer Res*, 65(12):5428–5438, Jun 2005.

[146] M. Hahne, D. Rimoldi, M. Schröter, P. Romero, M. Schreier, L. E. French, P. Schneider, T. Bornand, A. Fontana, D. Lienard, J. Cerottini, and J. Tschopp. Melanoma cell expression of fas(apo-1/cd95) ligand: implications for tumor immune escape. *Science*, 274(5291):1363–1366, Nov 1996.

[147] Alexander Greenhough, Helena J M. Smartt, Amy E. Moore, Heather R. Roberts, Ann C. Williams, Christos Paraskeva, and Abderrahmane Kaidi. The cox-2/pge2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis*, 30(3):377–386, Mar 2009.

[148] P. Chomarat, J. Banchereau, J. Davoust, and A. K. Palucka. Il-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol*, 1(6):510–514, Dec 2000.

[149] Nicolas A. Giraldo, Etienne Becht, Franck Pagès, Georgios Skliris, Virginie Verkarre, Yann Vano, Arnaud Mejean, Nicolas Saint-Aubert, Laetitia Lacroix, Ivo Natario, Audrey Lupo, Marco Alifano, Diane Damotte, Aurelie Cazes, Frederic Triebel, Gordon J. Freeman, Marie-Caroline Dieu-Nosjean, Stephane Oudard, Wolf H. Fridman, and Catherine Sautès-Fridman. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. *Clin Cancer Res*, 21(13):3031–3040, Jul 2015.

[150] John J. Engelhardt, Bijan Boldajipour, Peter Beemiller, Priya Pandurangi, Caitlin Sorensen, Zena Werb, Mikala Egeblad, and Matthew F. Krummel. Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific t cells. *Cancer Cell*, 21(3):402–417, Mar 2012.

[151] J. Alcalay and M. L. Kripke. Antigen-presenting activity of draining lymph node cells from mice painted with a contact allergen during ultraviolet carcinogenesis. *J Immunol*, 146(6):1717–1721, Mar 1991.

[152] M. P. Tas, P. J. Simons, F. J. Balm, and H. A. Drexhage. Depressed monocyte polarization and clustering of dendritic cells in patients with head and neck cancer: in vitro restoration of this immunosuppression by thymic hormones. *Cancer Immunol Immunother*, 36(2):108–114, 1993.

[153] D. I. Gabrilovich, S. Nadaf, J. Corak, J. A. Berzofsky, and D. P. Carbone. Dendritic cells in antitumor immune responses. ii. dendritic cells grown from bone marrow precursors, but not mature dc from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. *Cell Immunol*, 170(1):111–119, May 1996.

[154] P. Chaux, M. Moutet, J. Faivre, F. Martin, and M. Martin. Inflammatory cells infiltrating human colorectal carcinomas express hla class ii but not b7-1 and b7-2 costimulatory molecules of the t-cell activation. *Lab Invest*, 74(5):975–983, May 1996.

[155] T. Oyama, S. Ran, T. Ishida, S. Nadaf, L. Kerr, D. P. Carbone, and D. I. Gabrilovich. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa b activation in hemopoietic progenitor cells. *J Immunol*, 160(3):1224–1232, Feb 1998.

[156] Douglas R. Green, Thomas Ferguson, Laurence Zitvogel, and Guido Kroemer. Immunogenic and tolerogenic cell death. *Nat Rev Immunol*, 9(5):353–363, May 2009.

[157] Noelia Casares, Marie O. Pequignot, Antoine Tesniere, François Ghiringhelli, Stéphan Roux, Nathalie Chaput, Elise Schmitt, Ahmed Hamai, Sandra Hervas-Stubbs, Michel Obeid, Frédéric Coutant, Didier Métivier, Evelyne Pichard, Pierre Aucouturier, Gérard Pierron, Carmen Garrido, Laurence Zitvogel, and Guido Kroemer. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med*, 202(12):1691–1701, Dec 2005.

[158] Augusto C. Ochoa, Arnold H. Zea, Claudia Hernandez, and Paulo C. Rodriguez. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res*, 13(2 Pt 2):721s–726s, Jan 2007.

[159] Paulo C. Rodriguez, Claudia P. Hernandez, David Quiceno, Steven M. Dubinett, Jovanny Zabaleta, Juan B. Ochoa, Jill Gilbert, and Augusto C. Ochoa. Arginase i in myeloid suppressor cells is induced by cox-2 in lung carcinoma. *J Exp Med*, 202(7):931–939, Oct 2005.

[160] Magali Terme, Simon Pernot, Elie Marcheteau, Federico Sandoval, Nadine Benhamouda, Orianne Colussi, Olivier Dubreuil, Antoine F. Carpentier, Eric Tartour, and Julien Taieb. Vegfa-vegfr pathway blockade inhibits tumor-induced regulatory t-cell proliferation in colorectal cancer. *Cancer Res*, 73(2):539–549, Jan 2013.

[161] Jürgen C. Becker, Mads Hald Andersen, David Schrama, and Per Thor Straten. Immune-suppressive properties of the tumor microenvironment. *Cancer Immunol Immunother*, 62(7):1137–1148, Jul 2013.

[162] Andrea Facciabene, Xiaohui Peng, Ian S. Hagemann, Klara Balint, Andrea Barchetti, Li-Ping Wang, Phyllis A. Gimotty, C Blake Gilks, Priti Lal, Lin Zhang, and George Coukos. Tumour hypoxia promotes tolerance and angiogenesis via ccl28 and t(reg) cells. *Nature*, 475(7355):226–230, Jul 2011.

[163] Kiavash Movahedi, Damya Laoui, Conny Gysemans, Martijn Baeten, Geert Stangé, Jan Van den Bossche, Matthias Mack, Daniel Pipeleers, Peter In't Veld, Patrick De Baetselier, and Jo A. Van Ginderachter. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from ly6c(high) monocytes. *Cancer Res*, 70(14):5728–5739, Jul 2010.

[164] Thibault Voron, Orianne Colussi, Elie Marcheteau, Simon Pernot, Mevyn Nizard, Anne-Laure Pointet, Sabrina Latreche, Sonia Bergaya, Nadine Benhamouda, Corinne Tanchot, Christian Stockmann, Pierre Combe, Anne Berger, Franck Zinzindohoue, Hideo Yagita, Eric Tartour, Julien Taieb, and Magali Terme. Vegf-a modulates expression of inhibitory checkpoints on cd8+ t cells in tumors. *J Exp Med*, 212(2):139–148, Feb 2015.

[165] Hélène Salmon, Katarzyna Franciszkiewicz, Diane Damotte, Marie-Caroline Dieu-Nosjean, Pierre Validire, Alain Trautmann, Fathia Mami-Chouaib, and Emmanuel Donnadieu. Matrix architecture defines the preferential localization and migration of t cells into the stroma of human lung tumors. *J Clin Invest*, 122(3):899–910, Mar 2012.

[166] Matthew Kraman, Paul J. Bambrough, James N. Arnold, Edward W. Roberts, Lukasz Magiera, James O. Jones, Aarthi Gopinathan, David A. Tuveson, and Douglas T. Fearon. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science*, 330(6005):827–830, Nov 2010.

[167] Sylvie Séguier, Eric Tartour, Coralie Guérin, Ludovic Couty, Mathilde Lemitre, Laetitia Lallement, Marysette Folliguet, Samah El Naderi, Magali Terme, Cécile Badoual, Antoine Lafont, and Bernard Coulomb. Inhibition of the differentiation of monocyte-derived dendritic cells by human gingival fibroblasts. *PLoS One*, 8(8):e70937, 2013.

[168] Simon Jones, Nicole Horwood, Andrew Cope, and Francesco Dazzi. The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells. *J Immunol*, 179(5):2824–2831, Sep 2007.

[169] Chiara Bocelli-Tyndall, Andrea Barbero, Christian Candrian, Rhodri Ceredig, Alan Tyndall, and Ivan Martin. Human articular chondrocytes suppress in vitro proliferation of anti-cd3 activated peripheral blood mononuclear cells. *J Cell Physiol*, 209(3):732–734, Dec 2006.

[170] Muzlifah A. Haniffa, Xiao-Nong Wang, Udo Holtick, Michelle Rae, John D. Isaacs, Anne M. Dickinson, Catharien M U. Hilkens, and Matthew P. Collin. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. *J Immunol*, 179(3):1595–1604, Aug 2007.

[171] Irina V. Pinchuk, Jamal I. Saada, Ellen J. Beswick, Gushyalatha Boya, Sumin M. Qiu, Randy C. Mifflin, Gottumukkala S. Raju, Victor E. Reyes, and Don W. Powell. Pd-1 ligand expression by human colonic myofibroblasts/fibroblasts regulates cd4+ t-cell activity. *Gastroenterology*, 135(4):1228–1237, 1237.e1–2, Oct 2008.

[172] Giao Q. Phan, James C. Yang, Richard M. Sherry, Patrick Hwu, Suzanne L. Topalian, Douglas J. Schwartzentruber, Nicholas P. Restifo, Leah R. Haworth, Claudia A. Seipp, Linda J. Freezer, Kathleen E. Morton, Sharon A. Mavroukakis, Paul H. Duray, Seth M. Steinberg, James P. Allison, Thomas A. Davis, and Steven A. Rosenberg. Cancer regression and autoimmunity induced by cytotoxic t lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*, 100(14):8372–8377, Jul 2003.

[173] Antoni Ribas, Luis H. Camacho, Gabriel Lopez-Berestein, Dmitri Pavlov, Cecile A. Bulanhagui, Robert Millham, Begoña Comin-Anduix, James M. Reuben, Elisabeth Seja, Charla A. Parker, Amarnath Sharma, John A. Glaspy, and Jesus Gomez-Navarro. Antitumor activity in melanoma and anti-self responses in a phase i trial with the anti-cytotoxic t lymphocyte-associated antigen 4 monoclonal antibody cp-675,206. *J Clin Oncol*, 23(35):8968–8977, Dec 2005.

[174] Kimberly E. Beck, Joseph A. Blansfield, Khoi Q. Tran, Andrew L. Feldman, Marybeth S. Hughes, Richard E. Royal, Udai S. Kammula, Suzanne L. Topalian, Richard M. Sherry, David Kleiner, Martha Quezado, Israel Lowy, Michael Yellin, Steven A. Rosenberg, and James C. Yang. Enterocolitis in patients with cancer after antibody blockade of cytotoxic t-lymphocyte-associated antigen 4. *J Clin Oncol*, 24(15):2283–2289, May 2006.

[175] Antoni Ribas. Clinical development of the anti-ctla-4 antibody tremelimumab. *Semin Oncol*, 37(5):450–454, Oct 2010.

[176] F Stephen Hodi, Steven J. O'Day, David F. McDermott, Robert W. Weber, Jeffrey A. Sosman, John B. Haanen, Rene Gonzalez, Caroline Robert, Dirk Schadendorf, Jessica C. Hassel, Wallace Akerley, Alfons J M. van den Eertwegh, Jose Lutzky, Paul Lorigan, Julia M. Vaubel, Gerald P. Linette, David Hogg, Christian H. Ottensmeier, Celeste Lebbé, Christian Peschel, Ian Quirt, Joseph I. Clark, Jedd D. Wolchok, Jeffrey S. Weber, Jason Tian, Michael J. Yellin, Geoffrey M. Nichol, Axel Hoos, and Walter J. Urba. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*, 363(8):711–723, Aug 2010.

[177] Julie R. Brahmer, Charles G. Drake, Ira Wollner, John D. Powderly, Joel Picus, William H. Sharfman, Elizabeth Stankevich, Alice Pons, Theresa M. Salay, Tracee L. McMiller, Marta M. Gilson, Changyu Wang, Mark Selby, Janis M. Taube, Robert Anders, Lieping Chen, Alan J. Korman, Drew M. Pardoll, Israel Lowy, and Suzanne L. Topalian. Phase i study of single-agent anti-programmed death-1 (mdx-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*, 28(19):3167–3175, Jul 2010.

[178] Stephen M. Ansell, Alexander M. Lesokhin, Ivan Borrello, Ahmad Halwani, Emma C. Scott, Martin Gutierrez, Stephen J. Schuster, Michael M. Millenson, Deepika Cattry, Gordon J. Freeman, Scott J. Rodig, Bjoern Chapuy, Azra H. Ligon, Lili Zhu, Joseph F. Grosso, Su Young Kim, John M. Timmerman, Margaret A.

Shipp, and Philippe Armand. Pd-1 blockade with nivolumab in relapsed or refractory hodgkin's lymphoma. *N Engl J Med*, 372(4):311–319, Jan 2015.

[179] Dung T. Le, Jennifer N. Uram, Hao Wang, Bjarne R. Bartlett, Holly Kemberling, Aleksandra D. Eyring, Andrew D. Skora, Brandon S. Luber, Nilofer S. Azad, Dan Laheru, Barbara Biedrzycki, Ross C. Donehower, Atif Zaheer, George A. Fisher, Todd S. Crocenzi, James J. Lee, Steven M. Duffy, Richard M. Goldberg, Albert de la Chapelle, Minori Koshiji, Feriyl Bhaijee, Thomas Huebner, Ralph H. Hruban, Laura D. Wood, Nathan Cuka, Drew M. Pardoll, Nickolas Papadopoulos, Kenneth W. Kinzler, Shibin Zhou, Toby C. Cornish, Janis M. Taube, Robert A. Anders, James R. Eshleman, Bert Vogelstein, and Luis A Diaz, Jr. Pd-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med*, 372(26):2509–2520, Jun 2015.

[180] Raanan Berger, Rinat Rotem-Yehudar, Gideon Slama, Shimon Landes, Abraham Kneller, Merav Leiba, Maya Koren-Michowitz, Avichai Shimoni, and Arnon Nagler. Phase i safety and pharmacokinetic study of ct-011, a humanized antibody interacting with pd-1, in patients with advanced hematologic malignancies. *Clin Cancer Res*, 14(10):3044–3051, May 2008.

[181] Philippe Armand, Arnon Nagler, Edie A. Weller, Steven M. Devine, David E. Avigan, Yi-Bin Chen, Mark S. Kaminski, H Kent Holland, Jane N. Winter, James R. Mason, Joseph W. Fay, David A. Rizzieri, Chitra M. Hosing, Edward D. Ball, Joseph P. Uberti, Hillard M. Lazarus, Markus Y. Mapara, Stephanie A. Gregory, John M. Timmerman, David Andorsky, Reuven Or, Edmund K. Waller, Rinat Rotem-Yehudar, and Leo I. Gordon. Disabling immune tolerance by programmed death-1 blockade with pidilizumab after autologous hematopoietic stem-cell transplantation for diffuse large b-cell lymphoma: results of an international phase ii trial. *J Clin Oncol*, 31(33):4199–4206, Nov 2013.

[182] Jason R. Westin, Fuliang Chu, Min Zhang, Luis E. Fayad, Larry W. Kwak, Nathan Fowler, Jorge Romaguera, Fredrick Hagemeister, Michelle Fanale, Felipe Samaniego, Lei Feng, Veerabhadran Baladandayuthapani, Zhiqiang Wang, Wencai Ma, Yanli Gao, Michael Wallace, Luis M. Vence, Laszlo Radvanyi, Tariq Muzzafar, Rinat Rotem-Yehudar, R Eric Davis, and Sattva S. Neelapu. Safety and activity of pd1 blockade by pidilizumab in combination with rituximab in patients with relapsed follicular lymphoma: a single group, open-label, phase 2 trial. *Lancet Oncol*, 15(1):69–77, Jan 2014.

[183] Drew M. Pardoll. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*, 12(4):252–264, Apr 2012.

[184] Alexander M. Lesokhin, Margaret K. Callahan, Michael A. Postow, and Jedd D. Wolchok. On being less tolerant: enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of t cell activation. *Sci Transl Med*, 7(280):280sr1, Mar 2015.

[185] Julie R. Brahmer, Scott S. Tykodi, Laura Q M. Chow, Wen-Jen Hwu, Suzanne L. Topalian, Patrick Hwu, Charles G. Drake, Luis H. Camacho, John Kauh, Kunle Odunsi, Henry C. Pitot, Omid Hamid, Shailender Bhatia, Renato Martins, Keith Eaton, Shuming Chen, Theresa M. Salay, Suresh Alaparthy, Joseph F. Grosso, Alan J. Korman, Susan M. Parker, Shruti Agrawal, Stacie M. Goldberg, Drew M. Pardoll, Ashok Gupta, and Jon M. Wigginton. Safety and activity of anti-pd-l1

antibody in patients with advanced cancer. *N Engl J Med*, 366(26):2455–2465, Jun 2012.

[186] Roy S. Herbst, Jean-Charles Soria, Marcin Kowanetz, Gregg D. Fine, Omid Hamid, Michael S. Gordon, Jeffery A. Sosman, David F. McDermott, John D. Powderly, Scott N. Gettinger, Holbrook E K. Kohrt, Leora Horn, Donald P. Lawrence, Sandra Rost, Maya Leabman, Yuanyuan Xiao, Ahmad Mokatrin, Hartmut Koeppen, Priti S. Hegde, Ira Mellman, Daniel S. Chen, and F Stephen Hodi. Predictive correlates of response to the anti-pd-l1 antibody mpdl3280a in cancer patients. *Nature*, 515(7528):563–567, Nov 2014.

[187] Thomas Powles, Joseph Paul Eder, Gregg D. Fine, Fadi S. Braiteh, Yohann Loriot, Cristina Cruz, Joaquim Bellmunt, Howard A. Burris, Daniel P. Petrylak, Siew-leng Teng, Xiaodong Shen, Zachary Boyd, Priti S. Hegde, Daniel S. Chen, and Nicholas J. Vogelzang. Mpdl3280a (anti-pd-l1) treatment leads to clinical activity in metastatic bladder cancer. *Nature*, 515(7528):558–562, Nov 2014.

[188] Stefan Löb, Alfred Königsrainer, Derek Zieker, Björn L D M. Brücher, Hans-Georg Rammensee, Gerhard Opelz, and Peter Terness. Ido1 and ido2 are expressed in human tumors: levo- but not dextro-1-methyl tryptophan inhibits tryptophan catabolism. *Cancer Immunol Immunother*, 58(1):153–157, Jan 2009.

[189] Cristina Iclozan, Scott Antonia, Alberto Chiappori, Dung-Tsa Chen, and Dmitry Gabrilovich. Therapeutic regulation of myeloid-derived suppressor cells and immune response to cancer vaccine in patients with extensive stage small cell lung cancer. *Cancer Immunol Immunother*, 62(5):909–918, May 2013.

[190] Yana G. Najjar and James H. Finke. Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer. *Front Oncol*, 3:49, 2013.

[191] Eric Tartour, H. Pere, B. Maillere, M. Terme, N. Merillon, J. Taieb, F. Sandoval, F. Quintin-Colonna, K. Lacerda, A. Karadimou, C. Badoual, A. Tedgui, W. H. Fridman, and S. Oudard. Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. *Cancer Metastasis Rev*, 30(1):83–95, Mar 2011.

[192] John Maher, Renier J. Brentjens, Gertrude Gunset, Isabelle Rivière, and Michel Sadelain. Human t-lymphocyte cytotoxicity and proliferation directed by a single chimeric tcrzeta /cd28 receptor. *Nat Biotechnol*, 20(1):70–75, Jan 2002.

[193] Shivani Srivastava and Stanley R. Riddell. Engineering car-t cells: Design concepts. *Trends Immunol*, Jul 2015.

[194] The International Agency for Research on Cancer. *Pathology and Genetics of Tumours of Soft Tissue and Bone (IARC WHO Classification of Tumours)*. World Health Organization, 2006.

[195] The International Agency for Research on Cancer. *Pathology and Genetics of Skin Tumours (IARC WHO Classification of Tumours)*. World Health Organization, 2005.

[196] The International Agency for Research on Cancer. *Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs (IARC WHO Classification of Tumours)*. World Health Organization, 2004.

[197] The International Agency for Research on Cancer. *Pathology and Genetics of Head and Neck Tumours (IARC WHO Classification of Tumours)*. World Health Organization, 2005.

[198] The International Agency for Research on Cancer. *Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart (IARC WHO Classification of Tumours)*. World Health Organization, 2004.

[199] The International Agency for Research on Cancer. *Pathology and Genetics of Tumours of the Digestive System*. IARC Press, 2000.

[200] The International Agency for Research on Cancer. *Pathology and Genetics of Tumours of the Breast and Female Genital Organs (IARC WHO Classification of Tumours)*. World Health Organization, 2003.

[201] N. Howlader, A. M. Noone, M. Krapcho, J. Garshell, N. Neyman, S. F. Altekruse, C. L. Kosary, M. Yu, J. Ruhl, Z. Tatalovich, H. Cho, A. Mariotto, Chen H. S. Lewis DR, and Feuer EJ. Seer cancer statistics review, 1975-2010, national cancer institute. bethesda, md, http://seer.cancer.gov/csr/1975_2010/, based on november 2012 seer data submission, posted to the seer web site, 2013.

[202] *TNM Classification of Malignant Tumours*. Wiley-Blackwell, 2009.

[203] Britta Weigelt, Felipe C. Geyer, and Jorge S. Reis-Filho. Histological types of breast cancer: how special are they? *Mol Oncol*, 4(3):192–208, Jun 2010.

[204] Justin Pijpe, Gerben H Torn Broers, Boudewijn E Ch Plaat, M. Hundeiker, F. Otto, Mirjam F. Mastik, Harald J. Hoekstra, Winette T A. van der Graaf, Eva van Den Berg, and Willemina M. Molenaar. The relation between histological, tumorbiological and clinical parameters in deep and superficial leiomyosarcoma and leiomyoma. *Sarcoma*, 6(3):105–110, 2002.

[205] Hiromitsu Hatakeyama, Tadashi Kondo, Kiyonaga Fujii, Yukihiro Nakanishi, Hoichi Kato, Satoshi Fukuda, and Setsuo Hirohashi. Protein clusters associated with carcinogenesis, histological differentiation and nodal metastasis in esophageal cancer. *Proteomics*, 6(23):6300–6316, Dec 2006.

[206] G. N. CALKINS. Zur frage der entstehung maligner tumoren. *Science*, 40(1041):857–859, Dec 1914.

[207] O. T. Avery, C. M. Macleod, and M. McCarty. Studies on the chemical nature of the substance inducing transformation of pneumococcal types : Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type iii. *J Exp Med*, 79(2):137–158, Feb 1944.

[208] T. G. Krontiris and G. M. Cooper. Transforming activity of human tumor dnas. *Proc Natl Acad Sci U S A*, 78(2):1181–1184, Feb 1981.

[209] C. Shih, L. C. Padhy, M. Murray, and R. A. Weinberg. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature*, 290(5803):261–264, Mar 1981.

[210] J. D. Rowley. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and giemsa staining. *Nature*, 243(5405):290–293, Jun 1973.

[211] E. P. Reddy, R. K. Reynolds, E. Santos, and M. Barbacid. A point mutation is responsible for the acquisition of transforming properties by the t24 human bladder carcinoma oncogene. *Nature*, 300(5888):149–152, Nov 1982.

[212] C. J. Tabin, S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, and E. H. Chang. Mechanism of activation of a human oncogene. *Nature*, 300(5888):143–149, Nov 1982.

[213] Cristian Tomasetti and Bert Vogelstein. Cancer etiology. variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science*, 347(6217):78–81, Jan 2015.

[214] Michael R. Stratton, Peter J. Campbell, and P Andrew Futreal. The cancer genome. *Nature*, 458(7239):719–724, Apr 2009.

[215] Sylvanie Surget, Marie P. Khoury, and Jean-Christophe Bourdon. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. *Onco Targets Ther*, 7:57–68, 2013.

[216] N. S. Fearnhead, M. P. Britton, and W. F. Bodmer. The abc of apc. *Hum Mol Genet*, 10(7):721–733, Apr 2001.

[217] Brian J. Druker. Translation of the philadelphia chromosome into therapy for cml. *Blood*, 112(13):4808–4817, Dec 2008.

[218] Thuy Vu and Francois X. Claret. Trastuzumab: updated mechanisms of action and resistance in breast cancer. *Front Oncol*, 2:62, 2012.

[219] Astrid Lièvre, Jean-Baptiste Bachet, Delphine Le Corre, Valérie Boige, Bruno Landi, Jean-François Emile, Jean-François Côté, Gorana Tomasic, Christophe Penna, Michel Ducreux, Philippe Rougier, Frédérique Penault-Llorca, and Pierre Laurent-Puig. Kras mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res*, 66(8):3992–3995, Apr 2006.

[220] Giovanni Ciriello, Ethan Cerami, Chris Sander, and Nikolaus Schultz. Mutual exclusivity analysis identifies oncogenic network modules. *Genome Res*, 22(2):398–406, Feb 2012.

[221] Chen-Hsiang Yeang, Frank McCormick, and Arnold Levine. Combinatorial patterns of somatic gene mutations in cancer. *FASEB J*, 22(8):2605–2622, Aug 2008.

[222] N. L. Harris, E. S. Jaffe, H. Stein, P. M. Banks, J. K. Chan, M. L. Cleary, G. Delsol, C. De Wolf-Peeters, B. Falini, and K. C. Gatter. A revised european-american classification of lymphoid neoplasms: a proposal from the international lymphoma study group. *Blood*, 84(5):1361–1392, Sep 1994.

[223] A. A. Alizadeh, M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J Hudson, Jr, L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson,

M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown, and L. M. Staudt. Distinct types of diffuse large b-cell lymphoma identified by gene expression profiling. *Nature*, 403(6769):503–511, Feb 2000.

[224] A predictive model for aggressive non-hodgkin's lymphoma. the international non-hodgkin's lymphoma prognostic factors project. *N Engl J Med*, 329(14):987–994, Sep 1993.

[225] C. M. Perou, T. Sørlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lønning, A. L. Børresen-Dale, P. O. Brown, and D. Botstein. Molecular portraits of human breast tumours. *Nature*, 406(6797):747–752, Aug 2000.

[226] T. Sørlie, C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P. E. Lønning, and A. L. Børresen-Dale. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, 98(19):10869–10874, Sep 2001.

[227] Pilar Eroles, Ana Bosch, J Alejandro Pérez-Fidalgo, and Ana Lluch. Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. *Cancer Treat Rev*, 38(6):698–707, Oct 2012.

[228] Sherene Loi, Benjamin Haibe-Kains, Christine Desmedt, Françoise Lallemand, Andrew M. Tutt, Cheryl Gillet, Paul Ellis, Adrian Harris, Jonas Bergh, John A. Foekens, Jan G M. Klijn, Denis Larsimont, Marc Buyse, Gianluca Bontempi, Mauro Delorenzi, Martine J. Piccart, and Christos Sotiriou. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. *J Clin Oncol*, 25(10):1239–1246, Apr 2007.

[229] M. Guedj, L. Marisa, A. de Reynies, B. Orsetti, R. Schiappa, F. Bibeau, G. MacGrogan, F. Lerebours, P. Finetti, M. Longy, P. Bertheau, F. Bertrand, F. Bonnet, A. L. Martin, J. P. Feugeas, I. Bièche, J. Lehmann-Che, R. Lidereau, D. Birnbaum, F. Bertucci, H. de Thé, and C. Theillet. A refined molecular taxonomy of breast cancer. *Oncogene*, 31(9):1196–1206, Mar 2012.

[230] T. Sørlie, C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P. E. Lønning, and A. L. Børresen-Dale. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, 98(19):10869–10874, Sep 2001.

[231] Zhiyuan Hu, Cheng Fan, Daniel S. Oh, J. S. Marron, Xiaping He, Bahjat F. Qaqish, Chad Livasy, Lisa A. Carey, Evangeline Reynolds, Lynn Dressler, Andrew Nobel, Joel Parker, Matthew G. Ewend, Lynda R. Sawyer, Junyuan Wu, Yudong Liu, Rita Nanda, Maria Tretiakova, Alejandra Ruiz Orrico, Donna Dreher, Juan P. Palazzo, Laurent Perreard, Edward Nelson, Mary Mone, Heidi Hansen, Michael Mullins, John F. Quackenbush, Matthew J. Ellis, Olufunmilayo I. Olopade, Philip S. Bernard, and Charles M. Perou. The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics*, 7:96, 2006.

[232] Alan Mackay, Britta Weigelt, Anita Grigoriadis, Bas Kreike, Rachael Natrajan, Roger A'Hern, David S P. Tan, Mitch Dowsett, Alan Ashworth, and Jorge S. Reis-Filho. Microarray-based class discovery for molecular classification of breast cancer: analysis of interobserver agreement. *J Natl Cancer Inst*, 103(8):662–673, Apr 2011.

[233] Tatsuya Ando, Miyuki Suguro, Takeshi Kobayashi, Masao Seto, and Hiroyuki Honda. Multiple fuzzy neural network system for outcome prediction and classification of 220 lymphoma patients on the basis of molecular profiling. *Cancer Sci*, 94(10):906–913, Oct 2003.

[234] Andreas Rosenwald, George Wright, Wing C. Chan, Joseph M. Connors, Elias Campo, Richard I. Fisher, Randy D. Gascoyne, H Konrad Muller-Hermelink, Erlend B. Smeland, Jena M. Giltnane, Elaine M. Hurt, Hong Zhao, Lauren Averett, Liming Yang, Wyndham H. Wilson, Elaine S. Jaffe, Richard Simon, Richard D. Klausner, John Powell, Patricia L. Duffey, Dan L. Longo, Timothy C. Greiner, Dennis D. Weisenburger, Warren G. Sanger, Bhavana J. Dave, James C. Lynch, Julie Vose, James O. Armitage, Emilio Montserrat, Armando López-Guillermo, Thomas M. Grogan, Thomas P. Miller, Michel LeBlanc, German Ott, Stein Kvaloy, Jan Delabie, Harald Holte, Peter Krajci, Trond Stokke, Louis M. Staudt, and Lymphoma/Leukemia Molecular Profiling Project . The use of molecular profiling to predict survival after chemotherapy for diffuse large-b-cell lymphoma. *N Engl J Med*, 346(25):1937–1947, Jun 2002.

[235] Sarah Barton, Eliza A. Hawkes, Andrew Wotherspoon, and David Cunningham. Are we ready to stratify treatment for diffuse large b-cell lymphoma using molecular hallmarks? *Oncologist*, 17(12):1562–1573, 2012.

[236] Andreas Schlicker, Garry Beran, Christine M. Chresta, Gael McWalter, Alison Pritchard, Susie Weston, Sarah Runswick, Sara Davenport, Kerry Heathcote, Denis Alferez Castro, George Orphanides, Tim French, and Lodewyk F A. Wessels. Subtypes of primary colorectal tumors correlate with response to targeted treatment in colorectal cell lines. *BMC Med Genomics*, 5:66, 2012.

[237] Anguraj Sadanandam, Costas A. Lyssiotis, Krisztian Homicsko, Eric A. Collisson, William J. Gibb, Stephan Wullschleger, Liliane C Gonzalez Ostos, William A. Lannon, Carsten Grotzinger, Maguy Del Rio, Benoit Lhermitte, Adam B. Olshen, Bertram Wiedenmann, Lewis C. Cantley, Joe W. Gray, and Douglas Hanahan. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med*, 19(5):619–625, May 2013.

[238] Paul Roepman, Andreas Schlicker, Josep Tabernero, Ian Majewski, Sun Tian, Victor Moreno, Mireille H. Snel, Christine M. Chresta, Robert Rosenberg, Ulrich Nitsche, Teresa Macarulla, Gabriel Capella, Ramon Salazar, George Orphanides, Lodewyk F A. Wessels, Rene Bernards, and Iris M. Simon. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *Int J Cancer*, 134(3):552–562, Feb 2014.

[239] Laetitia Marisa, Aurélien de Reyniès, Alex Duval, Janick Selves, Marie Pierre Gaub, Laure Vescovo, Marie-Christine Etienne-Grimaldi, Renaud Schiappa, Dominique Guenot, Mira Ayadi, Sylvain Kirzin, Maurice Chazal, Jean-François Fléjou, Daniel Benchimol, Anne Berger, Arnaud Lagarde, Erwan Pencreach, Françoise Piard, Dominique Elias, Yann Parc, Sylviane Olschwang, Gérard Milano, Pierre Laurent-Puig,

and Valérie Boige. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. *PLoS Med*, 10(5):e1001453, 2013.

[240] Felipe De Sousa E Melo, Xin Wang, Marnix Jansen, Evelyn Fessler, Anne Trinh, Laura P M H. de Rooij, Joan H. de Jong, Onno J. de Boer, Ronald van Leersum, Maarten F. Bijlsma, Hans Rodermond, Maartje van der Heijden, Carel J M. van Noesel, Jurriaan B. Tuynman, Evelien Dekker, Florian Markowetz, Jan Paul Medema, and Louis Vermeulen. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med*, 19(5):614–618, May 2013.

[241] Eva Budinska, Vlad Popovici, Sabine Tejpar, Giovanni D'Ario, Nicolas Lapique, Katarzyna Otylia Sikora, Antonio Fabio Di Narzo, Pu Yan, John Graeme Hodgson, Scott Weinrich, Fred Bosman, Arnaud Roth, and Mauro Delorenzi. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. *J Pathol*, 231(1):63–76, Sep 2013.

[242] A Rose Brannon, Scott M. Haake, Kathryn E. Hacker, Raj S. Pruthi, Eric M. Wallen, Matthew E. Nielsen, and W Kimryn Rathmell. Meta-analysis of clear cell renal cell carcinoma gene expression defines a variant subgroup and identifies gender influences on tumor biology. *Eur Urol*, 61(2):258–268, Feb 2012.

[243] A Rose Brannon, Anupama Reddy, Michael Seiler, Alexandra Arreola, Dominic T. Moore, Raj S. Pruthi, Eric M. Wallen, Matthew E. Nielsen, Huiqing Liu, Katherine L. Nathanson, Börje Ljungberg, Hongjuan Zhao, James D. Brooks, Shridar Ganesan, Gyan Bhanot, and W Kimryn Rathmell. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. *Genes Cancer*, 1(2):152–163, Feb 2010.

[244] Ruty Shai, Tao Shi, Thomas J. Kremen, Steve Horvath, Linda M. Liau, Timothy F. Cloughesy, Paul S. Mischel, and Stanley F. Nelson. Gene expression profiling identifies molecular subtypes of gliomas. *Oncogene*, 22(31):4918–4923, Jul 2003.

[245] Catherine L. Nutt, D. R. Mani, Rebecca A. Betensky, Pablo Tamayo, J Gregory Cairncross, Christine Ladd, Ute Pohl, Christian Hartmann, Margaret E. McLaughlin, Tracy T. Batchelor, Peter M. Black, Andreas von Deimling, Scott L. Pomeroy, Todd R. Golub, and David N. Louis. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. *Cancer Res*, 63(7):1602–1607, Apr 2003.

[246] Sandrine Boyault, David S. Rickman, Aurélien de Reyniès, Charles Balabaud, Sandra Rebouissou, Emmanuelle Jeannot, Aurélie Hérault, Jean Saric, Jacques Belghiti, Dominique Franco, Paulette Bioulac-Sage, Pierre Laurent-Puig, and Jessica Zucman-Rossi. Transcriptome classification of hcc is related to gene alterations and to new therapeutic targets. *Hepatology*, 45(1):42–52, Jan 2007.

[247] Derek Y. Chiang, Augusto Villanueva, Yujin Hoshida, Judit Peix, Philippa Newell, Beatriz Minguez, Amanda C. LeBlanc, Diana J. Donovan, Swan N. Thung, Manel Solé, Victoria Tovar, Clara Alsinet, Alex H. Ramos, Jordi Barretina, Sasan Roayaie, Myron Schwartz, Samuel Waxman, Jordi Bruix, Vincenzo Mazzaferro, Azra H. Ligon, Vesna Najfeld, Scott L. Friedman, William R. Sellers, Matthew Meyerson,

and Josep M. Llovet. Focal gains of vegfa and molecular classification of hepatocellular carcinoma. *Cancer Res*, 68(16):6779–6788, Aug 2008.

[248] Yujin Hoshida, Sebastian M B. Nijman, Masahiro Kobayashi, Jennifer A. Chan, Jean-Philippe Brunet, Derek Y. Chiang, Augusto Villanueva, Philippa Newell, Kenji Ikeda, Masaji Hashimoto, Goro Watanabe, Stacey Gabriel, Scott L. Friedman, Hiromitsu Kumada, Josep M. Llovet, and Todd R. Golub. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res*, 69(18):7385–7392, Sep 2009.

[249] Ju-Seog Lee, In-Sun Chu, Jeonghoon Heo, Diego F. Calvisi, Zongtang Sun, Tania Roskams, Anne Durnez, Anthony J. Demetris, and Snorri S. Thorgeirsson. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology*, 40(3):667–676, Sep 2004.

[250] Anne Biton, Isabelle Bernard-Pierrot, Yinjun Lou, Clémentine Krucker, Elodie Chapeaublanc, Carlota Rubio-Pérez, Nuria López-Bigas, Aurélie Kamoun, Yann Neuzillet, Pierre Gestraud, Luca Grieco, Sandra Rebouissou, Aurélien de Reyniès, Simone Benhamou, Thierry Lebret, Jennifer Southgate, Emmanuel Barillot, Yves Allory, Andrei Zinovyev, and François Radvanyi. Independent component analysis uncovers the landscape of the bladder tumor transcriptome and reveals insights into luminal and basal subtypes. *Cell Rep*, 9(4):1235–1245, Nov 2014.

[251] Scott A. Tomlins, Mohammed Alshalalfa, Elai Davicioni, Nicholas Erho, Kasra Yousefi, Shuang Zhao, Zaid Haddad, Robert B. Den, Adam P. Dicker, Bruce J. Trock, Angelo M. DeMarzo, Ashley E. Ross, Edward M. Schaeffer, Eric A. Klein, Cristina Magi-Galluzzi, R Jeffrey Karnes, Robert B. Jenkins, and Felix Y. Feng. Characterization of 1577 primary prostate cancers reveals novel biological and clinicopathologic insights into molecular subtypes. *Eur Urol*, May 2015.

[252] H J M. de Jonge, G. Huls, and E S J M. de Bont. Gene expression profiling in acute myeloid leukaemia. *Neth J Med*, 69(4):167–176, Apr 2011.

[253] J. R. Downing. Acute leukemia: subtype discovery and prediction of outcome by gene expression profiling. *Verh Dtsch Ges Pathol*, 87:66–71, 2003.

[254] Krzysztof Mrózek, Michael D. Radmacher, Clara D. Bloomfield, and Guido Marcucci. Molecular signatures in acute myeloid leukemia. *Curr Opin Hematol*, 16(2):64–69, Mar 2009.

[255] Fernando P G. Silva, Sigrid M A. Swagemakers, Claudia Erpelinck-Verschueren, Bas J. Wouters, Ruud Delwel, Harry Vrieling, Peter van der Spek, Peter J M. Valk, and Micheline Giphart-Gassler. Gene expression profiling of minimally differentiated acute myeloid leukemia: M0 is a distinct entity subdivided by runx1 mutation status. *Blood*, 114(14):3001–3007, Oct 2009.

[256] Roel G W. Verhaak, Bas J. Wouters, Claudia A J. Erpelinck, Saman Abbas, H Berna Beverloo, Sanne Lugthart, Bob Löwenberg, Ruud Delwel, and Peter J M. Valk. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica*, 94(1):131–134, Jan 2009.

[257] Joyce N. Barlin, Qin C. Zhou, Mario M. Leitao, Maria Bisogna, Narciso Olvera, Karin K. Shih, Anders Jacobsen, Nikolaus Schultz, William D. Tap, Martee L. Hensley, Gary K. Schwartz, Jeff Boyd, Li-Xuan Qin, and Douglas A. Levine. Molecular

subtypes of uterine leiomyosarcoma and correlation with clinical outcome. *Neoplasia*, 17(2):183–189, Feb 2015.

[258] Xiangqian Guo, Vickie Y. Jo, Anne M. Mills, Shirley X. Zhu, Cheng-Han Lee, Inigo Espinosa, Marisa R. Nucci, Sushama Varma, Erna Forgó, Trevor Hastie, Sharon Anderson, Kristen Ganjoo, Andrew H. Beck, Robert B. West, Christopher D. Fletcher, and Matt van de Rijn. Clinically relevant molecular subtypes in leiomyosarcoma. *Clin Cancer Res*, Apr 2015.

[259] Aurélien de Reyniès, Marie-Claude Jaurand, Annie Renier, Gabrielle Couchy, Ilir Hysi, Nabila Elarouci, Françoise Galateau-Sallé, Marie-Christine Copin, Paul Hofman, Aurélie Cazes, Pascal Andujar, Sandrine Imbeaud, Fabien Petel, Jean-Claude Pairon, Francoise Le Pimpec-Barthes, Jessica Zucman-Rossi, and Didier Jean. Molecular classification of malignant pleural mesothelioma: identification of a poor prognosis subgroup linked to the epithelial-to-mesenchymal transition. *Clin Cancer Res*, 20(5):1323–1334, Mar 2014.

[260] Guillaume Assié, Eric Letouzé, Martin Fassnacht, Anne Jouinot, Windy Luscap, Olivia Barreau, Hanin Omeiri, Stéphanie Rodriguez, Karine Perlemoine, Fernande René-Corail, Nabila Elarouci, Silviu Sbiera, Matthias Kroiss, Bruno Allolio, Jens Waldmann, Marcus Quinkler, Massimo Mannelli, Franco Mantero, Thomas Papathomas, Ronald De Krijger, Antoine Tabarin, Véronique Kerlan, Eric Baudin, Frédérique Tissier, Bertrand Dousset, Lionel Groussin, Laurence Amar, Eric Clauser, Xavier Bertagna, Bruno Ragazzon, Felix Beuschlein, Rossella Libé, Aurélien de Reyniès, and Jérôme Bertherat. Integrated genomic characterization of adrenocortical carcinoma. *Nat Genet*, 46(6):607–612, Jun 2014.

[261] Cancer Genome Atlas Research Network , Cyriac Kandoth, Nikolaus Schultz, Andrew D. Cherniack, Rehan Akbani, Yuexin Liu, Hui Shen, A Gordon Robertson, Itai Pashtan, Ronglai Shen, Christopher C. Benz, Christina Yau, Peter W. Laird, Li Ding, Wei Zhang, Gordon B. Mills, Raju Kucherlapati, Elaine R. Mardis, and Douglas A. Levine. Integrated genomic characterization of endometrial carcinoma. *Nature*, 497(7447):67–73, May 2013.

[262] Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*, 489(7417):519–525, Sep 2012.

[263] Cancer Genome Atlas Network . Comprehensive molecular portraits of human breast tumours. *Nature*, 490(7418):61–70, Oct 2012.

[264] Cancer Genome Atlas Research Network . Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature*, 499(7456):43–49, Jul 2013.

[265] Cancer Genome Atlas Research Network . Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature*, 507(7492):315–322, Mar 2014.

[266] Cancer Genome Atlas Research Network . Comprehensive molecular profiling of lung adenocarcinoma. *Nature*, 511(7511):543–550, Jul 2014.

[267] Cancer Genome Atlas Research Network . Comprehensive molecular characterization of gastric adenocarcinoma. *Nature*, 513(7517):202–209, Sep 2014.

[268] Cancer Genome Atlas Research Network . Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*, 517(7536):576–582, Jan 2015.

[269] Cancer Genome Atlas Research Network . Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*, 368(22):2059–2074, May 2013.

[270] Cancer Genome Atlas Research Network , Daniel J. Brat, Roel G W. Verhaak, Kenneth D. Aldape, W K Alfred Yung, Sofie R. Salama, Lee A D. Cooper, Esther Rheinbay, C Ryan Miller, Mark Vitucci, Olena Morozova, A Gordon Robertson, Houtan Noushmehr, Peter W. Laird, Andrew D. Cherniack, Rehan Akbani, Jason T. Huse, Giovanni Ciriello, Laila M. Poisson, Jill S. Barnholtz-Sloan, Mitchel S. Berger, Cameron Brennan, Rivka R. Colen, Howard Colman, Adam E. Flanders, Caterina Giannini, Mia Grifford, Antonio Iavarone, Rajan Jain, Isaac Joseph, Jaegil Kim, Katayoon Kasaian, Tom Mikkelsen, Bradley A. Murray, Brian Patrick O'Neill, Lior Pachter, Donald W. Parsons, Carrie Sougnez, Erik P. Sulman, Scott R. Vandenberg, Erwin G. Van Meir, Andreas von Deimling, Hailei Zhang, Daniel Crain, Kevin Lau, David Mallery, Scott Morris, Joseph Paulauskis, Robert Penny, Troy Shelton, Mark Sherman, Peggy Yena, Aaron Black, Jay Bowen, Katie Dicostanzo, Julie Gastier-Foster, Kristen M. Leraas, Tara M. Lichtenberg, Christopher R. Pierson, Nilsa C. Ramirez, Cynthia Taylor, Stephanie Weaver, Lisa Wise, Erik Zmuda, Tanja Davidsen, John A. Demchok, Greg Eley, Martin L. Ferguson, Carolyn M. Hutter, Kenna R. Mills Shaw, Bradley A. Ozenberger, Margi Sheth, Heidi J. Sofia, Roy Tarnuzzer, Zhining Wang, Liming Yang, Jean Claude Zenklusen, Brenda Ayala, Julien Baboud, Sudha Chudamani, Mark A. Jensen, Jia Liu, Todd Pihl, Rohini Raman, Yunhu Wan, Ye Wu, Adrian Ally, J Todd Auman, Miruna Balasundaram, Saianand Balu, Stephen B. Baylin, Rameen Beroukhim, Moiz S. Bootwalla, Reanne Bowlby, Christopher A. Bristow, Denise Brooks, Yaron Butterfield, Rebecca Carlsen, Scott Carter, Lynda Chin, Andy Chu, Eric Chuah, Kristian Cibulskis, Amanda Clarke, Simon G. Coetzee, Noreen Dhalla, Tim Fennell, Sheila Fisher, Stacey Gabriel, Gad Getz, Richard Gibbs, Ranabir Guin, Angela Hadjipanayis, D Neil Hayes, Toshinori Hinoue, Katherine Hoadley, Robert A. Holt, Alan P. Hoyle, Stuart R. Jefferys, Steven Jones, Corbin D. Jones, Raju Kucherlapati, Phillip H. Lai, Eric Lander, Semin Lee, Lee Lichtenstein, Yussanne Ma, Dennis T. Maglinte, Harshad S. Mahadeshwar, Marco A. Marra, Michael Mayo, Shaowu Meng, Matthew L. Meyerson, Piotr A. Mieczkowski, Richard A. Moore, Lisle E. Mose, Andrew J. Mungall, Angeliki Pantazi, Michael Parfenov, Peter J. Park, Joel S. Parker, Charles M. Perou, Alexei Protopopov, Xiaojia Ren, Jeffrey Roach, Thaís S. Sabedot, Jacqueline Schein, Steven E. Schumacher, Jonathan G. Seidman, Sahil Seth, Hui Shen, Janae V. Simons, Payal Sipahimalani, Matthew G. Soloway, Xingzhi Song, Huandong Sun, Barbara Tabak, Angela Tam, Donghui Tan, Jiabin Tang, Nina Thiessen, Timothy Triche, Jr, David J. Van Den Berg, Umadevi Veluvolu, Scot Waring, Daniel J. Weisenberger, Matthew D. Wilkerson, Tina Wong, Junyuan Wu, Liu Xi, Andrew W. Xu, Lixing Yang, Travis I. Zack, Jianhua Zhang, B Arman Aksoy, Harindra Arachchi, Chris Benz, Brady Bernard, Daniel Carlin, Juok Cho, Daniel DiCara, Scott Frazer, Gregory N. Fuller, Jianjiong Gao, Nils Gehlenborg, David Haussler, David I. Heiman, Lisa Iype, Anders Jacobsen, Zhenlin Ju, Sol Katzman, Hoon Kim, Theo Knijnenburg, Richard Bailey Kreisberg, Michael S. Lawrence, William Lee, Kalle Leinonen, Pei Lin, Shiyun Ling, Wenbin Liu, Yingchun Liu, Yuexin Liu, Yiling Lu, Gordon Mills, Sam Ng, Michael S. Noble, Evan Paull, Arvind Rao, Sheila Reynolds, Gordon Saksena, Zack Sanborn, Chris Sander, Nikolaus Schultz, Yasin Senbabaoglu, Ronglai Shen, Ilya Shmulevich, Rileen Sinha, Josh Stuart, S Onur Sumer, Yichao Sun, Natalie Tasman, Barry S. Taylor, Doug Voet, Nils Weinhold, John N. Weinstein, Da Yang, Kosuke Yoshihara, Siyuan Zheng, Wei Zhang, Lihua Zou, Ty Abel, Sara Sadeghi, Mark L. Cohen, Jenny

Eschbacher, Eyas M. Hattab, Aditya Raghunathan, Matthew J. Schniederjan, Dina Aziz, Gene Barnett, Wendi Barrett, Darell D. Bigner, Lori Boice, Cathy Brewer, Chiara Calatozzolo, Benito Campos, Carlos Gilberto Carlotti, Jr, Timothy A. Chan, Lucia Cuppini, Erin Curley, Stefania Cuzzubbo, Karen Devine, Francesco DiMeco, Rebecca Duell, J Bradley Elder, Ashley Fehrenbach, Gaetano Finocchiaro, William Friedman, Jordonna Fulop, Johanna Gardner, Beth Hermes, Christel Herold-Mende, Christine Jungk, Ady Kendler, Norman L. Lehman, Eric Lipp, Ouida Liu, Randy Mandt, Mary McGraw, Roger Mclendon, Christopher McPherson, Luciano Neder, Phuong Nguyen, Ardene Noss, Raffaele Nunziata, Quinn T. Ostrom, Cheryl Palmer, Alessandro Perin, Bianca Pollo, Alexander Potapov, Olga Potapova, W Kimryn Rathmell, Daniil Rotin, Lisa Scarpace, Cathy Schilero, Kelly Senecal, Kristen Shimmel, Vsevolod Shurkhay, Suzanne Sifri, Rosy Singh, Andrew E. Sloan, Kathy Smolenski, Susan M. Staugaitis, Ruth Steele, Leigh Thorne, Daniela P C. Tirapelli, Andreas Unterberg, Mahitha Vallurupalli, Yun Wang, Ronald Warnick, Felicia Williams, Yingli Wolinsky, Sue Bell, Mara Rosenberg, Chip Stewart, Franklin Huang, Jonna L. Grimsby, Amie J. Radenbaugh, and Jianan Zhang. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. *N Engl J Med*, 372(26):2481–2498, Jun 2015.

[271] Cameron W. Brennan, Roel G W. Verhaak, Aaron McKenna, Benito Campos, Houtan Noushmehr, Sofie R. Salama, Siyuan Zheng, Debyani Chakravarty, J Zachary Sanborn, Samuel H. Berman, Rameen Beroukhim, Brady Bernard, Chang-Jiun Wu, Giannicola Genovese, Ilya Shmulevich, Jill Barnholtz-Sloan, Lihua Zou, Rahulsimham Vegesna, Sachet A. Shukla, Giovanni Ciriello, W. K. Yung, Wei Zhang, Carrie Sougnez, Tom Mikkelsen, Kenneth Aldape, Darell D. Bigner, Erwin G. Van Meir, Michael Prados, Andrew Sloan, Keith L. Black, Jennifer Eschbacher, Gaetano Finocchiaro, William Friedman, David W. Andrews, Abhijit Guha, Mary Iacocca, Brian P. O'Neill, Greg Foltz, Jerome Myers, Daniel J. Weisenberger, Robert Penny, Raju Kucherlapati, Charles M. Perou, D Neil Hayes, Richard Gibbs, Marco Marra, Gordon B. Mills, Eric Lander, Paul Spellman, Richard Wilson, Chris Sander, John Weinstein, Matthew Meyerson, Stacey Gabriel, Peter W. Laird, David Haussler, Gad Getz, Lynda Chin, and T. C. G. A Research Network . The somatic genomic landscape of glioblastoma. *Cell*, 155(2):462–477, Oct 2013.

[272] Cancer Genome Atlas Network. Electronic address: irwatson@mdanderson.org and Cancer Genome Atlas Network . Genomic classification of cutaneous melanoma. *Cell*, 161(7):1681–1696, Jun 2015.

[273] National Cancer Institute at NIH 31 Center Drive Bldg. 31 Suite 3A20 Bethesda MD 20892 USA. giordano@umich.edu , Cancer Genome Atlas Research NetworkCancer Genome Atlas Program Office. Integrated genomic characterization of papillary thyroid carcinoma. *Cell*, 159(3):676–690, Oct 2014.

[274] B. Vogelstein, E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, M. Leppert, Y. Nakamura, R. White, A. M. Smits, and J. L. Bos. Genetic alterations during colorectal-tumor development. *N Engl J Med*, 319(9):525–532, Sep 1988.

[275] Tannaz Armaghany, Jon D. Wilson, Quyen Chu, and Glenn Mills. Genetic alterations in colorectal cancer. *Gastrointest Cancer Res*, 5(1):19–27, Jan 2012.

[276] Maria S. Pino and Daniel C. Chung. The chromosomal instability pathway in colon cancer. *Gastroenterology*, 138(6):2059–2072, Jun 2010.

[277] William M. Grady and Sanford D. Markowitz. The molecular pathogenesis of colorectal cancer and its potential application to colorectal cancer screening. *Dig Dis Sci*, 60(3):762–772, Mar 2015.

[278] Jean-Pierre Issa. Cpg island methylator phenotype in cancer. *Nat Rev Cancer*, 4(12):988–993, Dec 2004.

[279] Peter A. Jones and Stephen B. Baylin. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*, 3(6):415–428, Jun 2002.

[280] Stephen B. Gruber and Wendy Kohlmann. The genetics of hereditary non-polyposis colorectal cancer. *J Natl Compr Canc Netw*, 1(1):137–144, Jan 2003.

[281] Cancer Genome Atlas Network . Comprehensive molecular characterization of human colon and rectal cancer. *Nature*, 487(7407):330–337, Jul 2012.

[282] R. Dienstmann, J. Guinney, M. Delorenzi, A. De Reynies, P. Roepman, A. Sadanandam, L. Vermeulen, A. Schlicker, E. Missiaglia, C. Soneson, and et al. O-0025 * colorectal cancer subtyping consortium (CRCSC) identifies consensus of molecular subtypes. *Annals of Oncology*, 25(suppl 2):ii115–ii115, Jun 2014.

[283] Christudas Morais, Glenda Gobe, David W. Johnson, and Helen Healy. The emerging role of nuclear factor kappa b in renal cell carcinoma. *Int J Biochem Cell Biol*, 43(11):1537–1549, Nov 2011.

[284] Paul Cairns. Renal cell carcinoma. *Cancer Biomark*, 9(1-6):461–473, 2010.

[285] Yusuke Sato, Tetsuichi Yoshizato, Yuichi Shiraishi, Shigekatsu Maekawa, Yusuke Okuno, Takumi Kamura, Teppei Shimamura, Aiko Sato-Otsubo, Genta Nagae, Hiromichi Suzuki, Yasunobu Nagata, Kenichi Yoshida, Ayana Kon, Yutaka Suzuki, Kenichi Chiba, Hiroko Tanaka, Atsushi Niida, Akihiro Fujimoto, Tatsuhiko Tsunoda, Teppei Morikawa, Daichi Maeda, Haruki Kume, Sumio Sugano, Masashi Fukayama, Hiroyuki Aburatani, Masashi Sanada, Satoru Miyano, Yukio Homma, and Seishi Ogawa. Integrated molecular analysis of clear-cell renal cell carcinoma. *Nat Genet*, 45(8):860–867, Aug 2013.

[286] Ignacio Varela, Patrick Tarpey, Keiran Raine, Dachuan Huang, Choon Kiat Ong, Philip Stephens, Helen Davies, David Jones, Meng-Lay Lin, Jon Teague, Graham Bignell, Adam Butler, Juok Cho, Gillian L. Dalgliesh, Danushka Galappaththige, Chris Greenman, Claire Hardy, Mingming Jia, Calli Latimer, King Wai Lau, John Marshall, Stuart McLaren, Andrew Menzies, Laura Mudie, Lucy Stebbings, David A. Largaespada, L F A. Wessels, Stephane Richard, Richard J. Kahnoski, John Anema, David A. Tuveson, Pedro A. Perez-Mancera, Ville Mustonen, Andrej Fischer, David J. Adams, Alistair Rust, Waraporn Chan-on, Chutima Subimerb, Karl Dykema, Kyle Furge, Peter J. Campbell, Bin Tean Teh, Michael R. Stratton, and P Andrew Futreal. Exome sequencing identifies frequent mutation of the swi/snf complex gene pbrm1 in renal carcinoma. *Nature*, 469(7331):539–542, Jan 2011.

[287] Jonathan M. Rhodes and Barry J. Campbell. Inflammation and colorectal cancer: Ibd-associated and sporadic cancer compared. *Trends Mol Med*, 8(1):10–16, Jan 2002.

[288] T. A. Brentnall, D. A. Crispin, P. S. Rabinovitch, R. C. Haggitt, C. E. Rubin, A. C. Stevens, and G. C. Burmer. Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology*, 107(2):369–378, Aug 1994.

[289] Yanhong Shi, Zhenfeng Li, Wei Zheng, Xia Liu, Chenyi Sun, Jann-Birger Laugsand, Zhanju Liu, and Guanglin Cui. Changes of immunocytic phenotypes and functions from human colorectal adenomatous stage to cancerous stage: Update. *Immunobiology*, Jun 2015.

[290] G. Steinbach, P. M. Lynch, R. K. Phillips, M. H. Wallace, E. Hawk, G. B. Gordon, N. Wakabayashi, B. Saunders, Y. Shen, T. Fujimura, L. K. Su, B. Levin, L. Godio, S. Patterson, M. A. Rodriguez-Bigas, S. L. Jester, K. L. King, M. Schumacher, J. Abbruzzese, R. N. DuBois, W. N. Hittelman, S. Zimmerman, J. W. Sherman, and G. Kelloff. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med*, 342(26):1946–1952, Jun 2000.

[291] Francesco Mariani, Paola Sena, and Luca Roncucci. Inflammatory pathways in the early steps of colorectal cancer development. *World J Gastroenterol*, 20(29):9716–9731, Aug 2014.

[292] C. Koudougou, M. Bonneville, T. Matysiak-Budnik, and Y. Touchefeu. Review article: antitumoural immunity in colorectal cancer - current and potential future implications in clinical practice. *Aliment Pharmacol Ther*, 38(1):3–15, Jul 2013.

[293] Katsuhiko Nosho, Yoshifumi Baba, Noriko Tanaka, Kaori Shima, Marika Hayashi, Jeffrey A. Meyerhardt, Edward Giovannucci, Glenn Dranoff, Charles S. Fuchs, and Shuji Ogino. Tumour-infiltrating t-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol*, 222(4):350–366, Dec 2010.

[294] Paul Salama, Michael Phillips, Fabienne Grieu, Melinda Morris, Nik Zeps, David Joseph, Cameron Platell, and Barry Iacopetta. Tumor-infiltrating foxp3+ t regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol*, 27(2):186–192, Jan 2009.

[295] Daniel M. Frey, Raoul A. Droeser, Carsten T. Viehl, Inti Zlobec, Alessandro Lugli, Urs Zingg, Daniel Oertli, Christoph Kettelhack, Luigi Terracciano, and Luigi Tornillo. High frequency of tumor-infiltrating foxp3(+) regulatory t cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer*, 126(11):2635–2643, Jun 2010.

[296] Marie Tosolini, Amos Kirilovsky, Bernhard Mlecnik, Tessa Fredriksen, Stéphanie Mauger, Gabriela Bindea, Anne Berger, Patrick Bruneval, Wolf-Herman Fridman, Franck Pagès, and Jérôme Galon. Clinical impact of different classes of infiltrating t cytotoxic and helper cells (th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res*, 71(4):1263–1271, Feb 2011.

[297] Pin Wu, Dang Wu, Chao Ni, Jun Ye, Wuzhen Chen, Guoming Hu, Zhen Wang, Changrong Wang, Zhigang Zhang, Wenjie Xia, Zhigang Chen, Ke Wang, Tao Zhang, Jinghong Xu, Yuehua Han, Ting Zhang, Xianguo Wu, Jianwei Wang, Weihua Gong, Shu Zheng, Fuming Qiu, Jun Yan, and Jian Huang. $\gamma\delta$t17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity*, 40(5):785–800, May 2014.

[298] V. De Simone, E. Franzè, G. Ronchetti, A. Colantoni, M. C. Fantini, D. Di Fusco, G. S. Sica, P. Sileri, T. T. MacDonald, F. Pallone, G. Monteleone, and C. Stolfi. Th17-type cytokines, il-6 and tnf-α synergistically activate stat3 and nf-kb to promote colorectal cancer cell growth. *Oncogene*, 34(27):3493–3503, Jul 2015.

[299] R. Dolcetti, A. Viel, C. Doglioni, A. Russo, M. Guidoboni, E. Capozzi, N. Vecchiato, E. Macrì, M. Fornasarig, and M. Boiocchi. High prevalence of activated intraepithelial cytotoxic t lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol*, 154(6):1805–1813, Jun 1999.

[300] M. Guidoboni, R. Gafà, A. Viel, C. Doglioni, A. Russo, A. Santini, L. Del Tin, E. Macrì, G. Lanza, M. Boiocchi, and R. Dolcetti. Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis. *Am J Pathol*, 159(1):297–304, Jul 2001.

[301] David Tougeron, Emilie Fauquembergue, and Jean-Baptiste Latouche. [immune response and colorectal cancer]. *Bull Cancer*, 100(3):283–294, Mar 2013.

[302] Nicolas J. Llosa, Michael Cruise, Ada Tam, Elizabeth C. Wicks, Elizabeth M. Hechenbleikner, Janis M. Taube, Richard L. Blosser, Hongni Fan, Hao Wang, Brandon S. Luber, Ming Zhang, Nickolas Papadopoulos, Kenneth W. Kinzler, Bert Vogelstein, Cynthia L. Sears, Robert A. Anders, Drew M. Pardoll, and Franck Housseau. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov*, 5(1):43–51, Jan 2015.

[303] Yanping Xiao and Gordon J. Freeman. The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy. *Cancer Discov*, 5(1):16–18, Jan 2015.

[304] Lorenzo Galluzzi, Laura Senovilla, Laurence Zitvogel, and Guido Kroemer. The secret ally: immunostimulation by anticancer drugs. *Nat Rev Drug Discov*, 11(3):215–233, Mar 2012.

[305] Lee M. Ellis. Mechanisms of action of bevacizumab as a component of therapy for metastatic colorectal cancer. *Semin Oncol*, 33(5 Suppl 10):S1–S7, Oct 2006.

[306] Shigeo Koido, Toshifumi Ohkusa, Sadamu Homma, Yoshihisa Namiki, Kazuki Takakura, Keisuke Saito, Zensho Ito, Hiroko Kobayashi, Mikio Kajihara, Kan Uchiyama, Seiji Arihiro, Hiroshi Arakawa, Masato Okamoto, Jianlin Gong, and Hisao Tajiri. Immunotherapy for colorectal cancer. *World J Gastroenterol*, 19(46):8531–8542, Dec 2013.

[307] Suzanne L Topalian, F Stephen Hodi, Julie R Brahmer, Scott N Gettinger, David C Smith, David F McDermott, John D Powderly, Richard D Carvajal, Jeffrey A Sosman, Michael B Atkins, et al. Safety, activity, and immune correlates of anti–pd-1 antibody in cancer. *New England Journal of Medicine*, 366(26):2443–2454, 2012.

[308] Janis M Taube, Alison Klein, Julie R Brahmer, Haiying Xu, Xiaoyu Pan, Jung H Kim, Lieping Chen, Drew M Pardoll, Suzanne L Topalian, and Robert A Anders. Association of pd-1, pd-1 ligands, and other features of the tumor immune microenvironment with response to anti–pd-1 therapy. *Clinical Cancer Research*, 20(19):5064–5074, 2014.

[309] Momoe Itsumi and Katsunori Tatsugami. Immunotherapy for renal cell carcinoma. *Clinical and Developmental Immunology*, 2010, 2011.

[310] Osamu Nakano, Makoto Sato, Yoshitaka Naito, Kenichi Suzuki, Seiichi Orikasa, Masataka Aizawa, Yasuyoshi Suzuki, Ichirou Shintaku, Hiroshi Nagura, and Haruo Ohtani. Proliferative activity of intratumoral cd8+ t-lymphocytes as a prognostic factor in human renal cell carcinoma clinicopathologic demonstration of antitumor immunity. *Cancer research*, 61(13):5132–5136, 2001.

[311] Romain Remark, Marco Alifano, Isabelle Cremer, Audrey Lupo, Marie-Caroline Dieu-Nosjean, Marc Riquet, Lucile Crozet, Hanane Ouakrim, Jeremy Goc, Aurélie Cazes, et al. Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. *Clinical Cancer Research*, 19(15):4079–4091, 2013.

[312] K Hotta, M Sho, K Fujimoto, K Shimada, I Yamato, S Anai, N Konishi, Y Hirao, K Nonomura, and Y Nakajima. Prognostic significance of cd45ro+ memory t cells in renal cell carcinoma. *British journal of cancer*, 105(8):1191–1196, 2011.

[313] Katharina Geissler, Paolo Fornara, Christine Lautenschläger, Hans-Jürgen Holzhausen, Barbara Seliger, and Dagmar Riemann. Immune signature of tumor infiltrating immune cells in renal cancer. *OncoImmunology*, 4(1):e985082, 2015.

[314] Nicolas A Giraldo, Etienne Becht, Romain Remark, Diane Damotte, Catherine Sautès-Fridman, and Wolf H Fridman. The immune contexture of primary and metastatic human tumours. *Current opinion in immunology*, 27:8–15, 2014.

[315] Juliane S Stickel, Natalie Stickel, Jörg Hennenlotter, Karin Klingel, Arnulf Stenzl, Hans-Georg Rammensee, and Stefan Stevanović. Quantification of hla class i molecules on renal cell carcinoma using edman degradation. *BMC urology*, 11(1):1, 2011.

[316] Simone P Sittig, Tania Køllgaard, Kirsten Grønbæk, Manja Idorn, Jörg Hennenlotter, Arnulf Stenzl, Cécile Gouttefangeas, and Per Thor Straten. Clonal expansion of renal cell carcinoma-infiltrating t lymphocytes. *Oncoimmunology*, 2(9):e26014, 2013.

[317] Olivier Adotevi, Helene Pere, Patrice Ravel, Nacilla Haicheur, Cecile Badoual, Nathalie Merillon, Jacques Medioni, Severine Peyrard, Stephane Roncelin, Virginie Verkarre, et al. A decrease of regulatory t cells correlates with overall survival after sunitinib-based antiangiogenic therapy in metastatic renal cancer patients. *Journal of immunotherapy*, 33(9):991–998, 2010.

[318] James H Finke, Brian Rini, Joanna Ireland, Patricia Rayman, Amy Richmond, Ali Golshayan, Laura Wood, Paul Elson, Jorge Garcia, Robert Dreicer, et al. Sunitinib reverses type-1 immune suppression and decreases t-regulatory cells in renal cell carcinoma patients. *Clinical Cancer Research*, 14(20):6674–6682, 2008.

[319] Jacob Lokich. Spontaneous regression of metastatic renal cancer: case report and literature review. *American journal of clinical oncology*, 20(4):416–418, 1997.

[320] Steven A Rosenberg, James C Yang, Suzanne L Topalian, Douglas J Schwartzentruber, Jeffrey S Weber, David R Parkinson, Claudia A Seipp, Jan H Einhorn, and

Donald E White. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *Jama*, 271(12):907–913, 1994.

[321] R. I. Fisher, S. A. Rosenberg, and G. Fyfe. Long-term survival update for high-dose recombinant interleukin-2 in patients with renal cell carcinoma. *Cancer J Sci Am*, 6 Suppl 1:S55–S57, Feb 2000.

[322] R. I. Fisher, S. A. Rosenberg, M. Sznol, D. R. Parkinson, and G. Fyfe. High-dose aldesleukin in renal cell carcinoma: long-term survival update. *Cancer J Sci Am*, 3 Suppl 1:S70–S72, Dec 1997.

[323] S. D. Fosså. Interferon in metastatic renal cell carcinoma. *Semin Oncol*, 27(2):187–193, Apr 2000.

[324] Jacob S. Thomas and Fairooz Kabbinavar. Metastatic clear cell renal cell carcinoma: A review of current therapies and novel immunotherapies. *Critical Reviews in Oncology/Hematology*, Jul 2015.

[325] Asim Amin and Richard L White, Jr. High-dose interleukin-2: is it still indicated for melanoma and rcc in an era of targeted therapies? *Oncology (Williston Park)*, 27(7):680–691, Jul 2013.

[326] C. Coppin, F. Porzsolt, A. Awa, J. Kumpf, A. Coldman, and T. Wilt. Immunotherapy for advanced renal cell cancer. *Cochrane Database Syst Rev*, (1):CD001425, 2005.

[327] Christina Canil, Sebastien Hotte, Linda A. Mayhew, Tricia S. Waldron, and Eric Winquist. Interferon-alfa in the treatment of patients with inoperable locally advanced or metastatic renal cell carcinoma: a systematic review. *Can Urol Assoc J*, 4(3):201–208, Jun 2010.

[328] Suzanne L. Topalian, Charles G. Drake, and Drew M. Pardoll. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*, 27(4):450–461, Apr 2015.

[329] Robert J. Motzer, Brian I. Rini, David F. McDermott, Bruce G. Redman, Timothy M. Kuzel, Michael R. Harrison, Ulka N. Vaishampayan, Harry A. Drabkin, Saby George, Theodore F. Logan, Kim A. Margolin, Elizabeth R. Plimack, Alexandre M. Lambert, Ian M. Waxman, and Hans J. Hammers. Nivolumab for metastatic renal cell carcinoma: Results of a randomized phase ii trial. *J Clin Oncol*, 33(13):1430–1437, May 2015.

[330] Jérémy Goc, Claire Germain, Thi Kim Duy Vo-Bourgais, Audrey Lupo, Christophe Klein, Samantha Knockaert, Luc de Chaisemartin, Hanane Ouakrim, Etienne Becht, Marco Alifano, Pierre Validire, Romain Remark, Scott A. Hammond, Isabelle Cremer, Diane Damotte, Wolf-Herman Fridman, Catherine Sautès-Fridman, and Marie-Caroline Dieu-Nosjean. Dendritic cells in tumor-associated tertiary lymphoid structures signal a th1 cytotoxic immune contexture and license the positive prognostic value of infiltrating cd8+ t cells. *Cancer Res*, 74(3):705–715, Feb 2014.

[331] Etienne Becht, Jeremy Goc, Claire Germain, Nicolas A. Giraldo, Marie-Caroline Dieu-Nosjean, Catherine Sautès-Fridman, and Wolf-Herman Fridman. Shaping of

an effective immune microenvironment to and by cancer cells. *Cancer Immunol Immunother*, 63(10):991–997, Oct 2014.

[332] W. H. Fridman, R. Remark, J. Goc, N. A. Giraldo, E. Becht, Scott A. Hammond, D. Damotte, M-C. Dieu-Nosjean, and Catherine Sautès-Fridman. The immune microenvironment: a major player in human cancers. *Int Arch Allergy Immunol*, 164(1):13–26, 2014.

[333] Marie-Caroline Dieu-Nosjean, Jérémy Goc, Nicolas A. Giraldo, Catherine Sautès-Fridman, and Wolf Herman Fridman. Tertiary lymphoid structures in cancer and beyond. *Trends Immunol*, 35(11):571–580, Nov 2014.

[334] Aravind Subramanian, Pablo Tamayo, Vamsi K. Mootha, Sayan Mukherjee, Benjamin L. Ebert, Michael A. Gillette, Amanda Paulovich, Scott L. Pomeroy, Todd R. Golub, Eric S. Lander, and Jill P. Mesirov. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*, 102(43):15545–15550, Oct 2005.

[335] Neeraj Lal, Andrew D. Beggs, Benjamin E. Willcox, and Gary W. Middleton. An immunogenomic stratification of colorectal cancer: Implications for development of targeted immunotherapy. *Oncoimmunology*, 4(3):e976052, Mar 2015.

[336] Shai S. Shen-Orr and Renaud Gaujoux. Computational deconvolution: extracting cell type-specific information from heterogeneous samples. *Curr Opin Immunol*, 25(5):571–578, Oct 2013.

[337] D. Venet, F. Pecasse, C. Maenhaut, and H. Bersini. Separation of samples into their constituents using gene expression data. *Bioinformatics*, 17 Suppl 1:S279–S287, 2001.

[338] Dirk Repsilber, Sabine Kern, Anna Telaar, Gerhard Walzl, Gillian F. Black, Joachim Selbig, Shreemanta K. Parida, Stefan H E. Kaufmann, and Marc Jacobsen. Biomarker discovery in heterogeneous tissue samples -taking the in-silico deconfounding approach. *BMC Bioinformatics*, 11:27, 2010.

[339] Timo Erkkilä, Saara Lehmusvaara, Pekka Ruusuvuori, Tapio Visakorpi, Ilya Shmulevich, and Harri Lähdesmäki. Probabilistic analysis of gene expression measurements from heterogeneous tissues. *Bioinformatics*, 26(20):2571–2577, Oct 2010.

[340] Alexandre Kuhn, Doris Thu, Henry J. Waldvogel, Richard L M. Faull, and Ruth Luthi-Carter. Population-specific expression analysis (psea) reveals molecular changes in diseased brain. *Nat Methods*, 8(11):945–947, Nov 2011.

[341] Yi Zhong, Ying-Wooi Wan, Kaifang Pang, Lionel M L. Chow, and Zhandong Liu. Digital sorting of complex tissues for cell type-specific gene expression profiles. *BMC Bioinformatics*, 14:89, 2013.

[342] Jaeil Ahn, Ying Yuan, Giovanni Parmigiani, Milind B. Suraokar, Lixia Diao, Ignacio I. Wistuba, and Wenyi Wang. Demix: deconvolution for mixed cancer transcriptomes using raw measured data. *Bioinformatics*, 29(15):1865–1871, Aug 2013.

[343] Gerald Quon, Syed Haider, Amit G. Deshwar, Ang Cui, Paul C. Boutros, and Quaid Morris. Computational purification of individual tumor gene expression profiles leads to significant improvements in prognostic prediction. *Genome Med*, 5(3):29, 2013.

[344] Robert O. Stuart, William Wachsman, Charles C. Berry, Jessica Wang-Rodriguez, Linda Wasserman, Igor Klacansky, Dan Masys, Karen Arden, Steven Goodison, Michael McClelland, Yipeng Wang, Anne Sawyers, Iveta Kalcheva, David Tarin, and Dan Mercola. In silico dissection of cell-type-associated patterns of gene expression in prostate cancer. *Proc Natl Acad Sci U S A*, 101(2):615–620, Jan 2004.

[345] Harri Lähdesmäki, Llya Shmulevich, Valerie Dunmire, Olli Yli-Harja, and Wei Zhang. In silico microdissection of microarray data from heterogeneous cell populations. *BMC Bioinformatics*, 6:54, 2005.

[346] Shai S. Shen-Orr, Robert Tibshirani, Purvesh Khatri, Dale L. Bodian, Frank Staedtler, Nicholas M. Perry, Trevor Hastie, Minnie M. Sarwal, Mark M. Davis, and Atul J. Butte. Cell type-specific gene expression differences in complex tissues. *Nat Methods*, 7(4):287–289, Apr 2010.

[347] Peng Lu, Aleksey Nakorchevskiy, and Edward M. Marcotte. Expression deconvolution: a reinterpretation of dna microarray data reveals dynamic changes in cell populations. *Proc Natl Acad Sci U S A*, 100(18):10370–10375, Sep 2003.

[348] Debashis Ghosh. Mixture models for assessing differential expression in complex tissues using microarray data. *Bioinformatics*, 20(11):1663–1669, Jul 2004.

[349] Min Wang, Stephen R. Master, and Lewis A. Chodosh. Computational expression deconvolution in a complex mammalian organ. *BMC Bioinformatics*, 7:328, 2006.

[350] Alexander R. Abbas, Kristen Wolslegel, Dhaya Seshasayee, Zora Modrusan, and Hilary F. Clark. Deconvolution of blood microarray data identifies cellular activation patterns in systemic lupus erythematosus. *PLoS One*, 4(7):e6098, 2009.

[351] Ting Gong, Nicole Hartmann, Isaac S. Kohane, Volker Brinkmann, Frank Staedtler, Martin Letzkus, Sandrine Bongiovanni, and Joseph D. Szustakowski. Optimal deconvolution of transcriptional profiling data using quadratic programming with application to complex clinical blood samples. *PLoS One*, 6(11):e27156, 2011.

[352] Ting Gong and Joseph D. Szustakowski. Deconrnaseq: a statistical framework for deconvolution of heterogeneous tissue samples based on mrna-seq data. *Bioinformatics*, 29(8):1083–1085, Apr 2013.

[353] Aaron M. Newman, Chih Long Liu, Michael R. Green, Andrew J. Gentles, Weiguo Feng, Yue Xu, Chuong D. Hoang, Maximilian Diehn, and Ash A. Alizadeh. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods*, 12(5):453–457, May 2015.

[354] Michael S. Rooney, Sachet A. Shukla, Catherine J. Wu, Gad Getz, and Nir Hacohen. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*, 160(1-2):48–61, Jan 2015.

[355] Mihaela Angelova, Pornpimol Charoentong, Hubert Hackl, Maria L. Fischer, Rene Snajder, Anne M. Krogsdam, Maximilian J. Waldner, Gabriela Bindea, Bernhard Mlecnik, Jerome Galon, and Zlatko Trajanoski. Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. *Genome Biol*, 16:64, 2015.

[356] A. R. Abbas, D. Baldwin, Y. Ma, W. Ouyang, A. Gurney, F. Martin, S. Fong, M. van Lookeren Campagne, P. Godowski, P. M. Williams, A. C. Chan, and H. F. Clark. Immune response in silico (iris): immune-specific genes identified from a compendium of microarray expression data. *Genes Immun*, 6(4):319–331, Jun 2005.

[357] Tatyana Chtanova, Rebecca Newton, Sue M. Liu, Lilach Weininger, Timothy R. Young, Diego G. Silva, Francesco Bertoni, Andrea Rinaldi, Stephane Chappaz, Federica Sallusto, Michael S. Rolph, and Charles R. Mackay. Identification of t cell-restricted genes, and signatures for different t cell responses, using a comprehensive collection of microarray datasets. *J Immunol*, 175(12):7837–7847, Dec 2005.

[358] Chana Palmer, Maximilian Diehn, Ash A. Alizadeh, and Patrick O. Brown. Cell-type specific gene expression profiles of leukocytes in human peripheral blood. *BMC Genomics*, 7:115, 2006.

[359] Jason E. Shoemaker, Tiago J S. Lopes, Samik Ghosh, Yukiko Matsuoka, Yoshihiro Kawaoka, and Hiroaki Kitano. Cten: a web-based platform for identifying enriched cell types from heterogeneous microarray data. *BMC Genomics*, 13:460, 2012.

[360] Kosuke Yoshihara, Maria Shahmoradgoli, Emmanuel Martínez, Rahulsimham Vegesna, Hoon Kim, Wandaliz Torres-Garcia, Victor Treviño, Hui Shen, Peter W. Laird, Douglas A. Levine, Scott L. Carter, Gad Getz, Katherine Stemke-Hale, Gordon B. Mills, and Roel G W. Verhaak. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun*, 4:2612, 2013.

[361] Bernhard Mlecnik, Marie Tosolini, Pornpimol Charoentong, Amos Kirilovsky, Gabriela Bindea, Anne Berger, Matthieu Camus, Mélanie Gillard, Patrick Bruneval, Wolf-Herman Fridman, Franck Pagès, Zlatko Trajanoski, and Jérôme Galon. Biomolecular network reconstruction identifies t-cell homing factors associated with survival in colorectal cancer. *Gastroenterology*, 138(4):1429–1440, Apr 2010.

[362] Luc de Chaisemartin, Jérémy Goc, Diane Damotte, Pierre Validire, Pierre Magdeleinat, Marco Alifano, Isabelle Cremer, Wolf-Herman Fridman, Catherine Sautès-Fridman, and Marie-Caroline Dieu-Nosjean. Characterization of chemokines and adhesion molecules associated with t cell presence in tertiary lymphoid structures in human lung cancer. *Cancer Res*, 71(20):6391–6399, Oct 2011.

[363] Alexandre Calon, Enza Lonardo, Antonio Berenguer-Llergo, Elisa Espinet, Xavier Hernando-Momblona, Mar Iglesias, Marta Sevillano, Sergio Palomo-Ponce, Daniele V F. Tauriello, Daniel Byrom, Carme Cortina, Clara Morral, Carles Barceló, Sebastien Tosi, Antoni Riera, Camille Stephan-Otto Attolini, David Rossell, Elena Sancho, and Eduard Batlle. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet*, 47(4):320–329, Apr 2015.

[364] Claudio Isella, Andrea Terrasi, Sara Erika Bellomo, Consalvo Petti, Giovanni Galatola, Andrea Muratore, Alfredo Mellano, Rebecca Senetta, Adele Cassenti, Cristina Sonetto, Giorgio Inghirami, Livio Trusolino, Zsolt Fekete, Mark De Ridder, Paola Cassoni, Guy Storme, Andrea Bertotti, and Enzo Medico. Stromal contribution to the colorectal cancer transcriptome. *Nat Genet*, 47(4):312–319, Apr 2015.

[365] J-Y. Feng, X-W. Diao, M-Q. Fan, P-X. Wang, Y. Xiao, X. Zhong, R-H. Wu, and C-B. Huang. Screening of feature genes of the renal cell carcinoma with dna microarray. *Eur Rev Med Pharmacol Sci*, 17(22):2994–3001, Nov 2013.

[366] Weiqi Tan, Michelle A T. Hildebrandt, Xia Pu, Maosheng Huang, Jie Lin, Surena F. Matin, Pheroze Tamboli, Christopher G. Wood, and Xifeng Wu. Role of inflammatory related gene expression in clear cell renal cell carcinoma development and clinical outcomes. *J Urol*, 186(5):2071–2077, Nov 2011.

[367] Magdalena B. Wozniak, Florence Le Calvez-Kelm, Behnoush Abedi-Ardekani, Graham Byrnes, Geoffroy Durand, Christine Carreira, Jocelyne Michelon, Vladimir Janout, Ivana Holcatova, Lenka Foretova, Antonin Brisuda, Fabienne Lesueur, James McKay, Paul Brennan, and Ghislaine Scelo. Integrative genome-wide gene expression profiling of clear cell renal cell carcinoma in czech republic and in the united states. *PLoS One*, 8(3):e57886, 2013.

[368] Elzbieta Zodro, Marcin Jaroszewski, Agnieszka Ida, Tomasz Wrzesinski, Zbigniew Kwias, Hans Bluyssen, and Joanna Wesoly. Fut11 as a potential biomarker of clear cell renal cell carcinoma progression based on meta-analysis of gene expression data. *Tumour Biol*, 35(3):2607–2617, Mar 2014.

[369] Zisan Zeng, Tengcheng Que, Jiange Zhang, and Yanling Hu. A study exploring critical pathways in clear cell renal cell carcinoma. *Exp Ther Med*, 7(1):121–130, Jan 2014.

[370] Tsung Wen Chong, Fera Yiqian Goh, Mei Yi Sim, Hong Hong Huang, Daw Aye Aye Thike, Weng Khong Lim, Bin Tean Teh, and Puay Hoon Tan. Cd1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol*, 68(3):200–205, Mar 2015.

[371] Katherine A. Hoadley, Christina Yau, Denise M. Wolf, Andrew D. Cherniack, David Tamborero, Sam Ng, Max D M. Leiserson, Beifang Niu, Michael D. McLellan, Vladislav Uzunangelov, Jiashan Zhang, Cyriac Kandoth, Rehan Akbani, Hui Shen, Larsson Omberg, Andy Chu, Adam A. Margolin, Laura J. Van't Veer, Nuria Lopez-Bigas, Peter W. Laird, Benjamin J. Raphael, Li Ding, A Gordon Robertson, Lauren A. Byers, Gordon B. Mills, John N. Weinstein, Carter Van Waes, Zhong Chen, Eric A. Collisson, Cancer Genome Atlas Research Network , Christopher C. Benz, Charles M. Perou, and Joshua M. Stuart. Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. *Cell*, 158(4):929–944, Aug 2014.

[372] Andrew J. Gentles, Aaron M. Newman, Chih Long Liu, Scott V. Bratman, Weiguo Feng, Dongkyoon Kim, Viswam S. Nair, Yue Xu, Amanda Khuong, Chuong D. Hoang, Maximilian Diehn, Robert B. West, Sylvia K. Plevritis, and Ash A. Alizadeh. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*, Jul 2015.

[373] Matthew N. McCall, Harris A. Jaffee, and Rafael A. Irizarry. frma st: frozen robust multiarray analysis for affymetrix exon and gene st arrays. *Bioinformatics*, 28(23):3153–3154, Dec 2012.

[374] Matthew N. McCall, Benjamin M. Bolstad, and Rafael A. Irizarry. Frozen robust multiarray analysis (frma). *Biostatistics*, 11(2):242–253, Apr 2010.

[375] Bernhard Mlecnik, Gabriela Bindea, Helen K. Angell, Maria Stella Sasso, Anna C. Obenauf, Tessa Fredriksen, Lucie Lafontaine, Amelie M. Bilocq, Amos Kirilovsky, Marie Tosolini, Maximilian Waldner, Anne Berger, Wolf Herman Fridman, Arash

Rafii, Viia Valge-Archer, Franck Pagès, Michael R. Speicher, and Jérôme Galon. Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. *Sci Transl Med*, 6(228):228ra37, Mar 2014.

[376] G. Nilsson, T. Blom, M. Kusche-Gullberg, L. Kjellén, J. H. Butterfield, C. Sundström, K. Nilsson, and L. Hellman. Phenotypic characterization of the human mast-cell line hmc-1. *Scand J Immunol*, 39(5):489–498, May 1994.

[377] Sarah Fox, Andrew E. Leitch, Rodger Duffin, Christopher Haslett, and Adriano G. Rossi. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *J Innate Immun*, 2(3):216–227, 2010.

[378] Yosra Messai, Muhammad Zaeem Noman, Meriem Hasmim, Bassam Janji, Andrés Tittarelli, Marie Boutet, Véronique Baud, Elodie Viry, Katy Billot, Arash Nanbakhsh, Thouraya Ben Safta, Catherine Richon, Sophie Ferlicot, Emmanuel Donnadieu, Sophie Couve, Betty Gardie, Florence Orlanducci, Laurence Albiges, Jerome Thiery, Daniel Olive, Bernard Escudier, and Salem Chouaib. Itpr1 protects renal cancer cells against natural killer cells by inducing autophagy. *Cancer Res*, 74(23):6820–6832, Dec 2014.

[379] F. A. Vyth-Dreese, J. Sein, W. van de Kasteele, T A M. Dellemijn, C. van den Bogaard, W. J. Nooijen, G. C. de Gast, J B A G. Haanen, and A. Bex. Lack of anti-tumour reactivity despite enhanced numbers of circulating natural killer t cells in two patients with metastatic renal cell carcinoma. *Clin Exp Immunol*, 162(3):447–459, Dec 2010.

[380] Petra U. Prinz, Anna N. Mendler, Dorothee Brech, Ilias Masouris, Ralph Oberneder, and Elfriede Noessner. Nk-cell dysfunction in human renal carcinoma reveals diacylglycerol kinase as key regulator and target for therapeutic intervention. *Int J Cancer*, 135(8):1832–1841, Oct 2014.

[381] Patrick Chames and Daniel Baty. Bispecific antibodies for cancer therapy: the light at the end of the tunnel? *MAbs*, 1(6):539–547, 2009.

[382] Franck Pagès, Amos Kirilovsky, Bernhard Mlecnik, Martin Asslaber, Marie Tosolini, Gabriela Bindea, Christine Lagorce, Philippe Wind, Florence Marliot, Patrick Bruneval, Kurt Zatloukal, Zlatko Trajanoski, Anne Berger, Wolf-Herman Fridman, and Jérôme Galon. In situ cytotoxic and memory t cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol*, 27(35):5944–5951, Dec 2009.

[383] Domenico Coppola, Michael Nebozhyn, Farah Khalil, Hongyue Dai, Timothy Yeatman, Andrey Loboda, and James J. Mulé. Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by immune gene array profiling. *Am J Pathol*, 179(1):37–45, Jul 2011.