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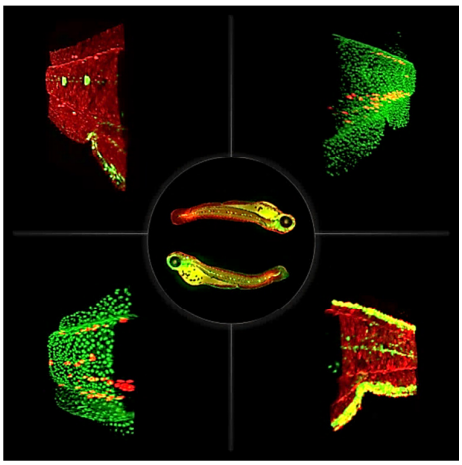
HOW HYDROGEN PEROXIDE AND CXCL8 MODULATE NEUTROPHIL RECRUITMENT *IN VIVO*



Sofia Ludovina Novais de Oliveira

PhD in Biomedical Sciences

Speciality in Molecular and Cellular Biology

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HOW HYDROGEN PEROXIDE AND CXCL8 MODULATE NEUTROPHIL RECRUITMENT *IN VIVO*

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FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

Para a minha Mãe,

o meu irmão Nelson, a minha irmã Vânia

e para ti Tiago...

For my Mother ,

my brother Nelson, my sister Vânia

and for you Tiago...

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¡Muchas gracias a todos!

List of Abbreviations

μm	Micrometer
ADAM17	A disintegrin and metalloproteinase domain 17
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AS	Antisense
auf	Arbitrary units of fluorescence
BCRs	B cell receptors
cDNA	Complementary deoxyribonucleic acid
CHT	Caudal hematopoietic tissue
COX2b	Cyclooxygenase 2b
DAMPs	Damage-associated molecular patterns
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpf	Days post-fertilization
DPI	Dibenziodolium chloride
Duox1	Dual oxidase 1
Fw	Forward oligonucleotide
FACS	Fluorescence activated cell system
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
hpf	Hours post-fertilization

hpi	Hours post-injection
hpw	Hours post-wound
IBD	Inflammatory bowel disease
iCa^{2+}	Intracellular Calcium
Ig	Immunoglobulin
IL1β	Interleukin 1 beta
IL1R	Interleukine 1 Receptor
IL6	Interleukine 6
IL8	Interleukine 8
IL10	Interleukine 10
LTB4	Leukotriene B4
mAb	Monoclonal antibody
mM	Milimolar
MHC	Major histocompatibility complexes
MMPs	Matrix matalloproteinases
MO	Morpholino
MPO	Mieloperoxidase
mRNA	Messenger ribonucleic acid
nd	Not detected
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa beta
Nox	NADPH oxidases
ns	Not significant
Rv	Reverse primer
RFP	Red fluorescent protein
ROS	Reactive oxygen species

rps11	Ribosomal protein S11 gene
PAMPs	Pathogen-associated molecular patterns
PLC	Phospholipase C
PRRs	Pattern recognition receptors
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
S.E.M	Standard error of the mean
SFKs	Src family kinases
Std C	Standard control
TCRs	T cell receptors
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Tnfa	Tumor necrosis factor alpha
Tnfr's	Tumor necrosis factor receptors
Tnfr1	Tumor necrosis factor receptor 1
Tnfr2	Tumor necrosis factor receptor 2
*Va*DNA	*Vibrio anguillarum* genomic deoxyribonucleic acid
WIHC	Whole mount immunohistochemistry
ZIRC	Zebrafish International Resource Center

Abstract

Neutrophils are one of the major pathogen-fighting immune cells that belong to the innate branch of the immune system. These cells play an important role in inflammation due to their outstanding ability to be quickly recruited to sites of infection or injury and there, to recognize and phagocyte threats and then to kill pathogens through a combination of cytotoxic mechanisms. During the last decade neutrophils have been shown not to be simply terminally differentiated cells with a short life-span with a microbicidal function, but also crucial modulators of the adaptive immune response by acting as antigen-presenting cells, B cell "helpers" or T cell suppressors. Additionally the idea that neutrophils are "dead end" cells whose ultimate fate is death within inflamed tissues is currently under dispute by strong *in vivo* evidences of reverse neutrophil migration, both in zebrafish and mice. Importantly, neutrophils are involved in several autoimmune and chronic inflammatory diseases with increasing rates of incidence in our society. In this regard, dysregulation of the mechanisms that control the inflammatory response are often observed to be associated with persistent neutrophilic tissue infiltration and extensive host tissue damage in several diseases. Taken all this, it is currently crucial to expand our knowledge of the mechanisms governing neutrophil migration so as to more appropriately target specific key components and further advance in the development of more effective treatments for those diseases.

In this thesis, we have focused our studies onto two main neutrophil chemoattractant molecules, the Cxcl8 and hydrogen peroxide (H_2O_2). CXCL8 is the prototypical member of the CXCL8/IL8-chemokine sub-family of CXC chemokines that acts on CXCR1 and CXCR2 receptors. CXCL8 was actually one of the first chemokine to be discovered and to due to its important biological role gave name to this sub-family. CXCL8 is mostly known as a potent chemoattractant for neutrophils, although it has also been shown to be involved in other processes such as leukocyte biology and function, angiogenesis and cancer. As for H_2O_2, this molecule was until recently extensively studied as one of the most important components of the neutrophil oxidative burst response against threats. In 2010, a new role was discovered for this harmful molecule in the zebrafish inflammation. Upon production by the Dual oxidase 1 (Duox1), H_2O_2 was shown to be released by the wounded tissue as an initial signal responsible for early neutrophil recruitment. Taken all this we aimed in this thesis to address the mechanisms underlying Cxcl8 and H_2O_2 expression/production and function in zebrafish inflammation and most importantly, to understand whether and how the action of these signals may be concerted so as to modulate neutrophil recruitment during the inflammatory response.

Zebrafish is nowadays a very powerful and promising animal model. In particular, it has several features that make this small fish an amazing, if not unique, model for the study of neutrophil recruitment by non-invasive methods. The most important ones are: a complete sequenced genome; a high homology with humans; an immune system that is remarkably similar to

mammalian counterparts after 4-5 weeks and that enable the study of innate immune responses independently of adaptive ones at earlier developmental stages; and the most captivating one, the transparency of their embryos that along with the large availability of transgenic lines allow *in vivo* tracking of specific cells. Importantly, the zebrafish has two true homologues of the mammalian CXCL8, namely Cxcl8-l1 and Cxcl8-l2, which are absent from the mouse.

We started by asking if the zebrafish Cxcl8s had the same biological role in the inflammatory response as in mammals. By using 3 days post-fertilization larvae and tail fin wound as an inflammatory stimulus we verified that both chemokines are induced after wounding and that both are crucial for normal neutrophil recruitment and inflammatory resolution. Additionally, using the open-source MATLAB package, PhagoSight, we found that Cxcl8s absence increase neutrophil recruitment speed. We postulated that this effect may be due to the existence of different neutrophil subpopulations with perhaps different functions that could respond differentially to CXCL8 chemokines and other chemoattractants expressed locally during inflammatory process thus exhibiting distinct migratory behaviors. In addition, we further addressed the role of Cxcl8-l1 and Cxcl8-l2 in the inflammatory response after infection. First, we found that both Cxcl8s are also induced upon different infectious stimuli. In the *Salmonella enterica* serovar Typhimurium (*Salmonella* Thyphimurium) infection model, we further observed that larvae survival, neutrophil recruitment and bacteria clearing are significantly reduced when compared to normal conditions. Altogether these results demonstrate that both Cxcl8 chemokines seem to be important for inflammatory response in zebrafish either against tissue damage or infection. Our findings fully establish the use of zebrafish as an ideal animal model to study Cxcl8 biological functions but most importantly we have expanded the knowledge concerning the chemokine function *in vivo*

Besides being a modulator of early neutrophil recruitment, Duox1-derived H_2O_2 has been also shown to affect tissue regeneration what suggested to us that this molecule might also be involved in the modulation of the inflammatory response and could further be involved in inflammatory disorders. In this respect, our first goal was to address which signals would be able to activate Duox1 and further H_2O_2 production upon wounding. We demonstrated that, as an early danger signal released after wounding, ATP is able to activate purinergic P2Y receptors, and to further modulate Duox1 activity through phospholipase C (PLC) and intracellular calcium signaling. In addition, we also proved that the Duox1-derived H_2O_2 is able to trigger NF-κB signaling pathway in the wound-associated inflammatory response.

In view of the distinct temporal expression of these two chemotactic cues, we next hypothesized that H_2O_2 could be able to influence Cxcl8 signaling and by this way modulate later neutrophil recruitment and inflammatory response. Further studies led us to confirm that the early H_2O_2 signal is a key regulatory factor in the *in vivo* wound response by crosstalking with the later Cxcl8 signaling pathway. In particular, we showed that Duox1-derived H_2O_2 modulates Cxcl8-l2 expression by a complex mechanism involving SFKs, p38 and Jnk/AP1 signaling pathways, as well as histone 3 modifications and AP1 binding to *cxcl8-l2* proximal promoter. Our findings support

the idea that H_2O_2 is an early signal involved not only in initial neutrophil recruitment but is also essential for later neutrophil responses as well as other processes central to inflammation and tissue regeneration by modulating important signaling pathways like Cxcl8-l2, NF-κB and Jnk/AP1.

Finally, we were also able to particularly present Duox1/H_2O_2 as a key modulator in skin inflammatory diseases. Here, we developed a skin inflammatory model, based on the perturbation of the role of the Tnfa/ Tnfr2 signaling in the maintenance of zebrafish skin homeostasis. In this, we observed that the blockade of this signaling pathway induces the production of H_2O_2 by Duox1 which in turn, activates NF-κB and results in the upregulation of genes encoding pro-inflammatory mediators (such as Il1β and Cxcl8s) and neutrophil infiltration in skin tissues. Most importantly, we found that DUOX1 is induced in skin lesions of psoriasis and lichen planus patients, indicating the involvement of H_2O_2 and DUOX1 in human skin inflammation. Importantly, it is very likely that the H_2O_2-based mechanism here described is as well present in several other autoimmune/chronic inflammatory diseases. In this respect, pharmacologic and genetic therapies that target Duox1/H_2O_2 could provide innovative approaches to the management of not only psoriasis or lichen planus but also of other important inflammatory diseases.

Altogether, the data reported in this thesis unraveled in our opinion important new findings in the field of inflammation not only regarding the Cxcl8 *in vivo* function in neutrophil recruitment and behavior, but also regarding H_2O_2 physiological function as an early inflammatory signal. Importantly, we clarified relevant issues involving Duox1 activation *in vivo* by showing the role of ATP and intracellular calcium in this matter. Additionally, we demonstrated the ability of H_2O_2 to modulate key inflammatory signaling pathways like NF-κB and Jnk/ AP1 and the existence of a crosstalk between early H_2O_2 and later Cxcl8-l2 signals. At the end we also were able to establish the involvement of H_2O_2 and DUOX1 in human and zebrafish skin inflammation. Overall this study has undoubtedly confirmed that the zebrafish is an ideal model to study the complex processes involved in neutrophil recruitment and in the inflammatory response.

Resumo

Os neutrófilos são uma das principais células imunitárias de combate a agentes patogénicos que pertencem ao ramo inato do sistema imunitário. Estas células desempenham um papel importante na inflamação, devido à sua notável capacidade de ser rapidamente recrutadas para locais de infecção ou lesão e de reconhecer e fagocitar as ameaças, que em seguida mata através de uma combinação de mecanismos de citotoxicidade. Durante a última década, os neutrófilos demonstraram não ser simplescélulas diferenciadas, com um tempo de vida curto e com uma função microbicida, mas também moduladores importantes da resposta imune adaptativa, agindo como células apresentadoras de antigénio, "ajudantes" de celulas B e supressores de celulas T. Além disso, a ideia de que os neutrófilos são células "*dead-end* ", cujo destino final é a morte dentro dos tecidos inflamados, está atualmente a perder poder devido a fortes evidências *in vivo* da existência de um mechanismo de migração reversa de neutrófilos, tanto em peixes-zebra como em ratos. É importante salientar que os neutrófilos estão envolvidos em várias doenças inflamatórias auto-imunes e crónicas, com taxas de incidência cada vez maiores na nossa sociedade. A este respeito, a desregulação dos mecanismos que controlam a resposta inflamatória em diversas doenças é frequentemente associada à infiltração de neutrofílos persistente no tecido e aos extensos danos provocados. Tendo em conta tudo isto é atualmente crucial expandir o nosso conhecimento sobre os mecanismos que regem a migração de neutrófilos, de modo a atingir de forma mais adequada componentes principais específicos e a avançar no desenvolvimento de tratamentos mais eficazes para essas doenças.

Nesta tese, focamos os nossos estudos em duas principais moléculas conhecidas como tendo um elevado poder quimiotáctico sobre os neutrófilos, o CXCL8 e o peróxido de hidrogénio (H_2O_2). CXCL8 é o membro prototípico da sub- família CXCL8/IL8-chemokine de quimiocinas CXC, que atua sobre os receptores CXCR1 e CXCR2. CXCL8 foi uma das primeira quimiocina a ser descoberta e devido ao seu papel biológico importante deu nome a esta sub- família. CXCL8 é conhecida principalmente por ser um quimioatrator potente para neutrófilos, embora também tenha sido demonstrado estar envolvida em outros processos, tais como na biologia e na função dos leucócitos, angiogénese e cancro. Quanto ao H_2O_2, foi até recentemente extensivamente estudado como um dos componentes mais importantes da resposta oxidativa de neutrófilos. Em 2010, uma nova função foi descoberta para essa molécula na inflamação em peixe-zebra. Após a sua produção por dual oxidase 1 (Duox1), o H_2O_2 demonstrou ser libertado pelo tecido lesado como um sinal responsável pelo recrutamento inicial de neutrófilos. Tendo em conta toda esta informação o nosso objetivo nesta tese foi abordar os mecanismos subjacentes à expressão/produção e função de CXCL8 e de H_2O_2 na inflamação do peixe-zebra e, mais importante entender se e como a ação destes sinais pode ser conciliada de modo a modular o recrutamento de neutrófilos durante a resposta inflamatória.

O peixe-zebra é hoje em dia um modelo animal muito poderoso e promissor. Em particular, ele tem várias características que tornam este pequeno peixe num incrível, senão único, modelo para o estudo do recrutamento de neutrófilos através de métodos não-invasivos. As mais importantes são: genoma totalmente sequenciado; elevada homologia com os seres humanos; sistema imunológico que é notavelmente semelhante ao seu homólogo em mamíferos (após 4-5 semanas), permite o estudo da resposta imune inata independentemente da presença do sistema imune adaptado em estadios de desenvolvimento inicial; e talvez uma das mais interessantes características, a transparência dos seus embriões que, juntamente com a grande disponibilidade de linhas transgénicas permitem o rastreio *in vivo* de células específicas. Mais importante, o peixe-zebra tem dois verdadeiros homólogos da CXCL8 de mamíferos, nomeadamente Cxcl8-l1 e Cxcl8-l2, que estão ausentes do rato.

A nossa primeira questão foi se Cxcl8s tinham a mesma função biológica na resposta inflamatória no peixe-zebra que nos mamíferos. Usando larvas de 3dias após-fertilização e o corte de cauda como estímulo inflamatório verificou-se que ambas as quimiocinas são induzidas após o ferimento e que ambas são cruciais para o recrutamento normal de neutrófilos e resolução da inflamação. Além disso, usando o pacote desenvolvido em código-aberto de MATLAB, PhagoSight, descobrimos que a velocidade de recrutamento dos neutrófilos aumenta na ausência de Cxcl8s. Proposemos então que este efeito se deve à existência de diferentes subpopulações de neutrófilos, talvez com diferentes funções, que poderão responder diferencialmente a quimiocinas CXCL8 e a outras moléculas quimioatrativas expressas localmente durante o processo inflamatório exibindo assim comportamentos migratórios distintos. De seguida estudámos a função de Cxcl8-l1 e Cxcl8-l2 na resposta inflamatória após a infecção. Em primeiro lugar, verificámos que ambos os Cxcl8s são induzidos mediante diferentes estímulos infecciosos. Usando o modelo de infecção por Salmonella Typhimurium, observámos ainda que a taxa de sobrevivência, o recrutamento dos neutrófilos e a limpeza das bactérias são significativamente reduzidos uma vez comparados com as condições normais. Em conjunto estes resultados demonstram que ambas as quimiocinas CXCL8 parecem ser importante para a resposta inflamatória no peixe-zebra tanto provocados por danos no tecido como por infecção. As nossas descobertas estabelecem o uso do peixe-zebra como um modelo animal ideal para estudar as funções biológicas de CXCL8, mas a descoberta mais importante foi termos conseguido ampliar o conhecimento sobre a função de quimiocinas *in vivo*.

Além de ser um modulador inicial de recrutamento de neutrófilos, o H_2O_2 derivado de Duox1 também afecta a regeneração dos tecidos sugerindo que esta molécula pode estar envolvida na modulação da resposta inflamatória e ser importante em doenças inflamatórias. A este respeito, o primeiro objectivo foi descobrir que sinais libertados pela lesão seriam capazes de activar Duox1 e induzir a produção de H_2O_2. Demonstramos que o sinal de perigo, ATP , libertado logo após lesão, é capaz de ativar os receptores purinérgicos P2Y, e de modular a atividade de Duox1 através da fosfolipase C (PLC) e sinalização intracelular de cálcio. Além disso, também demonstrámos que o H_2O_2 derivado de Duox1 é capaz de desencadear a activação da via de sinalização NF-κB.

Em vista da expressão temporal distinto destes dois sinais quimiotáticos, postulámos a hipótese de que o H_2O_2 pode ser capaz de influenciar a sinalização CXCL8 e desta forma modular o recrutamento tardio de neutrófilos e da resposta inflamatória. Estudos posteriores levaram-nos a confirmar que o sinal inicial de H_2O_2 é um factor chave na regulação da resposta *in vivo* á lesão de tecidos através do "crosstalk" com a posterior via de sinalização de Cxcl8. Em particular, demonstrámos que o H_2O_2 modula a expressão do Cxcl8-l2 por um mecanismo complexo que envolve SFKs , p38 e vias de sinalização Jnk/AP1 , bem como modificações da histona 3 e ligação de AP1 ao promotor proximal de Cxcl8- l2 . Os nossos resultados suportam a ideia de que o H_2O_2 é um sinal precoce envolvido não só no recrutamento inicial de neutrófilos, mas também essencial para a resposta de neutrófilos posterior, bem como para outros processos fundamentais na inflamação e regeneração dos tecidos, modulando vias de sinalização importantes como Cxcl8-l2, NF -kB e Jnk/AP1.

Por fim, fomos capazes de apresente Duox1/H_2O_2 como um modulador chave em doenças inflamatórias da pele. Desenvolvemos um modelo inflamatório da pele, que tem por base a perturbação da função da sinalização de TNFa/TNFR2 na manutenção da homeostase da pele do peixe-zebra. Observámos que o bloqueio desta via de sinalização induz a produção de H_2O_2 por Duox1 e que por sua vez activa NF-κB, resultando na indução de genes que codificam mediadores pró-inflamatórios (tais como Il1β e Cxcl8s) e a infiltração de neutrófilos na pele. Mais importante, descobrimos que DUOX1 é induzida em lesões de pele de pacientes com psoríase e líquen plano, indicando o envolvimento de H_2O_2 e DUOX1 na inflamação da pele humana. É muito provável que o mecanismo baseado em H_2O_2 aqui descrito esteja presentes em várias outras doenças inflamatórias auto-imunes/crónicas. A este respeito, terapias farmacológicas e genéticas contra Duox1/H_2O_2 poderiam proporcionar abordagens inovadoras para a gestão não apenas de psoríase ou líquen plano, mas também de outras doenças inflamatórias importantes.

Ao todo, os dados relatados nesta tese expõem na nossa opinião importantes novas descobertas no campo da inflamação, não só em relação à função de CXCL8 *in vivo* no recrutamento e comportamento dos neutrófilos, mas também em relação a função fisiológica do H_2O_2 como um sinal inflamatório inicial. Mais importante, esclarecemos questões relevantes que envolvem a ativação Duox1 *in vivo*, mostrando o papel do ATP e do cálcio intracelular nesta matéria. Além disso, foi demonstrada a capacidade do H_2O_2 para modular principais vias de sinalização inflamatórias, como o NF -kB e Jnk / AP1 e a existência de um "crosstalk" entre H_2O_2 e Cxcl8-l2. No final, também fomos capazes de estabelecer o envolvimento de H_2O_2 e DUOX1 na inflamação da pele humana e de peixe-zebra. Em geral, este estudo confirmou que, sem dúvida, o peixe-zebra é um modelo ideal para estudar os processos complexos envolvidos no recrutamento dos neutrófilos e na resposta inflamatória.

Chapter 1

General Introduction

General Introduction

Immunity

All living things- animal, plants, and even bacteria- can possibly be threaten by infectious stimuli , so in order to defend themselves and survive from this threats host organisms developed an immune system, that according to its complexity can be more or less complex (1-3). Immune system is composed by several different cell types, molecules and tissues. The collective and coordinated response through time against these foreign substances constitutes the immune response.

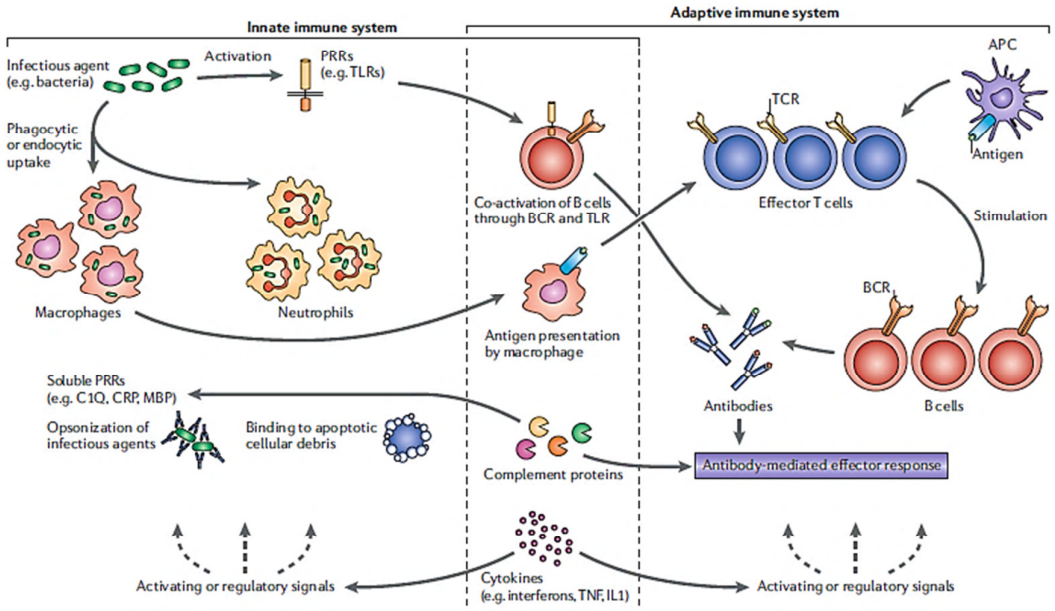

Figure 1: The innate and adaptive immune systems crosstalk. Innate immune mechanisms generally involve immediate, nonspecific responses to foreign infectious agents, and include cellular functions such as phagocytosis and endocytosis by macrophages and neutrophils. Adaptive immune mechanisms involve the engagement of receptors that are selected for reactivity with specific antigens (T-cell receptors (TCRs) and immunoglobulin receptors on B cells). Innate and adaptive immune systems crosstalk occurs by means that have not been fully understood. (Adapted from (4)).

Besides natural barriers, immune system has two main branches, the innate immune system and the adaptive immune system (Figure 1) (5). Most organism survive due to innate immune mechanisms alone, just vertebrates had created an alternative pathogen recognition and elimination system called adaptive immunity (5, 6). For many years adaptive immune system has captivated immunologists' attention basically because innate immunity was considered by many as "primitive" or "rough". (6). Nowadays it is commonly accepted that, in higher organisms the development of an effective immune response will always depend on the careful interplay and

regulation between innate and adaptive immunity (5). A complex system like this requires therefore sophisticated mechanisms to control and regulate its activity as well as to avoid the possibility of developing immune responses against the self. Such mechanisms may still sometimes fail allowing the development of severe autoimmune disorders, such rheumatoid arthritis, psoriasis or lupus (4).

Natural barriers

The first line of defense of an organism is the natural barriers, included by some authors within the innate immune system (Figure 2). These barriers are responsible for preventing infectious agents from invading the host or for providing a hostile environment against them. Among these physical barriers that are also able to secrete anti-microbial substances we can find epithelia from skin, lung, airways and the gastrointestinal and urogenital tracts that (2).

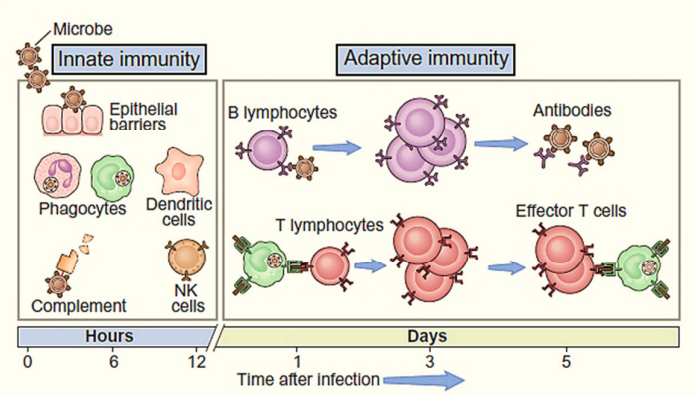

Figure 2: Main intervenient in innate and adaptive immune systems. (Adapted from (1).

The innate immune system

The innate immune system appeared millions of years before the adaptive one, the innate immune response is mainly orchestrated by myeloid cells, such as macrophages and neutrophils, and soluble molecules, such as complement and acute phase proteins (Figure 2) (1, 2, 7). These professional phagocytes are responsible for the detection, engulfment and clearance of host threats. To accomplish these functions, these cells need to be able to detect threats in order to trigger alarm signals and further immune response (2, 8, 9). For such, innate immune cells express pattern recognition receptors (PRRs) (Figure 1) that are able to recognize conserved features of infectious agents called pathogen-associated molecular patterns (PAMPs). Many PRRs can also be engaged by damage-associated molecular patterns (DAMPs) which are released after tissue damage or by necrotic cells during sterile inflammation (2, 5, 8, 9). As part of the still growing PRR family, we highlight toll-like receptors (TLRs) which display different ligand-binding specificities (2, 5, 10).

The adaptive immune system

During evolution, vertebrates have evolved more complex immune systems that further comprise an adaptive or acquired immune system in order to respond more specifically to threats. This system is composed of highly specialized cells, lymphocytes, responsible for eliminating or preventing pathogen outgrowth (Figure 1 and 2). Acquired immunity further enables the creation of an immunological memory upon a first contact with a specific pathogen that ensures the rapid assembly of an immune response of greater magnitude to subsequent encounters with that microbe (2). There are two types of adaptive immunity, the humoral immunity and the cell-mediated immunity (Figure 3). These responses are mediated by different lymphocytes and molecules designed to provide defense against extracellular microbes (the humoral immunity based on the B lymphocyte-mediated antibody production) and intracellular microbes (the cell-mediated immunity based on the action of T lymphocytes)(1, 2, 9).

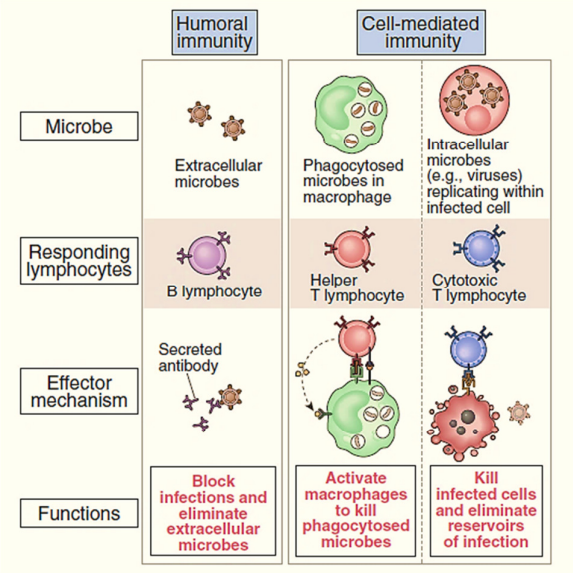

Figure 3: Types of adaptive immunity. Adaptive immune system has two main arms, the humoral and the cell-mediated response. According to the detected threat specific mechanisms are activated in order to trigger different immune responses (Adapted from (2)

As mentioned before innate immune cells express PRRs that enable them to discriminate signs of infection or tissue injury and stress. The adaptive immune cells are able to recognize foreign antigens via by specifically tailored lymphocyte antigen receptors, the B cell receptors (BCRs) in B lymphocytes and T cell receptors (TCRs) in T lymphocytes (Figure 1) (1, 2). BCRs have evolved to recognize components of infectious agents that are derived from the extracellular compartments of the body, including the tissue fluids. Such infectious agents include, for example, free viruses and bacteria in the blood or in extracellular fluids. In contrast to B cells, T cells are able to recognize foreign antigens presented in the context of major histocompatibility complexes (MHC) molecules by the antigen-presenting cells, such as for example, phagocytes that have internalized bacteria or cells that have been infected by viruses (1, 2, 9).

The generation of both these antigen receptors, TCRs and BCRs, involves DNA recombination processes that ensure the creation of an impressive array of receptors, capable of recognizing every possible antigen, including those present in our own body components - self antigens. Usually lymphocytes expressing these autoreactive receptors are eliminated or their action restrained by tolerance mechanisms. However, if immunological tolerance fails then autoimmune disorders may develop.(4, 9).

Inflammation

In response to infections or tissue damage, stress or malfunction and upon activation of TLRs and other PRRs the innate immune system launches a pathophysiological response, termed as inflammation, with the main purpose of neutralizing the causative agent of the threat (5, 11-14). Upon detection of an insult, the host inflammatory response is quickly initiated by epithelial cells and tissue resident macrophages and further involving the migration of monocytes (that later on differentiates into macrophages) and neutrophils, among other cells, towards the site of inflammation (5, 14-16). Among other functions, tissue resident macrophages, phagocytic cells residing within many tissues, are importantly responsible for the production of high levels of inflammatory cytokines, like IL1β, TNFα or IL6, and chemokines like CXCL8 that further convey a "red alert" message of the threat onto other cellular players (5, 16). The local release of these mediators and others such as histamine or LTB4 immediately induce a diversity of changes at the inflamed area that comprise for instance, increased vascular permeability, increased expression of adhesion molecules by the endothelium and other cellular players and the recruitment of leukocytes, mainly neutrophils (Figure 4) (5, 8).

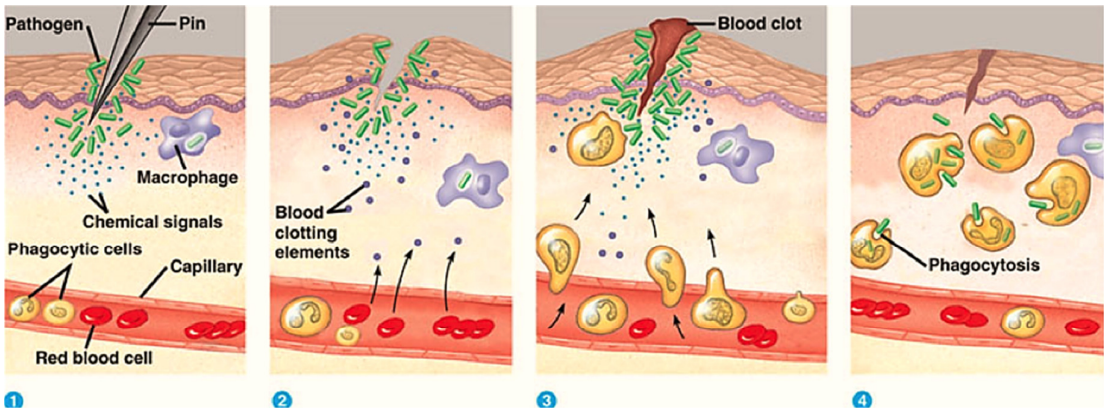

Figure 4: Scheme for inflammatory response cascade of events. Inflammatory responses initiates with the detection of the threat by resident cells that entering in a "red alert" state release several chemical signals that are sensed by endothelial cells initiating a cascade of events to favor leukocyte recruitment. Next neutrophils are recruited by extravasation process and migrate towards the tissue following chemotactic cues until the inflammatory locus. Once in the inflamed tissue neutrophils initiate very quickly clearing process in order to restore tissue homeostasis. (Adapted from http://www.studyblue.com/notes/note/n/biol-1202-spring-2012-final-exam-flashcards/deck/2723216).

As mentioned before, the inflammatory response combines a series of events that occur through time (Figure 5) and it is characterized by a coordinated activation of several signaling pathways that regulate the expression of both pro- and anti-inflammatory mediators. Among the most important ones, one can highlight the action of IL1/IL1R, TLR, TNFα/TNFRs, CXCL8/CXCR1/2, NF-κB and AP1 signaling pathways (17). Some of these have actually been used as targets for the development of anti-inflammatory treatments.

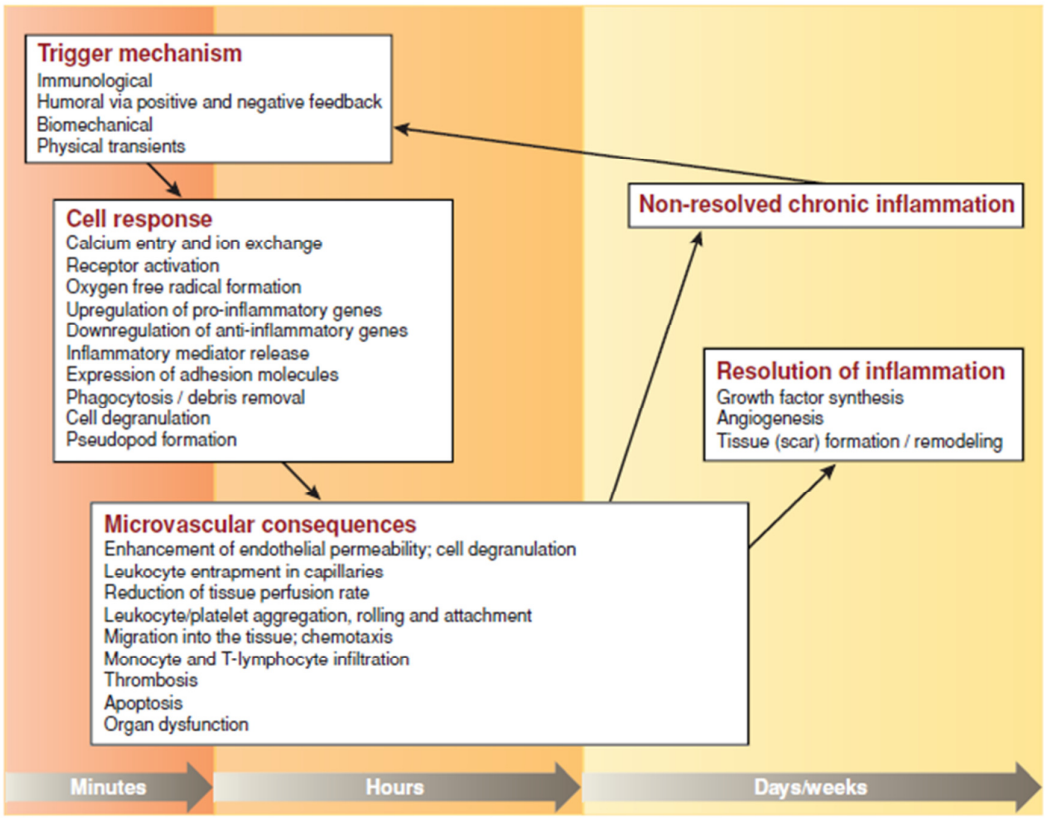

Figure 5: A schematic timeline of selected steps in the inflammatory cascade. Inflammatory response may leads to resolution and final repair of the initial injury, or under the influence of continued stimulation it may progress into a chronic inflammatory cascade. (Adapted from (14))

Inflammation can be divided in two types, acute and chronic, according to the duration of the inflammatory response and consequently, to the types of cells involved in it. Acute inflammation (Figure 4) comprehends a series of coordinated events that result in diverse systemic effects such as, changes in temperature, blood flow, monocyte and neutrophil trafficking and serum protein levels (5, 14). Ultimately an acute inflammatory process has to be necessarily resolved (figure 5) (11), via the shutdown of pro-inflammatory pathways and the activation and release of several anti-inflammatory and pro-resolution molecules, like IL10, resolvins and lipoxins (11, 18-20). In the case acute inflammation does not achieve successful resolution, the inflammatory process is further prolonged and progresses into a chronic phase (21). Most features of acute inflammation continue once inflammation becomes chronic, including increased blood flow and

increased capillary permeability. Accumulation of white blood cells in the inflamed area also persists, but the cellular composition of this infiltrate is importantly different. In chronic inflammation the main players are not only macrophages and neutrophils but also lymphocytes. The presence of these cells leads to the activation of a whole new series of local mechanisms and molecules release that are responsible to start an "un-shut" process. Among most common chronic disorders we have diabetes, rheumatoid arthritis, and psoriasis (17). The incidence of these diseases is rapidly increasing worldwide, mainly in the most developed countries, already causing a great impact for their national health systems. Actually, chronic inflammatory diseases lead the list of causes of mortality in most developed countries.

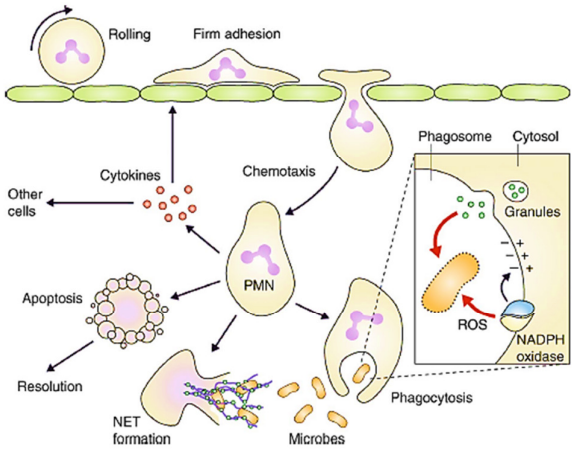

Figure 6: Main neutrophil functions until early 2000s. After the extravasion process neutrophils phagocyte and kill the invading pathogens. Pagocitosis includes the enclosure of threats creating phagosomes inside the neutrophil, further fusion of granules or delivery of ROS lead to microorganisms killing. This response can lead neutrophils to different fates such as apoptosis and further resolution or NETosis(Adapted from (22))

Neutrophil, a multifaceted cell

Neutrophils are the most abundant white-blood cells circulating in the human blood (they represent around 70% of all blood circulating leukocytes (refs)) and are the main pathogen-fighting immune cells of the innate immune system (8, 22-24). As for other blood cells, neutrophils are produced from haematopoietic progenitors in the bone marrow. Importantly and in case of emergency the granulocyte colony-stimulating factor (G-CSF) can induce neutrophil production up to 10 times in a process termed as "danger mobilization" (22, 23, 25). Mobilization of these cells from the bone marrow to the blood circulation is mediated by increased levels of CXCL8, CXCL1 and CXCL2 (23) acting via CXCR2 binding in mice, humans and also zebrafish (23, 26). Once neutrophils get to the blood, the neutrophil recruitment cascade can start (Figure 6) (27). This

process involves their interaction with the activated endothelium in the vicinity of inflamed regions of the body through the action of specific components, such as adhesion molecules and integrins, and ultimately leads to their extravasation into tissues (22, 23, 25, 27). Leukocyte recruitment has been shown to involve several stages, namely tethering, rolling, adhesion, crawling and transmigration (8, 22, 23, 25). Once within tissues neutrophils are guided by gradients of chemoattractants up to the focus of inflammation, such as H_2O_2 or chemokines like CXCL8. Such gradients are produced in the affected area by tissue cells like epithelial cells or fibroblasts, or by tissue resident cells like macrophages and mast cells that also act as sentinels (23, 25).

Neutrophils are crucial players in the early immune response against microbial pathogens. In response to such inflammatory stimuli neutrophils are activated so as to mediate their eradication by four main mechanisms of defence: phagocytosis, production of reactive oxygen species (ROS), degranulationm and neutrophil extracellular traps (NETs) (Figure 6) (8, 22, 23, 25). Phagocytosis involves the pathogen enclosure in a phagosome and its subsequent destruction inside this structure via the fusion with specific cytoplasmic granules or the action of ROS. Additionally, phagosome formation also triggers cytokine and chemokine production in order to further promote the recruitment of other immune cells (8, 23).

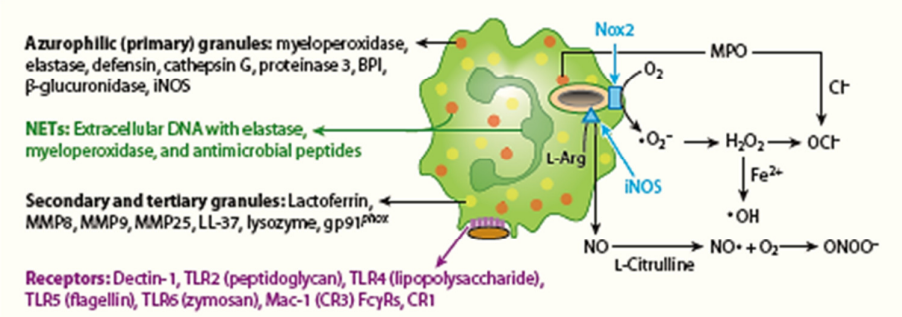

Figure 7: Scheme showing main tools of neutrophil to kill pathogens. The O_2-dependent mechanism involve the activation of Nox and further ROS formation, on the other side neutrophils also have granules with different contents of proteases that are release at different stages of inflammatory response. In addition, neutrophils also can promote decondensed DNA extrusion together with MPO or antimicrobial peptides. (Adapted from (8))

Pathogen killing processes of neutrophils are based on O_2-dependent and O_2-independent mechanisms. The O_2-dependent mechanisms exploit the ability of NADPH oxidase (Nox) to produce tremendously destructive ROS that induce pathogen killing once released into the phagosome or to the extracellular matrix (8, 23). The O_2-independent killing processes involve proteinases and antimicrobial peptides that are stored in cytoplasmic granules and that can be released into the phagosome upon membrane fusion. In alternative, these granules can be fused with the plasma membrane thus causing the extracellular release of their microbicidal content (8, 23). There are three types of granules: i) azurophil/primary granules which mainly contain myeloperoxidase (MPO) as well as elastase, cathepsin G and proteinase 3; ii) secondary granules that contain lactoferrin, lysozyme and several matrix metalloproteinases (MMPs), such as MMP8,

MMP9 and MMP25; iii) tertiary granules with a content similar to that of secondary ones but lacking lactoferrin (Figure 7) (8, 23). By far the most controversial killing mechanism of the neutrophil, but quite accepted nowadays, relates with the formation of NETs, currently coined as NETosis. This consists of the extrusion of decondensed chromatin associated to granule-derived products such as antimicrobial peptides, MPO and elastase. NETs are responsible for pathogens immobilization thus preventing them from spreading and enabling their phagocytosis, or their destruction by antimicrobial peptides and proteases (8, 23, 25, 28)

In response to inflammatory stimuli neutrophils can undergo different destinies at the inflammatory foci. Besides generating NETs, activated neutrophils can ultimately undergo apoptosis or reverse migration (Figure 8) (8, 25, 29-32). Uptake of apoptotic neutrophils by macrophages is extremely important to achieve inflammation resolution not only due to the clearance of neutrophils from the inflamed areas but nost importantly by inducing the production of anti-inflammatory and pro-resolution mediators (8, 11, 19, 20, 25, 28). Importantly a controlled balance between apoptosis and NETosis needs to occur although these mechanisms are still unknown and need further attention (8). Moreover, the classic idea that death is the ultimate fate of activated neutrophils is currently less robust. Several *in vivo* studies have shown not only in zebrafish but also in mice and humans, that neutrophils are as well able to do reverse migration, i.e., to migrate away from the site of inflammation (25, 28, 29, 31-35). Although the mechanisms and biological functions behind this process need to be further clarified, it seems that at least, some neutrophils can migrate to lymph nodes (through the lymphatic vessels or the blood circulation) where they may present antigens to T cells and thus contribute for inducing an adaptive immune response(28, 34-36).

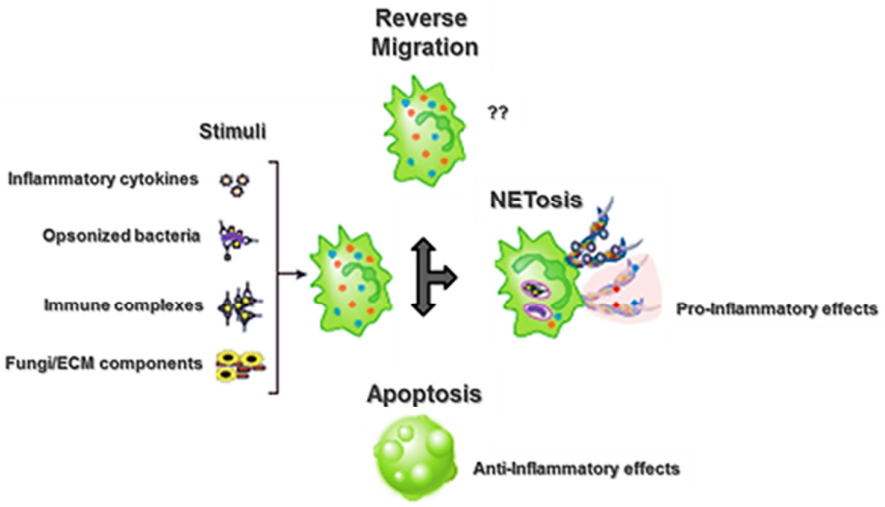

Figure 8: Neutrophil fates after activation. In contact with different stimuli neutrophils can undergo different fates like apoptosis, NETosis or reverse migration. Effects promoted by apoptosis and NETosis are known, although the effects induced by reverse migration are still unknown. (Adapted from (8)).

This latter finding among others has collectively contributed to challenge the classical view of the neutrophil function in the immune response. During the last 10 year several groups have

demonstrated that neutrophils are not only involved in the killing of extracellular pathogens but are also key immunomodulators of both of innate and adaptive immune responses by crosstalking with other cells (Figure 9) such as platelets, endothelial and epithelial cells, macrophages, dendritic cells (DC), natural killer (NK) cells, mesenchymal stem cells and lymphocytes (8, 22, 28). Among other functions, neutrophils have been implicated for example in DC maturation, antigen presentation to T cells, T cell suppression or activation, NK cell regulation and B cell activation (8, 22, 28).

At this point there are not clear answers for this neutrophil plasticity as well as for the causes and mechanisms underlying it. One of the major current lines of thinking is that these distinct fate choices and biological functions may actually be correlated with the existence of different neutrophil subpopulations (22, 25).

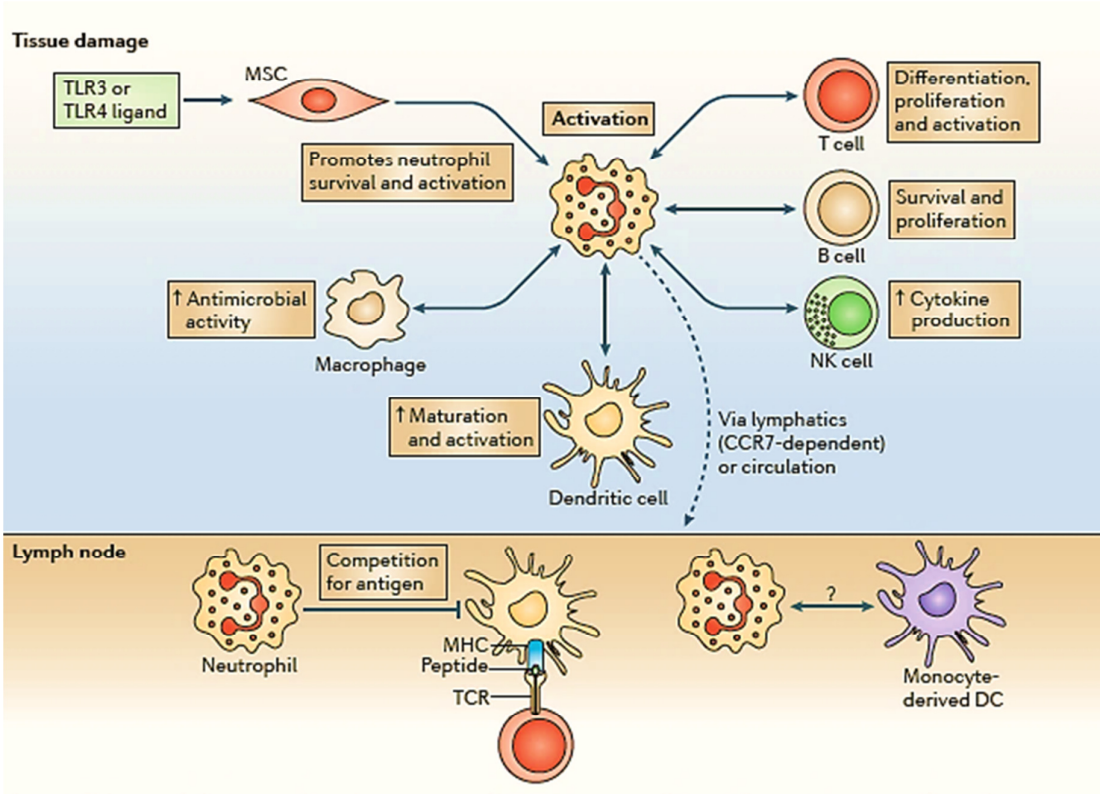

Figure 9: Neutrophils crosstalk with immune and non-immune cells. After stimulation neutrophils crosstalk with platelets and endothelial cells and next they leave circulation. Later once in inflamed tissue neutrophils crosstalk with resident and recruited cells like mesenchymal stem cells, macrophages, DCs, NK cells and B and T cells. Ultimately neutrophils can migrate to lymph nodes and interact with DCs or T cells. (Adapted from (28)).

Several studies have further implicated neutrophils in the induction or promotion of several disease states. In this regard, neutrophils have been shown to be involved in several autoimmune and chronic immune diseases with worldwide high rates of incidence, such as: type 2 diabetes (22), atherosclerosis (8, 22), thrombus formation and vasculitis (22, 24), cancer (8, 23, 28), inflammatory bowel disease (8), psoriasis (23, 24), rheumatoid arthritis or transfusion-related acute lung injury (23, 24).

Chemoatractants: Cxcl8 and H_2O_2

Neutrophil recruitment towards areas of inflammation is the result of a cascade of events modulated partly by the concerted action of several chemoattractive signals like chemokines (37, 38), H_2O_2.(39-41), leukotriene B4 (LTB4) (42-45), and most recently, osmotic changes (46). Among these, a major focus will be given here to CXCL8 and H_2O_2.

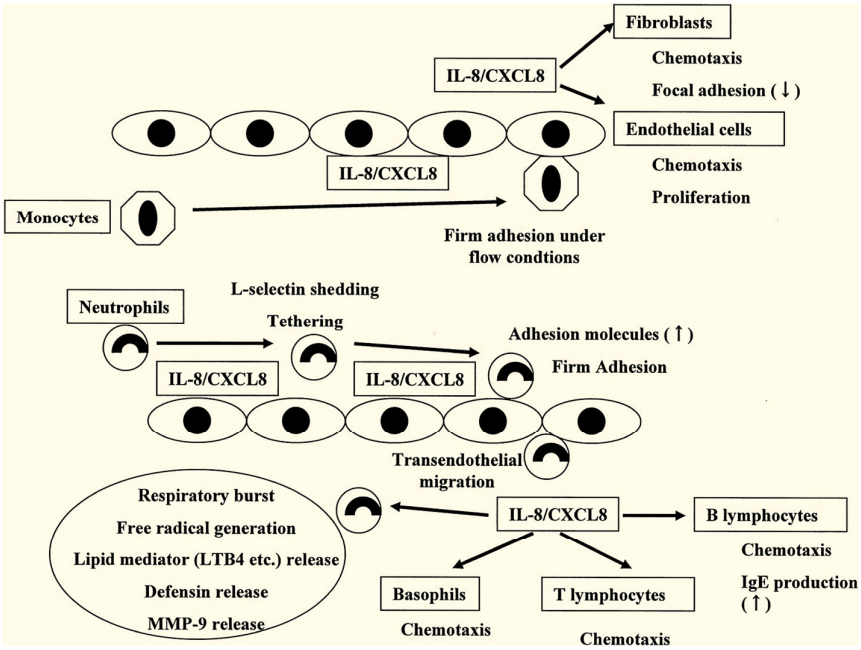

Figure 12: Major biological activities of IL-8/CXC8. CXCL8 modulates neutrophil action in inflammation at several stages from the recruitment process to its function in the inflamed area. (Adapted from (47))

CXCL8

CXCL8 is a small chemokine, belonging to the CXCL8/IL8-chemokine sub-family of CXC chemokines. It was actually one of the first chemokines to be discovered more than two decades ago (48). CXCL8 contains a conserved sequence motif (glutamic acid-leucine-arginine), named ELR motif, that immediately precedes the first cysteine residue near the amino-terminal end and is critical for receptor binding and for chemotactic activity (38, 49). This small cytokine is considered as one of the most potent neutrophil chemoattractants in inflammation (50, 51), and it has also been shown to be involved in angiogenesis and cancer development (52). The two G protein-coupled receptors expressed on leukocytes responsible for CXCL8 signal are CXCR1 and CXCR2 (26, 51, 53, 54). CXCL8 binds to CXCR1 and CXCR2 by different mechanisms, thus activating neutrophils and inducing specific intracellular signaling cascades that result in rapid neutrophil recruitment and further pathogen killing (Figure 12) (38, 55-57). Since neutrophils play early key

roles in the activation and regulation of innate and adaptive immunity (22, 28), CXCL8 and other chemokines are consistently among the first signals to be expressed and released by various cell types involved in the inflammatory response, such as epithelial and endothelial cells, macrophages, mast cells and even by neutrophils themselves so as to direct and amplify their recruitment (Figure 12) (37, 38).

In accordance to their function, the list of inflammatory diseases which have been shown to be associated with CXCL8 and its receptors is impressively extensive. Among these, one can find diseases with an high worldwide health impact, such as atherosclerosis, cancer, inflammatory bowel disease, sepsis, cystic fibrosis, rheumatoid arthritis and psoriasis (51). Currently there are 38 clinical trials evaluating the potential use of drugs targeting CXCL8, CXCR1 and/or CXCR2 to treat different diseases (51).

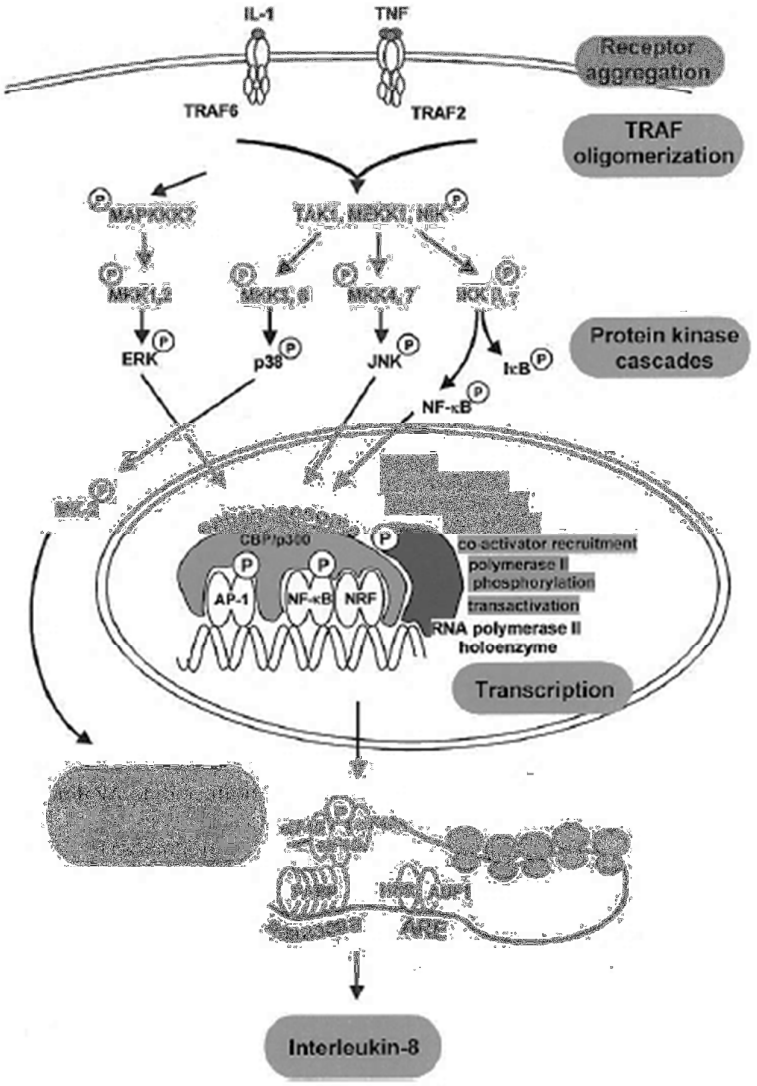

Figure 13: Essential signal transduction steps in cytokine-mediated regulation of IL-8/CXCL8 gene expression. (Adapted from (50).

Several signaling pathways have been shown, mostly *in vitro*, to be important for CXCL8 production and release. In particular, CXCL8 transcription has been shown to be quickly induced after activation of several mitogen-activated protein kinases (MAP kinases) signaling pathways, like those involving extracellular signal regulator kinase 1 and 2 (ERK1/ERK2), c-Jun N-terminal kinases (JNK) and p38, as well as nuclear factor kappa B (NF-kB) signaling pathways (Figure 13) (50, 58, 59). Among other possible effects, these pathways activate several transcription factors such as NF-κB, the activator protein 1 (AP1) and the cAMP response element-binding protein (CREB) that control CXCL8 transcription (60, 61).

Despite these and other advances, the *in vivo* study of CXCL8 in neutrophil biology and, more precisely, in neutrophil recruitment in inflammation has been hampered by the lack of true CXCL8 homologues in the most widely used animal models, such as mice and rats (47, 62). Importantly however, teleost fish have been shown to express members of the various chemokine families, including CXC chemokines (63, 64). Among these, two CXCL8 lineages have been identified in these fish, including in zebrafish (63, 65) further enabling the *in vivo* study of CXCL8 biological functions in these animal models. Although lacking the ELR motif typically present in the human CXCL8, teleost Cxcl8s possess high homology with this chemokine (65, 66). In the carp, both lineages were shown to be differentially expressed during early phases of inflammation (65, 66) but so far in the zebrafish only Cxcl8-l1 has been proven to be up-regulated in the inflammatory response elicited upon infection (54).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) was known and studied for several years as one of the main molecules produced by neutrophils that form the leukocyte oxidative burst responsible by host defense (Figure 14) (67). Recently this old molecule won a new role in inflammation since it has been shown to be produced after wounding (39, 41, 68) by the Dual oxidase 1 (DUOX1), a member of the NADPH oxidase (Nox) protein family (69-72).

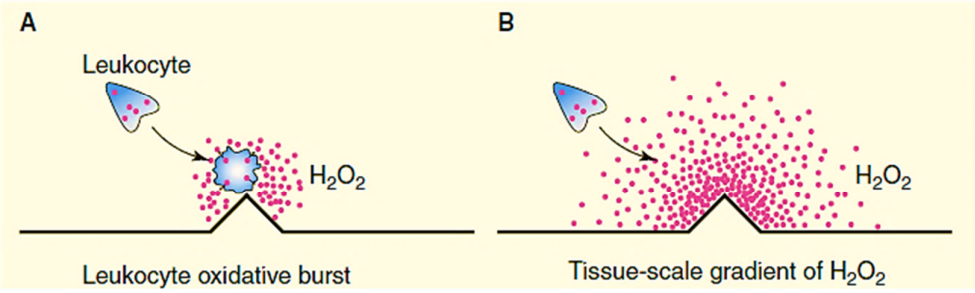

Figure 14: Different functions for H_2O_2. (A) Leukocytes once activated migrate to wounds and secrete reactive oxygen species, including H_2O_2 which play an important antimicrobial function. (B) Tissue injury induces H_2O_2 production and formation of tissue-scale gradient, responsible for leukocytes guidance to the wound. (Adapted from (67).

The formed tissue gradient has been demonstrated to act as a main trigger of early leukocyte recruitment and to be later on required for tissue regeneration. The Src-family kinases (SFKs) Lyn and Fyn b have been identified as the H_2O_2-redox sensors in leukocytes and epithelial cells respectively (40, 41, 68). Importantly, it was also reported that the neutrophil-delivered myeloperoxidase is responsible for the rapid clearance of H_2O_2 after injury (73), thus preventing excessive tissue damage (Figure 15).

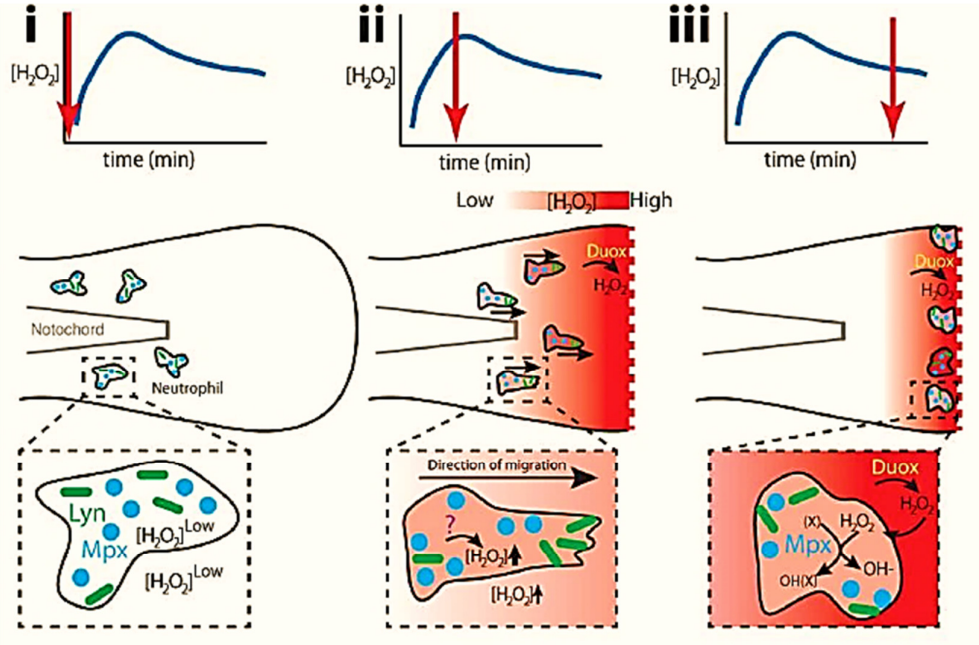

Figure 15: Model of the H_2O_2/Myeloperoxidase Interaction after Wounding. Schematic diagrams at three timepoints of the early inflammatory response to wounding: before (i), just after (ii) and 60-90 min after (iii) wounding. For each time point, it is shown its stage in the post-wounding H_2O_2 burst (upper panels), a whole tail overview (middle panels), and a detailed representation of the molecular events involved in the H_2O_2 function within the neutrophil (lower panels). Before wounding (i), intra-neutrophil and surrounding tissue H_2O_2 concentration ($[H_2O_2]$) is low. Neutrophils express the redox sensor Lyn (green), of the Src-family kinase, as well as myeloperoxidase (Mpx; in blue). Immediately after wounding (ii), a tissue-scale H_2O_2 concentration gradient (red) is generated by Dual oxidase (Duox; in yellow). Upon sensing H_2O_2 via Lyn, neutrophils initiate their migration. Initially, the net outcome of intracellular $[H_2O_2]$ is higher than in its surroundings due to the sum of their own endogenously produced H_2O_2 and of the diffusing tissue-sourced H_2O_2.. Clearance of tissue gradient H_2O_2 (iii), coincides with neutrophil arrival and is dependent on their own supply of enzymatically-active Mpx that interacts intracellularly with H_2O_2. Of the neutrophils approaching the wound, some sustain a lower intracellular $[H_2O_2]$ in comparison to the extracellular one: these are persistent net consumers of endogenous and tissue-sourced H_2O_2, thus functioning as intracellular H_2O_2 sumps. At the same time, other wound-proximate neutrophils display higher internal $[H_2O_2]$ than their surroundings. (Adapted from (74)

Although in the last few years the knowledge regarding this novel function of H_2O_2 has expanded tremendously, the mechanisms that regulate *in vivo* DUOX1 activation are far from being consensually understood. In the injured epidermis of Drosophila embryos, it has been reported that calcium flashes orchestrate the wound inflammatory response after DUOX1 activation, through its EF-hand calcium-binding motif (68), whereas in zebrafish this does not seem to be the case (40). The generation of calcium waves (75-77), and the release of ATP are main early danger signals triggered upon tissue damage (78-80). Importantly, the activation of purinergic receptors by

extracellular ATP is one of the main biological mechanisms responsible for epithelial intracellular calcium (iCa^{2+}) mobilization (80-83) and ATP was demonstrated *in vitro* in different cell lines to directly correlate with DUOX1 activation (84-88).

Another important function reported for the redox processes was the regulation of oxygen-sensitive transcription factors by their direct modification (89-92) like in the case of NF-κB (93, 94), AP1 or CREB (92). In addition, H_2O_2 has further been identified as a regulator of several important players of the inflammatory response like SFKs, ERK1/2 (86), matrix metalloproteinase-9 (MMP9), and ADAM17 (88). Importantly, an increasing number of evidences collected from in vitro studies suggest that H_2O_2 is not only a generic modulator of gene expression via its effect on transcription factors and signaling molecules, but that it may also act as a specific regulator of particular individual genes (95-99) with CXCL8 being potentially one of them (100, 101).

Wounding and inflammation

Tissue repair after injury occurs in all organisms and is a dynamic process that involves complex interactions of extracellular matrix molecules, soluble mediators, various tissue resident cells and infiltrating leukocytes (102). The immediate goal in this response is to restore tissue integrity and homeostasis after injury. According to the tissue, wound size, age or organism, wound healing can lead to complete tissue regeneration or at least to the formation of a tissue scar (40). In general, mammals have lost the ability to fully regenerate most part of their tissues after birth, whereas several other organisms like the teleosts and the amphibians are able to completely regenerate most parts of their bodies throughout their lives (40, 103). The wound healing process involves three different phases that overlap in time and space, which are inflammation, tissue formation and tissue remodeling (Figure 16) (102, 104).

After wounding several mechanisms are quickly activated including inflammatory phase (Figure 17). During this phase platelets aggregation in the wound is followed by the infiltration of leukocytes, first of neutrophils and then of macrophages and mast cells, emigrating from nearby tissues and from the circulation (102). Undoubtedly, this inflammatory response is crucial for preventing the entry of infectious agents through the wound and must have been selected for during the course of evolution so that tissue damage did not inevitably lead to death through septicemia. Despite this, several studies have suggested that depletion of one or more of these inflammatory cell lineages can actually enhance healing (102, 105). As such, the role of inflammation in wound healing is currently a controversial issue far from being solved.

During this immediate response, a clot or scab is temporarily formed on top of the wound as a protective barrier while further restoring the function of the skin. The next phase is tissue formation that involves cell proliferation, migration, and contraction within the wound to achieve permanent closure of the wound gap and replenishment of the lost tissue (Figure 17) (104). These processes initiate within hours after wounding, but the time required for their completion is highly

variable and depends on the size and location of the wound as well as on the age and health of the tissue (102). The last phase in the wound healing process is tissue remodeling which aims the restoration of the full functionality and the "normal" appearance of the tissue. Importantly, resolution of wound repair is essential for tissue remodeling. In the particular case of a cutaneous wound, migrating and proliferating keratinocytes confront among each other at the wound edge as it seals, until it stops allowing further restratification of skin (102, 104).

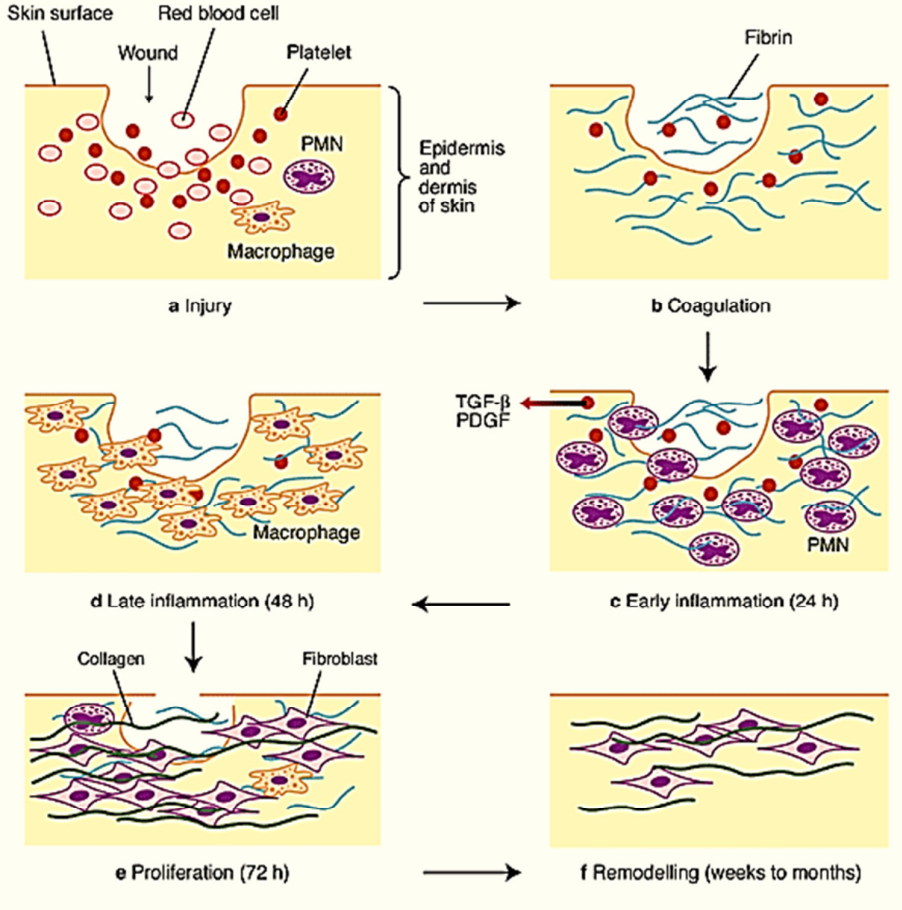

Figure 16: Wound healing phases. Tissue repair is a complex and dynamic process that involves three main phases overlapping in time and space which are inflammation (a-d), tissue formation (e) and tissue remodeling (f). (Adapted from http://www.pilonidal.org/aftercare/wound_healing_indepth.php)

Wound healing may sometimes fail due to dysfunctions occurring during the healing process (Figure 17). In patients suffering from chronic diseases, such as coronary artery disease, peripheral vascular disease, cancer and diabetes mellitus, wound healing can be compromised. As such, the increasing incidence of these chronic diseases has turned the inability for wound repair into a currently major clinical problem (103). Additionally, chronic wounds are also associated to an increased risk of neoplastic progression and of developing squamous cell carcinomas (102).

The role of transcription-independent damage signals in the initiation of epithelial wound healing has been recently revised by Cordeiro and Jacinto (78). Among these signals, calcium

waves, H_2O_2 gradients and the cellular release of ATP have been shown to regulate subsequent events during wound healing in vertebrates and invertebrates (78). Zebrafish has been one of the animal models of election to study tissue regeneration for almost 25 years, our current knowledge of its immune system has added a new perspective on to the study of this scientific field. The use of zebrafish has been instrumental in the study of transcription-independent damage signals role not just in inflammation but also in wound healing. In particular, early H_2O_2 tissue release has so far been shown to be implicated in tissue regeneration, blastema formation and axon regrowth indicating a key regulatory role for this signal in the wound healing process (40, 107, 108) .

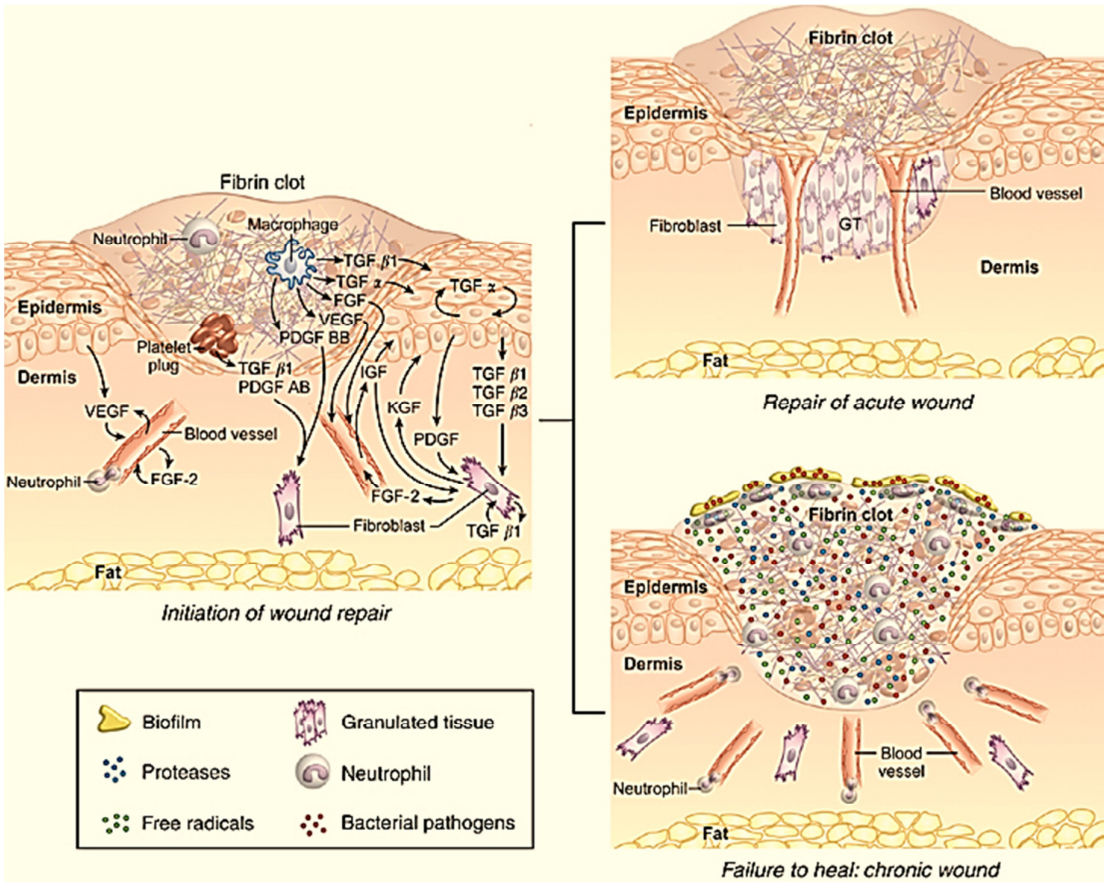

Figure 17: Schematic diagram of the possible outcomes in wound healing. Wounding leads to clotting formation, platelet aggregation, and migration of leukocytes, to the site of injury. Initially, the blood clot is composed of fibrin and fibronectin. It provides a scaffold for cellular migration. Under normal physiologic conditions the wound continues to heal with an ingrowth of granulation tissue composed of fibroblasts, additional macrophages, and neovasculature. New epidermal cells migrate over the newly forming tissue. Several proteases are important during this phase. Growth factors released by platelets and secreted by macrophages and sequestered in the provisional matrix during the first phase of healing stimulate tissue cells to move into the wound. Underlying pathologies or microorganism invasion can interrupt the healing process and further lead to a failure in wound healing and to the establishment of a chronic wound (ulcer). Among the several pathologies known to interfere with wound healing one can refer for example, venous insufficiency, diabetes mellitus or arterial occlusion. (Adapted from (106)).

Zebrafish: a XXI century Animal Model

Zebrafish (*Danio rerio*) is a small teleost fish (maximum size of 60 mm) that belongs to the family Cyprinidae and is originally from India and Pakistan regions (109). For many decades, zebrafish has been a very popular aquarium fish and also an important research model in toxicology and developmental biology. Since its introduction in the science laboratory more than 30 years ago, its popularity in biomedical research has increased tremendously. Zebrafish has captured scientists' attention due to an array of amazing and unquestionable advantages in respect to other vertebrate models (29, 110-115). Among them are:

- Its small size and low maintenance cost;
- Its robustness patent in a high resistance to pathogens;
- Its high fecundity enabling a large production of embryos (around 200 eggs/female/week);
- Its short generation time, typically 3 to 4 months, making it suitable for selection experiments (Figure 10);
- Its rapid development, with precursors to all major organs developing within 36 hours, and fully developed larvae available after 48 hours after hatching;
- Its transparency in the embryo and larval stages;
- Its easy tractability for genetic and embryological manipulation allowing large drug screenings of therapeutic drugs at lower cost compared to other models;
- The increasing availability of powerful genetic tools like reverse genetic approaches using zinc finger nucleases (ZFNs) and transposon-based strategies that enable in a relatively easy way the generation of transgenic and mutant zebrafish;
- The increasing availability of transgenic lines, enabling *in vivo* tracking of different cell types;
- Its completed sequenced genome and its remarkable homology with humans;
- Its amenability for studies modulating gene expression via gene-specific knockdown techniques such as the morpholinos or via the overexpression of proteins by mRNA or plasmid microinjection.

All these excellent advantages allied with a world that needed to adapted to a new way of do science, with lower funds each year and higher need to publish good results, suddenly turned zebrafish into an ideal vertebrate model not just for the study of development biology but also of

the immune system (113), hematopoiesis (116), vascular development (117), neurogenesis (118) and cancer research (119) and vertebrate behavior (120).

In the last years, the zebrafish has proven to be a versatile model organism for the study of inflammatory immune responses. Zebrafish immune system closely resembles its counterpart in mammals, but with the advantage that the adaptive immunty is not morphologically neither functionally mature until 4-6 weeks post-fertilization. At lower development stages zebrafish immune system relies on the innate immune system that is fully competent at early embryological stages. This chronological separation provides an amazing window of opportunity to study the vertebrate innate immune response *in vivo*, without the presence of adaptive immune system (113, 115, 121-123).

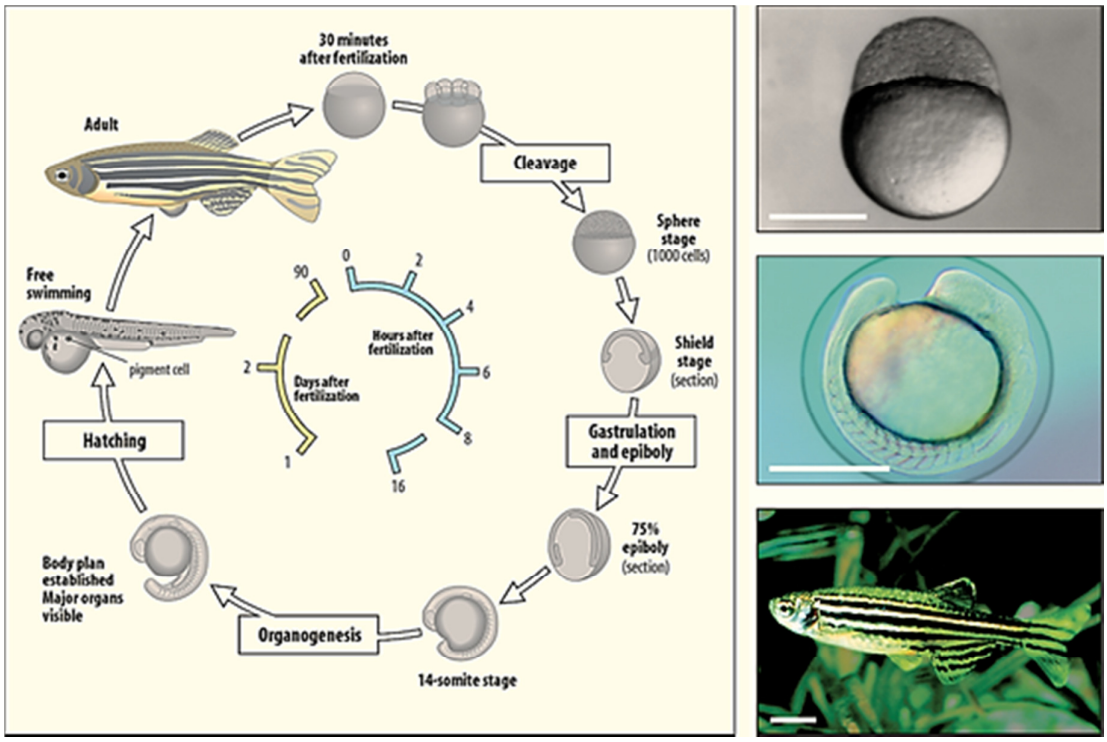

Figure 10: Diagram of zebrafish life-cycle (Adapted from http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_03/devo_03.html)

The appearance of specific transgenic zebrafish lines expressing fluorescent proteins under specific promoters, like for example neutrophils (124, 125) and macrophages (126) allowed the study in vivo of mechanisms responsible for leukocyte recruitment, behavior and function under diverse inflammatory conditions (45, 127). In addition, zebrafish also demonstrated to be useful as model system to study chronic inflammation and to visualize the immune responses implicated *in vivo* (128).

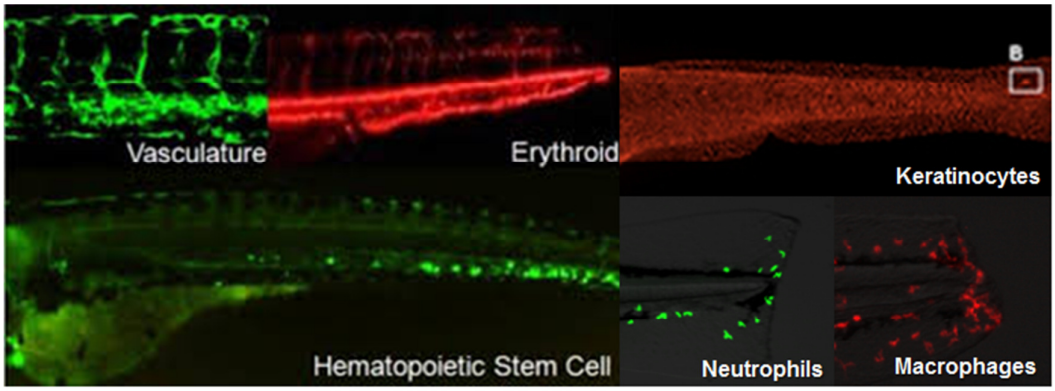

Figure 11: Zebrafish transgenic lines for the in vivo study of specific cellular types. Transgenic lines were generated to express fluorescent reporter proteins under the control of cell-specific promotors for different cellular lineages. This development has allowed the dissection of hematopoiesis and immunology in the zebrafish by using non-invasive methodologies what has resulted in an extraordinary advantage over other animal models. (Adapted from http://www.med.hku.hk/corefac/zebrafish.htm)

Aims

Neutrophils are important key players of the innate immune response that have been shown to be involved in several inflammatory diseases of worldwide impact. In consequence, there is currently a growing need to expand our knowledge regarding this cell. The CXCL8 chemokine and H_2O_2 are two major players involved in neutrophil recruitment. CXCL8 has importantly been reported to be associated with several diseases such as rheumatoid arthritis, psoriasis and cancer. At the time this work was initiated, the zebrafish had been recently shown to express two true homologues of the mammalian CXCL8 in clear opposition with the mouse that interestingly does not have any. However, there were no studies addressing the role of the zebrafish Cxcl8s in inflammation and more precisely, in neutrophil recruitment. On the other hand, an old player of the inflammatory response, the H_2O_2, had been recently shown to play a novel role in early neutrophil recruitment in zebrafish inflammation, although the mechanisms underlying its production were then unknown. Importantly, it was also unclear whether and how the action of these two chemotactic cues could be concerted in neutrophil recruitment in inflammation. Having all this in mind, the main objectives of this work were:

- To address the role of zebrafish Cxcl8s in neutrophil recruitment and behavior and tissue resolution under different inflammatory conditions (tissue damage and infection);

- To identify the early danger signals as well as the signaling pathways that are required for Duox1 activity *in vivo,* in the inflammatory response upon wounding and address the involvement of Duox1/H_2O_2 in the activation of the NF-κB inflammatory signaling pathway;

- To address the existence of a possible crosstalk between early wound signal H_2O_2 and later Cxcl8 signals in the modulation of the neutrophil recruitment and the inflammatory response;

- To study the role of Duox1/H_2O_2 in a zebrafish skin inflammatory model and address the involvement of the human DUOX1 role in skin lesions of psoriasis and lichen planus human patients.

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Chapter 2

Cxcl8 (Interleukin-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response

Cxcl8 (Interleukin-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response

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Abstract

Neutrophils play a pivotal role in the innate immune response. The small cytokine CXCL8 (also known as interleukin-8 or IL-8) is known to be one of the most potent chemoattractant molecules which, among several other functions, is responsible for guiding neutrophils through the tissue matrix until they reach sites of injury. Unlike mice and rats that lack a CXCL8 homologue, zebrafish has two distinct CXCL8 homologues: Cxcl8-l1 and Cxcl8-l2. Cxcl8-l1 is known to be up-regulated under inflammatory conditions caused by bacterial or chemical insult but until now, the role of Cxcl8s in neutrophil recruitment has not been studied. Here, we show that both Cxcl8 genes are up-regulated in response to an acute inflammatory stimulus, and that both are crucial for normal neutrophil recruitment to the wound and normal resolution of inflammation. Additionally, we have analyzed neutrophil migratory behavior through tissues to the site of injury *in vivo*, using open-access phagocyte tracking software, *PhagoSight*. Surprisingly, we observed that in the absence of these chemokines, the speed of the neutrophils migrating to the wound was significantly increased in comparison to control neutrophils, although the directionality was not affected. Our analysis suggests that zebrafish may possess a sub-population of neutrophils whose recruitment to inflamed areas occurs independently of Cxcl8 chemokines. Moreover, we report that Cxcl8-l2 signaled through Cxcr2 for inducing neutrophil recruitment. Our study, therefore, confirms the zebrafish as an excellent *in vivo* model to shed light on the roles of CXCL8 in neutrophil biology.

Introduction

Neutrophils are known to be one of the first lines of defense against invading microbes, playing a pivotal role in antimicrobial host defense by recognizing microorganisms through various receptor systems. In order to fulfill this function, they are the first leukocytes to be recruited towards areas of inflammation (*1*). Neutrophil recruitment towards areas of inflammation is considered to be the result of the concerted action of several chemoattractants including chemokines (*2, 3*). The first chemokine to be discovered more than 20 years ago was CXCL8 (*4*) and nowadays stands as the prototypical member of the family of CXC chemokines. CXCL8 is considered as one of the most potent neutrophil chemoattractants in inflammation (*5*) and which binds to two different chemokine receptors on leukocytes: the G protein-coupled receptors CXCR1 and CXCR2 (*6*). By using different mechanisms of activation, CXCL8 binding activates these receptors and thus induces specific intracellular signaling cascades that result in rapid neutrophil recruitment (*3, 7-9*). As neutrophils are initial players in acute inflammation, CXCL8 and other chemokines are consistently among the first signals to be expressed and released by the various cell types involved in inflammation (*2, 3*).

The *in vivo* study of the role of CXCL8 in neutrophil biology and, more precisely, in neutrophil recruitment in inflammation has been hampered by the lack of true CXCL8 homologues in most widely used animal models, such as mice and rats (*10, 11*). As discussed below, this may now have been circumvented by the identification of CXCL8 homologues in another model organism, the zebrafish. Remarkably, the zebrafish immune system resembles that of mammals. In particular, zebrafish development offers a window of opportunity for the study of innate immunity during initial larval stages independently of the adaptive component, which is only active after 4 weeks post fertilization (*12, 13*). In addition, the optical transparency of zebrafish and the development of fluorescent cell-specific transgenic lines have enabled the *in vivo* study of leukocyte biology (*14, 15*). Zebrafish neutrophils have now been extensively studied in inflammation (*16-23*), infection (*24-27*) and tumor progression (*28-30*).

Importantly, the zebrafish has been shown to express chemokines from the various families, including CXC chemokines (*31, 32*). Recently, two Cxcl8 lineages were identified in teleosts. In the carp, members of both lineages were shown to be differentially expressed during early phases of inflammation (*33, 34*). Although lacking the ELR motif, teleost Cxcl8s possess high homology with human CXCL8 (*33, 34*). Despite the description of both lineages in zebrafish, so far it has only been reported that the *cxcl8-l1* gene is induced in inflammation (*35*). To date, neither the expression of *cxcl8-l2* in this context nor the involvement of these chemokines in neutrophil recruitment has been addressed in the zebrafish.

The objective of this study was to understand *in vivo* the role of zebrafish Cxcl8s on neutrophil behavior and function in the inflammation elicited by tissue injury. Firstly, our analysis indicated that both *cxcl8-l1* and *cxcl8-l2* were up-regulated in wound inflammation. Importantly, we observed that recruitment of neutrophils towards the wound was significantly reduced in the

absence of these Cxcl8s. By analyzing *in vivo* neutrophil migration and behavior with new open source tracking algorithms, *PhagoSight*, we have unexpectedly observed that in the absence of these chemokines, the velocity of the neutrophils migrating to the wound was significantly increased in comparison to normal controls. Furthermore, we have found that Cxcr2 mediated neutrophil recruitment to wounds and that Cxcl8-l2 signaled through this receptor to promote neutrophil recruitment *in vivo*. Overall, these observations led us to propose that zebrafish Cxcl8 chemokines are both required for efficient neutrophil recruitment in inflammation. Our data further support the idea that the zebrafish may possess, at least at the larval stage, a sub-population of neutrophils whose recruitment to inflamed areas occurs independently of Cxcl8 chemokines.

Materials and Methods

Characterization of zebrafish CXCL8

A search for cxcl8 was performed at the Ensembl (http://www.ensembl.org/index.html) zebrafish database (zv8 and zv9) in order to check for the localization of CXCL8s in the zebrafish genome. Further genomic DNA analysis was performed in order to obtain accurate exon/intron sequences. Protein sequence alignments of zebrafish Cxcl8-l1 (XP_001342606) and Cxcl8-l2 (HF674400) with human CXCL8 (NP_000575) were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Zebrafish Husbandry

All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU) and the Bioethical Committee of the University of Murcia (approval number #333/2008). Zebrafish fertilized eggs were obtained from natural spawning of wild-type (obtained from the Zebrafish International Resource Center), and the *Tg(mpx:gfp)i114* (*22*) line held at our facilities following standard husbandry practices. Animals were maintained in a 12 hr light/dark cycle at 28.5°C.

Table 1: MOs used in this study

Gene	Morpholino`s name	MO Sequence	Target	[Mo] ng/egg
cxcl8-l1	***MO cxcl8-l1-E1/I1***	**5'-GGTTTTGCATGTTCACTTACCTTCA-3'**	**E1/I1**	**4**
cxcl8-l1	***MOcxcl8-l1 E2/I2***	**5'-TTAGTTTGAAAACTCACATGATCTC-3'**	**E2/I2**	**6**
cxcl8-l2	***MO cxcl8-l2 E1/I1***	**5'-TTAGTATCTGCTTACCCTCATTGGC-3'**	**E1/I1**	**4**
cxcl8-l2	***MO cxcl8-l2 I2/E3***	**5'-GGCGCTGTTGAAAACAGATGTAAAA-3'**	**I2/E3**	**2**

Morpholino knockdown

The following splice blocking morpholino-modified antisense oligonucleotides (morpholinos (MO), Gene Tools) were injected into 1-cell-stage fertilized eggs (2-6 ng/egg): MO *cxcl8-l1* E1/I1 (4 ng/egg), MO *cxcl8-l1* E2/I2 (6 ng/egg), MO *cxcl8-l2* E1/I1 (4 ng/egg), MO *cxcl8-l1* I2/E3 (2 ng/egg) (see Table 1).

For assessment of morphant efficacy, total RNA was prepared from 3 days post-fertilization (dpf) whole-larvae using TRIzol reagent and purified with PureLink RNA MiniKit (Invitrogen), following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/µg RNA; Invitrogen). The SuperScript III RNase H- reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo(dT)18 primer from 1 µg of total RNA at 50°C for 50 min. To confirm MO efficiency, semi-quantitative PCR was performed using specific primers for each cxcl8 gene (see Table 2). After gel electrophoresis, bands of amplified products were extracted and sequenced.

In the subsequent experiments employing the above mentioned MOs, standard control MOs (MO StdC) purchased from GeneTools were used in parallel to control for the specificity of the identified MO-mediated effects.

Table 2: Primers used to analyze gene expression in this study

Gene	Acession number	Name	Nucleotide sequence	Use
bactin2	**AF025305**	**F** **R**	**5'-GTGCCCATCTACGAGGGTTA-3'** **5'-TCTCAGCTGTGGTGGTGAAG-3'**	**PCR**
cxcl8-l1	**XM_001342570**	**F** **R** **F1** **R1**	**5´-CCAGCTGAACTGAGCTCCTC-3'** **5'-GGAGATCTGTCTGGACCCCT-3'** **5'-GTCGCTGCATTGAAACAGAA-3'** **5'-CTTAACCCATGGAGCAGAGG-3'**	**PCR/qPCR**
cxcl8-l2	**HF674400**	**F1** **R1** **F3** **R3**	**5´-GCTGGATCACACTGCAGAAA-3'** **5'- TGCTGCAAACTTTTCCTTGA -3'** **5´-CCACACACACTCCACACACA-3'** **5'- TGATGAAAGGACAATTCAGTGG -3'**	**PCR/qPCR**
il-1b	**NM_212844**	**F5** **R5**	**5'-GGCTGTGTGTTTGGGAATCT-3'** **5'-TGATAAACCAACCGGGACA-3'**	**qPCR**
ptgs2b	**NM_001025504**	**F2** **R2**	**5'-CCCCAGAGTACTGGAAACCA-3'** **5'-ACATGGCCCGTTGACATTAT-3'**	**qPCR**
rps11	**NM_213377**	**F** **R**	**5'-ACAGAAATGCCCCTTCACTG-3'** **5'-GCCTCTTCTCAAAACGGTTG-3'**	**qPCR**

Zebrafish tail tissue sample collection and gene expression analysis

At 3 dpf, larvae were anesthetized in embryo medium with 0.16 mg/ml tricaine (ethyl 3-aminobenzoate, Sigma Aldrich) and complete transection of the tailfin tip was performed with a

disposable sterile scalpel. Larvae were recovered in embryo medium at 28.5ºC. At the time-points indicated, larvae were anesthetized again with 0.16 mg/ml tricaine and, using a sterile scalpel, the body portion between the cloaca and the wounded tail tip was excised from 80 larvae at each time-point. Tail tissue samples were then pooled and frozen in liquid nitrogen. Total RNA was extracted from cell pellets with TRIzol reagent (Invitrogen) and purified with RNAquous Micro Kit, total RNA purification system (Ambion), following the manufacturer's instructions and first-strand cDNA synthesized as above. Real-time PCR was performed with an ABIPrism 7500 instrument (Applied Biosystems) using SYBR-Green (AppliedBiosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C, and 15 s at 95°C. For each mRNA, gene expression was normalized against the expression of ribosomal protein S11 (rps11) in each sample. The primers used are shown in Table 2. In all cases, PCR was performed with triplicate samples and repeated at least twice. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test in Graph Prism5 software.

Tail fin wounding

At 3 dpf, larvae were anesthetized in embryo medium with 0.16 mg/ml tricaine. Then, complete transection of the tailfin tip was performed with a disposable sterile scalpel and fish were mounted in 1% (w/v) agarose low melting point (Sigma-Aldrich) dissolved in embryo medium supplemented with 0.16 mg/ml tricaine. The success of transection was immediately confirmed a fluorescence stereo microscope MZ16FA (Leica) equipped with green fluorescent filters. After solidification, embryo medium with 0.16 mg/ml tricaine solution was added in order to keep embryos hydrated during experiments. Thereafter, images were captured at the selected times while animals were kept in their agar matrixes with added medium at 28.5°C.

SB225002 pharmacological treatment

For CXCR2 inhibition assays, we choose to use a bath immersion method. Briefly, larvae were pre-incubated 1 hour at 28ºC in presence or absence of the selective non-peptide inhibitor SB225002 (Tocris) at a final concentration of 5 μM diluted in embryo medium supplemented with 1% DMSO. In tail fin wounding experiments, during recovery larvae were kept in embryo medium supplemented with 1% DMSO in presence or absence of SB225002, until imaging at 6 hours post wounding (hpw).

Production of recombinant Cxcl8-l2

Recombinant zebrafish Cxcl8-l2 was produced in *Escherichia coli.* Briefly, the open reading frame of *cxcl8-l2* encoding the mature protein without the signal peptide (residues 27-118) was synthesized, cloned in vector E3, produced as an N-terminal 6xHis fusion protein in *E. coli*, obtained from inclusion bodies with 6 M guanidine hydrochloride and purified by Ni-HiTrap column (GenScript).

Otic injection

At 3 dpf larvae were pre-incubated in presence or absence of SB225002 (Tocris) as described before, anesthetized in embryo medium with 0.16 mg/ml tricaine and mounted in 1% of agarose low melting point. Injection in the otic vesicle of 1 nl of PBS, Cxcl8-l2 recombinant protein at 30 μM or leukotriene B4 (LTB4) at 30 nM was performed. Embryo medium with 0.16 mg/ml tricaine solution supplemented with 1% DMSO and when required with 5 μM SB225002 was added on the top, in order to keep embryos hydrated during the experiments. Images were taken after 1 hour post-injection (hpi).

Image acquisition and processing

For each experiment 3 dpf morphant and control larvae were imaged in three independent experiments. Images were taken from wounded or control larvae mounted as described above. Different methods of image acquisition were used according to the requirements of each experiment. Briefly, for total neutrophil counts and number of neutrophils at site of injury/injection, images were taken using a Leica MZ16F fluorescence stereo microscope. Time lapse images from wounded tail fins were acquired using a Zeiss 5 Live confocal line-scanning microscope with a NA1/20x water immersion objective in z-stack mode, every 3 minutes until 6 hpw, and assembled into time lapse movies. For neutrophil *PhagoSight* analysis, time lapse images were taken using a Zeiss Axiovert200 fully motorized, inverted, wide field fluorescence microscope using an NA 0.8/20x objective, in z-stack mode, every 2 minutes until 6 hpw. All data were processed using Image J (http://rsb.info.nih.gov/ij/).

Neutrophil response analysis- PhagoSight

The time-lapse movies from morphant and control larvae were processed using *PhagoSight (http://www.phagosight.org.uk),* an open-source neutrophil tracking software developed in MATLAB. This software allowed us to generate quantitative measurements with which we were able to perform an unbiased comparison of neutrophil migratory behavior between *cxcl8* morphants and control larvae and between resting and inflammatory conditions. This was done by analyzing either neutrophil speed or velocity oriented/lateral with respect to the wound as well as distinguishing the movement before the neutrophil reached the wound (herein referred to as “in translation”) and after it reached the wound (herein referred to as “in exploration”). Among the many parameters compared, the following were analyzed: the meandering index, the number of neutrophils that leave the wound, the total number of tracks generated by the neutrophils, the number of tracks that enter the wound, the number of tracks that enter the wound and leave, and the time-point at which the neutrophils enter the wound.

Tail fin injury resolution assay

To address whether inflammation resolved normally in cxcl8-l1 morphants, 3 dpf morphant and control larvae were wounded as previously described and recovered at 28ºC in embryo medium until live imaging acquisition. Larvae were then mounted in 0.5% (w/v) low melting point agarose in embryo medium with 0.16 mg/ml tricaine. Tailfin images were taken using a Zeiss Axiovert200 fully motorized, inverted, wide field fluorescence microscope, using an NA 0.8/20x objective, in a z-stack mode at 6, 24, and 48 hpw. Larvae were allowed to recover between acquisitions at 28ºC in embryo medium. Images were processed with Image J and neutrophil counts were performed at the indicated time-points.

Statistical analysis

All error bars indicates standard error of the mean (SEM). For gene expression experiments data are shown as mean ± SEM of three separate experiments. One-way ANOVA with Bonferroni post-test was used for Figures 1, 2D, 3-5 and 7E, Supplemental Figure 1E, 4, 5A; two-tailed Mann-Whitney test was used for Figure 7B, and Two-way ANOVA with Bonferroni post-test for Figure 2B, 3 (*il1b* and *ptgs2b*) and 6B, Supplemental Figure 1D.

Results

Expression of both cxcl8 genes is up-regulated in wounded zebrafish tailfin tissue

In the carp, it has been shown that members of Cxcl8 lineages, namely Cxcl8-l1 and the Cxcl8-l2 play a role in acute inflammation. Consistently, the mRNA levels of both carp *Cxcl8* genes are up-regulated under these conditions (*33*). In view of this, we first addressed the expression of both zebrafish *cxcl8* at different time-points in 3 dpf *Tg(mpx:gfp)i114* larvae under acute inflammatory conditions. This expression analysis was performed by qPCR by using the primers presented in Table 2. Here, we made use of the tail fin transection model, a validated model to investigate acute inflammation (*16-23*). We found that the mRNA levels of *cxcl8-l1* and *cxcl8-l2* were significantly increased in injured tail fin tissue and peaked at 1 hpw (Figure 1). *il1b* and *ptgs2b* (*cox2b*) mRNA levels were used as positive controls of inflammation in qPCR experiments (Figure 1).

Genetic inhibition of both cxcl8 genes attenuate zebrafish neutrophil recruitment in acute inflammation

Next, we decided to knockdown both cxcl8 by using splice-blocking morpholinos. In order to design these morpholinos, we had to know the complete genomic sequence for both chemokines. While *cxcl8-l1* was already properly annotated in zv8 database, for *cxcl8-l2* first we needed to use an EST sequence (EH557944) to design primers in order to perform RT-PCR analysis and sequencing. Next, after confirming the full coding sequence, we performed a BLAST search of the

zebrafish genome from Ensembl using the zv9 database and we were able to identify the full *cxcl8-l2* sequence on chromosome 7, position 8658505:8665964, opposite strand, this gene is now annotated at Gene EMBL

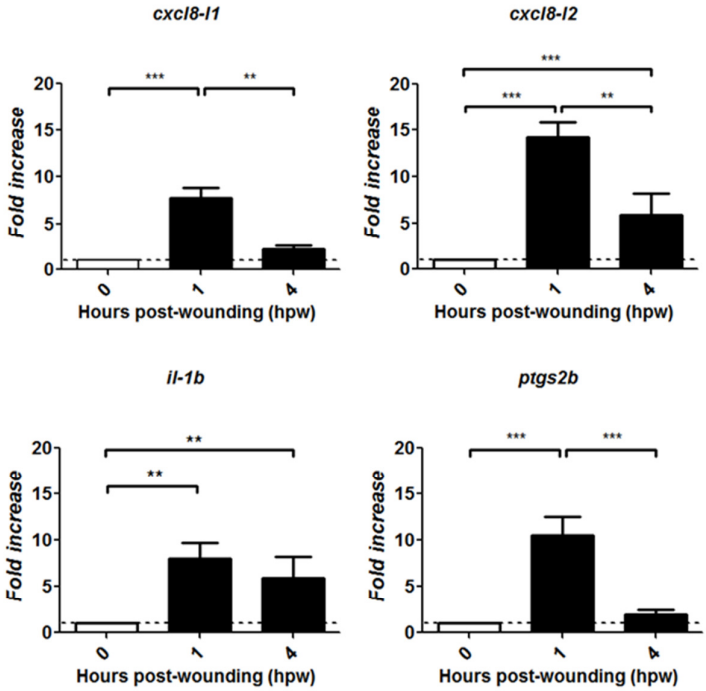

Figure 1: cxcl8-l1 and cxcl8-l2 mRNA levels are up-regulated in wounded zebrafish tail fin tissue. Tail fins from 3 dpf zebrafish larvae were wounded and mRNA levels of indicated genes were determined by qPCR in tail fin tissue at 0, 1 and 4 hpw (80 tail fins per time-point). Gene expression was normalized against rps11 and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the mean ± SEM of triplicated samples. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, *P<0.05, **P<0.01, and ***P<0.001.

with the accession number HF674400. Two different splice-blocking morpholinos were designed and tested for each gene, in order to generate viable *cxcl8-l1* and *cxcl8-l2* morphants. Thus, we used MO *cxcl8-l1* E1/I1 (referred to as MO *cxcl8-l1*) and MO *cxcl8-l1* E2/I2 to generate *cxcl8-l1* morphants and MO *cxcl8-l2* E1/I1 (referred to as MO *cxcl8-l2*) and MO *cxcl8-l2* I2/E3 for *cxcl8-l2* morphants (Supplemental Figure 1A-B). At 3 dpf *mpx:*GFP morphant larvae were injured and neutrophil recruitment was quantified at different time-points from 1 to 6 hpw. Our results show that *cxcl8-l1* and *cxcl8-l2* knockdown significantly attenuated neutrophil recruitment to injured zebrafish tail fins (Figure 2A-B, Supplemental Movie 1-3 and Supplemental Figure 1C-D). Furthermore, none of the morpholinos used to knockdown *cxcl8-l1* or *cxcl8-l2* affected the total neutrophil number at 3 dpf in whole larvae (Figure 2C-D and Supplemental Figure 1E-F). Although all the four morpholinos reduced neutrophil recruitment, we decided to use MO *cxcl8-l1* and MO *cxcl8-l2* for further studies, since they had higher efficiencies in CXCL8 knockdown and thus affected neutrophil recruitment more significantly than the other two.

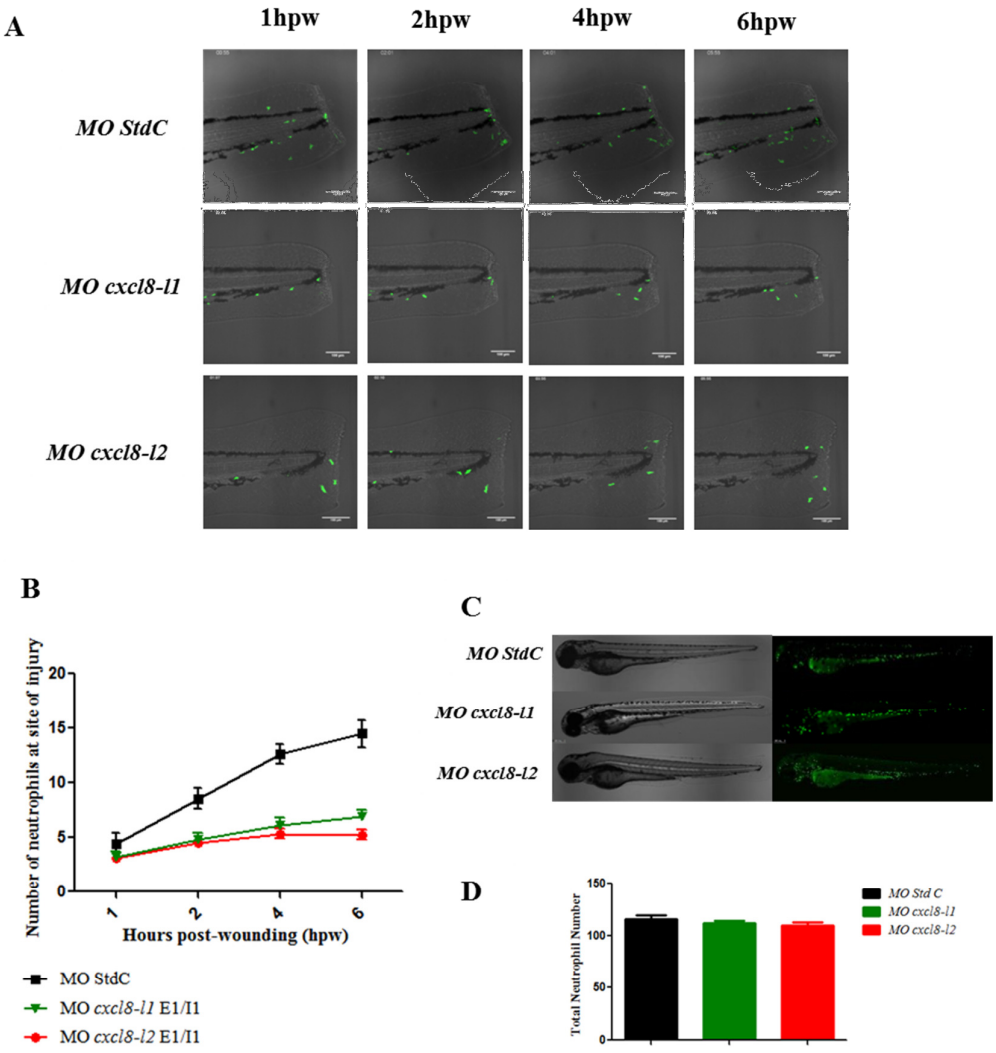

Figure 2: cxcl8 morphants display a reduced zebrafish neutrophil recruitment in acute inflammation. Tail fins of Tg(mpx:gfp)i114 previously microinjected with standard control morpholino (MO StdC), MO cxcl8-l1 and MO cxcl8-l2 were transected at 3 dpf. (A) Representative maximum intensity projections of 6 hours confocal time-lapse microscopy of 3 dpf Tg(mpx:gfp)i114 control and morphant larvae, acquired at 3 min intervals. This sequence is shown in Supplemental Movie 1. Scale bar=100 µm (B) Counts of fluorescent neutrophils at the wound were made at 1, 2, 4 and 6 hpw. Data are presented as means ± SEM (n=30 performed as 3 independent experiments). P values were calculated using two-way ANOVA and Bonferroni multiple comparison test. At 1 hpw no significant differences in neutrophil number were observed for both morphants in comparison to control larvae (P>0.05). For MO cxcl8-l1 significant decreases were observed at 2 hpw (P<0.01) and from 4 to 6 hpw (P<0.001). As for MO cxcl8-l2, we have observed a significant decrease from 2 to 6 hpw (P<0.001) (C) DIC and GFP wide field fluorescence microscope micrographs from 3 dpf control and morphant larvae. (D) Total neutrophil counts in whole-larvae for each condition. Each bar represents means ± SEM (n=20 performed as 2 independent experiments). P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (P>0.05, no significant differences were observed).

cxcl8-l2 knockdown impairs cxl8-l1 induction after wounding

Although both genes were important for neutrophil recruitment in zebrafish, *MO cxcl8-l2* caused a larger inhibition of the neutrophil response. Considering this, we further asked whether knockdown of a given *cxcl8* could impact on the expression of the other. By qPCR analysis, we observed that both morphants expressed higher basal levels of the other *cxcl8* gene (Figure 3).

Upon wounding, the *cxcl8-l2* mRNA levels were not affected in the *cxcl8-l1* morphant. In contrast, *cxcl8-l1* transcript levels were significantly reduced in *cxcl8-l2* morphants when compared to control conditions. Moreover, in both *cxcl8* morphants, we observed that the mRNA levels of two inflammation control markers, namely *il1b* and *ptgs2b*, were also significantly reduced (Figure 3).

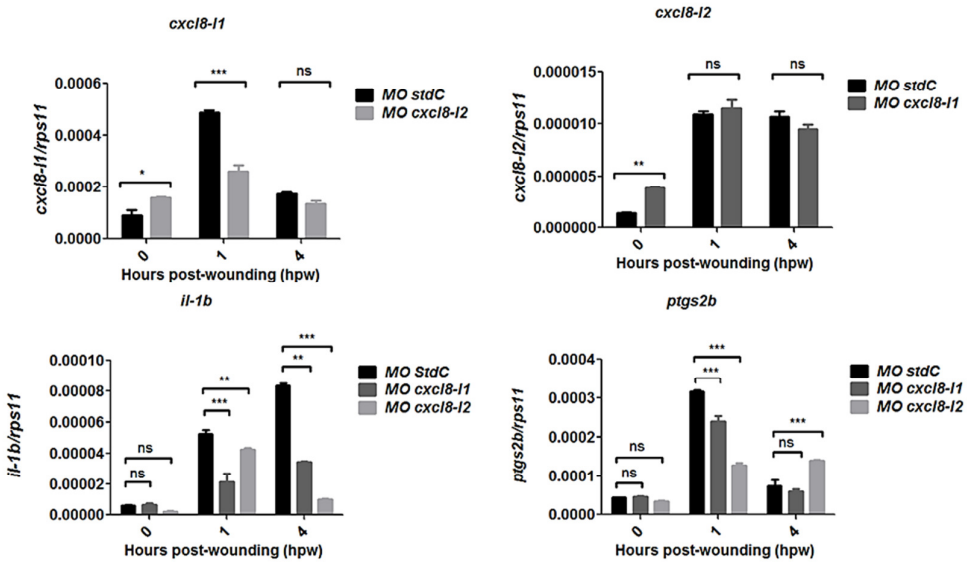

Figure 3: Expression of inflammatory genes in cxcl8 morphants. Tail fins from 3 dpf zebrafish control and morphants larvae were wounded and mRNA levels of the indicated genes were determined by qPCR in tail fin tissue at 0, 1 and 4 hpw. Gene expression was normalized against rps11. Each bar represents the mean ± SEM of triplicated samples. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, *P<0.05, **P<0.01, and ***P<0.001

Neutrophil migratory behavior is affected in the absence of either Cxcl8.

Our main objective was to study *in vivo* the function of Cxcl8s on the migratory behavior of neutrophils in acute inflammation. For such purpose, time-lapse images were acquired every 2 minutes from uninjured or previously injured 3 dpf larvae tail fins. Imaging was performed simultaneously for control conditions and for both *cxcl8* morphants. To inspect neutrophil migratory behavior in detail, the acquired data sets were further analyzed using *PhagoSight*, software developed in Matlab (http://www.phagosight.org.uk/). *PhagoSight* provides an array of measurements which capture neutrophil behavioral traits, the most important of which are presented in Figures 4-5, Supplemental Figure 2, and Supplemental Movies 4-6.

In wounded larvae, we first confirmed that in both *cxcl8* morphants, a significant lower number of neutrophils were being mobilized and recruited to the wound in the first 6 hpw (Figure 4A-C). Moreover, neutrophils in *cxcl8-l2* morphants were observed to enter the wound at significantly later time-points than in control and *cxcl8-l1* morphants (Figure 4E). The ratio between the total number of tracks and the number of tracks in the wound (Figure 4C), the number of tracks that crossed the wound and back (Figure 4D) and the number of neutrophil time-points in the wound (Figure 4F) were not significantly affected.

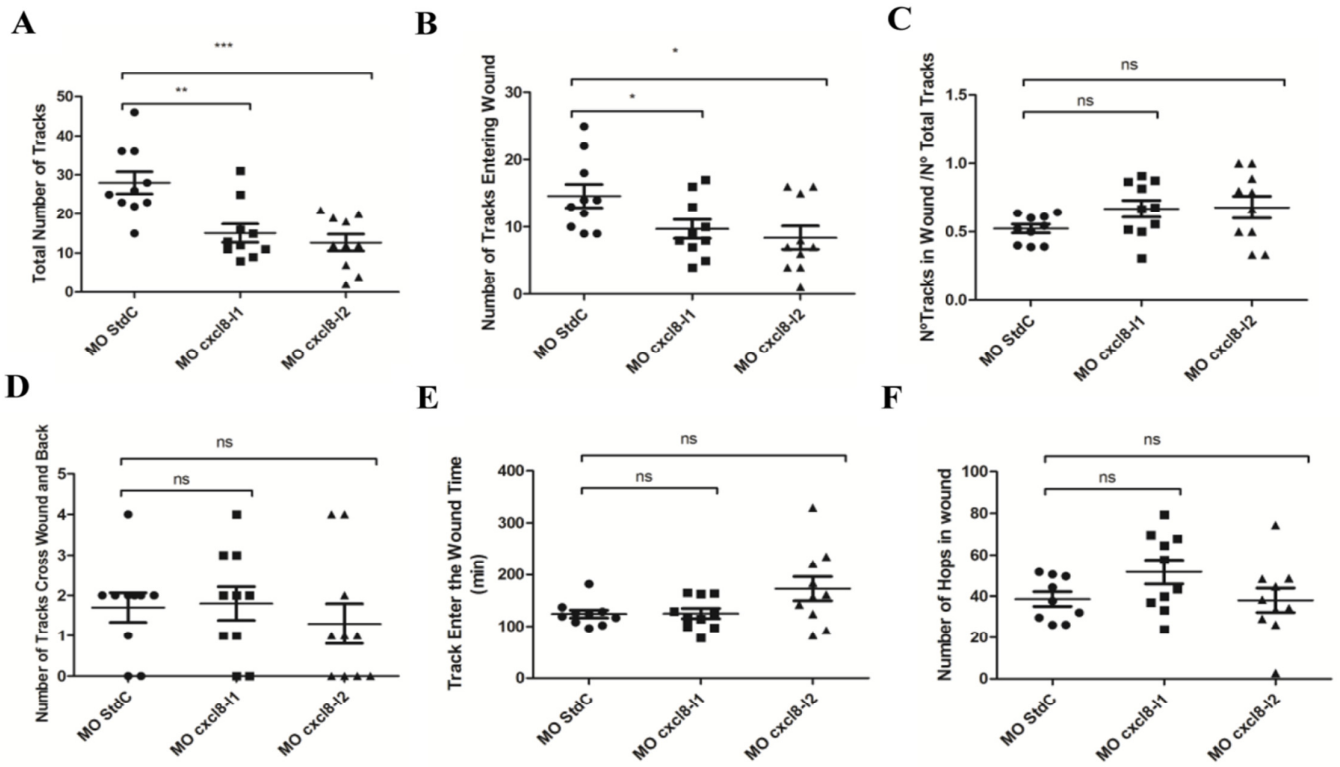

Figure 4: PhagoSight analysis confirms that CXCL8s morphants display a reduced zebrafish neutrophil recruitment in acute inflammation. Tailfins of Tg(mpx:gfp)i114 previously microinjected with MO StdC, MO cxcl8-l1 and MO cxcl8-l2 were transected at 3 dpf and time-lapse movies were performed under a wide field fluorescence microscope (MO StdC=270 tracks, MO cxcl8-l1=147 tracks and MO cxcl8-l2=128 tracks from n=10 larvae as 3 independent experiments) and further analyzed by PhagoSight. Distinct parameters were determined to study neutrophil migratory behavior namely: (A) Total Number of Tracks; (B) Number of Tracks Entering the Wound; (C) Ratio of Number of Tracks in Wound/Total Number of Tracks; (D) Number of Tracks Cross the Wound and Back; (E) Tracks Enter the Wound time; (F) Number of Hops in Wound. Longer horizontal bars represent the means and shorter horizontal bars represent SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, *P<0.05, **P<0.01, and ***P<0.001.

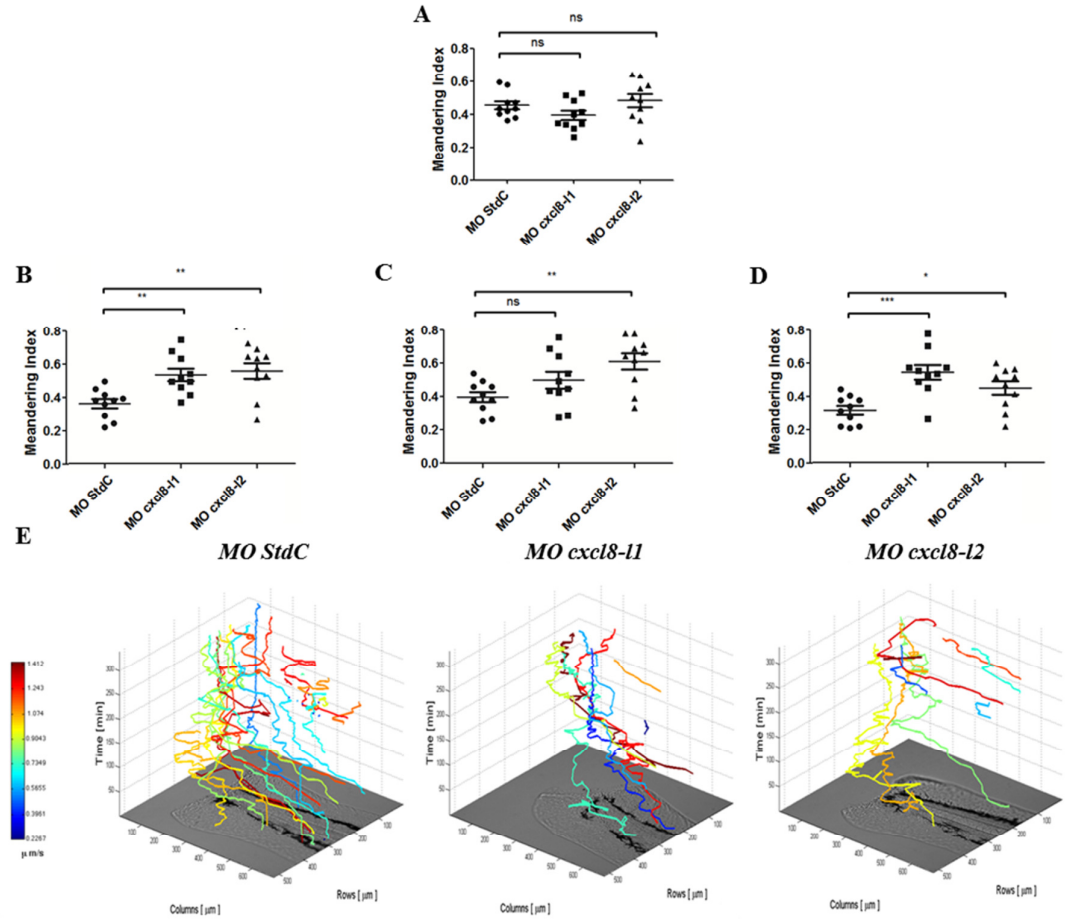

Figure 5: PhagoSight analysis reveals that cxcl8s morphants have an increased zebrafish neutrophil recruitment velocity in acute inflammation. Tailfins of Tg(mpx:gfp)i114 previously microinjected with MO StdC, MO cxcl8-l1 and MO cxcl8-l2 were transected at 3 dpf and time-lapse movies were made in wide field fluorescence microscope (MO StdC=270 tracks, MO cxcl8-l1=147 tracks and MO cxcl8-l2=128 tracks from n=10 larvae as 3 independent experiments) and further analyzed by PhagoSight. Distinct parameters were determined to study neutrophil migratory behavior such as: (A) Meandering Index; (B) Mean Speed; (C) Mean Speed inTranslation; (D) Mean Speed inExploration. Longer horizontal bars represent the mean values and shorter horizontal bars represent SEM. (E) 3D-Photomicrograph of a typical tracking experiment, with lines indicating the path of neutrophil movement over 6-hour time lapse during the recruitment phase of inflammation and colors indicating neutrophil velocity in µm/s. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, *P<0.05, **P<0.01, and ***P<0.001.

Interestingly, the knockdown of either *cxcl8-l1* or *cxcl8-l2* did not affect the meandering index of the neutrophil movement in the tail fin tissue under acute inflammatory conditions (Figure 5A). Unexpectedly, the speed of neutrophils in both *cxcl8* morphants was significantly higher comparing with the control condition (Figure 5B). Consistently, the mean speed from neutrophils moving towards the wound (referred here as “in translation”) was significantly increased in both *cxcl8* morphants (Figure 5C). Furthermore, the mean neutrophil speed was also significantly reduced for neutrophils moving within the wound (referred here as “in exploration”) in the absence of Cxcl8-l2 chemokine (Figure 5D). Both lateral and oriented velocities (Supplemental Figure 2A-F) were significantly increased in both *cxcl8* morphants, indicating that the overall neutrophil speed was increased and not just the speed towards the wound.

In Figure 5E, we present representative 3D tracking plots of wounded tail fins of 3 dpf larvae from control, *cxcl8-l1* and *cxcl8-l2* morphants. Tracks were plotted highlighting the faster ones. As mentioned above, a significantly lower number of neutrophils were recruited to the wounds at higher speeds in both *cxcl8* morphants in comparison to normal conditions. For the unwounded conditions, no significant differences were observed for neutrophil migratory speed, directionality or number of neutrophils migrating to the tail (Supplemental Figure 2G-L). In addition, neutrophils migrated extravascularly, through the tissue matrix, in both wild type and *cxcl8* morphants (Fig. 5E and Supplemental Movies 1-6).

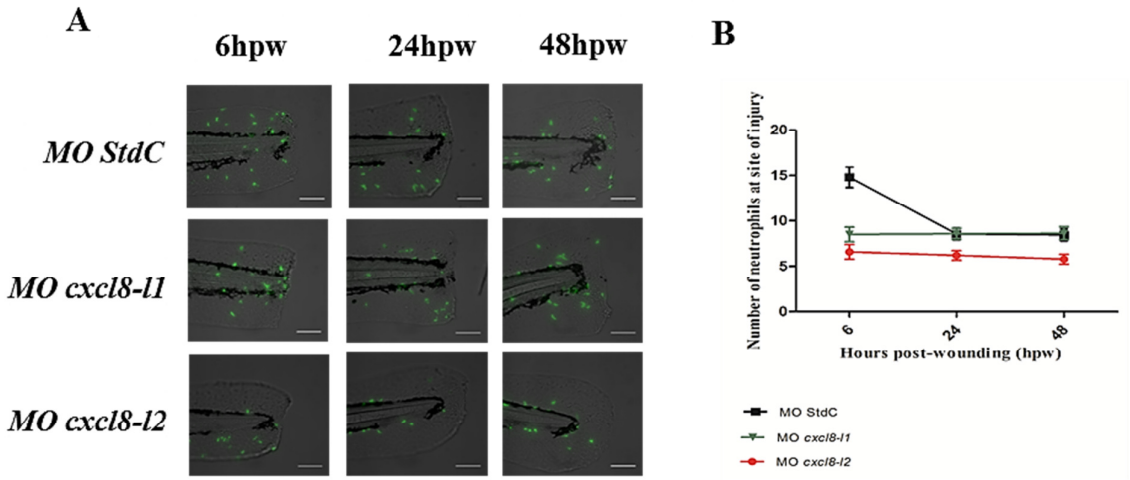

Figure 6: Resolution of the cellular component of inflammation is decreased in both cxcl8 morphant larvae. (A) Representative maximum intensity projections from wide field fluorescence microscope micrographs of 3 dpf Tg(mpx:gfp)i114 control and morphants larvae tail fins at 6, 24 and 48 hpw. Scale bar=100□m. (B) Counts of fluorescent neutrophils at the wound were made at 6, 24 and 48 hpw. Data is shown as mean ± SEM (n=40 performed as 3 independent experiments). P values were calculated using two-way ANOVA and Bonferroni multiple comparison test. For both CXCL8 morphants, significant differences were observed at 6 hpw in comparison to control larvae ($P<0.001$) but not at 24 or 48 hpw ($P>0.05$).

Inflammation resolution in wounded tail fin tissue is affected by the absence of Cxcl8 chemokines

Having established that neutrophil recruitment to wound heavily depends on both Cxcl8s, we further addressed whether inflammation resolution is affected by the absence of these chemokines. For these experiments, we wounded 3 dpf control and morphant larvae at the tail fins and counted the number of neutrophils present at the wound at 6, 24 and 48 hpw. Control larvae had a significantly reduced number of neutrophils at 24 hpw when compared to 6 hpw (Figure 6), indicating a successful inflammation resolution. However, in both *cxcl8* morphants, a reduction of the number of recruited neutrophils was not observed at either 24 or 48 hpw. Interestingly, *cxcl8-l2*, and to some extent *cxcl8-l1*, morphant larvae showed a faster healing and regenerative capacity than control larvae (data not shown).

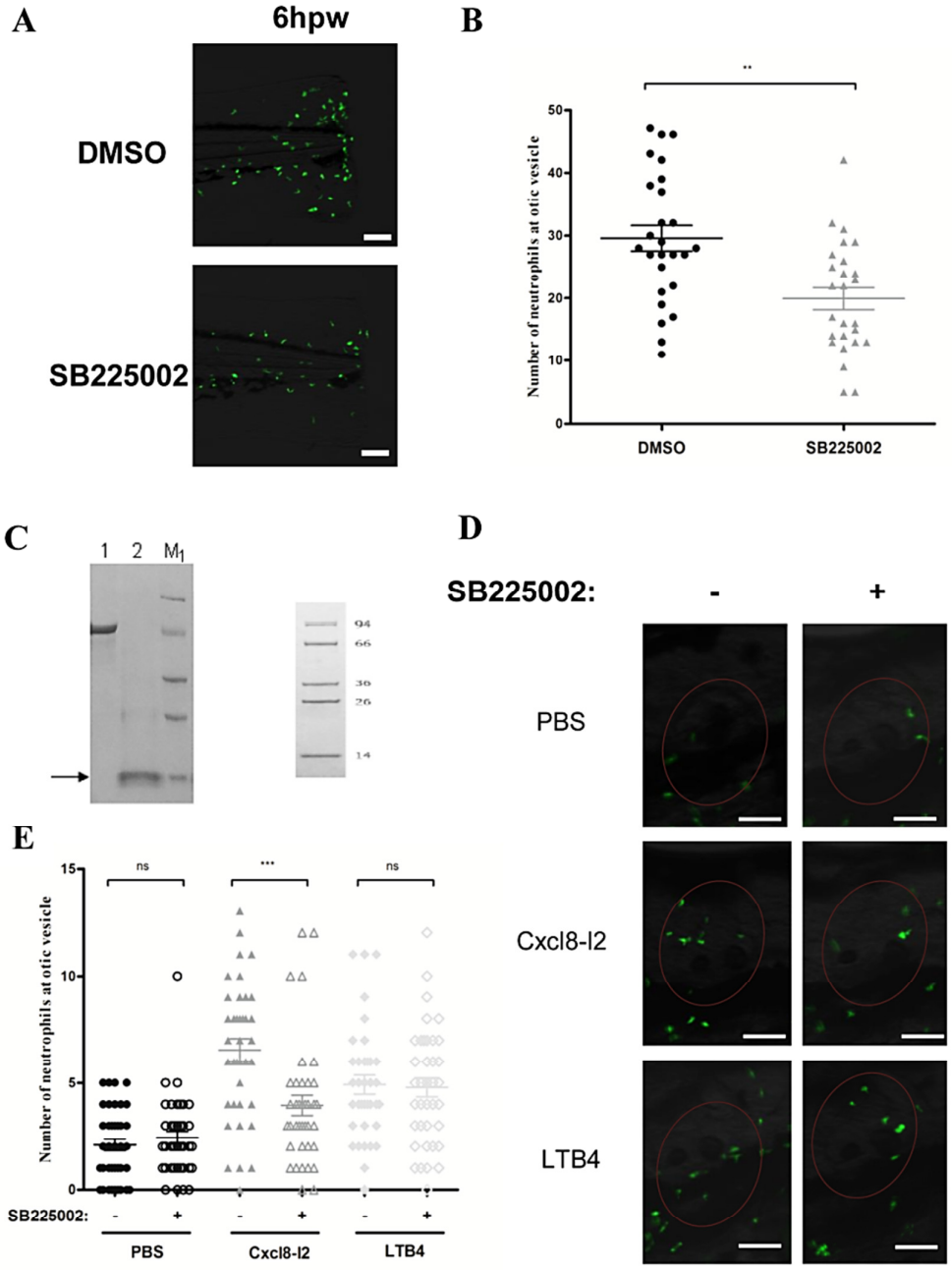

Figure 7: Pharmacological inhibition of Cxcr2 impaired neutrophil recruitment. (A) Representative wide field fluorescence microscope micrographs of 3 dpf Tg(mpx:gfp)i114 control and morphant larvae, pre-treated or not with 5 μM SB225002 followed by tail fin wounding. Scale bar=100 μm. (B) Counts of fluorescent neutrophils at the wound were made at 6 hpw. Data are presented as means ± SEM (n=25 performed as 3 independent experiments). P values were calculated using two-tailed Mann-Whitney test, ** P<0.01. (C) SDS-Page analysis of Cxcl8-l2 recombinant protein. Lane 1: BSA (2μg), lane 2: Cxcl8-l2 (2μg). (D) Representative wide field fluorescence microscope micrographs of 3 dpf Tg(mpx:gfp)i114 pre-treated with 5 μM SB225002 followed by otic vesicle injection of PBS, 30 μM Cxcl8-l2 and /or 30 nM LTB4. Images were taken at 1 hpi. Scale bar=100□m. (E) Counts of fluorescent neutrophils recruited to the ear (encircled) were made 1 hpi. Data indicate means ± SEM (n=32 performed as 3 independent experiments). P values were calculated using one-way ANOVA and Bonferroni multiple comparison, ***P<0.001.

Pharmacological inhibition of Cxcr2 impairs neutrophil recruitment to recombinant Cxcl8-l2

In order to address whether also in zebrafish Cxcl8s signal through Cxcr2 for inducing neutrophil recruitment, we designed two different *in vivo* recruitment assays using a human CXCR2 selective non-peptide inhibitor, SB225002 (*36*). The presence of SB225002 at 5 μM significantly reduced the number of neutrophils recruited to the wound upon tail fin injury (Figure 7A and B). Due to the fact that *cxcl8-l2* was induced at higher levels than *cxcl8-l1* in wounded tissue (Figure 1), recombinant Cxcl8-l2 protein was produced (Figure 7C). Consistently with its chemoattractant function, injection of the recombinant chemokine into the otic vesicle increased neutrophil recruitment to the ear in comparison to control conditions (Figure 7D and E). When recombinant protein injection was performed into the otic vesicle of larvae pre-treated with SB225002 so as to inhibit Cxcr2, the number of neutrophils recruited to the ear was reduced to a basal level (Figure 7D and E). In contrast, leukotriene B4 (LTB4)-induced neutrophil recruitment was unaffected by SB225002 (Figure 7D and E), confirming the specificity of this inhibitor.

Discussion

CXCL8 is an important chemokine that mediates neutrophil migration, accumulation and function at sites of inflammation (*37, 38*). Up to now, its *in vivo* study has been greatly hampered by the lack of true homologues in rats and mice (*11*). This difficulty can now be circumvented by using zebrafish models, as this teleost fish has been shown to express close CXCL8 homologues (*33-35, 39*). This is evidenced by the protein alignment (data not shown), which further allowed us to conclude that Cxcl8-l2 phylogenetically constitutes a closer homologue to human CXCL8 than Cxcl8-l1 (45.4% of identity between zebrafish Cxcl8-l2 and human CXCL8 *versus* 35.2% of identity between zebrafish Cxcl8-l1 and human CXCL8). As such, we aimed here to address *in vivo* the requirement of the zebrafish Cxcl8s for the recruitment and behavior of neutrophils in acute inflammation. As an inflammatory model, we used here the transection of the tail fin, which is currently one the best studied and most used models in zebrafish.

In this study, we hypothesized that zebrafish Cxcl8s play a role in neutrophil recruitment under acute inflammatory conditions. Consistently, we first demonstrated that the expression of both *cxcl8* genes is rapidly up-regulated upon wounding. A similar increase in expression of *cxcl8*s has also been observed in response to infectious stimuli (data not shown). These results are in agreement with those published previously for Cxcl8-l1 (*35*) as well as for the carp Cxcl8s (*33, 34*) and strongly suggest that both zebrafish Cxcl8s play a role in acute inflammation.

Considering the literature published on the inflammatory function of CXCL8, we have further addressed the requirement of zebrafish Cxcl8 lineages for neutrophil recruitment to sites of inflammation. For such, neutrophil recruitment to wounded tail fins was assayed in the absence of these chemokines via the use of specific MOs that enabled the knockdown of their expression. Our analysis revealed that both chemokines are necessary for a normal neutrophil response, as in their

absence, neutrophil recruitment to the wound was severely impaired. These findings are in agreement with the reduced neutrophil recruitment observed in inflammation in knockout mice for CXCR1, CXCR2 or CXCL1 (one of the mouse CXCL8 functional homologues) (*40-44*). Furthermore the mRNA levels of key pro-inflammatory mediators, such as IL-1β and PTGS2b, were affected in both morphants, strongly suggesting that in the absence of either Cxcl8 zebrafish acute inflammation is significantly attenuated. Similarly, infection of CXCL1-deficient mice with *Klebsiella pneumonia* has been shown to affect the expression of several chemokines and cytokines in the lungs in comparison with control animals (*44*). It is also important to point out that although Cxcl8-l2 deficiency impaired the induction of Cxcl8-l1 upon wounding, Cxcl8-l1 knockdown had no effect on Cxcl8-l2 induction. This crosstalk between the two zebrafish CXCL8 chemokines will require further investigation.

Having established the requirement of both Cxcl8s for neutrophil recruitment, we next investigated in detail by *PhagoSight* analysis how the absence of these chemokines affected this process. Inspection of the total numbers of neutrophil tracks and of tracks entering the wound allowed us first to confirm that neutrophil recruitment is significantly reduced in the absence of both Cxcl8s. Moreover, we also observed that the number of neutrophils that entered the wound and left until 6 hpw were similar in control and morphant larvae. These results suggest that the absence of both chemokines does not favor reverse migration that could perhaps function as a compensatory response to achieve resolution of inflammation. Additionally, our data reveal that in *cxcl8* morphants, the meandering index of migrating neutrophils was not significantly affected whereas, surprisingly, the mean neutrophil migratory speed, as well as the lateral and oriented velocities, increased. Moreover, a sensitivity analysis has been performed to demonstrate that all these measurements are not sensitive to variation in the selected size of the wound region, or to variation in track length (data not shown). A similar analysis performed for unwounded larvae allowed us to exclude the possibility that these results reflected an altered migratory pattern typical of *cxcl8* morphant neutrophils, regardless of any elicited inflammatory response. Altogether these experiments led us to conclude that while reducing neutrophil recruitment, the absence of both Cxcl8s increases the velocity of the neutrophils migrating to the wound area. As it is generally accepted that chemotactic cues guide leukocytes to sites of injury by increasing their velocity and directionality (usually measured by the meandering index) (*45, 46*), these findings are quite paradoxical. One possible scenario for explaining these controversial results would be that distinct neutrophil sub-populations may respond differentially to CXCL8 chemokines and other chemoattractants expressed locally at the site of inflammation and could thus display distinct migratory behaviors. Under normal inflammatory conditions, these neutrophil subpopulations could be mobilized via sensing distinct chemotactic cues to inflamed areas where they could perhaps exert different functions. In the absence of either Cxcl8 signal, only the Cxcl8-unresponsive neutrophils would then migrate towards the inflamed area, possibly with an increased velocity by responding to other chemoattractants, such as for example, LTB4 which induces neutrophil recruitment as demonstrated by us and others (*21, 47*). In agreement with a scenario of distinct functional populations, a recent study has proposed that zebrafish neutrophils

can be functionally classified either as consumers or producers of H_2O_2 (*48*). The idea that neutrophils have distinct functional populations is taking shape (*49, 50*), and the acceptance of different functional subpopulations of neutrophils will require additional work. In this respect, a differential neutrophil response to CXCL8 may actually constitute a pivotal criterion to discriminate between the identified neutrophil populations.

In order to restore the normal tissue function and homeostasis, it is crucial that an acute inflammatory process should be well resolved (*51*). Among other markers, inflammation resolution is characterized by a significant decrease in the number of neutrophils at the inflamed sites (*17, 52-55*). Taken the impact of both Cxcl8s in the recruitment of zebrafish neutrophils, we have also addressed whether and how resolution was affected in *cxcl8-l1* and *cxcl8-l2* morphants. As expected for a normal resolution, our results showed that at 24 hpw, control larvae presented a significantly reduced number of neutrophils comparing to 6 hpw. Such scenario was not observed for both morphants that were not able to reduce their neutrophil counts at the wound at 24 or even at 48 hpw. In fact, the number of neutrophils at wounds remained fairly constant in *cxcl8* morphants from 6 to 48 hpw. These results, together with the impaired induction of the pro-inflammatory mediators IL-1β and PTGS2b in Cxcl8-deficient larvae after wounding, suggest that inflammation resolution was compromised in these larvae. In addition, these results also demonstrate that CXCL8 chemokines can modulate the expression of other inflammatory mediators such as factors with a pro-resolution function that might be enrolled in neutrophil apoptosis signaling pathways, in macrophage-mediated phagocytosis or even in neutrophil reverse migration, among other events critical for inflammation resolution (*17, 23, 56, 57*). As such, the unavailability of these chemokines during acute inflammation would necessarily affect the resolution of the process. Nevertheless, it is surprising that *Cxcl8*-deficient larvae showed faster healing than control larvae, despite being unable to resolve the inflammation. Although the mechanisms involved in this process deserve further investigations, our results are in agreement with a recent study that reported the absence of neutrophils to contribute to a faster and more successful repair of the tail fin (*58*).

In mammals CXCL8 binds to CXCR1 and CXCR2 with different affinities and thus activates different cascade pathways (*3, 7-9*). These receptors have been proposed to exert different functions in the neutrophil inflammatory response (*7, 59*). Due to its intrinsic higher affinity towards its ligand chemokines, CXCR2 is believed to play a more active role in neutrophil recruitment and degranulation (*7, 36, 59*). As for CXCR1, it is thought to be more important for regulating cytotoxic events such as, ROS formation and protease release, at the injury site, (*7, 59, 60*). As zebrafish also have CXCR1 and CXCR2 homologues (*35*), we made here use of a human selective non-peptide CXCR2 inhibitor, SB225002, to establish that (i) Cxcr2 plays a prominent role in neutrophil recruitment to wound and (ii) neutrophil recruitment mediated by Cxcl8-l2 mainly depends on Cxcr2 signaling.

In summary, we report that zebrafish Cxcl8-l1 and Cxcl8-l2 are both important *in vivo* for the recruitment of neutrophils in the wound-elicited acute inflammation. In particular, we have

demonstrated that the presence of these chemokines is required for the maintenance of a normal neutrophil migratory behavior upon wounding. As evidenced in this report, there is still much work to be done in order to understand in detail how this small cytokine affects and modulates the neutrophil inflammatory response. In this respect, we believe that the zebrafish will undoubtedly stand as the optimal experimental animal model to pursue these issues and further to develop new therapeutic approaches strategically targeting CXCL8.

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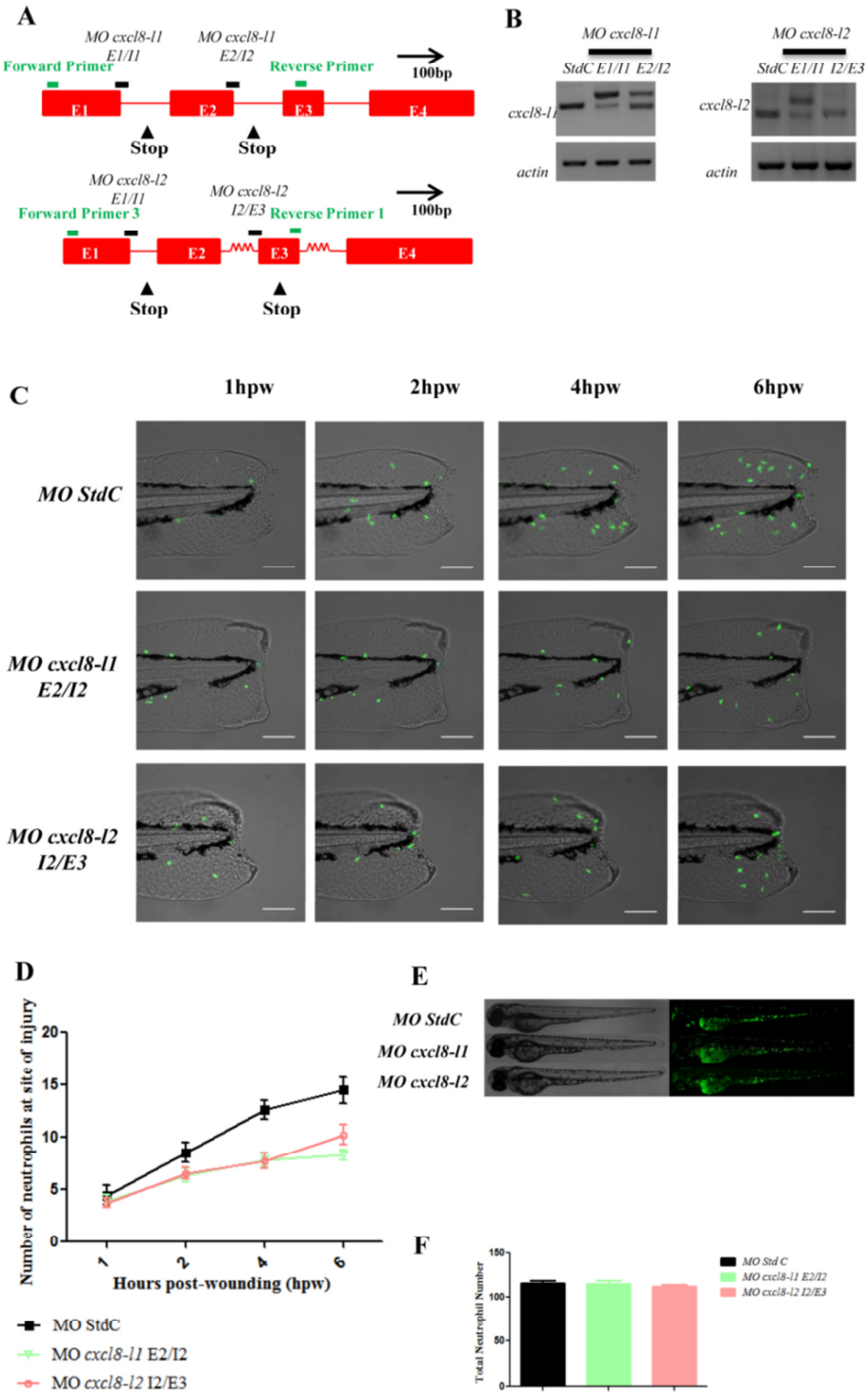
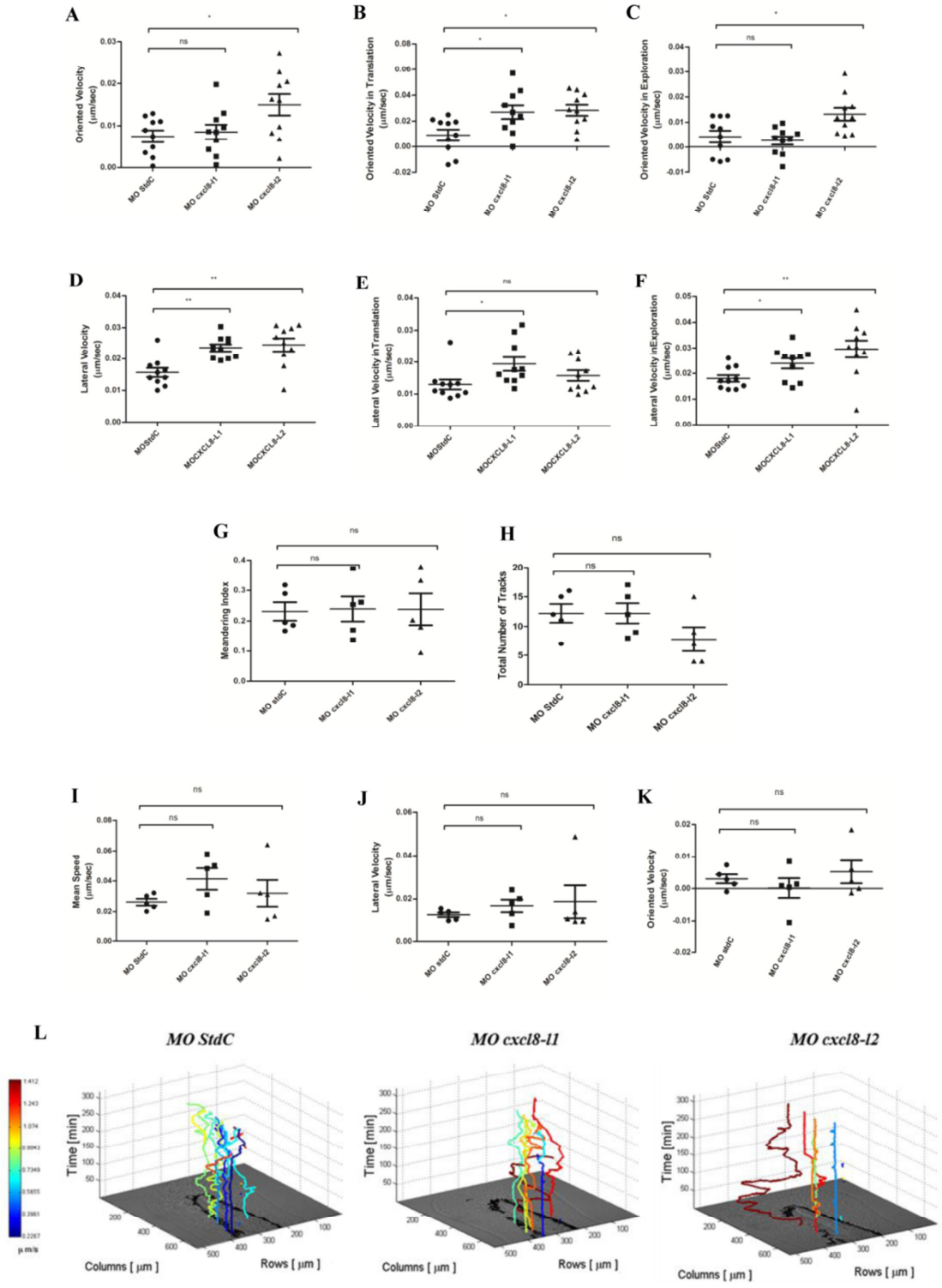
Supplementary Figures:



Supplemental Figure 1: Knock-down analysis of cxcl8-l1 and cxcl8-l2 using splice blocking morpholinos. Other two Cxcl8 morpholinos also reduces zebrafish neutrophil recruitment in acute inflammation. (A) Exon/Intron scheme of cxcl8 genes, MOs and Primers targeting sites. (B) RT-PCR of cxcl8-l1 and cxcl8-l2 transcripts detection, in 3dpf Tg(mpx:gfp)i114 control and morphant larvae. Insertion of intron 1 in MOs targeting E1/I1 boundary resulted in several premature stop codons and early truncated Cxcl8 proteins (22 of 98 amino acid for Cxcl8-l1 and 24 of 118 amino acids for Cxcl8-l2). (C) Tailfins of

Tg(mpx:gfp)i114 morphants previously microinjected with MO StdC, MO cxcl8-l1 E2/I2 and MO cxcl8-l2 I2/E3 were transected at 3 dpf. Representative maximum intensity projections from widefield fluorescence microscope micrographs of 3dpf Tg(mpx:eGFP)i114 control and morphant larvae. Scale bar = 100μm (D) Counts of fluorescent neutrophils at the wound were made at 1, 2, 4 and 6 hpw. Data indicate means ± SEM (n= 30 performed as 3 independent experiments). P values were calculated using two-way ANOVA and Bonferroni multiple comparison test. There were no significant differences in neutrophil number at 1 and 2 hpw for both morphants ($P>0.05$), but for 4 and 6 hpw a significant decrease were observed ($P<0.01$) both for MO cxcl8-l1 E2/I2 and MO cxcl8-l2 I2/E3 (E) DIC and GFP widefield fluorescence microscope micrographs from 3 dpf control and morphants larvae. (F) Total neutrophil counts in whole-larvae for each condition. Each bar represent means ± SEM (n= 20 performed as 2 independent experiments). P values were calculated using one-way ANOVA and Bonferroni multiple comparison test ($P>0.05$, no significant differences were observed)



Supplemental Figure 2: PhagoSight analysis reveals that CXCL8s morphants have increased zebrafish neutrophil recruitment velocities in acute inflammation. Tailfins of Tg(mpx:gfp)i114 previously microinjected with MO StdC, MO cxcl8-l1 and MO cxcl8-l2 were transacted or not at 3 dpf and 2 min time-lapse movies were made in widefield fluorescence microscope (n=10 larvae as 3 independent experiments for wound conditions (A-F), and n=5 larvae as 2 independent experiments for un-wound conditions (G-L)) followed by Phagotrack analysis, several distinct parameters were analyzed in order to study neutrophil migratory behavior in wound conditions: (A) Oriented Velocity; (B) Oriented Velocity inTranslation; (C) Oriented Velocity in Exploration; (D) Lateral Velocity; (E) Lateral Velocity inTranslation; (F) Lateral Velocity inExploration; in un-wound

conditions: (G) Meandering Index; (H) Total Number of Tracks; (I) Mean Speed; (J) Lateral Velocity; (K) Oriented Velocity; (L) 3DPhotomicrograph of a typical tracking experiment, with lines indicating the path of neutrophil movement over 6-hour time lapse in un-wound larvae tail fins, colors indicate neutrophil velocity in μm/s . P values were calculated using one-way ANOVA and Bonferroni multiplecomparison test, *P<0.05, **P<0.01, and ***P<0.001.

Supplemental Movie 1: Representative movie showing neutrophils recruitment to wound in a standard morphant larvae.

Supplemental Movie 2: Representative movie showing neutrophils recruitment to wound in a cxcl8-l1 morphant larvae.

Supplemental Movie 3: Representative movie showing neutrophils recruitment to wound in a cxcl8-l2 morphant larvae.

Supplemental Movie 4: Representative movie showing neutrophil tracks in a wounded starndard morphant larvae.

Supplemental Movie 5: Representative movie showing neutrophil tracks in a wounded cxcl8-l1 morphant larvae.

Supplemental Movie 6: Representative movie showing neutrophil tracks in a wounded cxcl8-l2 morphant larvae

Chapter 3

Cxcl8-l1 and Cxcl8-l2 are both essential in zebrafish defense against

S. Typhimurium

Cxcl8-l1 and Cxcl8-l2 are both required in the zebrafish defense against *Salmonella* Typhimurium

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Abstract

In the last few years zebrafish has emerged as an excellent model to study the Cxcl8 signaling pathway in inflammation elicited upon tissue damage or infection. Zebrafish has two true homologs of mammalian CXCL8, named Cxcl8-l1 and Cxcl8-l2. Previously, we have shown that in the wound-associated inflammation, these chemokines are up-regulated after wounding and are relevant for neutrophil recruitment. In infection, this knowledge is so far lacking as most studies performed in this matter in the zebrafish have focused mostly in Cxcl8-l1 even though Cxcl8-l2 shares higher homology with the human CXCL8.

In this study we aimed to address the biological function of both zfCxcl8s in infection in order to better understand their roles under different inflammatory conditions. Gene expression analysis first confirmed that Cxcl8-l1 and l2 are induced upon infection or in PAMP-elicited inflammatory processes. Upon *S.* Typhimurium infection, we further found that a significantly decreased number of *cxcl8* morphants was able to survive in comparison to controls in the first 72 hours post-infection (hpi). In *S.* Typhimurium otic ear infection, neutrophil recruitment was further shown to be significantly reduced at 6 hpi in both *cxcl8* morphants. Accordingly, these morphants showed a decreased bacterial clearance from the site of infection. As observed in mammals, our data show that both zebrafish Cxcl8s play important roles in neutrophil recruitment and in the inflammatory response elicited upon infection or tissue damage. These results suggest that even though divergence of zebrafish and humans from a common ancestor occurred millions of years ago, the basic principles of neutrophil recruitment are apparently conserved.

Introduction

Zebrafish was first used as an animal model to study chemokine biology in 2000 (1). In 2006, a first comprehensive analysis of the zebrafish chemokine system was published by *DeVries et. al* based on comparative whole genome analysis studies (2) and the first transgenic zebrafish lines with fluorescently labeled leukocytes (3-5), that revolutionized our ability to dissect the molecular mechanisms that regulate inflammation, were developed. This along with the remarkable similarity observed between the zebrafish and mammalian immune systems and the possibility of using non-invasive procedures to study inflammation *in vivo*, prompted zebrafish to emerge as an ideal model for the study of leukocyte recruitment. In particular, zebrafish has turned into an attractive and powerful new tool to study CXCL8 biology in inflammation (6-9)

Similarly as with the carp, the zebrafish expresses two homologues of the human CXCL8, the Cxcl8-l1 and the Cxcl8-l2 (10) and both chemokines have been shown to be induced under inflammatory conditions (6-10). In particular, we have previously shown that Cxcl8-l1 and l2 are induced after tail-fin wounding, being Cxcl8-l2 more potently induced than Cxcl8-l1 (6). In this wound-associated inflammatory model, both were equally observed to be essential for neutrophil recruitment to the inflamed area (6). In this respect, Cxcl8-l1 has been shown to be expressed in tissue-bound gradients *in vivo* by binding to heparan sulfate proteoglycans (HSPGs) so as to guide and control neutrophil velocity in order to promote an accurate recruitment to the inflammatory locus (8). Among the known Cxcl8 receptors, Cxcl8-l1-mediated neutrophil recruitment from the caudal hematopoietic tissue to sites of *Pseudomonas aeruginosa* infection has been further shown to be dependent of the function of Cxcr2 but not of Cxcr1 in zebrafish larvae (7). As in this latter study, zebrafish studies addressing the expression and/or role of Cxcl8 chemokines in infection have been mostly focused on Cxcl8-l1, overlooking the other homologue. At this point, we believed that in order to strengthen the use zebrafish as an animal model for the study of inflammation/immunity and more precisely, in neutrophil function, a full understanding of the biological roles of both Cxcl8-l1 and l2 in the zebrafish immune defense was lacking. In this regard, we considered of utmost relevance to understand if these two chemokines play or not different biological functions in infection-elicited inflammation. For such, we first made use of specific infection models, such as the Spring viraemia of carp virus (SVCV) infection or subjected zebrafish larvae to treatment with specific PAMPs, such as poly I:C or *Vibrio anguillarum* DNA (vDNA), known to produce inflammatory responses in the context of particular infection models. In these assays, we observed that the gene expression of both Cxcl8s was always up-regulated. To further address their role in infection, we have particularly focused on the *Salmonella* enterica serovar Typhimurium *(S.* Typhimurium*)* infection (11, 12) a well characterized infection model in zebrafish among several others (13). In this model, we have observed that *cxcl8s* morphants larvae were more susceptible than controls in the first 72 hours post-infection (hpi). Moreover, we have also shown in *S.* Typhimurium otic ear-infection that in relation to control larvae, the chemokine morphants presented: (i) a significantly reduced neutrophil recruitment at 6hpi, as well as (ii) a reduced bacteria clearance from site of infection at 24hpi. Taken together our findings demonstrate that

both chemokines should be important for larval immune defense against infection, as well as already demonstrated in the response to wounding (6).

Material and Methods

Zebrafish Husbandry

All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU) and the Bioethical Committee of the University of Murcia (approval number #537/2011). Zebrafish fertilized eggs were obtained from natural spawning of wild-type (obtained from the Zebrafish International Resource Center), the *Tg(mpx:gfp)*^{i114} (3) line and the Tg(*lyz:dsRED*)^{nz50} (5) held at our facilities following standard husbandry practices. Animals were maintained in a 12 hr light/dark cycle at 28.5°C.

Morpholino knockdown

The following splice blocking morpholino-modified antisense oligonucleotides (morpholinos (MO), Gene Tools) were injected into 1-cell-stage fertilized eggs: MO *cxcl8-l1* E1/I1 5'-GGTTTTGCATGTTCACTTACCTTCA-3' (4 ng/egg), MO *cxcl8-l2* E1/I1 5'-TTAGTATCTGCTTACCCTCATTGGC-3' (4 ng/egg) (6). Standard control MO (MO Std) purchased from GeneTools was used as control.

Analysis of gene expression

Total RNA was extracted from whole larvae and gene expression analysis was performed as previously reported (6). Gene expression was normalized to the ribosomal protein S11 (rps11) content in each sample according to the Pfaffl method (14). The primers used were: *rps11-Fw 5'-ACAGAAATGCCCCTTCACTG-3', rps11-Rv 5'-GCCTCTTCTCAAAACGGTTG-3', cxcl8-l1-Fw 5'-GTCGCTGCATTGAAACAGAA-3', cxcl8-l1-Rv 5'-CTTAACCCATGGAGCAGAGG-3', cxcl8-l1-Fw1 5'-GTCGCTGCATTGAAACAGAA-3', cxcl8-l1-Rv1 5'-CTTAACCCATGGAGCAGAGG-3', cxcl8-l2-Fw1 5´-CCACACACACTCCACACACA-3', cxcl8-l2-Rv1 5'-TGATGAAAGGACAATTCAGTGG-3', cxcl8-l2-Fw3 5´-CCACACACACTCCACACACA-3', cxcl8-l2-Rv3 5'- TGATGAAAGGACAATTCAGTGG -3'*. In all cases, each PCR was performed in triplicate samples and repeated at least with two independent experiments. Statistical analysis was performed using two-way ANOVA with Tukey post-test in Graph Pad Prism5 software.

SVCV, Poly(I:C) and vDNA assay

Spring viraemia of carp virus infections were made as previously described (15, 16). Repeats of molecular CpG motifs obtained as phenolextracted genomic *V. anguillarum* DNA (vDNA) (17) were used to stimulate 3dpf zebrafish larvae by bath immersion (18), as well as 25mg/ml Poly (I:C) (19). Incubation was carried out for 24hours for SVCV and 4hours for PAMPs, at 28.5 °C. Whole larvae were collected into TRIzol for RNA extraction and gene expression.

Salmonella Typhimurium

Wild type (WT) S. Typhimurium or S Typhimurium expressing DsRed (*S.* Typhimurium*:DsRed)* were inoculated in 5mL of LB (S. Typhimurium) or LB with 40µg/ml Kanamycin (for S Typhimurium:DsRed) incubated overnight at 37ºC at 250-300 rpm. In the next morning inocula were diluted 1/5 WT S. Typhimurium or 2/5 S. Typhimurium:DsRed in corresponding media, with 0.3M NaCl and incubated at 37ºC until 1.5 OD600nm. Bacteria were diluted in sterile PBS for further experimentation.

Infection survival assay

Larvae of 2dpf were anesthetized in embryo medium with 0.16 mg/ml tricaine and 50 bacteria of *S.* Typhimurium per larvae were microinjected into the yolk-salk. Larvae were let to recover in egg water at 28-29ºC, and monitored for clinical signs of disease or mortality over 48hours.

Neutrophil recruitment assay

In order to address neutrophil recruitment to a localized site of *S.* Typhimurium infection 2 dpf larvae were anesthetized in embryo medium with 0.16 mg/ml tricaine and mounted in 1% of agarose low melting point supplemented with 0.16 mg/ml tricaine. Injection at the otic ear vesicle of 0.5 nL of PBS or *S.* Typhimurium*:DsRED* suspension, supplemented with phenol red, was then performed. In the latter condition, 100 bacteria per larvae were microinjected into the otic ear. Embryo medium with 0.16 mg/ml tricaine solution was added on the top, in order to keep embryos hydrated during the experiments. Images from otic area were taken after 1, 6 and 24 hour post-injection (hpi). After 6hpi, agarose was taken out from tail fin area in order to avoid death of larvae due to repressed growth and imaging was made at 24hpi. Neutrophil counts and *S.* Typhimurium*:*DsRed fluorescence intensity were determined at 1, 6 and 24hpi. Images were acquired using a Leica MZ16F fluorescence stereo microscope and treated with ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical analysis

Data were analyzed by analysis of one or two-variance (ANOVA) with Tukey (qPCR) or Bonferroni multiple range test (neutrophil recruitment and *S.* Typhimurium fluorescence quantification) to determine differences between groups. The contingency graphs were analyzed by Log-Rank Mantel-Cox test

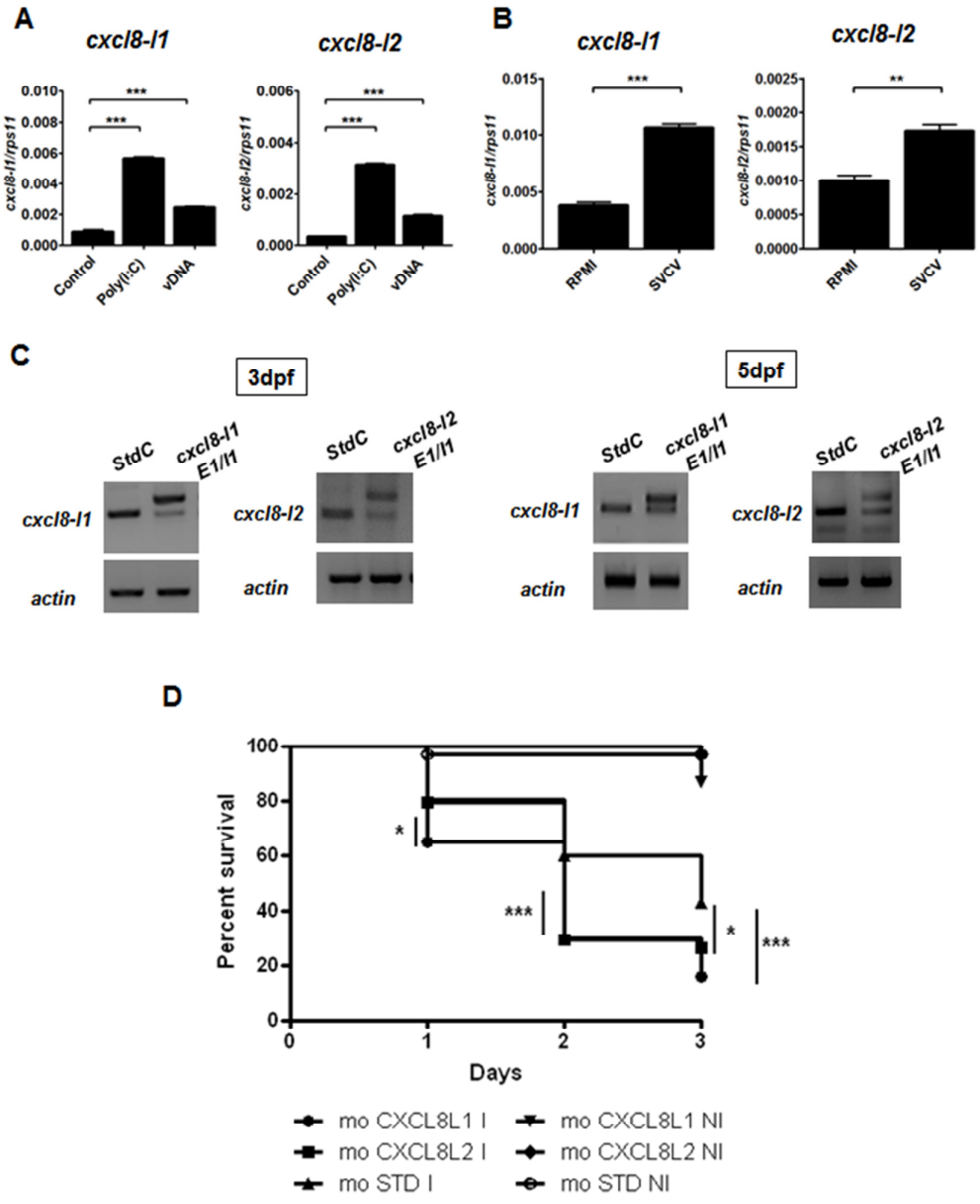

Figure 1: Cxcl8-l1 and Cxcl8-l2 are important for zebrafish survival upon infection. (A) Larvae were infected with SVCV for 24hours or stimulated with Poly(I:C) for 4hours. mRNA levels of the *cxcl8-l1 and cxcl8-l2* were determined by qPCR. Gene expression was normalized against *rps11*. Each bar represents the mean ± SEM of triplicated samples. P values were calculated using one-way ANOVA and Tukey multiple comparison test, **P<0.01, and ***P<0.001. **(B)** Zebrafish one-cell Tg(mpx:GFP)^{i114} or Tg(lyz:DsRED2)^{nz50} were microinjected with standard control (MO StdC) or *cxcl8-l1 or l2* morpholinos (MO Cxcl8-l1 or MO *cxcl8-l2*). RT-PCR of *cxcl8-l1* and *cxcl8-l2* transcripts detection, in 3 and 5dpf Tg(mpx:gfp)i114 control and morphant larvae. Insertion of intron 1 in MOs targeting E1/I1 boundary resulted in several premature stop codons and early truncated Cxcl8 proteins (22 of 98 amino acid for Cxcl8-l1 and 24 of 118

amino acids for Cxcl8-l2). **(C)** Survival curves of 2dpf larvae infected with *S.* Typhimurium from 0 to 3 days post infection (dpi). Each curve represents the mean of three independent experiments. P values were calculated using Log-Rank Mantel-Cox test, **P*<0.5, and ****P*<0.001.

Results and Discussion

Cxcl8s expression is induced in response to different PAMPs and infectious stimuli in zebrafish larvae

Despite the fact that the zebrafish possesses two distinct CXCL8 homologues and that Cxcl8-l2 shares a higher homology with the human CXCL8 (6, 10), previous studies have mostly addressed the expression and/or role of Cxcl8-l1 in infection overlooking that of the other homologue (6, 8-10, 20, 21). As such, it is currently not clear if Cxcl8-l2 plays a role in infection and in this case, if these two chemokines may play distinct roles in zebrafish infection as it could be suggested by the different expression profiles of the homologous carp Cxcl8s after infection (10). In order to address these issues we started by analyzing gene expression levels of both Cxcl8s. We have used larvae previously infected with SVCV (15) or incubated in the presence of two different PAMPs, namely the Poly(I:C), which is structurally similar to viral double-stranded RNAs and is sensed by TLR3, or with genomic DNA from the bacteria *V. anguillarum* (vDNA), which is recognized by TLR9 (22). We observed that the gene expression of both chemokines was significantly up-regulated in whole-larvae at the early stages of the associated inflammatory responses (Figure 1A and B), thus confirming that both Cxcl8s should be involved in the inflammatory processes elicited upon infection as previously observed upon wounding (6).

Cxcl8s are required for zebrafish larvae survival upon S. Typhimurium *infection*

After having established the induction of both Cxcl8s in response to different infectious stimuli, we next addressed the relevance of these chemokines to larvae survival upon bacterial infection. For such, survival curves were performed with 2dpf (days-post fertilization) *cxcl8* morphants and control larvae infected with *S.* Typhimurium, a gram-negative bacteria known to be involved in food poisoning or typhoid fever and having been used massively as an infection model (11, 12, 23). Although survival curves are usually made until 5 to 7 days after infection, we have here only focused on the early stages of infection (1-3 days post infection). This limitation was mainly due to two reasons. First and most importantly, the gene expression silencing efficiencies of both *cxcl8-l1* and *cxcl8-l2* splice-blocking morpholinos are greatly reduced after 5dpf (Figure 1C) restricting the use of these morphants in studies of shorter duration. A second line of reasoning relates to the fact that Cxcl8 is a pro-inflammatory molecule responsible for neutrophil recruitment, the first immune cells being recruited to the inflamed area, and thus should be mainly important in early stages of inflammation. By performing survival experiments until 3dpi, we observed that upon *S.* Typhimurium infection, both *cxcl8* morphant larvae were significantly more

susceptible of dying than controls (Figure 1D) demonstrating that both zebrafish Cxcl8s are important for the inflammatory response elicited to *S.* Typhimurium infection.

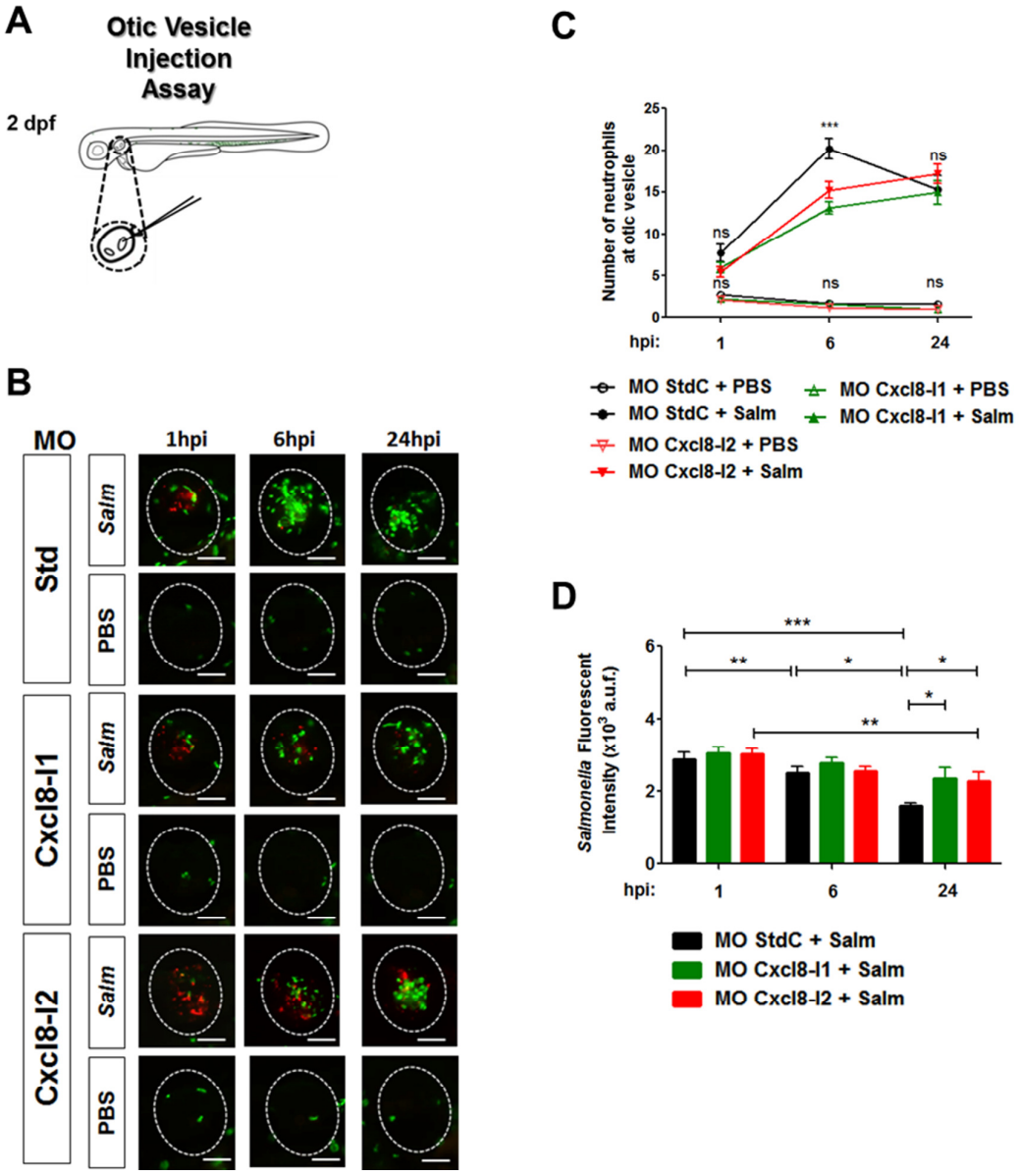

Figure 2: Neutrophil recruitment and bacterial clearance after *S.* Typhimurium infection is dependent of both Cxcl8s. Zebrafish one-cell Tg(mpx:GFP)^{i114} were microinjected with standard control (MO StdC) or *cxcl8-l1 or l2* morpholinos (MO Cxcl8-l1 or MO *cxcl8-l2*) and at 2 dpf, were further microinjected with PBS or *S.* Typhimurium*:DsRed* bacteria into the otic vesicle. **(A)** Schematic representation of the otic vesicle injection assay. **(B)** Representative images of red (*S.* Typhimurium*:DsRed)* and green (neutrophils) channels of otic vesicles **(C)** Counts of fluorescent neutrophils at the site of infection were made at 1, 6 and 24hpi. **(D)** Fluorescence intensity quantification of *S.* Typhimurium*:DsRed* bacteria at 1, 6 and 24hpi. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (**P*<0.5, ***P*<0.01; ****P*<0.001). Scale bars = 100µm.

Both Cxcl8s are required for neutrophil recruitment and bacteria clearance in S. Typhimurium *infection*

Next we hypothesized that the higher failure to survive to *S.* Typhimurium infection observed for both *cxcl8* morphants might be due to a deficient neutrophil recruitment. In order to test this, *cxcl8* morphants and control larvae were injected with DsRed expressing *S.* Typhimurium, into the otic vesicle (Figure 2A). Larvae from both *cxcl8* morphants presented a significant decrease in neutrophil recruitment to the infected ears at 6hpi when compared to PBS injected-control larvae (Figure 2B and C). Additionally we also observed that cxcl8 morphants presented a significant decrease in the ability to clear bacteria from the site of infection at 24hpi in comparison to control larvae (Figure 2B and D). This decrease in bacteria clearance may be due not only to the lower amounts of neutrophils recruited to site of infection but possibly also to the recruitment of a lower number of macrophages to this site, that could contribute to a reduction in bacteria phagocytosis. Moreover, these results strongly suggest to us that the higher susceptibility of cxcl8 morphants towards *S.* Typhimurium, infection presented up to the first 72hpi may mainly relate to a deficient neutrophil recruitment that directly or indirectly may further affect bacteria clearance.

Overall, we report here that both Cxcl8-l1 and l2 are important for larvae survival in the first 72 hours following *S.* Typhimurium infection by controlling neutrophil recruitment and modulating bacteria clearance at least in the first 24hpi, and thus affecting the inflammatory response against the bacterial infection. In line with our previous observations in the wound-associated inflammation model (6), both Cxcl8s seem to be important in zebrafish inflammatory response against infections. A plausible explanation for these results as well as for those previously reported by us in wounding (6), is that these two chemokines may be working as a heterodimer. Additional work will be required to better clarify this issue.

Undoubtedly, we can conclude that Cxcl8s play central roles in neutrophil recruitment and in the zebrafish inflammatory response against infection or tissue damage, similarly as it happens in mammals. These results taken along with the growing knowledge acquired in relation to neutrophil recruitment in zebrafish inflammation strongly indicate that the basic principles governing this process are very likely to be conserved between the zebrafish and humans even though these species are diverged from a common ancestor about 450 millions of years ago. Altogether, these considerations firmly place zebrafish in the frontline of the study of Cxcl8 biology in vivo over other experimental animal models.

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Chapter 4

ATP modulates acute inflammation in vivo through Duox1-derived H_2O_2 production and NF-κB activation

ATP modulates acute inflammation *in vivo* through Duox1-derived H_2O_2 production and NF-κB activation

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Abstract

Dual oxidase 1 (Duox1) is the NADPH oxidase responsible for the H_2O_2 gradient formed in tissues after injury to trigger the early recruitment of leukocytes. Little is known about the signals that modulate H_2O_2 release from DUOX1 and whether the H_2O_2 gradient can orchestrate the inflammatory response *in vivo*. Here, we report on a dominant negative form of zebrafish Duox1 that is able to inhibit endogenous Duox1 activity, H_2O_2 release and leukocyte recruitment after tissue injury, with none of the side effects associated with morpholino-mediated Duox1 knockdown. Using this specific tool, we found that ATP release following tissue injury activates purinergic P2Y receptors, and modulates Duox1 activity through phospholipase C (PLC) and intracellular calcium signaling *in vivo*. Furthermore, Duox1-derived H_2O_2 is able to trigger the NF-κB inflammatory signaling pathway. These data reveal that extracellular ATP acting as an early danger signal is responsible for the activation of Duox1 via a P2YR/PLC/Ca^{2+} signaling pathway and the production of H_2O_2, which, in turn, is able to modulate *in vivo* not only the early recruitment of leukocytes to the wound, but also the inflammatory response through activation of the NF-κB signaling pathway.

Introduction

Dual oxidase 1 (DUOX1), an NADPH oxidase and member of the NOX family (1-4) is responsible for the formation of hydrogen peroxide (H_2O_2) tissue gradient formed after wounding (5-7) . This gradient has been shown to be the main trigger of early leukocyte recruitment and is later required for tissue regeneration via oxidation of the Src-family kinases (SFKs), Lyn and Fyn b, respectively (6-8). Importantly, neutrophil-delivered myeloperoxidase is responsible for the rapid clearance of H_2O_2 after injury (9), preventing excessive tissue damage. In addition, DUOX1-derived H_2O_2 is essential in host defense by supporting lactoperoxidase-mediated antimicrobial defense mechanisms on mucosal surfaces (10). Despite our increased knowledge about H_2O_2 signaling, the mechanisms that regulate *in vivo* DUOX1 activation are poorly understood. In the injured epidermis of Drosophila embryos, it has recently been reported that calcium flashes orchestrate the wound inflammatory response through DUOX1 activation, via its EF-hand calcium-binding motif (7), whereas in zebrafish this does not seem to be the case (8). As in Drosophila (7), the zebrafish Duox1 (5, 7) also has two canonical EF-hands in an intracellular loop, which suggests that cytosolic calcium might regulate H_2O_2 production. However to date, little is known about the role of cytosolic calcium signaling in the modulation of DUOX1 activation and H_2O_2 production *in vivo*. Besides the generation of calcium waves (11-13), the release of ATP is an important early danger signal after tissue damage (14-16). Additionally, the activation of purinergic receptors by extracellular ATP is one of the main biological mechanisms responsible for epithelial intracellular calcium (iCa^{2+}) mobilization (16-19) and several *in vitro* studies have correlated this signal with DUOX1 activation (20-24).

Redox processes regulate different oxygen-sensitive transcription factors through the direct modification of proteins (25). Altering gene expression is a fundamental mechanism for cells to respond to changes in the extracellular environment. NF-κB is an important redox-sensitive transcription factor (25, 26), which regulates the expression of the genes involved in multiple biological functions, including inflammation, apoptosis and proliferation (27). However, there is controversy concerning whether H2O2 activates or inhibits NF-κB (28, 29). Furthermore, there is increasing evidence that H2O2 is not just a generic modulator of transcription factors and signaling molecules, but may also act as a specific regulator of individual genes (29-33).

Here we investigate whether ATP release acts as a danger signal to mobilize intracellular calcium, acting upstream of DUOX1 activation in vivo and further modulating NF-κB activation in acute inflammatory conditions. We show that zebrafish Duox1 activation and subsequent neutrophil recruitment are mediated by ATP acting through a P2Y receptor (P2YR)/phospholipase C (PLC)/calcium signaling pathway and that Duox1-derived H2O2 activates in vivo the NF-κB inflammatory signaling pathway.

Materials and Methods

Zebrafish Husbandry

All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU) and the Bioethical Committee of the University of Murcia (approval number #537/2011). Fertilized zebrafish eggs were obtained from natural spawning of wild-type (obtained from the Zebrafish International Resource Center), the Tg(mpx:gfp)^{i114} (34) and the Tg(lyz:DsRED2)^{nz50} (35) lines held at our facilities following standard husbandry practices. Tg(NFκB-RE:eGFP) (NF-κB:eGFP for even greater simplicity) line was generated with the method and constructs previously described (36) . Animals were maintained in a 12 hr light/dark cycle at 28.5°C.

DNA constructs

Zebrafish Duox1 amino acid sequence (XP_001919394.3) was submitted to domain analysis using the PFAM database (http://pfam.sanger.ac.uk/) (37). A truncated form of the wild type Duox1, which lacks the entire flavin domain (residues 1-1,232, dominant negative (DN) Duox1, Suppl. Fig. 1a), was chemically synthesized and cloned in pBluescript II KS+ (GenScript Corporation). CMV/T7:DN-Duox1:GFP was generated by MultiSite Gateway assemblies using LR Clonase II Plus (Life Technologies) according to standard protocols and using the Tol2kit vectors described previously (38).

Morpholino and mRNA injections

Specific morpholinos (Gene Tools) were resuspended in nuclease-free water. *In vitro*-transcribed DN-Duox1 (sense and anti-sense), DN-Duox1:GFP and GFP RNA were obtained following the manufacturer's instructions (mMESSAGE mMACHINE kit, Ambion). RNAs (300 pg/egg) and duox1 splice morpholino (5'-AGTGAATTAGAGAAATGCACCTTTT-3') (125 μM) with p53 morpholino (5'-GCGCCATTGCTTTGCAAGAATTG-3') (100μM) (5, 6) were mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into one-cell-stage embryos using a microinjector (Narishige) (0.5-1 nl per embryo). The same amounts of RNA and MOs were used in all experimental groups.

HEK293 cell transfection and western blot

Plasmid DNAs were prepared using the Midi-Prep procedure (Qiagen) and transfected into HEK293 cells with LyoVec transfection reagent (Invivogen), according to the manufacturer's instructions. At 48 hours after transfection, the cells were washed twice with PBS and lysed in 200 μl lysis buffer (10 mM Tris-HCl pH 7.4 and 1% SDS). The protein concentrations of cell lysates were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard. Cell extracts (50 μg

protein) were analyzed on 8% (DN-Duox1:GFP) and 15 % (GFP) SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). The blots were developed with mouse anti-GFP Ab (1/1000) (Clontech) and enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer's protocol.

Tail fin wounding

Essentially, tail fin amputation was performed as previously described (39). Briefly, Tg(lyz:DsRED2)nz50, Tg(mpx:gfp)i114, or Tg(NFkB:EGFP)nc1 larvae were anesthetized at 3 dpf in embryo medium with 0.16 mg/ml tricaine. Then, complete transection of the tailfin tip was performed with a disposable sterile scalpel and fish were mounted in 1% (w/v) low melting point agarose (Sigma-Aldrich) dissolved in embryo medium supplemented with 0.16 mg/ml tricaine. The success of transection was immediately confirmed by in a fluorescence stereo microscope MZ16FA (Leica) equipped with green fluorescent filters. After solidification, embryo medium with 0.16 mg/ml tricaine solution, pretreated or not with pharmacological inhibitors (see below), was added in order to keep the embryos hydrated during experiments. Then, images were captured at the selected times while animals were kept in their agar matrixes at 28.5°C.

Pharmacological treatment

All drug treatments were made using the bath immersion method. Briefly, 3 dpf Tg(lyz:DsRED2)^{nz50}, Tg(mpx:gfp)^{i114} or Tg(NFkB:EGFP)^{nc1} larvae were incubated for 1 hour at 28°C in the presence or absence of each of the following drugs: 100 µM dibenziodolium chloride (DPI), 1µM thapsigargin, 1µM U73122, 100 µM pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), 100 µM Suramin and 10 U/mL apyrase (all from Sigma-Aldrich) diluted in embryo medium supplemented (or not) with 1%DMSO. After tail fin amputation or otic injection, the larvae were kept in the corresponding treatments until imaging.

H_2O_2 imaging

H_2O_2 imaging using a live cell fluorescein dye was performed as previously described (40). Briefly, 3dpf Tg(lyz:DsRED2)^{nz50} larvae pre-treated with RNA, morpholinos or drugs and corresponding control siblings, were loaded for 30 min with 50 mM acetyl-pentafluorobenzene sulphonyl fluorescein (Cayman Chemical) in 1% DMSO in embryo medium, prior to tail fin amputation, carried out as described above. Larvae were left to recover in probe solution and imaging was made 30 min post wounding (minpw). The same number of larvae were left without wounding in each group/experiment and used as fluorescence background controls for mean fluorescence intensity quantification.

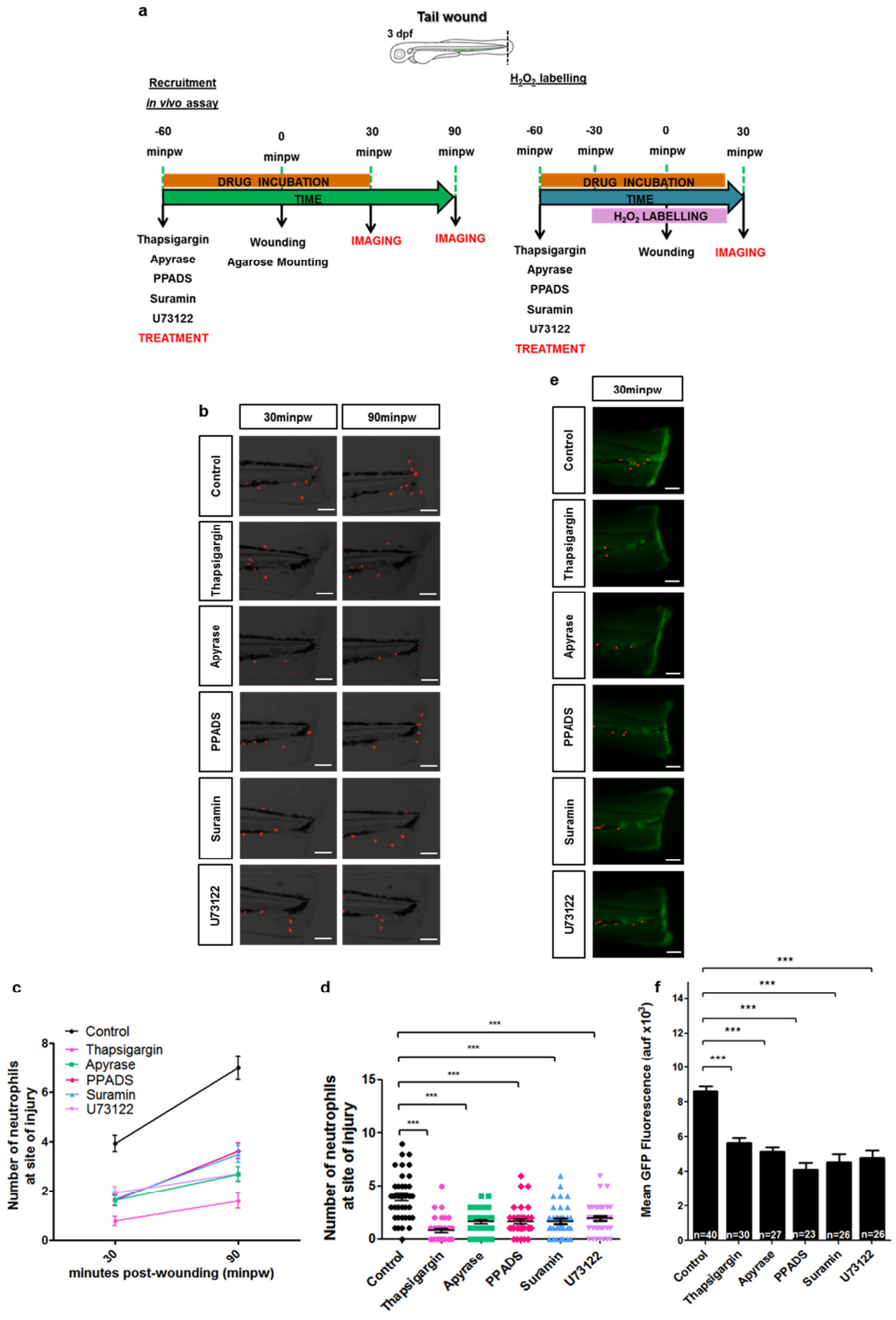

Fig. 1: ATP and calcium signaling modulate Duox1-derived H2O2 release and neutrophil recruitment in wounding. (a) Schematic representation of the neutrophil recruitment and

H2O2 visualization assays. (b-f) Un-injected 3dpf Tg(lyz:DsRED2)nz50 larvae were treated with 10 U/mL apyrase, 1 μM thapsigargin, 100 μM PPADS, 100 μM suramin or 1 μM U73122 and further tail fin larvae amputation was performed. For H2O2 labelling, larvae were incubated for 30 min with 50 mM acetylpentafluorobenzene sulphonyl fluorescein in 1% DMSO in embryo medium. (b) Representative overlay images of brightfield and green channels of wounded larvae tail fins at 30 and 90 minutes post wounding (minpw). (c, d) Counts of fluorescent neutrophils at the site of injury were made 30 minpw (c and d) and 90 minpw (c). (Control: n=33, apyrase : n=36, thapsigargin : n=35, PPADS : n=37, Suramin : n=38, U73122 : n=36). All treatments were able to significantly decrease neutrophil recruitment to wounds. (e) Representative maximum intensity projections of H2O2 production of red (neutrophils) and green (H2O2) channels and (f) wound fluorescence intensity quantification in wounded larvae tail fins at 30minpw labeled with acetyl-pentafluorobenzene sulphonyl fluorescein. (Control: n=27,apyrase: n=25, thapsigargin: n=28, PPADS : n=22, Suramin : n=22, U73122 : n=26). All treatments were able to significantly decrease H2O2 production in wounded tail fins. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (***,P<0.001). Scale bars = 100μm.

Otic injection

Otic vesicle injections of 3dpf Tg(lyz:DsRED2)^{nz50} larvae for different treatments were performed as previously described (39), 1 nl of 1xPBS, 10 μM H_2O_2, 100μM ATP, 100μM ADP, 1μM A23187 (Sigma-Aldrich) or 30nM leukotriene B4 (LTB4) (Cayman Chemical). Images were taken 30 minutes post-injection (minpi).

Image acquisition and processing

For each experiment 3 dpf morphant and control larvae were imaged in three independent experiments. Images were taken from wounded or control larvae mounted as described above. Briefly, for total neutrophil and neutrophils at the site of injury/injection counts, images were taken using a Leica MZ16F fluorescence stereo microscope equipped with green and red fluorescent filters while the animals were maintained in their agar matrixes at 28.5 °C. Stacked images were captured using 20 μm (neutrophil distribution, NF-κB activation and H_2O_2 formation) increments and deconvolved using Huygens Essential Confocal software (v 4.1 0p6b) by Scientific Volume Imaging. Stacks were then processed using the free source software Image J (http://rsbweb.nih.gov/ij) to obtain a maximum intensity projection of the xy axis, and mean fluorescence at the wound site was again quantified for each experiment. Mean fluorescence was measured in wounded epithelial cells and the corresponding background values of unwounded larvae tail fins were subtracted for experiments of NF-κB activation (same larvae were used at 0 and 30minpw) and H_2O_2 formation (same number of larvae wounded and unwounded were imaged 30minpw with the same time of probe incubation).

Statistical analysis

All error bars indicates standard error of the mean (SEM). A one-way ANOVA with Bonferroni post-test was used.

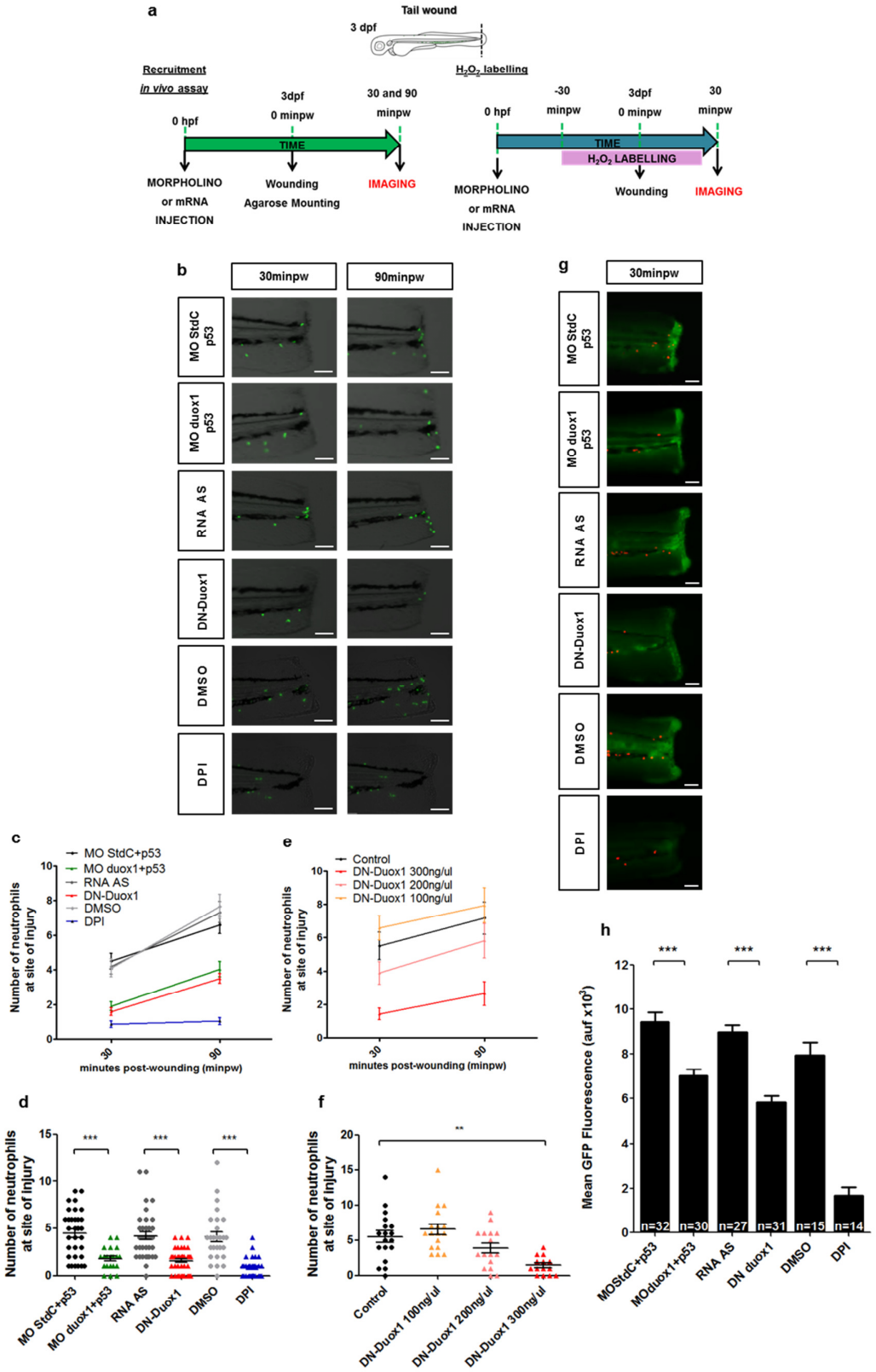

Fig. 2: Dominant negative Duox1 inhibits neutrophil recruitment and H_2O_2 release in wounding. (a) Schematic representation of the neutrophil recruitment and H2O2 labelling assays. (b-h) Zebrafish one-cell Tg(mpx:GFP)i114 or Tg(lyz:DsRED2)nz50 were microinjected with mRNA anti-sense (RNA AS) or sense (DN-Duox1), standard control morpholino (MO StdC) or Duox1 splice blocking morpholino (MO

duox1) both co-injected with p53 ATG morpholino (MO p53). Un-injected larvae were treated 3 days post-fertilization (dpf) with DPI or DMSO and used as controls. Tail fin larvae were amputated at 3dpf in all groups. For H2O2 labelling, larvae were incubated for 30 min with 50 mM acetylpentafluorobenzene sulphonyl fluorescein in 1% DMSO in embryo medium. (b) Representative overlay images of brightfield and green channels of wounded larvae tail fins 30 and 90 minutes post wounding (minpw). (c, d) Counts of fluorescent neutrophils at the site of injury were made at 30 minpw (c and d) and 90 minpw (c). (MO StdC+p53: n=32, MO duox1+p53: n=31, RNA AS: n=34, DN-Duox1: n=37, DPI: n=27, DMSO: n=29). DPI, DN-Duox1 and Duox1 morpholino significantly decreased neutrophil recruitment to wounds. (e, f) Counts of fluorescent neutrophils at the site of injury were made 30 minpw (e and f) and 90 minpw (e) in larvae microinjected with different doses of mRNA. (RNA AS 300 ng/ul: n=18, DN-Duox1 100ng/ul: n=17, DN-Duox1 200ng/ul: n=18, DN-Duox1 300ng/ul: n=17). mRNA of DN-Duox1 significantly decreased neutrophil recruitment to wounds in a dose-dependent manner. (g) Representative maximum intensity projections of H2O2 production of red (neutrophils) and green (H2O2) channels and (h) wound fluorescence intensity quantification of wounded larvae tail fins at 30minpw labeled with acetyl-pentafluorobenzene sulphonyl fluorescein. (MO StdC+p53: n=32, MO duox1 +p53: n=30, RNA AS: n=27, DN-Duox1: n=31, DPI: n=15, DMSO: n=14). DPI, DN-Duox1 and Duox1 morpholino were able to significantly decrease H2O2 production in wounded tail fins. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (**, P<0.01; ***,P<0.001). Scale bars = 100µm.

Results

ATP and calcium signaling modulate Duox1-derived H_2O_2 production and neutrophil recruitment in wounding.

We first focused on the signals that might be responsible for the activation/modulation of Duox1 *in vivo*. Extracellular ATP and Ca^{2+} appeared to be the strongest candidates to exert this function (11-14). Therefore, apyrase was used to degrade extracellular ATP (20) and thapsigargin, a SERCA Ca^{2+}ATPase inhibitor (8) (Figure 1a). It was seen that the inhibition of ATP or Ca^{2+} signaling significantly reduced early neutrophil recruitment to the wound 30 and 90 minutes post-wounding (minpw) (Fig. 1b-d) and also H_2O_2 production, assayed by the oxidation of the highly H_2O_2-specific probe acetyl-pentafluorobenzene sulphonyl fluorescein (5, 40), in tail fin wounded tissues 30minpw (Fig. 1e and f).

To further investigate the signaling pathway involved in the activation of Duox1 by ATP and Ca^{2+}, two general pharmacological inhibitors of purinergic receptors, suramin and PPADS(17, 20), and the PLC inhibitor U73122(8), were used (Figure 1a). The resulting data show that the inhibition of purinergic signaling and PLC significantly reduced early neutrophil recruitment to the wound 30 and 90minpw (Fig. 1b-d) and H_2O_2 production in tail fin wounded tissues 30minpw (Fig. 1e and f).

A Duox1 mutant lacking the flavin domain acts as a dominant negative in vivo.

Duox1 splice blocking morpholinos have been widely used to inhibit Duox1 in zebrafish but they needs to be tightly controlled and co-injected with a morpholino against p53 due to widespread cell death and developmental delay (5, 6, 41). For this reason, we developed a dominant negative form of Duox1 (DN-Duox1) lacking the carboxy-terminal flavin domain, which contains the binding sites for the cofactors NADPH and FAD (Suppl. Fig. 1a), as has been reported for NOX4 (42). The DN-Duox1 mRNA or the Duox1 morpholino was microinjected into one cell stage embryos and the total number of neutrophils and their development were assessed 3 days-post-fertilization (Suppl. Fig. 1b and c). As reported before (5, 6, 43), the Duox1 morphant larvae were smaller and showed

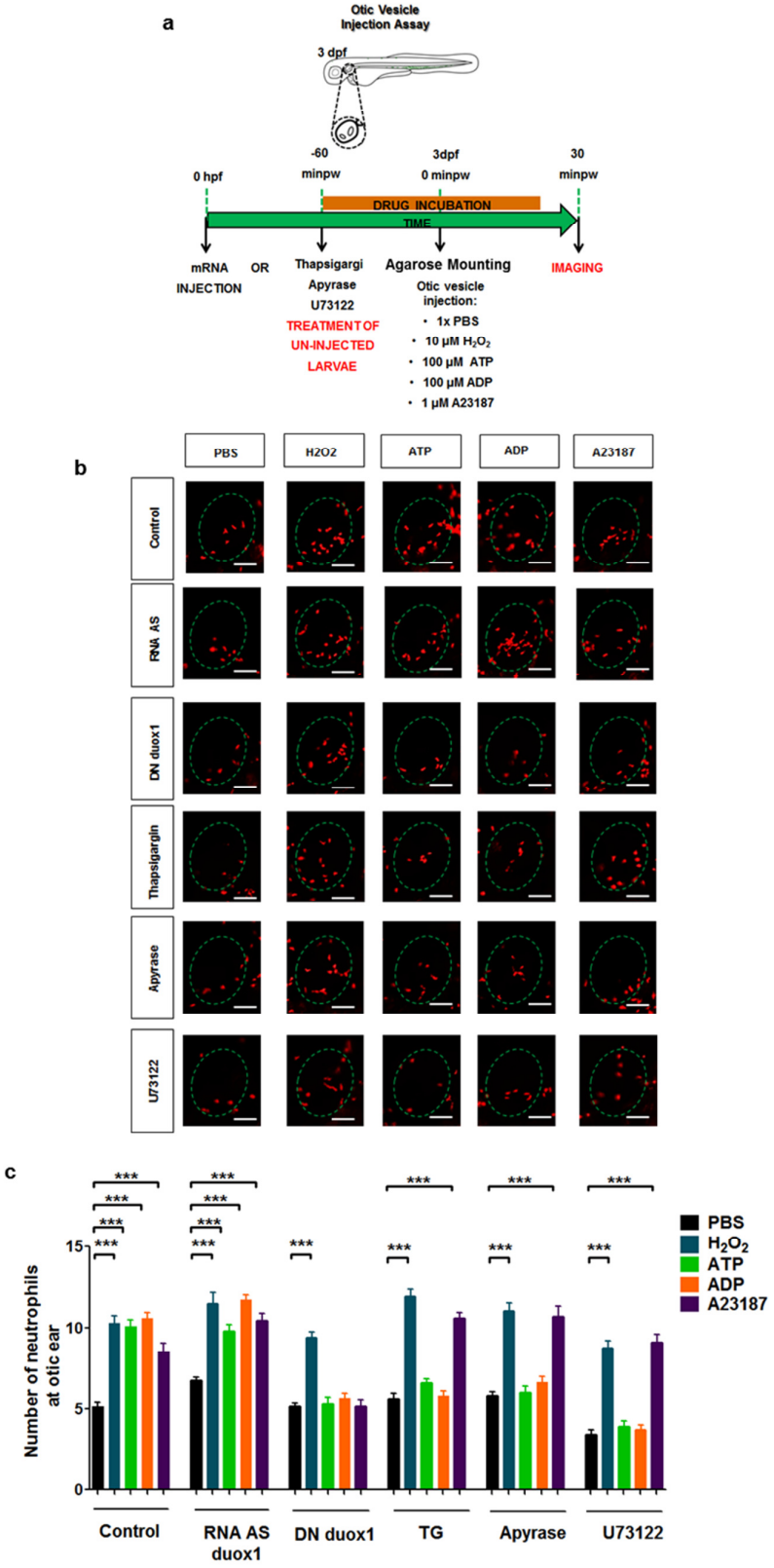

Fig. 3: ATP modulates Duox1 activity acting through P2Y/iCa2⁺ signaling pathway. (a) Schematic representation of the otic vesicle injection assay. (b - c) Zebrafish one-cell Tg(lyz:DsRED2)nz50 were microinjected with anti-sense (RNA AS) or sense (DN-Duox1) DN-Duox1 mRNA. At 3days post-fertilization (dpf) un-injected larvae were treated with thapsigargin, apyrase or U73122. Otic vesicle

microinjection of 1nL of PBS, 10 μM H_2O_2, 100μM ATP, 100μM ADP and 1μM A23187 was performed in each group. (b) Representative images of red channel of otic vesicles, 30 minutes post-injection (minpi). (c) Counts of fluorescent neutrophils at the site of injection were made 30 minpi. (Control/PBS: n=90, Control/H_2O_2: n=60, Control/ATP : n=60, Control/ADP: n=72, Control/ A23187 : n=70, RNA AS /PBS: n=71, RNA AS /H_2O_2: n=38, RNA AS /ATP : n=35, RNA AS /ADP: n=40, RNA AS / A23187 : n=40, DN-Duox1 /PBS: n=70, DN-Duox1 /H_2O_2: n=38, DN-Duox1 /ATP : n=40, DN-Duox1 /ADP: n=35, DN-Duox1 / A23187 : n=38, thapsigargin /PBS: n=40, thapsigargin /H_2O_2: n=40, thapsigargin /ATP : n=37, thapsigargin /ADP: n=37, thapsigargin / A23187 : n=40, apyrase /PBS: n=70, apyrase /H_2O_2: n=40, apyrase /ATP : n=37, apyrase /ADP: n=37, apyrase / A23187 : n=38, U73122 /PBS: n=37, U73122 /H_2O_2: n=41, U73122 /ATP: n=43, U73122 /ADP: n=45, U73122 / A23187: n=41). Note that all stimuli significantly increased the number of neutrophils recruited to the ear in Control and RNA AS samples but only H_2O_2 was able to significantly increase neutrophil recruitment to ear in DN-Duox1 larvae. In larvae pre-treated with thapsigargin, apyrase or U73122, H_2O_2 and the calcium ionophore A23187 were also the only stimuli able to significantly increase neutrophil recruitment. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (***,$P<0.001$). Scale bars = 100μm.

delayed development compared with larvae in control conditions or larvae microinjected with DN-Duox1 mRNA (Suppl. Fig. 1b). However, neither the Duox1 morpholino nor the DN affected total neutrophil numbers (Suppl. Fig. 1c). We noticed that Duox1 morphants presented an abnormal neutrophil distribution, so we quantified the number of neutrophils present above the notochord and in the caudal haematopoietic tissue (CHT), where most neutrophils are located at this developmental stage (44, 45), and calculated the percentage of neutrophils above the notochord. It was found that Duox1 morpholino significantly increased the percentage of neutrophils above notochord; that is, outside of the CHT, whereas the injection of DN-Duox1 mRNA did not (Suppl. Fig. 1d).

Next it was checked whether the DN-Duox1 was able to reduce neutrophil recruitment towards a wound and H_2O_2 production and release. For this, we performed in vivo neutrophil recruitment assays in 3dpf larvae (Figure 2a), using tail fin amputation as an acute inflammatory stimulus and diphenylene iodonium (DPI), a NADPH oxidase general inhibitor (5, 46), as a control. As expected, larvae microinjected with DN-Duox1 mRNA had a significantly lower number of neutrophils at the site of injury 30 and 90 minpw (Fig. 2b-d), and showed the same level of recruitment as the morphants. Additionally, the effect of the DN seemed to be specific since it was dose-dependent (Fig. 2e and f). Also, we observed that the DN-Duox1 RNA and the morpholino were both able to significantly reduce the amount of H_2O_2 produced by wounded tissue 30 minpw (Fig. 2g and h). Moreover, the effect of DN-Duox1 on H_2O_2-mediated neutrophil recruitment was specific, since it did not affect neutrophil recruitment to other stimuli, such as LTB4 (Suppl. Fig 2a).

To verify the expression of DN-Duox1, a GFP-tagged DN-Duox1 form was generated. The mRNA encoding DN-Duox1:GFP was microinjected into one cell stage Fig 2b-d), we were unable to detect a DN-Duox1:GFP signal by fluorescence microscopy (data not shown). However, we were able to detect GFP signal in HEK293 cells transfected with the same DN-Duox1:GFP construct driven by the CMV promoter (data not shown). Western blot analysis of HEK293 transfected with the DN-Duox1:GFP construct confirmed the expression of the chimeric protein with the expected molecular weight (Suppl. Fig 2e).

ATP modulates Duox1 activity through calcium signaling in vivo.

At this point we have shown that purinergic ATP signaling, probably through intracellular calcium rise, is able to modulate H_2O_2 production and neutrophil recruitment. However, whether these signals act in parallel or whether one is upstream of the other is still unknown. In an attempt resolve this issue, we microinjected the DN-Duox1 mRNA into one-cell stage embryos and pre-treated them 3dpf with apyrase, thapsigargin and U73122 for 60min, before being microinjected into the otic vesicle with different stimuli, including H_2O_2, ATP, ADP and the Ca^{2+} ionophore A23187 (Fig. 3a). In control conditions, all stimuli significantly increased neutrophil recruitment to the ear cavity of larvae, but, in the presence of the DN-Duox1 form, only H_2O_2 was able to significantly increase neutrophil recruitment (Fig. 3b,c), suggesting that purinergic and Ca^{2+} signaling were both upstream of Duox1 activation and H_2O_2 release. In addition, in the presence of apyrase, thapsigargin or U73122 (used to inhibit ATP, Ca^{2+} and PLC signaling respectively) only H_2O_2 and A23187 were able to significantly increase neutrophil recruitment to the ear cavity (Fig. 3b and c), indicating that purinergic signaling was upstream of Ca^{2+}, and strongly suggesting the involvement of a P2Y metabotropic receptor that would lead to increased intracellular Ca^{2+} (iCa^{2+}) due to depletion of the intracellular stores through PLC activation.

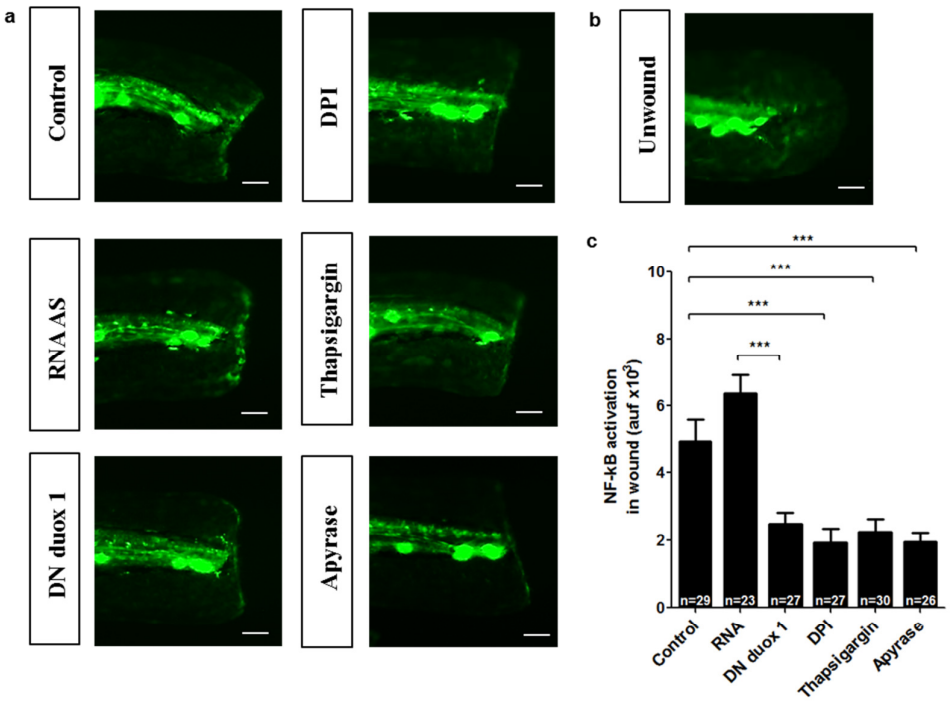

Fig. 4: H_2O_2 activates the NF-κB inflammatory signaling pathway. Zebrafish one-cell Tg(NF-κB:eGFP) were microinjected with anti-sense (RNA AS) or sense (DN-Duox1) DN-Duox1 mRNA. At 3 days post-fertilization (dpf) un-injected larvae were treated with thapsigargin or apyrase. Further tail fin larvae amputation was performed in all groups. **(a)** Representative maximum intensity projections of green channel of wounded larvae tail fins at 30 minutes post wounding (minpw). **(b)** Representative maximum intensity projections of green channel of unwounded larvae tail fin. **(c)** Wound fluorescence intensity quantification of wounded larvae tail fins at 30minpw. (Control: n=29 , RNA AS : n=23, DN-Duox1: n=27, DPI: n=27,thapsigargin: n=30, apyrase: n=26). NF-κB was activated in wounded larvae tail fin tissue. This signal suffers a significantly decrease in the presence of DN-Duox1, DPI, thapsigargin or apyrase. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (***,P<0.001). Scale bars = 100µm.

H_2O_2 activates the NF-κB inflammatory signaling pathway

NF-κB is a master regulator of inflammation, playing an essential role in the induction of several pro-inflammatory genes (47). Thus, we wanted to ascertain whether Duox1-derived H_2O_2 was able to activate this inflammatory signaling pathway *in vivo*. For this, we used the transgenic zebrafish line that expresses enhanced green fluorescent protein under transcriptional control of NF-κB in order to quantify NF-κB activation in 3dpf larvae after wounding in the presence of DN-Duox1, apyrase or thapsigargin. DPI was again used as a positive control for the experiment. As expected, the NF-κB signaling pathway was activated in wounded tail fin tissues (Fig. 4a, b). Additionally, when Duox1 was silenced by the use of the DN form or by inhibiting ATP or Ca^{2+} signaling with drugs, the NF-κB activation significantly decreased in wounded tissue (Fig. 4a,c). These results indicate that the activation of Duox1 by ATP and Ca^{2+} signaling leads to the release of H_2O_2, which, in turn, promotes the local activation of NF-κB.

Discussion

During recent years, the hydrogen peroxide tissue gradient has emerged as a very important process involved in several mechanisms such as leukocyte recruitment (5, 6, 8), tumor progression (48) and neuron regeneration (43). Tissue-scale H_2O_2 gradient and its roles have been extensively studied, although the upstream signals involved in early Duox1 activation and subsequent H_2O_2 production are still a controversial issue. In Drosophila, Ca^{2+} appears to play an important role in the activation of Duox1 *in vivo* (7), although in vertebrates it was reported that Duox1 activation and Ca^{2+} waves are two independent early signals that appear after tissue injury(8). ATP and Ca^{2+} are two of the main molecules involved in early signaling after tissue damage (11-16). Therefore, both ATP and Ca^{2+} might be acting as upstream signals of Duox1. In order to study this, several pharmacological inhibitors were used to affect intracellular Ca^{2+} (thapsigargin and U73122) and purinergic signaling (PPADS, Suramin and apyrase) at different levels in wounded tail fins. The findings show that both extracellular ATP and intracellular Ca^{2+} have a key role in Duox1 activation/H_2O_2 production *in vivo* and also in early neutrophil recruitment. PLC and purinergic receptor inhibition induced a significant decrease in both H_2O_2 release and neutrophil recruitment to the wound. Therefore, it is tempting to speculate that extracellular ATP acting through P2Y purinergic receptors might be triggering an increase in intracellular Ca^{2+}, which would then bind to the Duox1 EF-hands, inducing its activation. This is consistent with previous *in vitro* studies that demonstrated that Duox1 activation depended on calcium binding to functional EF-hand motifs (49).

In zebrafish, by knocking-down Duox1 using a splice blocking morpholino, it was shown that Duox1 is the main NADPH oxidase responsible for the formation of the H_2O_2 tissue gradient *in vivo* (5, 6, 41, 48). The use of this splice blocking morpholino involves a very precise microinjection of the eggs, since excessively high morpholino doses generally compromise larvae viability, inducing several problems in larvae development that require co-injection of the p53-blocking

morpholino to restore larvae viability (5). Moreover, in studies of signaling pathways the use of the p53 morpholino could interfere with the results, restricting the usefulness of this method to improve larvae viability. Due to these problems, it was felt necessary to develop another technique that would affect Duox1 activity in order to study the mechanisms involved in the generation of the tissue-scale H_2O_2 gradient. We hypothesized that a truncated form of Duox1 without the binding site for NADPH and FAD, the so-called flavin domain, would be able to compete with the native form of Duox1 and, therefore, act as a dominant negative form and decrease the H_2O_2 production, as has been found for NOX4 (42). Surprisingly, besides the negative aspects already reported for the use of Duox1 morpholinos (5, 6, 43), we found that they also induce an inflammatory process in 3dpf larvae, which is responsible for the mobilization of neutrophils from the CHT to other tissues. We speculated that this phenotype could be due to the effect of this morpholino on larvae development and the induction of cell death. Importantly, the developed DN-Duox1 does not affect development or neutrophil distribution. Using tail fin amputation, we were able to demonstrate that the DN-Duox1 is functionally able to decrease *in vivo* H_2O_2 production and subsequent neutrophil recruitment. Importantly, this effect was specific, since neutrophil recruitment to LTB4 was unaffected by DN-Duox1. Although further studies are required to understand the mechanism involved in the inhibition of endogenous Duox1 by the DN form lacking the flavin domain, it is tempting to speculate that the DN is competing with wild type form for the Duox1 maturation factor, called Duox activator 1 (Duoxa1), which is important for Duox1 trafficking to the plasma membrane and its full function (50). The low expression of GFP-tagged DN-Duox1 in larvae is consistent with this hypothesis, since Duox1 would be only properly expressed in epithelial tissues. Whatever case, our results demonstrate that the DN-Duox1 is a useful tool for future studies in the field, since it use avoids the problems of the morpholinos that have been used until now (5, 6, 48).

Our epistasis study by ear injection of different stimuli combined with pharmacological inhibition of purinergic or Ca^{2+} signaling allowed us to establish whether these signals act in parallel or whether one is upstream of the other. We observed that all the stimuli, including H_2O_2, ATP, ADP and the calcium ionophore A23187, were able to induce neutrophil recruitment in control conditions. Furthermore, in the presence of the DN-Duox1, only the H_2O_2 was able to induce neutrophil recruitment to the ear cavity, demonstrating that both ATP and iCa^{2+} are acting as upstream signals of Duox1. We also found that ATP and calcium are not two independent signals and that ATP is actually an upstream signal of iCa^{2+}, since H_2O_2 and the Ca^{2+} ionophore were the only two stimuli able to significantly increase neutrophil recruitment in larvae pre-treated with thapsigargin and the PLC inhibitor U73122. Taken together, these data strongly support the view that Duox1 activation involves metabotropic P2Y receptor signaling, rather than ionotropic P2X purinergic receptor activation, since P2X signaling is not triggered by ADP and does not involve PLC activation (51, 52).

NF-κB is a master regulator of inflammation and it controls the expression of several pro-inflammatory genes (47). Although there are still many inconsistencies concerning the influence of oxidative stress on NF-κB activity (53), several *in vitro* studies using H_2O_2 and cultured cells have shown that H_2O_2 can act as regulator of IκB kinases (IKKs) (54-57). Our results demonstrate that

the inhibition of Duox1-derived H_2O_2 production, by the direct genetic inhibition of Duox1 as a result of the overexpression of the DN-Duox1 or indirect pharmacological inhibition of upstream purinergic and Ca^{2+} signaling, decreased the NF-κB activation in wounded tissue. These data indicate, therefore, that NF-κB is a redox-sensitive transcription factor activated by H_2O_2 *in vivo* and indicate that the tissue-scale H_2O_2 gradient is a general player in the inflammatory process (Figure 5).

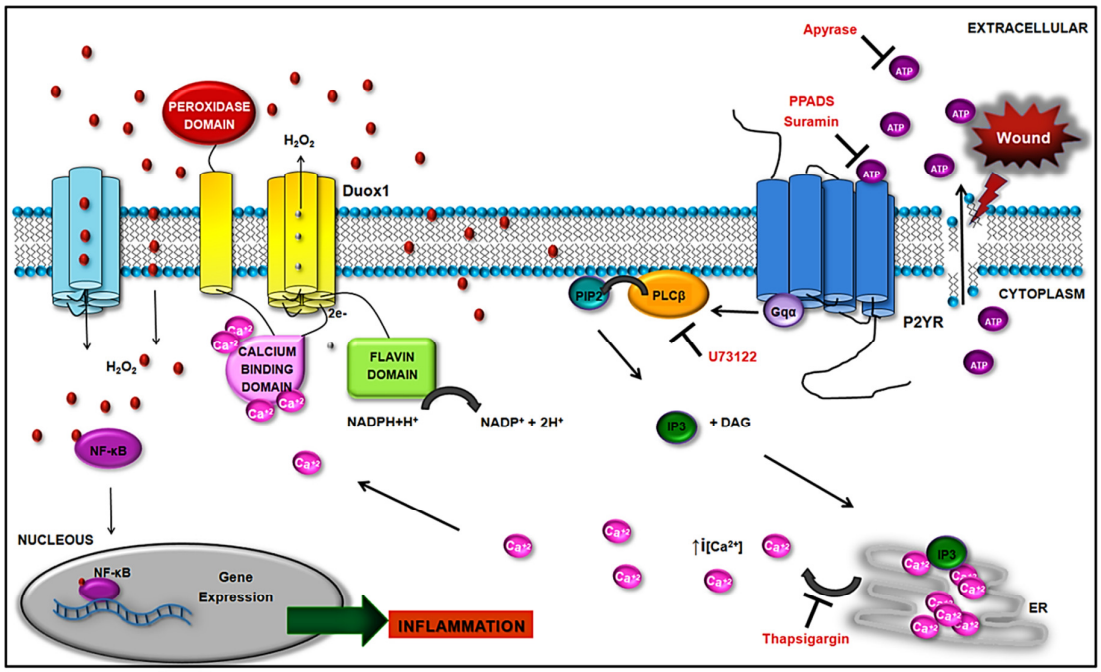

Fig. 5: Proposed model illustrating how early signals (ATP, calcium and H_2O_2) modulate inflammation via NF-κB activation. ATP released from damaged cells activates purinergic P2YR, which promotes the activation of PLC, the generation of IP3 and the release of Ca^{2+} from the endoplasmic reticulum. Ca^{2+} is then able to bind the EF-hands domain of Duox1, inducing its activation and the production of H_2O_2. Finally, H_2O_2 is able to activate the master inflammation transcription factor NF-κB, which induces the expression of target pro-inflammatory genes. The sites of action of the different pharmacological drugs used in this study are also indicated.

In summary, we have developed a new and promising DN form of zebrafish Duox1 which is able to inhibit endogenous Duox1 activity, affecting tissue-scale H_2O_2 gradient formation and early neutrophil recruitment to wounds. Using this tool, we have demonstrated that ATP released at wound triggers Duox1 activation and local H_2O_2 release via a P2YR/PLC/Ca^{2+} signaling pathway. Finally, we demonstrate for the first time that the early danger signals ATP, iCa^{2+} and H_2O_2 are necessary to activate *in vivo* the NF-κB inflammatory signaling pathway, uncovering an unprecedented role for these early signals in the modulation of wound inflammatory responses. Our study identifies new potential therapeutic targets for inflammatory diseases and paves the way for future studies on the mechanisms orchestrating H_2O_2 oxidative regulation of complex biological processes, such as inflammation, regeneration and cancer.

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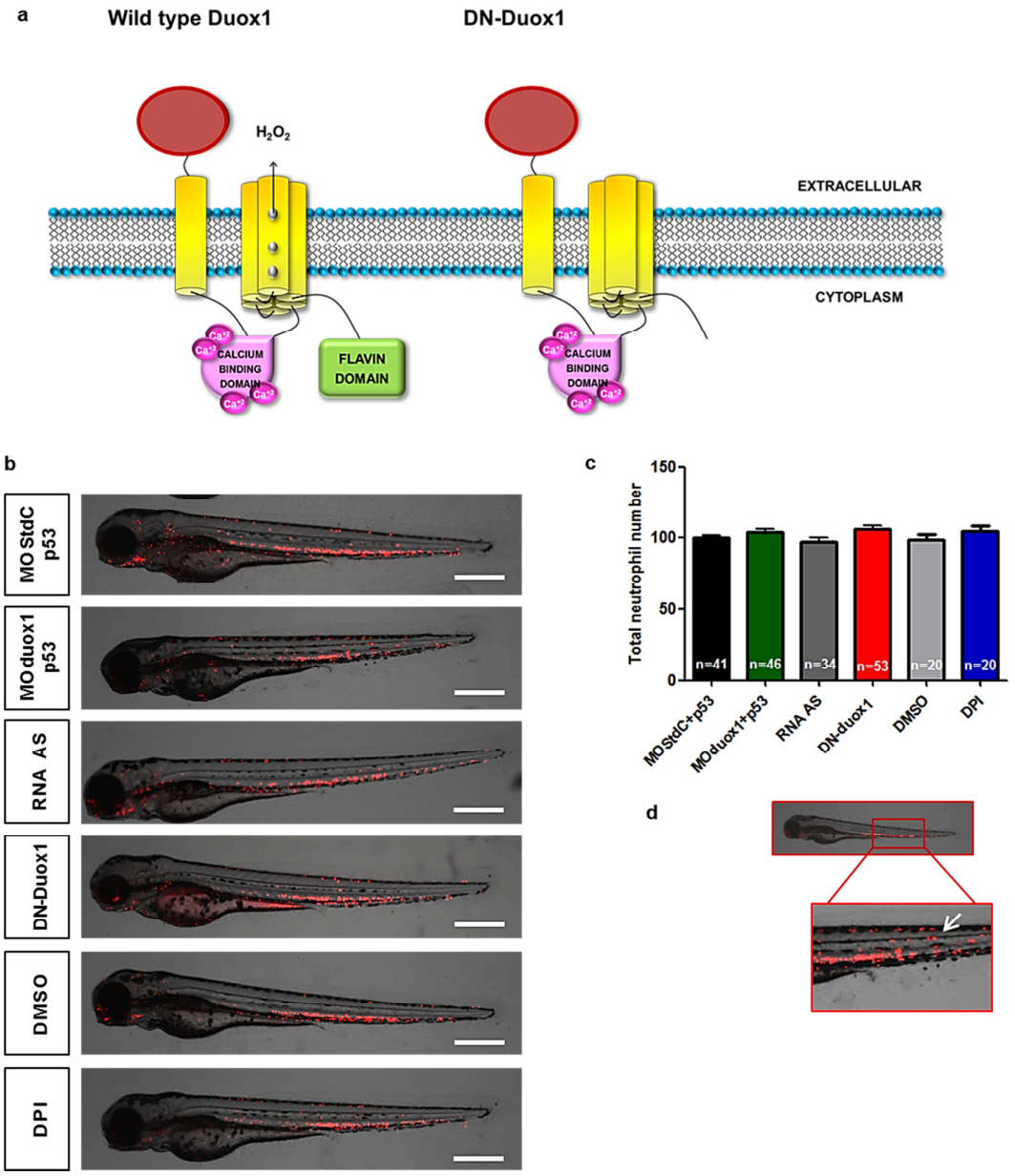
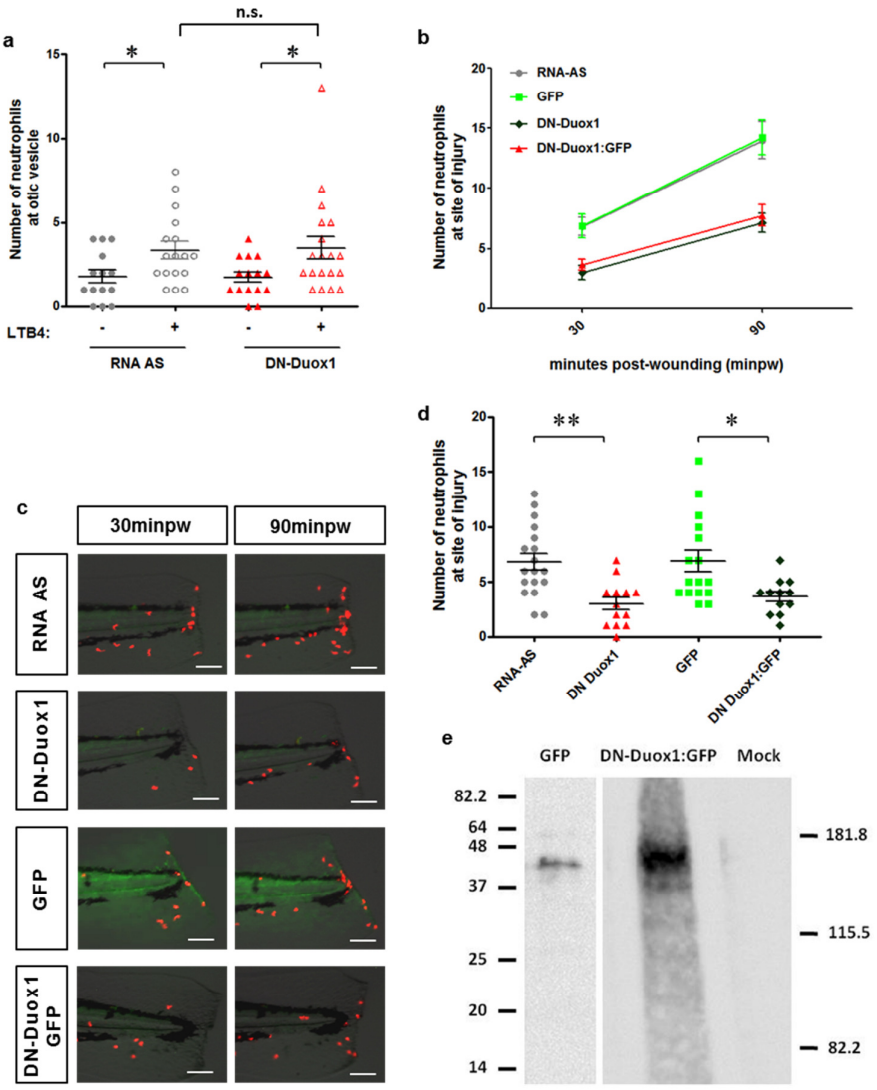
Supplementary Figures



Supplementary Figure 1: Duox1 morphants show an altered neutrophil tissue distribution pattern. (a) Scheme of Duox1 wild-type and dominant negative forms. The Duox1 truncated form lacks the flavin domain that contains the FAD (residues 1,233-1,331) and NADPH (residues 1,339-1,416) binding sites. Zebrafish one-cell Tg(lyz:DsRED2)nz50 were microinjected with mRNA anti-sense (RNA AS) or sense (DN-Duox1), standard control morpholino (MO StdC) or Duox1 splice blocking morpholino (MO duox1) both co-injected with p53 ATG morpholino (MO p53). Un-injected larvae were treated 3 days post-fertilization (dpf) with DPI or DMSO and used as controls. (b) Representative whole larvae overlay images of brightfield and red channels from 3 dpf larvae. (c) Total neutrophil counts in whole-larvae for each condition (MO StdC+p53: n=41, MO duox1+p53: n=46, RNA AS: n=34, DN-Duox1: n=53, DPI: n=20, DMSO: 20). No significant differences were found in total neutrophil numbers between groups. (d) Number of neutrophils above notochord (outside CHT) and total number of neutrophils (present in CHT and outside CHT) were quantified for all the groups in the larvae area indicated in the figure. Data are presented as the percentage of neutrophils above notochord (MO StdC+p53: n=20, MO duox1 +p53: n=29, RNA AS: n=17, DN-Duox1: n=29,

DPI: n=21, DMSO: n=20). Duox1 morphants showed significant increased percentage of neutrophils above the notochord. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (*, P<0.05). Scale bars = 200μm.



Supplementary Figure 2: DN-Duox1:GFP is expressed in 3dpf larvae and inhibits H2O2- but not LTB4-mediated neutrophil recruitment. (a-d) Zebrafish one-cell Tg(lyz:DsRED2)nz50 or Tg(mpx:GFP)i114 were microinjected with mRNA anti-sense (RNA AS), sense (DN-Duox1), DN-Duox1:GFP or GFP. (a) At 3days post-fertilization larvae were microinjected into otic vesicle with 1nL of PBS, or 30nM LTB4. LTB4 significantly increased neutrophil recruitment to otic vesicles both in DN-Duox1 and RNA-AS larvae. (b) At 3dpf larvae tail fins were amputated. Representative overlay images of brightfield, green and red channels of wounded tail fins at 30 and 90 minutes post wounding (minpw). (c, d) Counts of fluorescent neutrophils at the site of injury were made at 30 minpw (c and d) and 90 minpw (c). (RNA AS: n=18, DN-Duox1: n=13, DN-Duox1:GFP: n=12, GFP: n=16). Both DN-Duox1 forms, tagged and un-tagged, significantly decrease neutrophil recruitment to wounds. (e) Western Blot of lysates from HEK293 cells lysates expressing GFP or DN-Duox1:GFP. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (*, P<0.05; **,P<0.01). Scale bars = 100μm. n.s., not-significant.

Chapter 5

Duox1-derived H_2O_2 modulates Cxcl8 expression after wounding via JNK/c-Jun/AP1 signaling and chromatin modifications

Duox1-derived H_2O_2 modulates Cxcl8 expression after wounding via JNK/c-Jun/AP1 signaling and chromatin modifications

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Abstract

Inflammation is a major host defense process that involves complex signaling pathways in order to restore homeostasis. CXCL8 is one of the most potent chemoattractant molecules for neutrophils. Zebrafish has two distinct CXCL8 homologues, Cxcl8-l1 and Cxcl8-l2, which are both up-regulated under inflammatory conditions and crucial for normal neutrophil recruitment to wounds and for inflammation resolution. Another important neutrophil chemoattractant molecule is hydrogen peroxide (H_2O_2), produced by dual oxidase 1 (DUOX 1) upon wounding. A tissue gradient of H_2O_2 is responsible for the early leukocyte recruitment and later tissue regeneration via SFKs oxidation, Lyn and Fyn b, respectively. Moreover, our recent work has demonstrated that Duox1-derived H_2O_2 production contributes to the activation of NF-κB inflammatory signaling pathway, indicating that H_2O_2 might be able to modulate the overall inflammatory outcome.

Here, we show that Duox1-derived H_2O_2 modulates neutrophil recruitment to injuries not only at an early phase, as previously published by others, but also at a later phase by contributing for the induction of Cxcl8-l2 expression. The mechanism underlying this crosstalk seems to involve p38 and Jnk signaling pathways as well as the phosphorylation of the redox sensitive transcription factor AP1, but not Erk or NF-kB signaling. In addition, we were also able to show that H_2O_2 modulates the levels of histone 3 epigenetic markers as well as p-c-Jun binding at the *cxcl8-l2* promoter. Overall, we conclude that DUOX1-mediated H_2O_2 release is not only a key factor for the early neutrophil recruitment phase but also modulates neutrophil recruitment at a later phase by crosstalking with Cxcl8, therefore regulating the onset of inflammatory response in wound healing.

Introduction

Inflammation is a major host defense process that occurs after infection, injury or tissue stress and malfunction, and it involves the interplay of several molecular and cellular mediators so as to restore tissue homeostasis (1-3). Regardless of their controversial role in wound healing, neutrophils are the first leukocytes to be recruited to wounds (4, 5). Release of hydrogen peroxide (H_2O_2) from wounded tissues functions as an early signal for this neutrophil recruitment (6, 7), which is later on mediated by the action of chemokines, with CXCL8 being one of the most important ones in this respect (8-10).

The formation of a tissue gradient of H_2O_2 upon injury has been shown in zebrafish (6, 7, 11) and also in mice models (12). This gradient is not only an early signal for neutrophil recruitment but can also influence later phases of wound healing, such as tissue regeneration (11, 12). Additionally, it has been shown in vitro that H_2O_2 is able to modulate the function of redox sensitive transcription factors (13, 14) and to influence the gene expression of pro-inflammatory molecules, like IL1β or CXCL8 (15-18). Recently, we have further reported that Duox1-derived H_2O_2 is involved in NF-κB activation in wound healing (19). Importantly, we have also found that CXCL8 zebrafish homologues, Cxcl8-l1 and Cxcl8-l2, are crucial for neutrophil recruitment upon wounding and that are able to dictate neutrophil behavior and to influence later tissue regeneration in zebrafish larvae (10). As both H_2O_2 and Cxcl8 gradients mediate neutrophil recruitment, we hypothesized that the action of these two recruitment players could be concerted, *in vivo*, with H_2O_2 as an upstream factor for Cxcl8 expression and function. Using zebrafish larval tail fin injury we report that early wound-induced H_2O_2 is able to modulate Cxcl8-l2 expression and that this crosstalk involves p38 and Jnk signaling pathways as well as the phosphorylation of the transcription factor, AP1. Moreover, we also show that H_2O_2 modulates H3K4me3, H3K9ac and H3K9me3 levels as well as p-c-Jun binding at the *cxcl8-l2* gene promoter. Altogether these results explain, at least in part, how early H_2O_2 signal can modulate different neutrophil recruitment phases, directly via Lyn oxidation or indirectly by modulating *cxcl8-l2* gene expression. Overall, this work contributes for a better understanding of how an early signal as H_2O_2 can regulate the onset of the inflammatory response in wound healing and further supports H_2O_2 as a powerful drug target for anti-inflammatory therapies.

Material and Methods

Zebrafish Husbandry

All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU), the Spanish RD 53/2013, and the Bioethical Committee of the University of Murcia (approval number #537/2011). Fertilized zebrafish eggs were obtained from natural spawning of wild-type (obtained from the Zebrafish International Resource Center), the Tg(*mpx:gfp*)^{i114} (20) and the Tg(*lyz:DsRED2*)^{nz50} (21) lines held at our

facilities following standard husbandry practices. Animals were maintained in a 12 hr light/dark cycle at 28.5°C.

Morpholino and mRNA injections

Antisense (AS) and sense RNAs for the dominant negative form of Duox1 (DN-Duox1) (19) (300 pg/egg) and morpholinos (7, 10) were mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into one-cell-stage embryos using a microinjector (Narishige) (0.5-1 nl per embryo). The same amounts of RNA and MOs were used in all experimental groups. Splice-blocking morpholinos used in this study were the following: for *cxcl8-l2* 5'-TTAGTATCTGCTTACCCTCATTGGC-3' (10), for *lyn* 5'-TCAGACAGCAAATAGTAATCACCTT-3' (7).

Table 1: Quantitative PCR primer

Gene	Acession number	Name	Nucleotide sequence	ATG position	Use
bactin2	**ENSDARG 00000037870**	**F**	**5'-ACTATGAACTGAACCGACTG-3'**	**+939/**	**ChIP**
		R	**5'-CTGCGATCAATTACACAACC-3'**	**+1072**	**qPCR**
cxcl8-l2-PR	**HF674400**	**F1**	**5´-ATCTATCAGCCAGACGCCAG-3'**	**-381/**	
		R1	**5'- GTGCAACTAAATTGAGTGAGTGT -3'**	**-267**	**ChIP**
		F4	**5'-AACTCTGTCACGTTGTGTGGG-3'**	**-119/**	**qPCR**
		R4	**5'-CATCTTGAAGAGTTCTGGTGCAG-3'**	**+3**	
cxcl8-l2	**HF674400**	**F1**	**5´-GCTGGATCACACTGCAGAAA-3'**	**-**	**qPCR**
		R1	**5'- TGCTGCAAACTTTTCCTTGA -3'**		
rps11	**NM_213377**	**F**	**5'-ACAGAAATGCCCCTTCACTG-3'**	**-**	**qPCR**
		R	**5'-GCCTCTTCTCAAAACGGTTG-3'**		

Tail fin wounding

Tail fin amputation was performed as previously described (10) in wild type, Tg(*lyz:DsRED2*)^{nz50} or Tg(*mpx:gfp*)^{i114} 3dpf larvae. When required, pharmacological treatments were performed using the bath immersion method before and after wounding.

Pharmacological treatments

All drug treatments were performed using the bath immersion method. Briefly, 3 dpf wild type or Tg(*mpx:gfp*)^{i114} larvae were incubated for 15 minutes at 28°C in the presence or absence of each of the following drugs from Sigma-Aldrich: 100µM PD98059

(11), 10µM SB220025 (22) , 50µM SP600125 (23), 50nM Nai (24), 1µM wortmannin (25) and 20µM PP2 (11) diluted in embryo medium. After tail fin amputation, larvae were maintained in the corresponding treatments until experimental procedure.

H_2O_2 imaging

H_2O_2 imaging using 50 mM acetyl-pentafluorobenzene sulphonyl fluorescein, a live cell fluorescein dye was performed as previously described (19). Imaging was made 30 min post wounding (minpw).

Zebrafish tail tissue sample collection and gene expression analysis

At 3dpf tail tissue sample collection was performed as previously described (10). For each mRNA, gene expression was first normalized against the expression of the ribosomal protein S11 (*rps11)* in each sample and then, in relation to that determined for the control of the experiment. The primers used are shown in Table 1. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test in GraphPad Prism5 software.

Image acquisition and processing

For each assay 3 dpf larvae were imaged in three independent experiments. Images were taken from wounded larvae mounted as described above using a Leica MZ16F fluorescence stereo microscope equipped with green and red fluorescent filters while the animals were maintained in their agar matrixes at 28.5 °C. Mean fluorescence was measured in wounded epithelial cells and the corresponding background values of unwounded larvae epithelial cells were subtracted.

Whole-mount immunofluorescence

At 3 dpf larvae were fixed overnight at 4°C in 4% PFA after tail fin injury at 0, 1 or 5 hpw. Antibody labelling was performed as previously described (26). The following primary antibodies were used: a purified mouse mAb generated against recombinant zebrafish Cxcl8-l2 at 1:100 (GeneScript); rabbit anti-human phospho-p38 (#MA5-15177, Thermo Scientific) at 1:200 (27); mouse anti-human phospho-JNK (#MA5-15228, Thermo Scientific) 1:100 (28); mouse anti-human phospho-ERK1+ERK2 (#ab50011, Abcam) at 1:300 (11); anti-p-c-Jun (#3270, Cell Signaling) at 1:100 (29, 30) and Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary antibodies (Life Technologies) at 1:500.

Images were acquired using a Leica MZ16F fluorescence stereo microscope for DN-Duox1 or using a Zeiss Axiovert200 fully motorized, inverted, *wide field fluorescence microscope* using an NA 0.8/20x objective, in z-stack mode for *cxcl8-l2* morphants experiment. All data were processed using Image J (http://rsb.info.nih.gov/ij/).

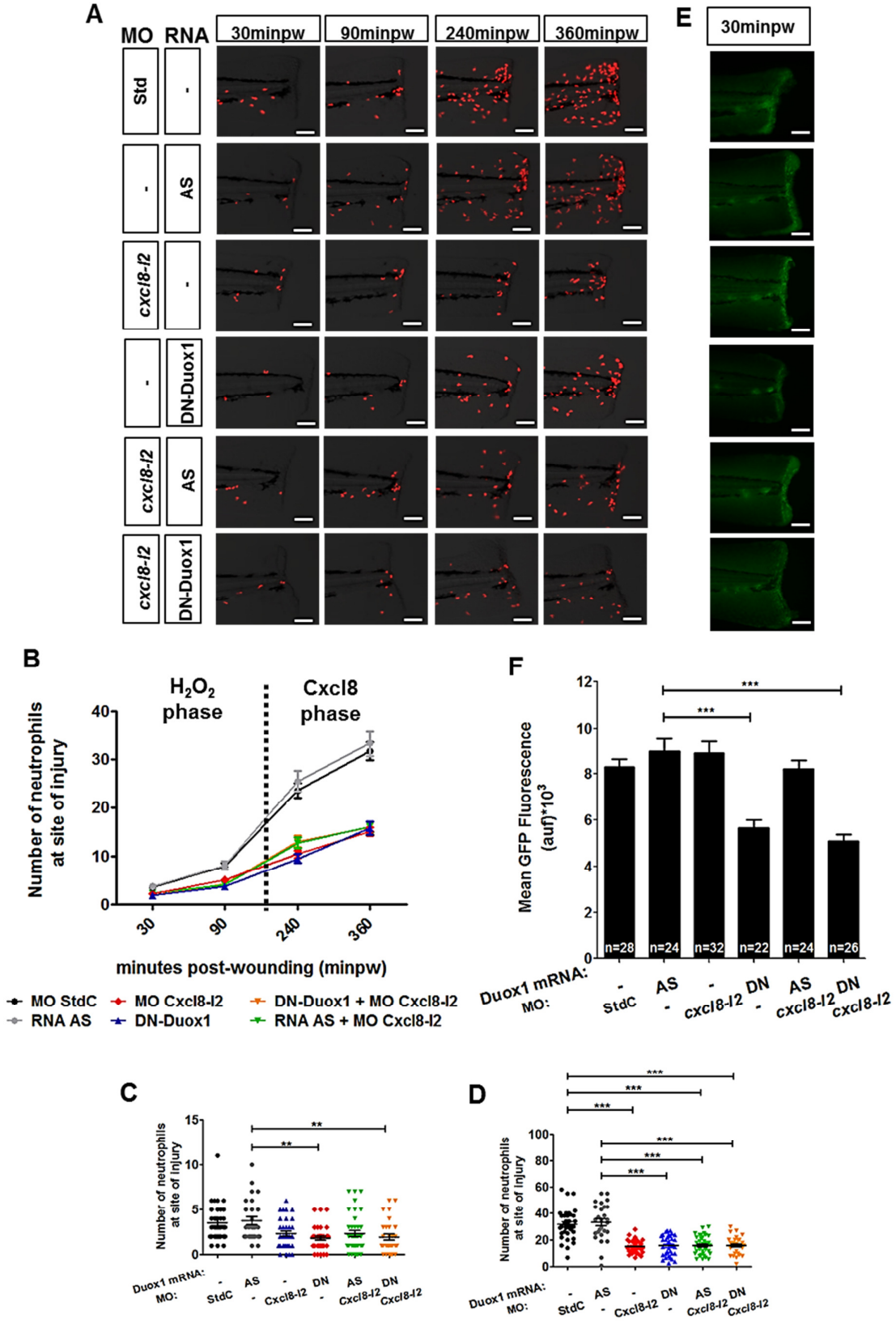

Figure 1: H2O2 modulates different neutrophil recruitment phases. Zebrafish one-cell wild-type or Tg(lyz:DsRED2)nz50 were microinjected with mRNA Duox1 anti-sense (AS), sense (DN-Duox1),

standard control (MO StdC) and/or cxcl8-l2 morpholino (MO cxcl8-l2). Tail fin larvae were amputated at 3 dpf in all groups. For H2O2 labelling, larvae were incubated for 30 min with 50 mM acetylpentafluorobenzene sulphonyl fluorescein in 1% DMSO in embryo medium. **(A)** Representative overlay images of brightfield and red channels of wounded larvae tail fins 30, 90, 240 and 360 minutes post wounding (minpw). **(B)** Counts of fluorescent neutrophils at the site of injury from 30 to 360minpw. **(C)** Counts of fluorescent neutrophils at the site of injury at 30 and **(D)** 360minpw. **(E)** Representative images of H2O2 production. **(F)** Wound fluorescence intensity quantification of wounded larvae tail fins at 30minpw labeled with acetyl-pentafluorobenzene sulphonyl fluorescein. The DN-Duox1 was able to significantly decrease H2O2 production in wounded tail fins. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (**, P<0.01; ***,P<0.001). Scale bars = 100μm.

Bioinformatic promoter analysis

The software tool Genomatix MatInspector Matrix library version 8.0 (www.genomatix.de) was employed for promoter analysis (31). 1,6 kb DNA sequence upstream of the predicted start codon was extracted for each gene from the zebrafish or human genome assembly (ENSEMBL). DNA sequences were analyzed using the matrixes for general core promoter elements for vertebrates and enhancer elements were used specifically for nuclear factor kappa B (NF-κB), signal transducer and activators for transcription (STAT), interferon regulatory factors (IRFs) and activating protein-1 (AP-1). Matrix matches with a similarity score > 0.80 were accepted and relevant elements were selected.

Chromatin Immunoprecipitation (ChIP)

Larvae with 72hpf previously microinjected with RNA AS or DN-Duox1 (400 per each ChIP biological replicate) were wounded and at 1 hour post-wounding processed for ChIP using the MAGnify Chromatin Immunoprecipitation System (Life Technologies) as described before (32). ChIP and input DNA were amplified by qPCR using specific primers for the 5′ upstream sequences of the different genes (Table 1). qChIP values were normalized to 10% Input and ChIP controls performed with mouse or rabbit anti-IgG. ChIPs were performed with at least two independent chromatin preparations. The antibodies used in this assay were: anti-H3K9ac (#ab1791;Abcam), anti-H3K4me3 (#ab1012; Abcam), anti-H3K9me3 (#06–942; Millipore), anti-p-c-Jun (#3270, Cell Signaling) and control mouse and rabbit anti-IgG provided by the MAGnify kit. All these antibodies have been previously validated in zebrafish (28, 30, 32).

Statistical analysis

All error bars indicate standard error of the mean (SEM). A Mann Whitney t-test, one or two-way ANOVA with Bonferroni post-test were used.

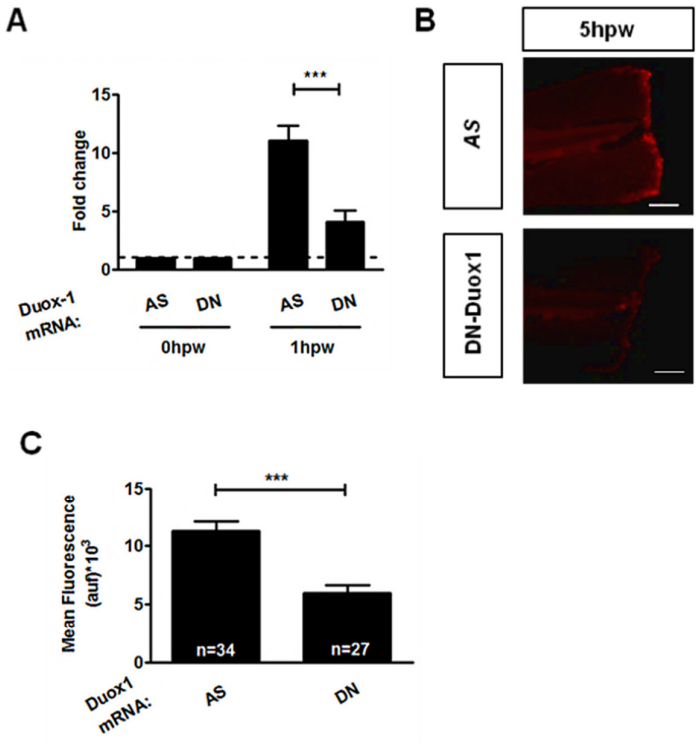

Figure 2: Duox1-derived H_2O_2 modulates Cxcl8-l2 expression both at the transcript and protein levels after wounding. Zebrafish one-cell Tg(*mpx:GFP*)^{i114} were microinjected with mRNA Duox1 anti-sense (AS) or sense (DN-Duox1). Tail fin larvae were amputated at 3dpf in all groups. **(A)** mRNA levels of *cxcl8-l2* were determined by qPCR in tail fin tissue at 0 and 1 hpw (40 tail fins per time-point). Gene expression was normalized against *rps11* and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the mean ± SEM of triplicated samples. P values were calculated using two-way ANOVA with Bonferroni multiple comparison test, ***P<0.001. **(B)** Representative overlay images of red (ZfCxcl8-l2) and green (neutrophils) channels of whole-mount immunofluorescence of zfCxcl8-l2 at 5hpw. **(C)** Wound fluorescence intensity quantification in wounded larvae tail fins at 5hpw. DN-duox1 form is able to significantly decrease Cxcl8-l2 protein levels in wounded tail fins. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using Mann Witney t-test (***,P<0.001). Scale bars = 100μm.

Results

H_2O_2 modulates different neutrophil recruitment phases

Tissue gradients of H_2O_2 were previously shown to be required for the early neutrophil recruitment upon wounding (6, 7, 11, 19). Previously, we have reported that by impairing H_2O_2 production, the dominant negative form of Duox1 (DN-Duox1) significantly reduces the recruitment of neutrophils towards wounds between 30-90 minutes post wounding (minpw) after tail fin transection of 3 dpf larvae in comparison to control antisense DN-Duox1 RNA (AS) injected larvae (19) (Figure 1A-C, E-F). Here by extending this analysis up to 6 hpw, we were found that expression of DN-Duox1 not only affects this early recruitment phase, but also significantly reduced neutrophil recruitment at later timepoints (Figure 1A,B and D).

Duox1-derived H_2O_2 is needed for Cxcl8-l2 expression after wounding

Besides H_2O_2, we have previously reported that the chemokine *cxcl8-l2* expression is induced after wounding and is actually crucial for normal neutrophil recruitment and behavior under this condition. Importantly, we further noticed that the DN-duox1 recruitment curve

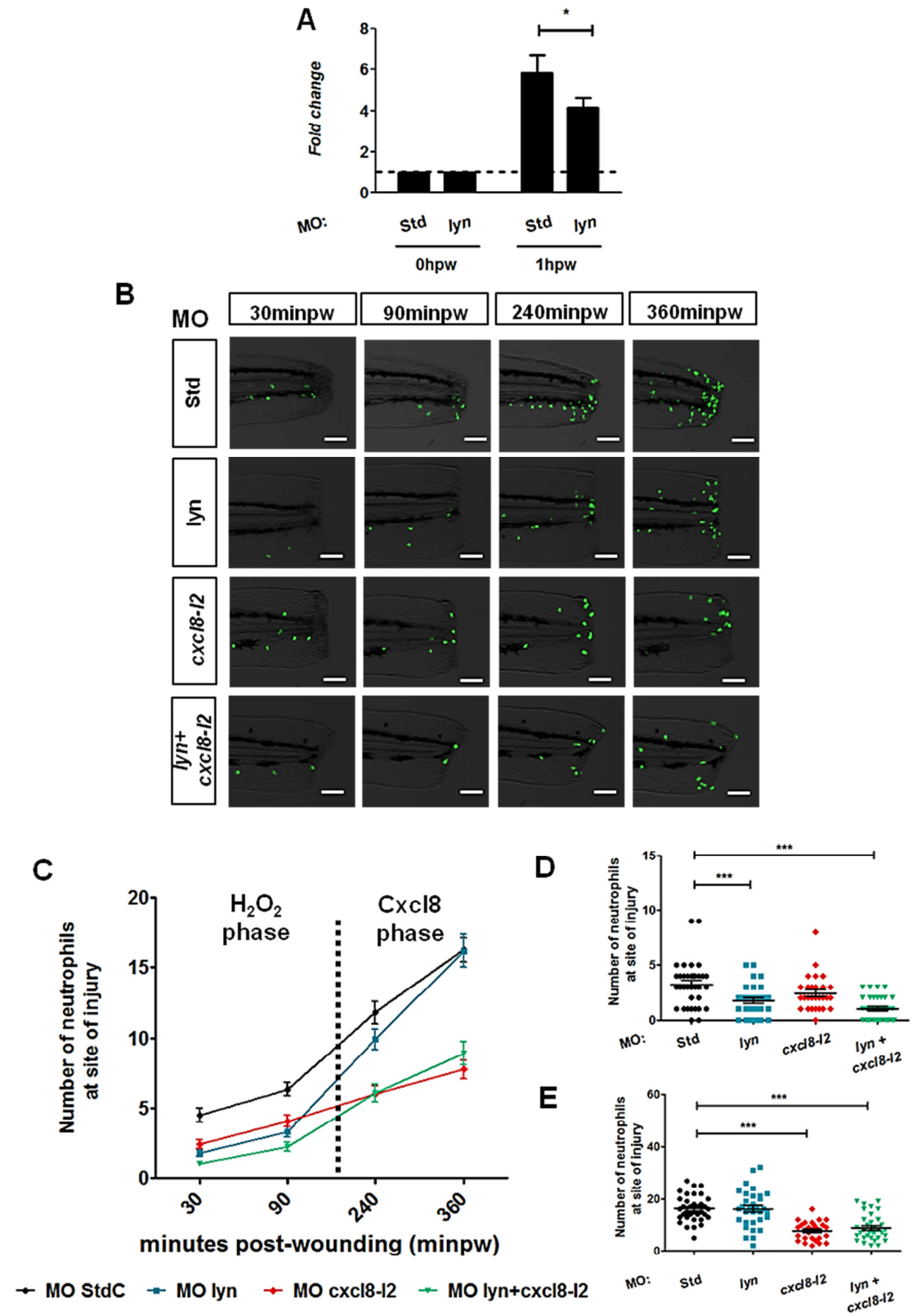

Figure 3: Lyn is important for early neutrophil recruitment and for *cxcl8-l2* gene expression. Zebrafish one-cell wild-type or Tg(*mpx:GFP*)^{i114} were microinjected with control (MO StdC), lyn (MO *lyn*) and/or *cxcl8-l2* morpholino (MO *cxcl8-l2*). Tail fin larvae were amputated at 3 dpf in all groups. **(A)**

cxcl8-l2 mRNA levels were determined by qPCR in tail fin tissue at 0 and 1 hpw (40 tail fins per time-point). Gene expression was normalized against *rps11* and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the mean ± SEM of triplicated samples. P values were calculated using two-way ANOVA with Bonferroni multiple comparison test, *P<0.05. **(B)** Representative overlay images of brightfield and green channels of wounded larvae tail fins 30, 90, 240 and 360 minutes post wounding (minpw). **(C)** Counts of fluorescent neutrophils at the site of injury from 30 to 360 minpw. **(D)** Counts of fluorescent neutrophils at the site of injury at 30 and **(E)** 360 minpw. *lyn* morpholino significantly decreased early neutrophil recruitment to wounds and *cxcl8-l2* significantly reduced neutrophil recruitment in both early and late phases. All data are represented as means ± SEM. P values were calculated using one or two-way ANOVA with Bonferroni multiple comparison test (**, P<0.01; ***,P<0.001). Scale bars = 100μm.

presented a kinetics very much similar to that obtained for *cxcl8-l2* morphants (10) (Figure 1B), suggesting that H_2O_2 might be modulating Cxcl8 signaling. Interestingly, *cxcl8-l2* morphants presented as well decreased neutrophil recruitment in the early phase (Figure 1A, B and C). However, H_2O_2 measurements showed no decrease in H_2O_2 levels as expected (Figure 1E and F). Altogether these results led us to address whether Duox1-derived H_2O_2 is involved in Cxcl8-l2 signaling. In the presence of the DN-Duox1, a decreased Cxcl8 expression was observed that both at mRNA (Figure 2A) and protein levels (Figure 2B and C) in tail wounded tissues in comparison to AS larvae, arguing that the H_2O_2 produced upon wounding is required for the induction of *cxcl8-l2* gene expression. To control for the specificity of anti-zfCxcl8-l2 antibody on whole mount immunofluorescence, we made use of *cxcl8-l2* morphants (Suppl. Figure 1). In these morphants, we could consistently observe a decreased Cxcl8-l2 protein signal at the wound margin in comparison to the control condition.

Lyn is important for normal neutrophil recruitment at early phase and also contributes for normal cxcl8-l2 gene expression

Lyn is a Src family kinase (SFK) previously identified by others as the neutrophil redox sensor responsible for early recruitment (7). So next, we wanted to address its involvement in H2O2-mediated Cxcl8-l2 expression. Upon tail fin transection lyn knockdown was able to significantly diminish cxcl8-l2 gene expression in comparison to the control condition (Figure 3A), thus suggesting that the neutrophil could contribute for this chemokine expression at the wound. Consistently, we further observed that *lyn* morphants presented a significant decrease in neutrophil recruitment towards wounds before 90 minpw (Figure 3A, B and C), but not at later time points (240 and 360 minpw) (Figure 3A, B and D).

SFKs, Erk, p38 and Jnk are crucial for Cxcl8s expression and normal neutrophil recruitment, but not for H_2O_2 production

To further understand the signaling mechanisms underlying H_2O_2-mediated Cxcl8-l2 expression, our next step was to unravel which signaling molecules might be implicated in this respect. For such, we made use of several pharmacological drugs to inhibit specifically distinct signaling pathways, namely SB220025 for p38 signaling; SP600125 for Jnk signaling; PD98059 for

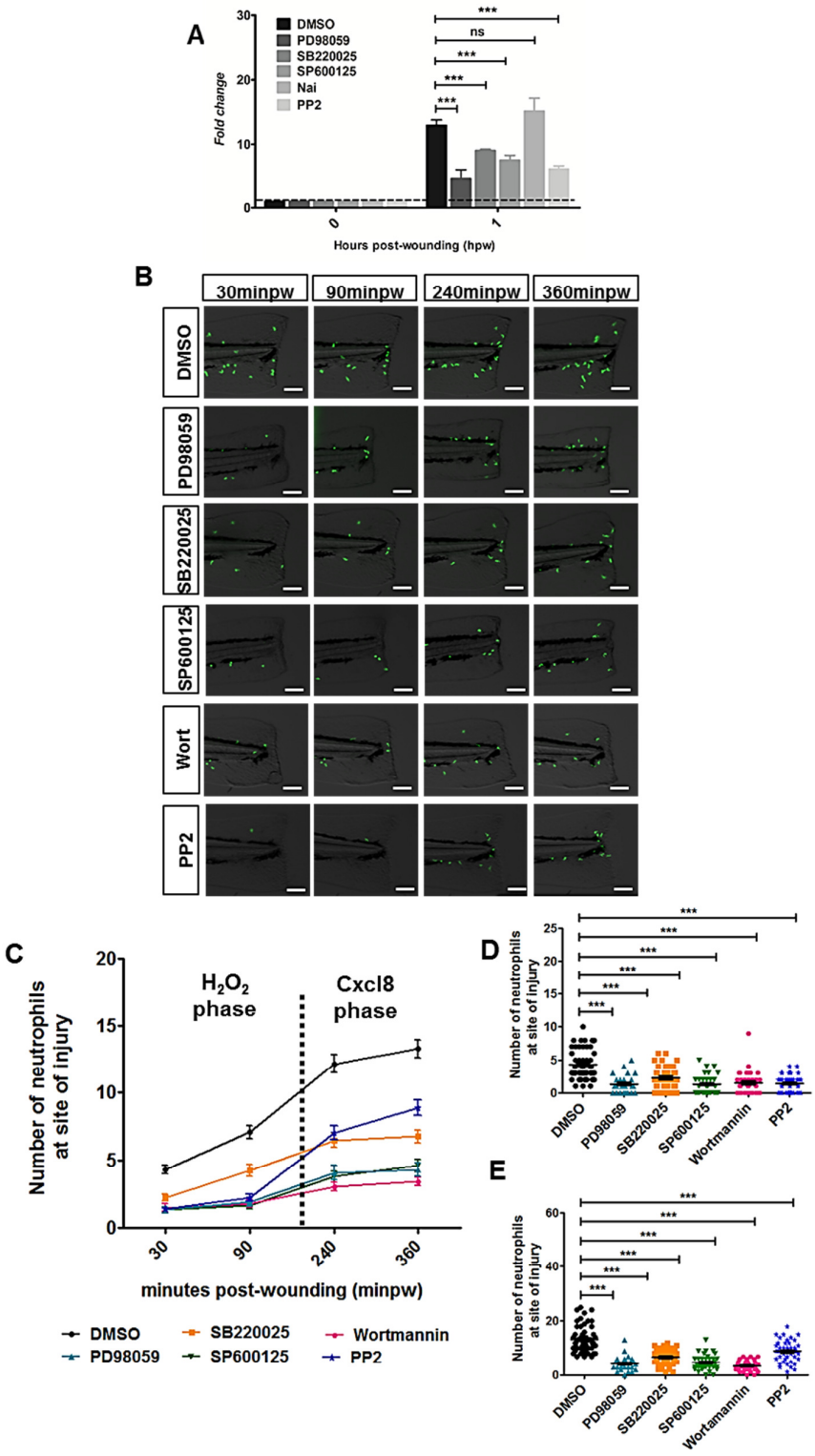

Figure 4: SFKs and Erk, p38 and Jnk signaling pathways are crucial for Cxcl8-l2 expression and for normal neutrophil recruitment. Zebrafish 3dpf Tg(mpx:GFP)^{i114} larvae were pre-treated for 15min with 100µM PD98059, 10µM SB220025, 50µM SP600125, 50nM Nai, 1µM wortmannin, 20µM PP2. Tail fin larvae were amputated in all groups; larvae were maintained with inhibitors during all the procedures. (A) *cxcl8-l2* mRNA levels were determined by qPCR in tail fin tissue at 0 and 1hpw (40 tail fins per time-point). Gene expression was normalized against *rps11* and expressed as fold change compared with transcript expression levels of 3 dpf tail fin tissue from unwounded larvae (0 hpw). Each bar represents the

mean ± SEM of triplicated samples. P values were calculated using two-way ANOVA with Bonferroni multiple comparison test, *P<0.05. (B) Representative overlay images of brightfield and green channels of wounded larvae tail fins 30, 90, 240 and 360 minutes post wounding (minpw). (C) Counts of fluorescent neutrophils at the site of injury from 30 to 360 minpw. (D) Counts of fluorescent neutrophils at the site of injury at 30 and (E) 360 minpw. All inhibitors significantly decreased neutrophil recruitment to wounds in both phases. All data are represented as means ± SEM. P values were calculated using two-way ANOVA with Bonferroni multiple comparison test (**, P<0.01; ***,P<0.001). Scale bars = 100µm.

Erk signaling; Nai for NF-kB signaling; Wortmannin for PI3K signaling and PP2, as a SFK inhibitor. Upon SFK inhibition or inhibition of p38, Jnk or Erk signaling pathways, *cxcl8-l2* gene expression was significantly diminished after wounding. In contrast, the Nai inhibitor failed to affect *cxcl8-l2* expression (Figure 4A).

We next addressed the effect of these pharmacological inhibitors on neutrophil recruitment in response to wounding. We observed that neutrophil recruitment was significantly reduced both at early and late recruitment phases in presence of all the inhibitors used (Figure 4B-E). Wortmannin was here used as a negative control of neutrophil recruitment taken the well-established requirement of the PI3K signaling for neutrophil chemotaxis (33). In order to verify if these inhibitors were affecting H2O2 tissue gradient formation, we have also measured H2O2 levels at 30 minpw in wounded tissues. Importantly, none of these drugs affected significantly H2O2 release (Figure 5A and B).

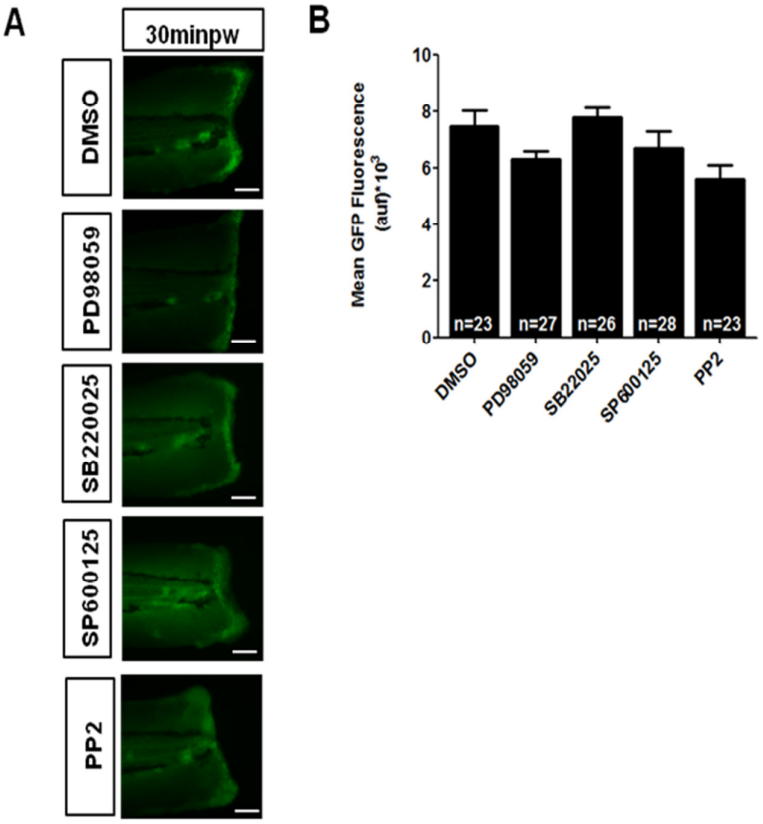

Figure 5: Inhibition of SFKs, Erk, p38 and Jnk signaling pathways do not affect H_2O_2 production. Zebrafish 3dpf wild type were pre-treated for 15min with 100µM PD98059, 10µM SB220025, 50µM SP600125, 20µM PP2. Tail fin larvae were amputated in all groups; larvae were maintained with inhibitors during all the procedures. **(A)** Representative images of H_2O_2 production. **(B)** Wound fluorescence intensity quantification of wounded larvae tail fins at 30minpw labelled with acetyl-pentafluorobenzene sulphonyl fluorescein. None of the inhibitors affected H_2O_2 levels in wounded tail fins. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (**, P<0.01; ***,P<0.001). Scale bars = 100µm.

H_2O_2 induces p38 and Jnk signaling and c-Jun phosphorylation but not Erk signaling, in wounded tail tissue

After establishing that Erk, p38 and Jnk signaling were required for normal cxcl8-l2 gene expression and neutrophil recruitment, we further asked if these effects were modulated by H2O2. For such, we next addressed if H2O2 was able to affect the phosphorylation levels of Erk, p38 and Jnk. Immunofluorescences of wounded larvae tail fins at 1 hpw showed that this was the case for p38, Jnk and c-Jun, but not for Erk, as the expression of the DN-Duox1 reduced the levels of phospho-p38, phospho-Jnk and phospho-c-Jun, but did not affect phospho-Erk levels in comparison to control larvae (Figure 6A and B).

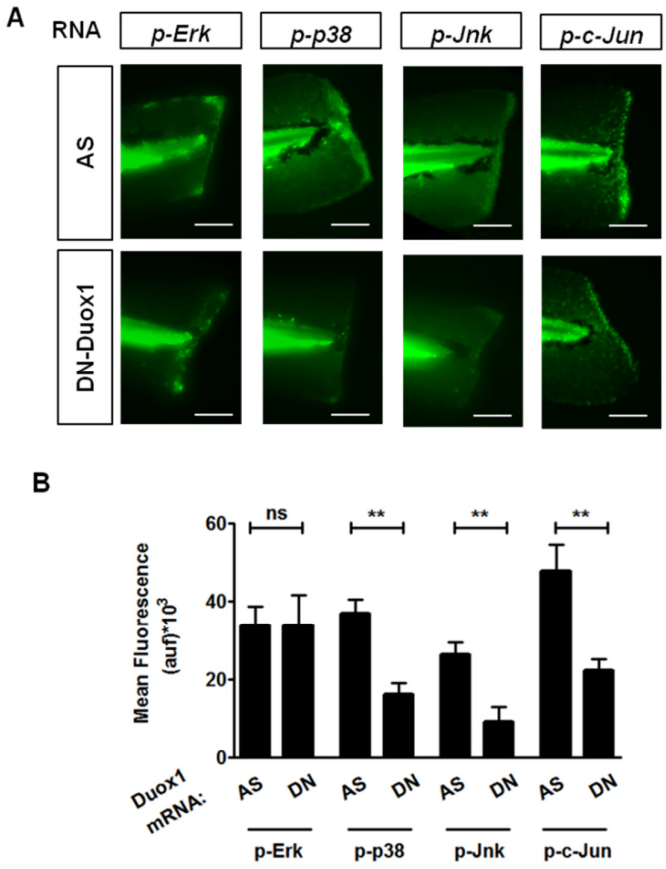

Figure 6: Duox1-derived H_2O_2 induces Jnk, p38 and c-jun phosphorylation, but not that of Erk, in wounded tail tissue. Zebrafish one-cell stage Tg(*lyz:DsRED2*)^{nz50} were microinjected with mRNA Duox1 anti-sense (AS) or sense (DN-Duox1). Tail fin larvae were amputated at 3 dpf. **(A)** Representative 3-D images of whole-mount immunofluorescence at 1 hpw **(B)** Wound fluorescence intensity quantification. H_2O_2 tissue gradients induce the phosphorylation of p38, Jnk and c-Jun but not that of Erk at wounded tissues. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test (***,P<0.001). Scale bars = 100µm.

H_2O_2 modulates histone 3 modification on proximal cxcl8-l2 promoter

H_2O_2 role is able to modulate gene expression by several modes of action (15-18). Among these, *in vitro* studies have suggested a possible role in modulating the acetylation/methylation status of histone core at gene promoters (13, 14, 18). To understand how H_2O_2 modulates Cxcl8-l2 expression, we decided to address *in vivo* whether H_2O_2 might contribute to histone 3 modifications in the *cxcl8-l2* gene proximal promoter, and more precisely at site I (region -119/+3)

as indicated in Figure 7A. For such, we made use of tail fin tissues after 1hour of amputation of 3 dpf larvae previously microinjected with the AS or DN-Duox1 RNA to perform ChIP analysis. In this study, we focused our attention on the following epigenetic markers: histone 3 lysine 4 tri-methylation (H3K4me3) and histone 3 lysine 9 acetylation (H3K9ac) as "activation" markers; and histone 3 lysine 9 tri-methylation (H3K9me3) as a "repression" marker (32). In the larvae injected with the DN-Duox1, we observed increased levels of H3K4me3 and H3K9ac markers and decreased levels of H3K9me3 levels at site I upon wounding in comparison to AS control larvae (Figure 7B). These results suggest that Duox1-derived H_2O_2 effect observed on *cxcl8-l2* gene expression upon wounding could in part be mediated by the modulation of the chromatin status at its promoter.

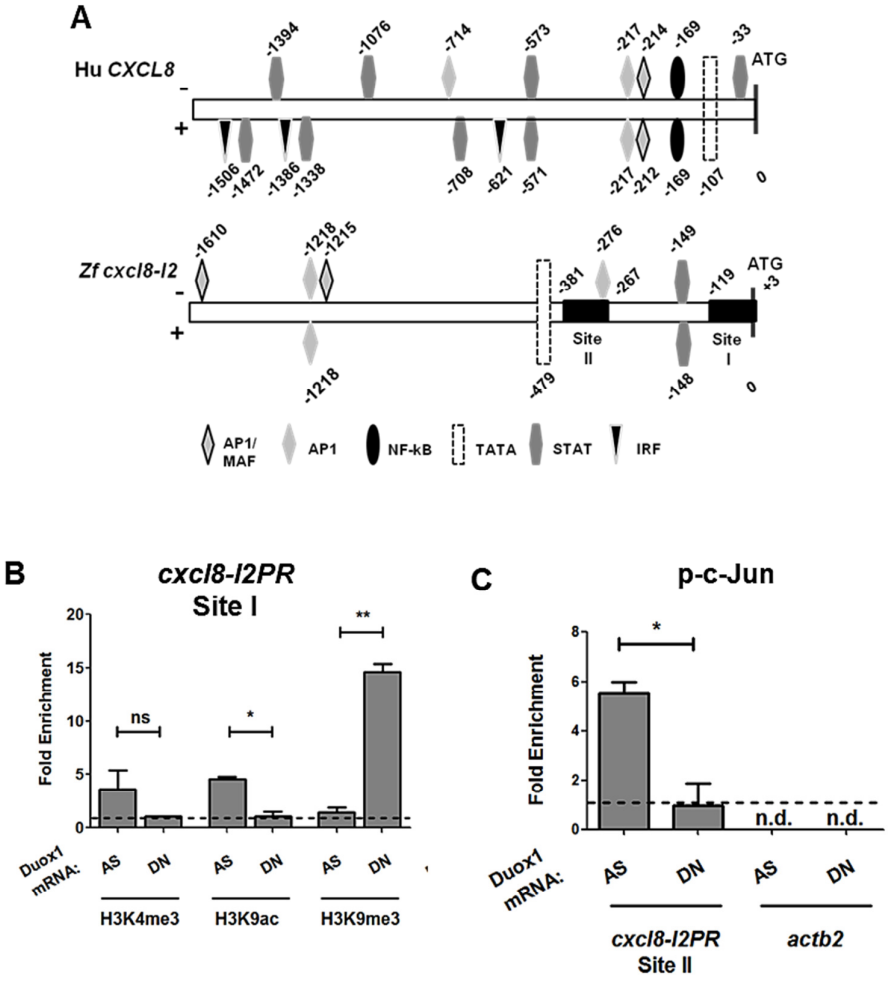

Figure 7: H_2O_2 modulates histone 3 covalent modifications and AP1 binding on the *cxcl8-l2* promoter. (A) Representation of transcription factors of human CXCL8 and zfCxcl8-l2. **(B and C)** Zebrafish one-cell stage wild type were microinjected with mRNA Duox1 anti-sense (AS) or sense (DN-Duox1). Tail fin larvae were amputated at 3 dpf and ChIP was performed from tissue of 400 larvae per group by using antibodies for H3K4me3, H3K9ac, H3K9me3 markers or for p-c-Jun. qPCR analysis was performed for site I to determine H3K4me3, H3K9ac, H3K9me3 levels **(B)** and for site II to inspect p-c-Jun binding levels **(C)**. H_2O_2 is able to induce H3K4me3, H3K9ac levels and p-c-Jun binding and also to decrease H3K9me3 levels in *cxcl8-l2* promoter. No changes were observed in the p-c-Jun binding to the actb2 promoter, which was used as

a control. All data are represented as means ± SEM. P values were calculated using Unpaired two-tailed Student's t-test (*, P<0.5; ***,P<0.001). n.d., non detected.

H_2O_2 promotes the binding of the transcription factor AP1 to cxcl8-l2 promoter

H_2O_2 has also been shown to modulate gene expression via the activation of redox sensible transcription factors, such as AP1 (Activator Protein-1) (13, 14). Promoter analysis for human *CXCL8* and zebrafish *cxcl8-l2* genes performed by using the software tool Genomatix MatInspector have allowed us to identify several binding sites for AP1, among other known transcription factors (Figure 7A). Of notice, this analysis further enabled us to conclude that the zf *cxcl8-l2* promoter does not have any consensus binding site for NF-κB (Figure 7A and Table S1) in agreement with the data obtained previously with the Nai inhibitor (Figure 4A). A ChIP assay for p-c-Jun, one of the two AP1 sub-units, was performed using wounded tail fin tissue, 1 hpw, from larvae microinjected with AS or DN-Duox1 mRNA. Through a qPCR analysis for site II (region -381/-267) of the zebrafish *cxcl8-l2* promoter we were able to show that a higher level of p-c-Jun is bound to the promoter in AS larvae than in larvae expressing the DN-Duox1 mutant (Figure 7C), arguing that H_2O_2 production is able to mediate AP1 recruitment to the *cxcl8-l2* gene promoter.

Discussion

During the last years zebrafish has been used to disclosure *in vivo* several aspects of neutrophil recruitment. One of the most important findings was the discovery that one of the molecules believed to be released after wounding, the H_2O_2, actually forms a tissue gradient (6) that is most importantly responsible for early leukocyte recruitment (7) and later tissue regeneration (11). Besides this huge advance, we (10) and others (34, 35) have further reported the importance of Cxcl8 gradients in neutrophil recruitment and behavior in zebrafish inflammation. Altogether, these and other findings clearly support nowadays the use of zebrafish as an important animal model for the *in vivo* study of neutrophil recruitment and function.

Overall, these previous studies have highlighted a role for H_2O_2 and Cxcl8 chemokines, among other chemotactic cues, in neutrophil recruitment in the zebrafish inflammation. A currently accepted view of neutrophil recruitment in this process proposes that it occurs by sequential phases in response to different inflammatory stimuli (36, 37). Taken the expression and function of H_2O_2 and Cxcl8 chemokines, these cues should act at different phases of neutrophil recruitment, with H_2O_2 being an early recruitment signal and the Cxcl8 chemokines guiding neutrophils in subsequent recruitment phases. Having this in mind, we have here hypothesized that early H_2O_2 production by wounded epithelial cells could crosstalk with Cxcl8 signaling pathway and in this way, indirectly modulate neutrophil recruitment at later timepoints.

To start tackling this issue, we first extended our neutrophil recruitment studies after tail fin transection up to 6 hpw under conditions in which H_2O_2 was impaired, namely in the presence of DN-Duox1 mutant. In agreement with our working hypothesis, reduction of H_2O_2 gradients

impacted not only at an early neutrophil recruitment phase, but also at a later phase. By modulating the production and release of H_2O_2 by Duox1, we could further observe that this early signal is in fact important for Cxcl8-l2 expression, both at transcript level (as observed at 1 hpw) as well as at the protein level (as observed at 5 hpw) after tail fin injury. As such, we conclude that H_2O_2 not only directs neutrophil recruitment at initial recruitment phases (30-90 minpw), but can also modulate the expression of Cxcl8-l2 gradients later on and thus, can indirectly contribute for neutrophil recruitment at later phases.

According to these results, we further propose a model for neutrophil recruitment in zebrafish inflammation. In this, an early recruitment phase (30-90 minpw) is directly dependent upon the function of H_2O_2 that further modulates the expression and action of Cxcl8 chemokines, which govern a later recruitment phase (from 90 minpw up to 6 hpw). This model is for sure an oversimplified version of neutrophil recruitment taken the role of other mediators and chemotactic cues, such as leukotriene B4, in this matter.

To further support this model, one important aspect will be to understand the molecular and cellular players involved in this H_2O_2-Cxcl8 crosstalk. In zebrafish, several cells and tissues involved in inflammatory response have been reported to be able to produce Cxcl8 (38). Among them, we can find tissues like the skin and specific cells like macrophages and neutrophils. Our data shows that early leukocyte recruitment, H_2O_2/Lyn dependent, seems to have a significant contribution on normal *cxcl8-l2* gene expression, but surprisingly not on later neutrophil recruitment. Such effect might be due to the increase of the total number of neutrophils previously reported (7) to take place upon *lyn* knockdown which could with time compensate for a reduced early recruitment.

To further dissect the H_2O_2-Cxcl8 crosstalk, we next attempted to identify putative molecular targets, like SFKs (7, 11), and signaling pathways, such as the Erk (11, 39, 40), p38 (39), Jnk (30) or NF-κB pathways (19, 40) that could be potentially modulated by H_2O_2, so as to regulate Cxcl8-l2 expression. For such, we combined a pharmacological approach to inhibit these molecular targets to the previously described assays. This way, we observed that almost all drug targets tested seem to be involved in *cxcl8-l2* expression. Surprisingly inhibition of NF-κB signaling was the only condition that did not affect this chemokine expression upon wounding. Accordingly, a *cxcl8-l2* gene promoter analysis confirmed the absence of binding sites for this transcription factor. Our next step was then to test if these drug-mediated decreases in *cxcl8-l2* expression would be correlated with a decrease in neutrophil recruitment. As expected all the inhibitors that have down-regulated *cxcl8-l2* expression were also able to significantly decrease neutrophil recruitment, both at early and later phases. Additionally, H_2O_2 measurements showed no changes in its levels indicating an upstream role for this molecule in the signaling cascade. Yoo *et. al.* have previously reported that H_2O_2 is able to induce SFKs phosphorylation upon wounding both in neutrophils (7) and in wounded epithelia tissue (11), modulating this way tissue regeneration. In this study we show that besides SFKs, H_2O_2 is also able to crosstalk with Cxcl8 signaling by modulating the phosphorylation levels of p38 and Jnk in wounded tissue. Additionally, phosphorylation levels of c-

Jun are also modulated by the presence of H_2O_2. Although inhibition of Erk signaling decreased as well *cxcl8-l2* gene expression and neutrophil recruitment, the activation of this pathway in the wounded tissue was observed not to be H_2O_2-dependent. This result is in accordance with the previous data reported by Yoo *et al.* (11) where Erk phosphorylation was observed to occur as an H_2O_2-independent early mechanism involved in later tissue regeneration. Altogether, our findings show that the mechanism underlying H_2O_2-Cxcl8-l2 crosstalk is partly based on the activation of p38 and Jnk signaling pathways as well as on c-Jun phosphorylation.

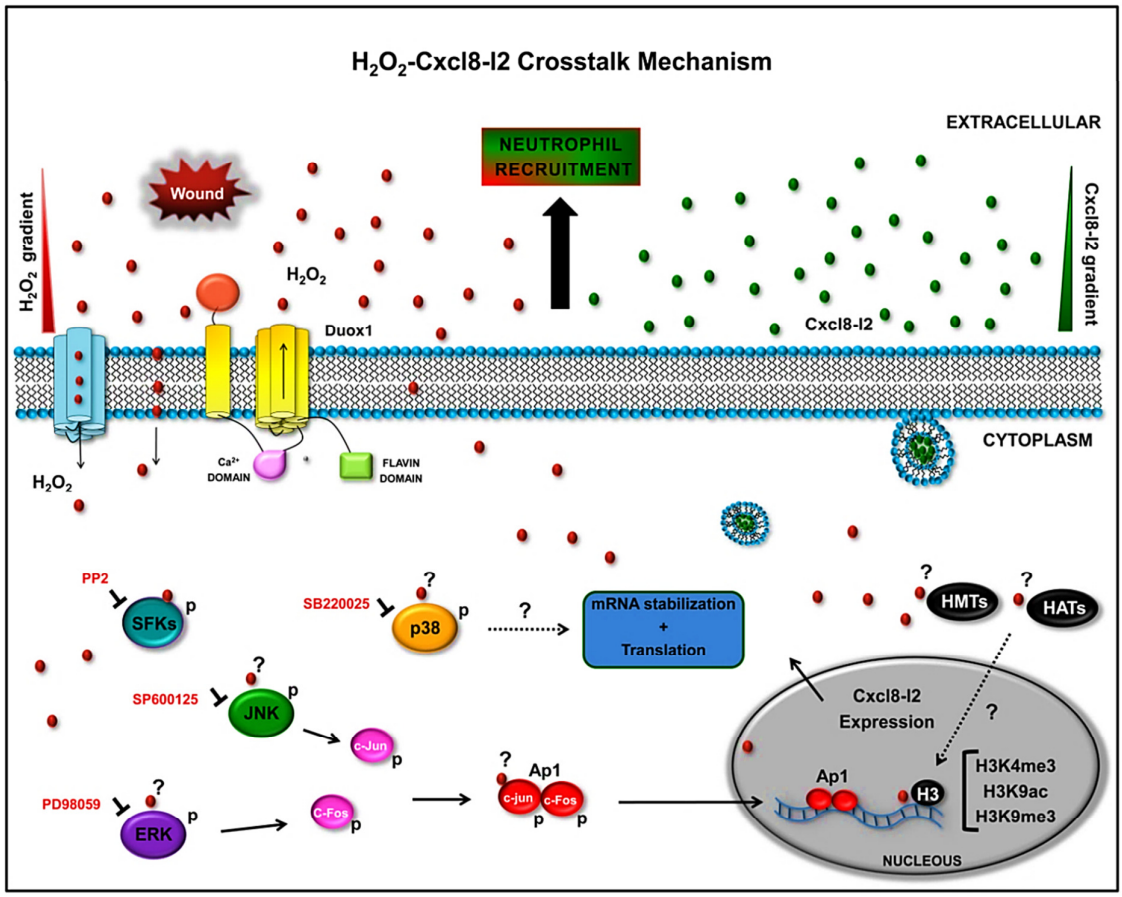

Figure 8: Proposed model illustrating how early H2O2 crosstalk with Cxcl8-l2. Tissue damage activates Duox1, inducing the production of H2O2. This H2O2 diffuses through tissue and enters the neighbouring cells activating SFKs, p38 Jnk and c-Jun phosphorylation. The mechanism of how this molecules are being activated by H2O2 is still undisclosed although autophosphorylation, as it occurs for the SFK Lyn, might be a possible scenario. Notice that Jnk and Erk are up-stream signals of the transcription factor AP1, so the activation of Jnk, by H2O2, and Erk upon wounding probably are the cause for the AP1 binding to *cxcl8-l2* promoter and further induction of gene expression detected in this study. Additionally H2O2 is also able to modulate Cxcl8-l2 expression via modifications of proximal *cxcl8-l2* promoter epigenetic markers, such as the "activation" marker H3K9ac and the "repression" marker H3K9me3. Altogether, this mechanism explains how early signals are able to crosstalk with later ones modulating inflammatory response and tissue regeneration. The sites of action of the different pharmacological drugs used in this study are also indicated.

Among other modes, H_2O_2 role is able to modulate gene expression by regulating several histone protein modifications (14, 18). Acetylation/deacetylation and methylation/demethylation of histone residues at the histone core, around which DNA is coiled, are important to modulate the

access of transcription factors to promoter elements and therefore, to modulate gene transcription. Nuclear histone acetylation/deacetylation and methylation/demethylation are reversible processes regulated by: acetyltransferases (HATs) which promote acetylation, deacetylases (HDACs) which promote deacetylation, histone methyltransferases (HMTs) which promote methylation and finally histone demethylases (HDMs) which promote demethylation (13, 14). By ChIP analysis, we found that H_2O_2 tissue gradient regulates the acetylation and methylation status of histone 3 at lysines 4 and 9 at the *cxcl8-l2* proximal promoter, after tissue injury. The levels of histone modifications obtained in the absence or presence of H_2O_2 tissue gradient were consistent with a role of H_2O_2 in inducing the transcriptional activation of *cxcl8-l2*.

H_2O_2 has also been shown to modulate gene transcription through controlling the function of redox sensitive transcription factors like NF-κB and AP1 (13, 14). By performing a ChIP analysis for p-c-Jun, one of the AP1 subunits, we could conclude that H_2O_2 can modulate activated AP1 binding to at least one of its potential binding sites in *cxcl8-l2* gene proximal promoter. Importantly, our findings are in accordance with a previous study that pointed out early Jnk signaling and Junb as essential players, both for macrophage recruitment and tail fin tissue regeneration in zebrafish (30). Additional work will be needed to better understand if this H_2O_2-mediated effect occurs directly at the transcription factor level, like it happens for example with Lyn oxidation (7), and/or indirectly by activating AP1 upstream factors like for example Jnk.

Overall, our study enabled us to conclude that H_2O_2 is a key regulatory factor in the *in vivo* wound response that crosstalks with Cxcl8 signal by a complex mechanism involving SFKs, p38 and Jnk signaling pathways, as well as histone 3 modifications and AP1 binding to *cxcl8-l2* proximal promoter (Figure 8). On the basis of several *in vitro* studies performed throughout the past decade, the existence of a complex signaling mechanism was previously proposed to explain how Duox1-derived H_2O_2 could modulate the expression of CXCL8 in mammalian cell culture systems. In this respect, our study provides for the first time *in vivo* evidence to support the existence of such crosstalk within an organism, the zebrafish, in the context of a particular biological response, the wound-associated inflammation. Moreover, this study has undoubtedly unraveled important issues regarding the *in vivo* modulation of Cxcl8 expression by H_2O_2. Still some unanswered questions will need further attention in the near future, such as for example, how H_2O_2 is able to modulate p38 and Jnk signaling and ultimately, c-Jun phosphorylation levels, or how it is able to impact on histone 3 epigenetic markers. Finally, it is clear that H_2O_2 is not only controlling the expression of *cxcl8-l2* gene, and it may be most probably able to modulate the expression and/or activity of several other pro-inflammatory and pro-resolution molecules. Once more, our studies highlight H_2O_2 as a major drug target for the development of new anti-inflammatory treatments.

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Author contributions

SdO, AC and VM conceived the study; SdO, AC and VM designed research; SdO and PB performed research; SdO, AC and VM analyzed data; and SdO wrote the paper with contribution from other authors.

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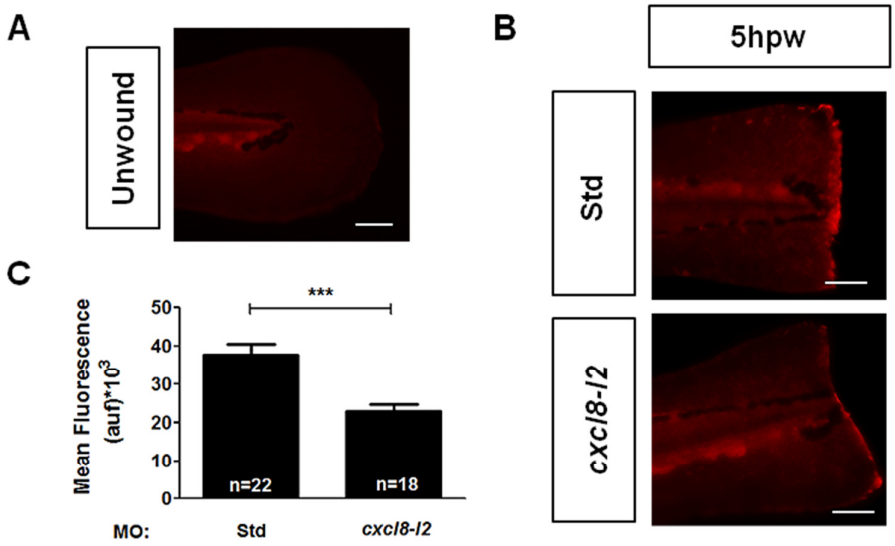
Supplementary Material:



Supplementary Figure 1: zf Cxcl8-l2 protein levels in *cxcl8-l2* morphants. Zebrafish one-cell Tg(*mpx:GFP*)^{i114} were microinjected with standard control (MO StdC) or *cxcl8-l2* morpholino (MO *cxcl8-l2*). Tail fin larvae were amputated at 3 dpf in all groups. **(A)** Representative 3D-projection whole-mount immunofluorescence of zfCxcl8-l2 before wounding. **(B)** Representative 3D-projection of red (ZfCxcl8-l2) channel of whole-mount immunofluorescence of zfCxcl8-l2 at 5 hpw. **(C)** Wound fluorescence intensity quantification in wounded larvae tail fins at 5hpw. The MO of *cxcl8-l2* is able to significantly decrease Cxcl8-l2 protein levels in wounded tail fins, confirming the specificity of the mAb used. auf: arbitrary units of fluorescence. All data are represented as means ± SEM. P values were calculated using Mann Witney t-test (***,P<0.001). Scale bars = 100µm.

Chapter 6

Tnfa signaling through Tnfr2 protects skin against oxidative stress-induced inflammation

Tnfa signaling through Tnfr2 protects skin against oxidative stress-induced inflammation

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Abstract

TNFα overexpression has been associated with several chronic inflammatory diseases, including psoriasis, lichen planus, rheumatoid arthritis and inflammatory bowel disease. Paradoxically, numerous studies have reported new-onset **psoriasis** and lichen planus following **TNFα** antagonist therapy.

Here, we show that genetic inhibition of Tnfa and Tnfr2 in zebrafish results in the mobilization of neutrophils to the skin. Using combinations of fluorescent reporter transgenes, fluorescence microscopy and flow cytometry, we identified the local production of dual oxidase 1 (Duox1)-derived H_2O_2 by Tnfa- and Tnfr2-deficient keratinocytes as a trigger for the activation of the master inflammation transcription factor NF-κB, which then promotes the induction of genes encoding pro-inflammatory molecules. In addition, pharmacological inhibition of Duox1 completely abrogated skin inflammation, placing Duox1-derived H_2O_2 upstream of this positive feedback inflammatory loop. Strikingly, DUOX1 was drastically induced in the skin lesions of psoriasis and lichen planus patients.

These results reveal a crucial role for TNFα/TNFR2 axis in the protection of the skin against DUOX1-mediated oxidative stress and could establish new therapeutic targets for skin inflammatory disorders.

Introduction

Tumor necrosis factor α (TNFα) is a multifunctional cytokine that mediates key roles in acute and chronic inflammation, antitumor responses and infection. TNFα binds TNF receptor 1 (TNFR1, also known as TNFRSF1A or P55) and TNFR2 (also known as TNFRSF1B or P75) for stimulation of two opposing signaling events [1]. In general, TNFR1 signaling results in the trigger of a cascade that can result in apoptosis [2]. This is dependent upon the cell type, the state of activation of the cell and the cell cycle. In contrast, a Tnfr2 signal induces cell survival pathways that can result in cell proliferation [2].

Enhanced TNFα synthesis is associated with the development of autoimmune/chronic inflammatory diseases, including psoriasis, lichen planus, rheumatoid arthritis and inflammatory bowel disease (IBD). The inhibition of TNFα activities in these diseases has been remarkably successful [3,4]. Paradoxically, however, numerous studies have reported new-onset psoriasis and lichen planus, or worsening of existing psoriasis, following TNFα antagonist therapy in adult patients [5-10]. Despite these clinical data pointing to an ambiguous function of TNFα in psoriasis and lichen planus, the role of TNFα, and in particular the contribution of each TNFR, in the regulation of skin inflammation has been scarcely studied. An earlier study using gene-targeted mutant mice lacking either TNFR1 or TNFR2 showed that skin inflammation induced indirectly by irritant chemicals or directly by intradermal administration of TNFα was greatly attenuated in TNFR1-deficient mice, while TNFR2-deficient siblings responded normally [11]. In addition, mice with arrested canonical NF-κB activation pathway in the keratinocytes develop a severe inflammatory skin disease shortly after birth, which is caused by TNFα- and macrophage-mediated, but T-cell-independent, mechanisms [12-16]. The characteristics of this complex disorder are strikingly similar to those associated with the human X-linked genodermatosis incontinentia pigmenti [17]. To the best of our knowledge, however, the role played by TNFα in the homeostasis of healthy skin has never been studied.

TNFα and TNFRs are conserved in all vertebrates. Recent studies have shown that in the zebrafish (*Danio rerio*) Tnfa functions as a pro-inflammatory cytokine [18] and TNFR signaling plays an important role in the homeostasis of endothelial cells [19]. In the present study, we have taken advantage of the strengths of the zebrafish embryo model to study the impact of Tnfa, Tnfr1 and Tnfr2 deficiencies in a whole vertebrate organism. We found that Tnfa and Tnfr2 are both crucial, while Tnfr1 is dispensable, for the homeostasis of the skin. Genetic inhibition of Tnfa and Tnfr2 promotes H_2O_2-mediated skin infiltration by neutrophils, increased keratinocyte proliferation and the local activation of the master inflammation transcription factor NF-κB, which then promotes the induction of genes encoding pro-inflammatory molecules. In addition, DUOX1 was strongly induced in keratinocytes of human psoriasis and lichen planus patients.

Materials and Methods

Ethics statement

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU). Experiments and procedures were performed as approved by the Bioethical Committee of the University of Murcia (approval number #537/2011) and Ethical Clinical Research Committee of The University Hospital Virgen de la Arrixaca (approval number #8/13).

Animals

Zebrafish (*Danio rerio* H.) were obtained from the Zebrafish International Resource Center and mated, staged, raised and processed as described [58]. The lines *Tg(mpx:eGFP)i114* [59], Tg(*lyz:dsRED*)^{nz50} [60] and *Tg(krt18:RFP)* [61] were previously described. *Tg(NFκB-RE*:eGFP) (*NF-κB:eGFP* for simplicity) line was generated with the method and constructs previously described [28].

Table S1. Morpholinos used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). ENA, European Nucleotide Archive (http://www.ebi.ac.uk/ena/).

Gene	ENA or Ensembl ID	Target	Sequence (5'→3')	[MO] (mM)	Reference
tnfa	**ENSDARG00000009511**	**e1/i1**	**GCAGGATTTTCACCTTATGGAGCGT**	**0.5**	**López-Muñoz *et al.*, 2011**
tnfr1	**ENSDARG00000018569**	**e6/i6**	**CTGCATTGTGACTTACTTATCGCAC**	**0.65**	**Espín *et al.*, 2013**
tnfr2	**ENSDARG00000070165**	**i1/e2**	**GGAATCTGTGAACACAAAGGGACAA**	**0.2**	**Espín *et al.*, 2013**
duox1	**ENSDARG00000062632**	**e8/i8**	**AGTGAATTAGAGAAATGCACCTTTT**	**0.125**	**Niethammer *et al.*, 2009**
p53	**NM_131327**	**atg/ 5'UTR**	**GCGCCATTGCTTTGCAAGAATTG**	**0.1**	**Niethammer *et al.*, 2009**
lyn	**ENSDARG00000031715**	**e6/i6**	**TCAGACAGCAAATAGTAATCACCTT**	**0.5**	**Yoo *et al.*, 2011**
il1b	**ENSDARG00000005419**	**e1/i1**	**CCCACAAACTGCAAAATATCAGCTT**	**0.6**	**López-Muñoz *et al.*, 2011**

Morpholino, RNA injection, and chemical treatments

Specific morpholinos (Gene Tools) were resuspended in nuclease-free water to 1mM (Table S1). *In vitro*-transcribed RNA was obtained following manufacturer's instructions (mMESSAGE mMACHINE kit, Ambion). Morpholinos and RNA (200 pg/egg) were mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into the yolk sac of

one- to eight-cell-stage embryos using a microinjector (Narishige) (0.5-1 nl per embryo). The same amount of MOs and/or RNA were used in all experimental groups. The efficiency of the MOS was checked by RT-PCR as described previously [19,27,31,34].

In some experiments, 1 dpf embryos were manually dechorionated and/or treated for 24h at 28ºC by bath immersion with the NADPH oxidase inhibitor dibenziodolium chloride (DPI, Sigma-Aldrich) at final concentration of 100 μM diluted in egg water supplemented with 1% DMSO.

Live imaging of zebrafish larvae

At 72 hpf, larvae were anesthetized in tricaine and mounted in 1% (wt/vol) low-melting-point agarose (Sigma-Aldrich) dissolved in egg water [62]. Images were captured with an epifluorescence Lumar V12 stereomicroscope equipped with green and red fluorescent filters while animals were kept in their agar matrixes at 28.5 °C. All images were acquired with the integrated camera on the stereomicroscope and were used for subsequently counting the number of neutrophils (mpx:eGFP), and examined their distribution. The activation of NF-κB was visualized and quantified using the line NF-κB::eGFP. Stacked images were captured using 1 μm (neutrophil infiltration into the skin) or 25 μm (neutrophil distribution, NF-κB activation and H_2O_2 formation) increments and deconvolved using Huygens Essential Confocal software (v 4.1 0p6b) by Scientific Volume Imaging. Stacks were processed using the free source software ImageJ (http://rsbweb.nih.gov/ij) to obtain a maximum intensity projection of the xy axis of the stack. For the quantification of neutrophil distribution and NF-κB activation, the maximum projection for each larva was then converted to a fluorescence value matrix where the value obtained for each pixel transversally was the mean ± S.E.M. for all the pixels for each row (15 larvae per treatment from 3 different experiments). In parallel, the activation of NF-κB in the skin was also quantified by the skin NF-κB activation index, which was defined as the fluorescence in the skin (a+b) relative to the total fluorescence of the larvae. For analysis of neutrophil infiltration into the skin, stacks from *mpx:eGFP*; *krt18*:*RFP* fish were processed using ImageJ to obtain a maximum intensity projection of the xy and zy axis of the stack.

H_2O_2 imaging using a live cell fluorogenic substrate was performed essentially as previously described [32].Briefly, 3-dpf TNFα and Tnfr2 morphants and their control siblings were loaded for 30 min with 50 μM acetyl-pentafluorobenzene sulphonyl fluorescein (Cayman Chemical) in 1% DMSO in egg water and imaged as above. As a positive control, complete transection of the tail of anesthetized 72 hpf larvae was performed with a disposable sterile scalpel [62].

Flow cytometry

At 3 dpf, approximately 300 to 500 *Tg(mpx:eGFP) and Tg(krt18:RFP)* larvae were anesthetized in tricaine, minced with a razor blade, incubated at 28°C for 30 min with 0.077 mg/ml Liberase (Roche) and the resulting cell suspension passed through a 40 μm cell strainer. Sytox (Life

Technologies) was used as a vital dye to exclude dead cells. Flow cytometric acquisitions were performed on a FACSCALIBUR (BD) and cell sorting was performed on a Coulter (Epics Altra). Analyses were performed using FlowJo software (Treestar).

Analysis of gene expression

Total RNA was extracted from whole embryos/larvae or sorted cell suspensions with TRIzol reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/µg RNA; Invitrogen). SuperScript III RNase H□ Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo(dT)18 primer from 1 µg of total RNA at 50°C for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C. For each mRNA, gene expression was normalized to the ribosomal protein S11 (*rps11)* content in each sample Pfaffl method [63]. The primers used are shown in Table S2. In all cases, each PCR was performed with triplicate samples and repeated at least with two independent samples.

Table S2. Primers used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). ENA, European Nucleotide Archive (http://www.ebi.ac.uk/ena/).

Gene	ENA ID	Name	Sequence (5'→3')	Use
rps11	**NM_213377**	**F1**	**GGCGTCAACGTGTCAGAGTA**	**RT-qPCR**
		R1	**GCCTCTTCTCAAAACGGTTG**	
tnfr1	**NM_213190**	**F5**	**AGCATTCCCCCAGTCTTTTT**	
		R5	**GCAGGTGACGATGACTGAGA**	
tnfr2	**NM_001089510**	**F14**	**CACACAAGAGATCCGAAGCA**	
		R14	**GGCATCTGTGATGGGAACTT**	
tnfa	**NM_212859**	**F2**	**GCGCTTTTCTGAATCCTACG**	
		R2	**TGCCCAGTCTGTCTCCTTCT**	
Il1b	**NM_212844**	**F5**	**GGCTGTGTGTTTGGGAATCT**	
		R5	**TGATAAACCAACCGGGACA**	
duox1	**AB255050**	**F**	**ACACATGTGACTTCATATCCAG**	
		R	**ATTATTAACTCATCCACATCCAG**	
ptgs2b	**NM_001025504**	**F2**	**CCCCAGAGTACTGGAAACCA**	
		R2	**ACATGGCCCGTTGACATTAT**	
gfp	**EF591490**	**F1**	**ACGTAAACGGCCACAAGTTC**	
		R1	**AAGTCGTGCTGCTTCATGTG**	
mpx	**NM_212779**	**F1**	**AGGGCGTGACCATGCTATAC**	
		R1	**AGGCTCAGCAACACCTCCTA**	
krt18	**NM_178437**	**F2**	**AGAACCTGAAAGGGTCACTGG**	
		R2	**GTAGGTGGCGATTTCTGCCT**	
p63	**NM_152986**	**F**	**CTCGGCAAGAACAAACTGCC**	
		R	**GAGGGGTCACTGAAGGAAGG**	

Histology and whole-mount immunohistochemistry (WIHC)

Larvae were fixed overnight in 4% paraformaldehyde solution (PFA), embedded in Paraplast Plus (Sherwood Medical) and sectioned at a thickness of 5 µm. After being dewaxed and rehydrated, they were stained with haematoxylin and eosin (H&E).

Tg(mpx:eGFP) or Tg(NF-κB:eGFP); Tg(krt18:RFP) 3dpf larvae were fixed overnight at 4°C in 4% PFA at room temperature, dehydrated in methanol/PBS solutions (25, 50, 75 and 100 %, 5 min each) and stored in 100 % methanol at –20°C. For staining, larvae were rehydrated in 75, 50 and 25 % methanol/PBT (PBS and 0.1 % Tween-20) solutions for 5 min each, washed three times for 5 min in PBT, incubated for 5min RT with 150mM Tris-HCl pH=9, followed by heating at 70°C for 15min [64]. After heating treatment larvae were directly washed twice in PBT for 10 min and twice in dH2O for 5 min. Subsequently, to enhance tissue permeabilization, larvae were incubated with cold acetone for 20 min at -20°C, washed twice in dH2O and twice in PBT (5 min each) followed by blocking with blocking solution (PDT=PBT+ 1% DMSO) supplemented with 5% FBS and 2 mg/ml BSA) for 2 h at 22°C. After blocking, embryos were incubated overnight at 4°C with primary antibodies diluted (1:200) in blocking buffer, washed three times in PDT (15 min each) and blocked again for 2 h at 22°C. Secondary antibodies staining was made for 2h RT at 1:500 dilution in blocking buffer, larvae were then washed five times in PBT (5 min each) and stored in Vectashield (Vector Labs) until image acquisition. The following primary antibodies were used: rabbit anti-phosphorylated-Histone H3 (Ser 10)-R (#SC8656-R, Santa Cruz Biotechnology) at 1:200, rabbit anti-human p63 (#SC8343, Santa Cruz Biotechnology) at 1:200. Mouse anti-RFP (#MA5-15257, Thermo Scientific), and Alexa Fluor 594 (#A11032) and Alexa Fluor 532 (#A11002) Goat Anti-Mouse IgG (H+L) (Life Technologies) were used as secondary antibodies at 1:500.

Confocal immunofluorescence images were acquired with a confocal microscope (LEICA TCS-SP2, Leica) using an NA 0.70/20x dry objective. Z-series were acquired using a 210-300µm pinhole. 2D, 3D maximum intensity projections and corresponding animation videos were made using ImageJ (http://rsb.info.nih.gov/ij/).

Human skin samples

Skin biopsies from healthy donors (n=10), and lichen planus (n=8) and psoriasis patients (n=15) were fixed in 4% paraformaldehyde, embedded in Paraplast Plus and sectioned at a thickness of 5 µm. After being dewaxed and rehydrated, the sections were incubated in 50 mM glycine-HCl buffer (pH 3.5) containing 0.01% ethylenediaminetetraacetic acid (EDTA) at 95 °C for 5 minutes and then at room temperature for 20 min to retrieve the antigen. Afterwards, they were immunostained with a 1/50 dilution of a goat polyclonal antibody to human DUOX1 (sc-48858, Santa Cruz Biotechnology) followed by ImmunoCruz™ goat ABC Staining System (sc-2023, Santa Cruz Biotechnology) following the manufacturer's recommendations. The specificity of the staining was confirmed by pre-incubating a 10-fold excess (in molarity) of a commercial blocking peptide (sc-48858 P, Santa Cruz Biotechnology) with the DUOX1 antibody overnight at 4°C.

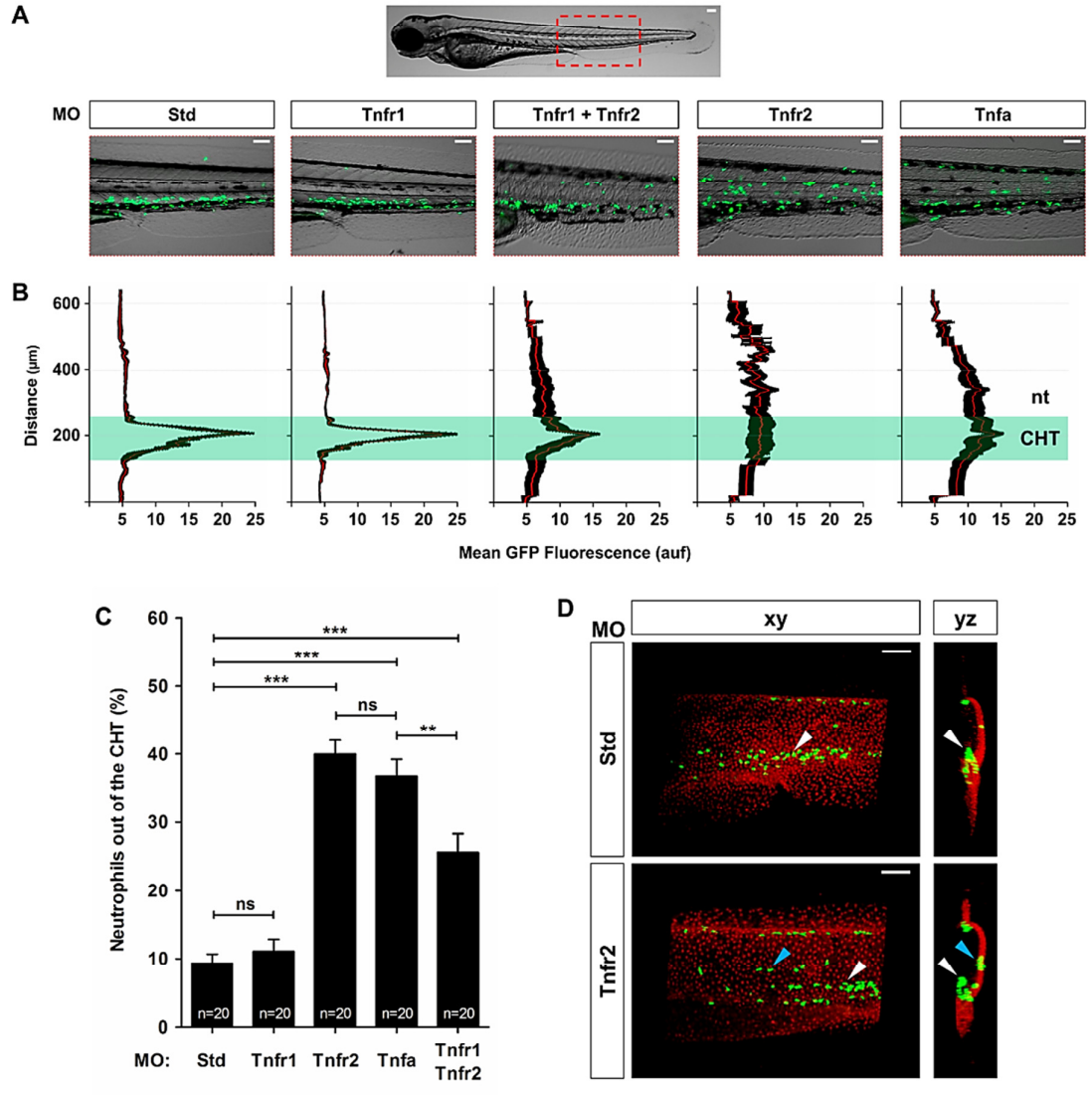

Figure 1. TNFa and Tnfr2 deficiencies result in neutrophil mobilization to the skin. Zebrafish one-cell mpx:eGFP and/or krt18:RFP embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Tnfr1+Tnfr2 morpholinos (MO). (A) Representative images, brightfield and green channels, of the morphants at 3 dpf showing the differences in the neutrophils distribution. (B) Fluorescence intensity was measured for all the groups in the area indicated A, which includes the caudal hematopoietic tissue (CHT), where most neutrophils are located in wild type larvae at 3 dpf. The images were converted to a fluorescence value matrix where the value obtained for each pixel transversally was the mean ☐ S.E.M. for all the pixels for each row (15 larvae per treatment from 3 different experiments). The area corresponding to the CHT has been labeled and highlighted. The notochord (nt) location has been indicated to facilitate the larval orientation. auf: arbitrary units of fluorescence. (C) The neutrophil mobilitation from the CHT in Tnfa- and Tnfr2-deficient larvae was quantified as the percentage of neutrophils outside the CHT in 20 larvae per group from 3 different experiments. The mean ± S.E.M. for each group is shown. (D) Representative frontal (xy) and lateral (yz) views of tridimensional reconstructions from confocal microscopy images of WIHC of mpx:eGFP larvae stained at 3 dpf with anti-p63 antibodies (basal keratinocyte marker, red) showing the neutrophils distribution in the CHT area of control and Tnfr2-deficient larvae. Note that most neutrophils (eGFP, green) are located in the CHT in control larvae (white arrowheads), while many of them infiltrate the skin (blue arrowheads) of Tnfr2-deficient larvae, while they are mainly located in the CHT in their wild type siblings. Scale bars: 100 µm (A) and 200 µm (D). ns, not significant. *p<0.05; **p<0.01; ***p<0.001.

No staining was observed in these conditions. Sections were finally examined under a Leica microscope equipped with a digital camera Leica DFC 280 and the photographs were processed with Leica QWin Pro software.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups. The differences between two samples were analyzed by the Student *t*-test. The contingency graphs were analyzed by the Chi-square (and Fisher's exact) test.

Results

Tnfa or Tnfr2 deficiency results in neutrophil mobilization to the skin

In wild type larvae, most neutrophils (approx. 90%) were located in the caudal hematopoietic tissue (CHT) [20] by 3 days post-fertilization (dpf) (Figs. 1A-1C), which is consistent with neutrophil localization patterns described previously [21,22]. However, in Tnfa- and Tnfr2-deficient larvae, approx. 40% of neutrophils were located outside the CHT (Figs. 1A-1C). In addition, Tnfr1-deficient animals showed a normal neutrophil distribution, while their double deficient siblings for both Tnfr1 and Tnfr2 showed also a distribution pattern more similar to single Tnfr2deficient fish (Figs. 1A-1C). The specificity of this phenotype was confirmed with a dominant negative (DN) Tnfr2 which is lacking the entire intracellular signaling domain, but is identical to full-length Tnfr2 in its transmembrane and extracellular domains, and, therefore, its trimerization with endogenous Tnfr2 extinguishes Tnfr2 signaling [19]. The results showed that the altered neutrophil distribution of Tnfr2 morphants was phenocopied by overexpression of DN-Tnfr2 (Fig. S1A). In addition, the scattered distribution of Tnfa- and Tnfr2-deficient larvae was partially rescued by overexpression of wild type Tnfa and Tnfr2 RNAs, respectively (Fig. S1B). These results prompted us to examine the distribution of macrophages in TNFα- and TNFR2-deficient fish and, surprisingly, macrophage distribution was apparently normal in all cases (Fig. S2). To ascertain the precise localization of neutrophils in Tnfa/Tnfr2-deficient larvae, we knocked-down Tnfr2 in transgenic *mpx:eGFP* animals followed by WIHC against p63 (basal keratinocyte marker) to visualize neutrophils (GFP^+) and skin keratinocytes ($p63^+$) at the same time in whole larvae. The results revealed that while neutrophils fromwild type animals were mainly located in the CHT, a high proportion of neutrophils were seen in close contact with keratinocytes in Tnfr2-deficient animals (Figs. 1D and Videos S1 and S2). Collectively, these results indicate that deficiency of either Tnfa or Tnfr2 specifically promotes neutrophil infiltration into the skin of zebrafish during early development.

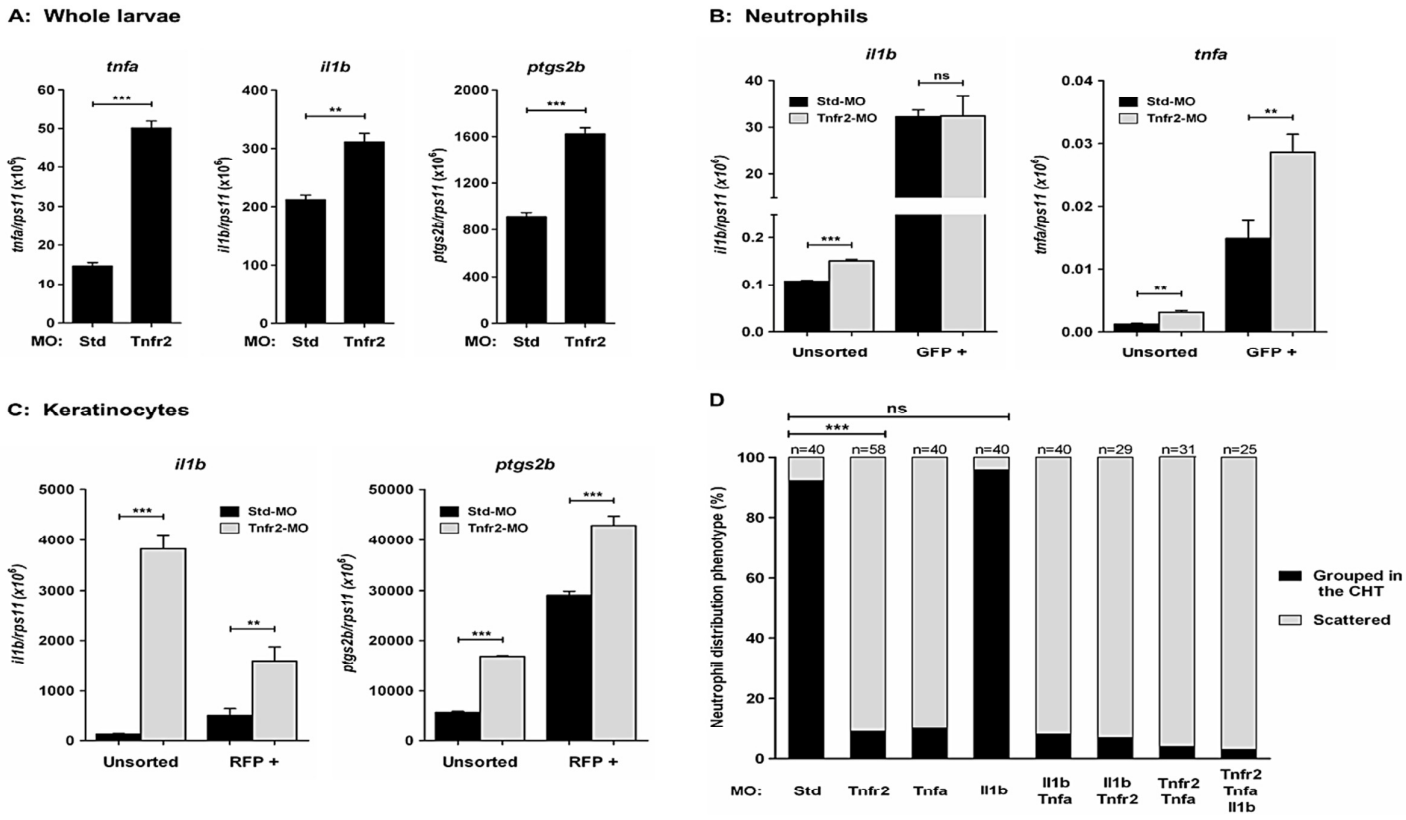

Figure 2. TNFa and Tnfr2 deficiencies trigger skin inflammation. Zebrafish one-cell mpx:eGFP or krt18:RFP embryos were injected with standard control (Std), Tnfr2, Il1b and/or Tnfa morpholinos (MO). The expression of tnfa, il1b and ptgs2b genes was measured by RT-qPCR in whole body (A), FACS-sorted neutrophils (B) and FACS-sorted keratinocytes (C) from Std and Tnfr2 morphants at 3 dpf. (D) The phenotype of 3 dpf morphant larvae was classified as neutrophils grouped in the CHT or scattered, as described in Fig. 1. Note that IL-1β knockdown failed to rescue the neutrophil mobilization in Tnfr2-deficient larvae. ns, not significant. *p<0.05; **p<0.01; ***p<0.001.

Tnfa or Tnfr2 deficiency triggers the induction of genes encoding pro-inflammatory mediators in keratinocytes

The phenotype of Tnfa- and Tnfr2-deficient fish is reminiscent of that of spint1a and clint1 mutant fish, which show chronic skin inflammation characterized by increased interleukin-1β (IL-1β) production and neutrophil infiltration [23-25]. This led us to examine the expression of three genes encoding major pro-inflammatory molecules, namely Tnfa itself, IL-1β and prostaglandin-endoperoxide synthase 2b (PTGS2b, also known as COX2b), in whole wild type and Tnfr2-deficient larvae at 3 dpf as well as in sorted mpx:eGFP+ cells, i.e. neutrophils. It was found that Tnfr2 deficiency triggered the expression of tnfa, il1b and ptgs2b genes (Fig. 2A). Although neutrophils highly expressed the genes encoding Tnfa and Il1b as well as both Tnfrs, they did not mediate the induction of il1b observed in Tnfr2-deficient fish (Figs. 2B, S3A and S4A). Nevertheless, the transcript levels of tnfa were higher in neutrophils from Tnfr2-deficient fish than in neutrophils from their wild type siblings (Fig. 2B), but this might reflect a positive feedback loop in response to Tnfr2 deficiency [19]. In addition, Tnfr2-deficient embryos showed higher transcript levels of il1b at 24 hpf (Fig. S5), soon after the development of the first neutrophils in the zebrafish embryo [21,22,26] and before hatching. We then sorted krt18+ cells from Tnfa- and Tnfr2-deficient animals at 3 dpf and found that they show much higher transcript levels of il1b and ptgs2b than krt18+ cells from wild type animals (Figs. 2C and S3B). Notably, krt18+ cells expressed both Tnf receptors (Fig. S4B) and the specific marker of basal keratinocytes p63 (Fig. S3B).

We next wondered whether knockdown of Il1b using a specific morpholino (MO) [27] might rescue the neutrophil dispersion of Tnfr2-deficient animals. As shown in Fig. 2D, genetic inhibition of Il1b failed to rescue the neutrophil dispersion observed in Tnfr2 morphants. These results taken together indicate that the Tnfa/Tnfr2 axis is required for skin homeostasis in zebrafish and that the deficiency of either ligand or receptor triggers an inflammatory response characterized by the induction of pro-inflammatory mediators and neutrophil infiltration.

Tnfa and Tnfr2 deficiencies induces NF-κB activation in the skin

The master regulator of inflammation NF-κB plays an essential role in the homeostasis of skin. Thus, genetic inhibition of the NF-κB pathway in keratinocytes triggers a severe inflammatory skin disease in newborn mice, which is completely rescued by Tnfa and Tnfr1 depletion [12-16]. We, therefore, use a NF-κB reporter line [28] to visualize the dynamics of NF-κB in whole Tnfr2-deficient larvae. Injection of bacterial DNA, which activated TLR9, resulted in a drastic activation of NF-κB in the whole larvae (Figs. 3A-3B), as expected from previous results [29,30]. Interestingly, Tnfr2 deficiency promoted a restricted activation of NF-κB in the skin (Figs. 3A-3E and Videos S3 and S4).

Furthermore, although skin integrity was unaffected up to 5 dpf in Tnfr2-deficient larvae as assayed by histology (Fig. S6), they showed increased keratinocyte proliferation, as assayed in

double transgenic NF-κB:eGFP; krt18:RFP animals and double WIHC with anti-RFP and anti-phosphorylated H3 (Fig. 4).

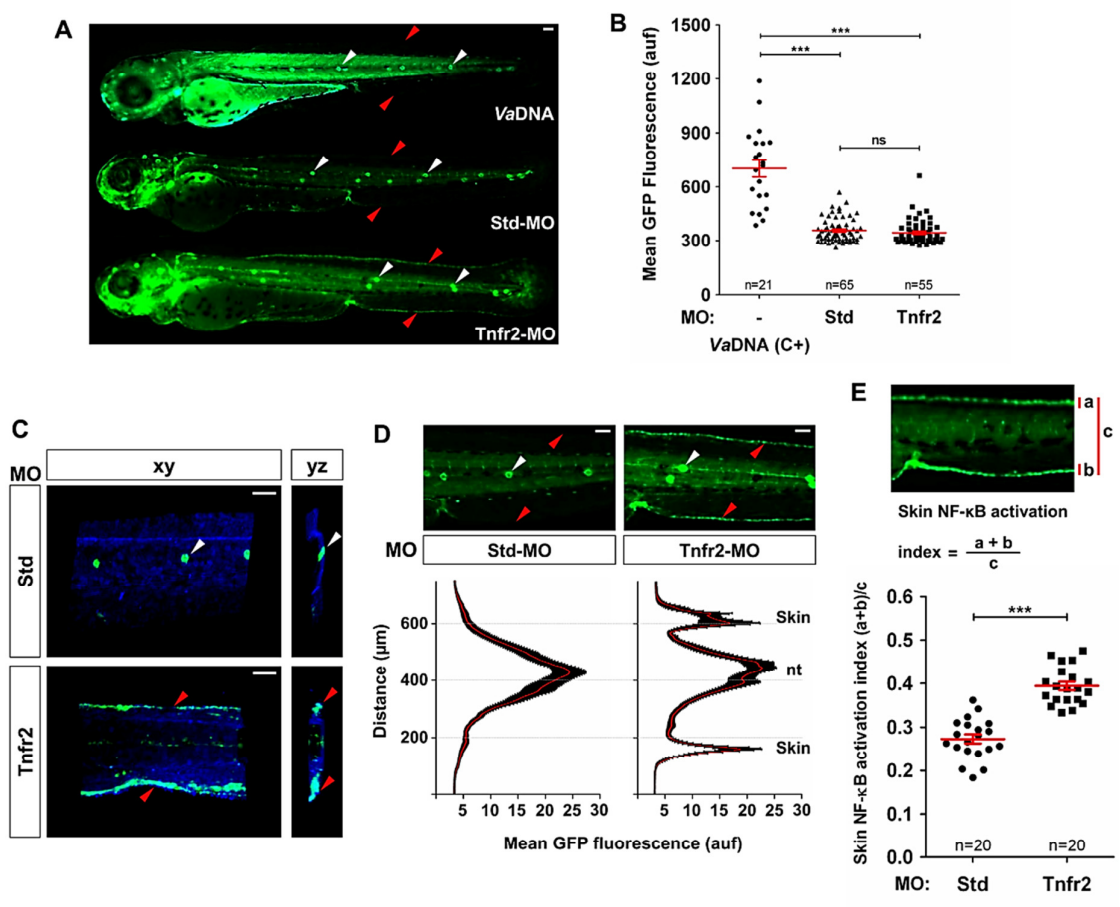

Figure 3. Tnfa and Tnfr2 deficiencies result in skin NF-κB activation. Zebrafish one-cell NF-κB:eGFP (A,B, D, E) or NF-κB:eGFP; krt18:RFP (C) embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO) alone or in the presence of 2.3 ng/egg of V. anguillarum genomic DNA (VaDNA), as a positive control for NF-κB activation. (A) Representative pictures showing the induction of NF-κB activation in the skin (red arrowheads) of Tnfr2-deficient larvae at 72 hpf and the ubiquitous, strong induction in their VaDNA-injected siblings. Note the strong expression of NF-κB in neuromasts of control larvae (white arrowheads). (B) The mean GFP fluorescence was quantified in whole larvae and no significant differences between Tnfr2-morphants and control larvae were observed. Each dot represents the mean GFP fluorescence per single larva. The mean ± S.E.M. of the whole GFP fluorescence for each group of larvae is also shown. (C) Representative frontal (xy) and lateral (yz) views of tridimensional reconstructions from confocal microscopy images of WIHC of NF-κB:eGFP; krt18:RFP larvae stained at 3 dpf with anti-RFP antibodies (keratinocytes, blue) showing the induction of NF-κB in the skin (eGFP, green) of Tnfr2-deficient larvae. (D, E) Quantification of NF-κB activation in the skin of Tnfr2-deficient larvae at 72 hpf. (D) Fluorescence intensity was measured in the area indicated of wild type and Tnfr2-deficient larvae, as explained in the legend to Figure 1 (15 larvae per treatment from 3 different experiments). The skin and the neuromasts have been labeled to facilitate the larval orientation. Note the activation of NF-κB in the skin of Tnfr2-deficient larvae. (E) Skin NF-κB activation index was defined as the fluorescence in the skin (a+b) relative to the total fluorescence of the whole larvae (c). Each dot represents the skin NF-κB activation index per single larva. The mean ± S.E.M. of the skin NF-κB activation index for each group of larvae is also shown. Scale bars: 100 µm. ns, not significant. auf: arbitrary units of fluorescence. *p<0.05; **p<0.01; ***p<0.001.

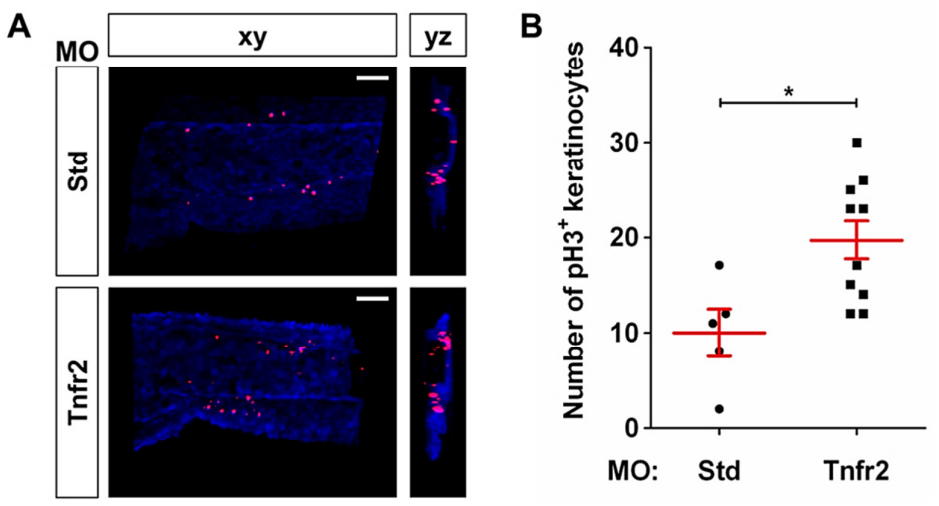

Figure 4. Tnfr2 deficiency results in increased proliferation of skin keratinocytes. Zebrafish one-cell krt18:RFP embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). (A) Representative frontal (xy) and lateral (yz) tridimensional reconstructions from confocal microscopy images of WIHC of krt18:RFP larvae stained at 3 dpf with anti-RFP (keratinocytes, blue) and anti-phosporylated H3 (pH3, proliferation marker) antibodies. (B) Quantification of the number of pH3+/RFP+ (i.e. proliferating keratinocytes) cells in the CHT area. Each dot represents one single larva and the mean ± S.E.M. for each group of larvae is also shown. Scale bars: 100 μm. *p<0.05.

Tnfa and Tnfr2 deficiencies trigger H_2O_2 production in the skin

Hydrogen peroxide gradients were recently shown to contribute to the early influx of neutrophils in wound [31] and tumor [32]. Interestingly, however, H_2O_2 is not required for neutrophil detection of localized infection [33]. These gradients are created by the dual oxidase 1 (Duox1) [31] and sensed by neutrophils through the tyrosine kinase Lyn [34]. Although identified and best studied in the zebrafish, H_2O_2 is likely to play the same function in human neutrophils [34]. We firstly analyzed the expression of the gene encoding Duox1 and found that Tnfr2-deficient keratinocytes showed higher transcript levels of *duox1* than wild type animals (Fig. 5A). Next, using an H_2O_2-detecting fluorescence probe, we observed that Tnfr2-deficient larvae also produced H_2O_2 in the skin (Figs. 5B, 5C). We observed similar levels of labeling with the H_2O_2 probe in Tnfr2-deficient keratinocytes and in local keratinocytes after wounding (Fig. S7). Notably, H_2O_2 production by Tnfr2-deficient keratinocytes preceded the activation of NF-κB (Fig. S8). Consistent with these observations, genetic inhibition of Duox1 with a specific MO [31] was able to partially prevent the infiltration of neutrophils into the skin of Tnfr2-deficient larvae (Figs. 5D, 5E).

To further confirm this result, we designed a dominant negative (DN) form of Duox1 [35] and, notably, overexpression of DN-Duox1 was also able to partially prevent neutrophil infiltration in Tnfr2-deficient larvae (Figs. S9A, S9B). Furthermore, we knocked-down the H_2O_2 sensor of neutrophils, Lyn [34], and found full prevention of neutrophil infiltration in both Tnfr2- and Tnfa-deficient animals (Figs. 5F, 5G).

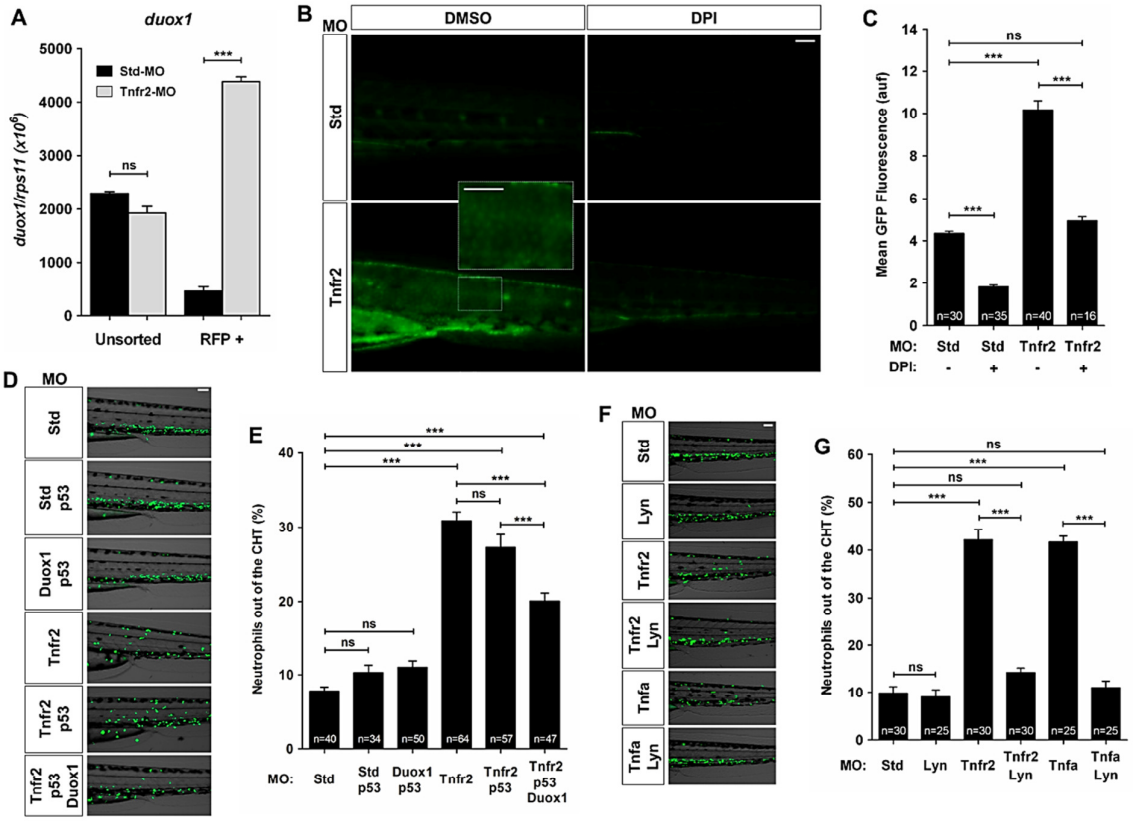

Figure 5. Tnfa and Tnfr2 deficiencies result in the Duox1-derived H_2O_2 production by keratinocytes. Zebrafish one-cell *krt18:RFP* (A), wild type (B, C) or *mpx:eGFP* (D-G) embryos were injected with standard control (Std), Tnfr2, Tnfa, Duox1/p53, and/or Lyn morpholinos (MO). (A) The expression of *duox1* gene was measured by RT-qPCR in FACS-sorted keratinocytes from 72 hpf wild type and Tnfr2-deficient larvae. (B,C) Wild type and Tnfr2-deficient larvae were dechorionated at 24 hpf and treated by immersion in 100 μM DPI or vehicle alone (DMSO) for 24 h and then labeled with 50 μM acetyl-pentafluorobenzene sulphonyl fluorescein. Representative images of green channels of Std and Tnfr2 morphants are shown. Note that single keratinocytes are labeled with the H_2O_2 probe in Tnfr2-deficient larvae (inset). (D-G) Rescues with Duox1 (D, E) and Lyn (F, G) MOs at 72 hpf. The differences in the neutrophils distribution (D, F) and quantification of neutrophil mobilization from the CHT to the skin in the indicated number of larvae per group from 3 different experiments (E, G) are shown. The mean ± S.E.M. for each group is shown. Scale bars: 100 μm. ns, not significant. ***p<0.001.

Pharmacological inhibition of Duox1 restores skin homeostasis in Tnfa- and Tnfr2-deficient animals

The above results prompted us to evaluate whether pharmacological inhibition of Duox1 using the NADPH oxidase inhibitor dibenziodolium chloride (DPI), which has been shown to inhibit Duox1 and H_2O_2 gradient formation in zebrafish [31,34], may restore skin homeostasis in Tnfa- and Tnfr2-deficient larvae. The results showed that DPI treatment completely inhibited the generation of H_2O_2 in the skin (Figs. 5B, 5C), the infiltration of neutrophils (Figs. 6A-6C) into this tissue and, more importantly, skin NF-κB activation (Figs. 6D-6F) in both Tnfa- and Tnfr2-deficient animals.

Collectively, these results demonstrate that the Tnfa/Tnfr2 axis is indispensable for skin homeostasis and its inhibition results in the release of Duox1-derived H_2O_2, local activation of NF-κB, induction of genes encoding Duox1 and pro-inflammatory mediators, and neutrophil infiltration.

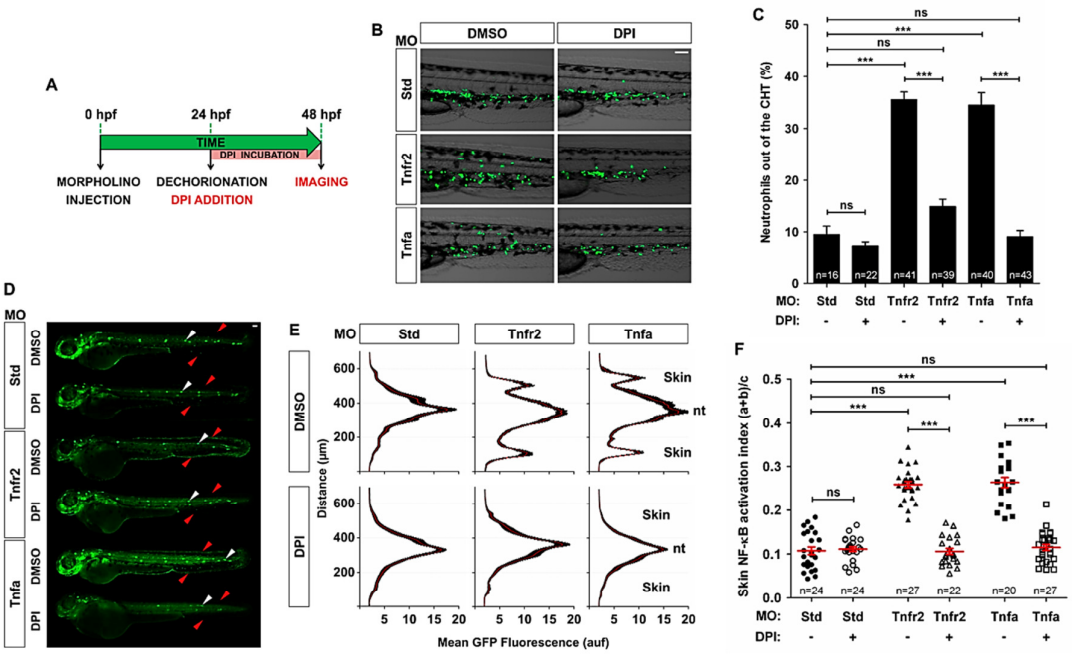

Figure 6. Pharmacological inhibition of Duox1 prevents skin inflammation in Tnfa- and Tnfr2-deficient zebrafish. Zebrafish one-cell *mpx:eGFP* (B,C) and *NF-κB:eGFP* (D-F) embryos were injected with standard control (Std), Tnfr2 or Tnfa morpholinos (MO). (A) Scheme showing the experimental procedure: embryos were dechorionated at 24 hpf and treated by immersion in 100 µM DPI or vehicle alone (DMSO) for 24 h. (B, C) Representative images of brightfield and green channels of the morphants at 48 hpf showing the differences in the neutrophils distribution (B) and quantification of neutrophil mobilization from the CHT to the skin in the indicated number of larvae per group from 3 different experiments (C). (D-F) Quantification of NF-κB activation in the skin of Tnfr2- and Tnfa-deficient larvae at 72 hpf. (E) Fluorescence intensity was measured for all the groups in the area indicated, as explained in the legend to Figure 1 (15 larvae per treatment from 3 different experiments). The skin and the notochord (nt) have been labeled to facilitate the larval orientation. Note the activation of NF-κB in the skin (red arrowheads) of Tnfr2-deficient larvae. Note the strong expression of NF-κB in neuromasts (white arrowheads). (F) Skin NF-κB activation index was defined as the fluorescence in the skin (a+b) relative to the total fluorescence of the whole larvae (c). Each dot represents the skin NF-κB activation index per single larva. The mean ± S.E.M. of the skin NF-κB activation index for each group of larvae is also shown. Scale bars: 100 µm. ns, not significant. ***p<0.001.

DUOX1 is induced in human psoriasis and lichen planus lesions

The crucial role of Duox1-generated H_2O_2 in the infiltration of neutrophils into the skin and the induction of NF-κB prompted us to investigate if this inflammatory signal may also play a role in human psoriasis. We analyzed by immunohistochemistry 10 healthy skins, and 8 lichen planus and 15 psoriasis lesions using an antibody to human DUOX1 (Fig. 7). The results showed that although DUOX1 was expressed at low levels in healthy epidermis, mainly in the granular layer, a drastic induction of this enzyme was obvious in the keratinocytes of the spinous layer of the epidermis from both psoriasis and lichen planus lesions. In some patients, the induction was

obvious in all keratinocytes of the spinous layer, while in others it was observed only in the upper layers of this stratrum. It was noticeable the localization of DUOX1 in the plasma membrane of psoriasis and lichen planus keratinocytes but also in their cytoplasm where it was accumulated in the upper side of these cells, i.e. facing the cornified layer. Although this particular distribution deserves further investigations, these results strongly suggest a role for DUOX1 in psoriasis and lichen planus.

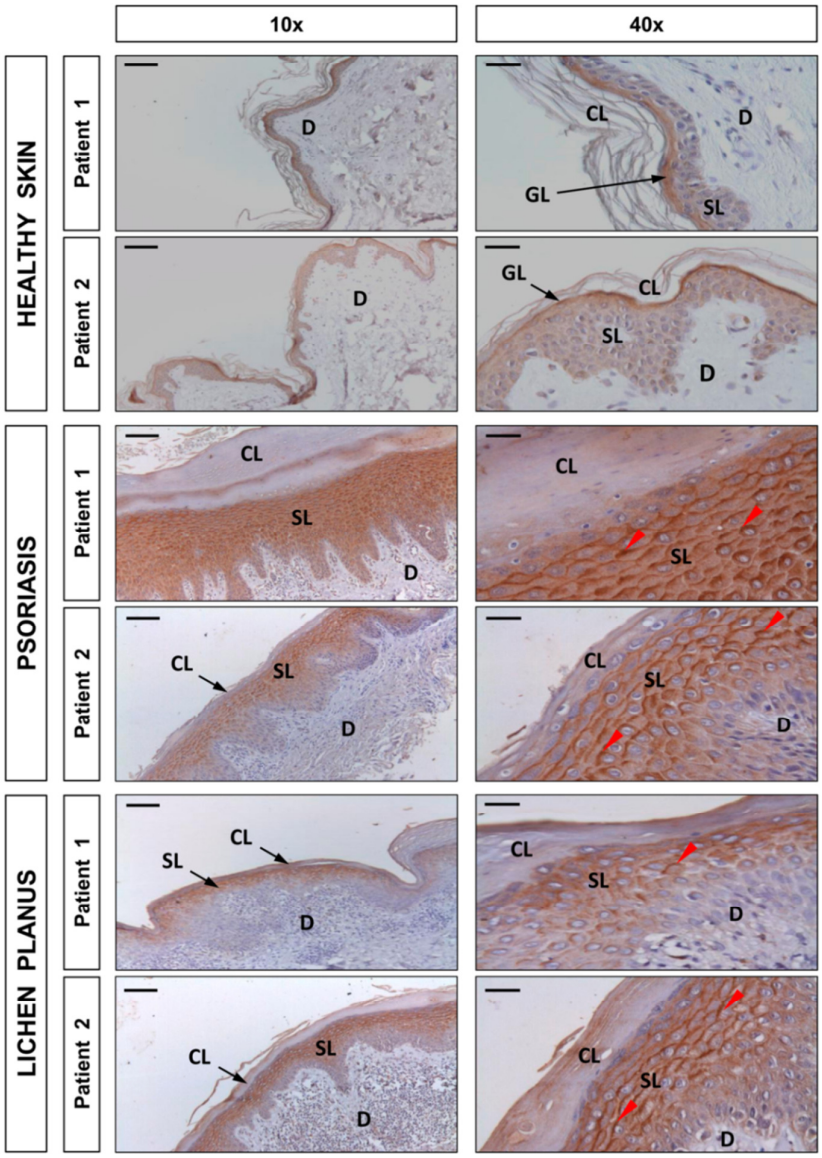

Figure 7. DUOX1 is induced in human psoriasis and lichen planus lesions. Representative images of sections from two healthy, two psoriatic and two lichen planus skin biopsies that have been immunostained with an anti-DUOX1 goat polyclonal antibody and then slightly counterstained with hematoxilin. Note that DUOX1 is weakly expressed in healthy epidermis, mainly in the granular layer (GL), while it is strongly expressed (red arrowheads) in the spinous layer (SL) of both psoriasis and lichen planus lesions. CL, cornified layer. D, dermis. Scale bars: 100 μm (left panel) and 30 μm (right panel).

Discussion

Increased production of TNFα is associated with the development of autoimmune/chronic inflammatory diseases, including psoriasis, lichen planus, rheumatoid arthritis and IBD. We have used the unique advantages of the zebrafish embryo for in vivo imaging and cell tracking to demonstrate that the genetic depletion of Tnfa or Tnfr2, but not Tnfr1, caused the infiltration of neutrophils into the skin and hyperproliferation of keratinocytes through the activation of an H2O2/NF-κB/Duox1 positive feedback inflammatory loop (Fig. 8). Strikingly, neutrophils, but not macrophages, are rapidly attracted to the skin. However, the activation of NF-κB and the induction of the gene encoding Il1b in the skin occurred before the appearance of the first neutrophils in the developing embryo. More importantly, DUOX1 was also strongly induced in the skin lesions of psoriasis and lichen planus patients. Collectively, these results (i) indicate a critical role of Tnfa/Tnfr2 signaling in the protection of the skin against oxidative stress, (ii) might explain the appearance of psoriasis and lichen planus in patients treated with anti-TNFα therapies [5-10], and (iii) support the idea that specific inhibition of the TNFα/Tnfr1 signaling axis while leaving TNFα/Tnfr2 signaling unaffected would inhibit the pathological effects of TNFα but reducing the side effects associated with this therapy [19,36]. This apparent discrepancy with TNFα-deficient mice, which do not show skin inflammation, may be due to developmental and/or physiological compensations, which probably do not exist in the human [37-39].

One of the most intriguing observations from this study is that impaired Tnfr2 signaling led to the induction of duox1 and the production of H2O2 by keratinocytes. H2O2 gradient was recently shown to contribute to the early influx of neutrophils in wound [31] and tumor [32], although it seems to be dispensable for neutrophil detection of localized infection [33]. To the best of our knowledge, this is the first study showing a role for Duox1-derived H2O2 in the induction of NF-κB in the skin in vivo, suggesting that H2O2 might play a critical role in the initiation and maintenance of chronic inflammatory diseases in both zebrafish and human. These observations suggest that antioxidants or inhibition of Duox1 might be therapeutic for the treatment of patients suffering from psoriasis, lichen planus and other inflammatory diseases. Supporting this notion, several studies using psoriasis and IBD mouse models have shown that transgenic overexpression of endogenous antioxidant genes promotes protection, while antioxidant gene knockout promotes sensitization (reviewed by [40,41]). Even more importantly, the antioxidant levels and the oxidative stress biomarkers are usually correlated with the disease severity and the extent of inflammation in the psoriasis and IBD patients [40-42]. Therefore, all these results taken together suggest that antioxidants should be considered as part of a more specific and effective therapy for the treatment of inflammatory skin diseases, including psoriasis and lichen planus. The ability of Duox1 inhibition by pharmacological approaches, but not of IL-1β, to restore skin homeostasis in Tnfa- and Tnfr2-deficient zebrafish embryos further supports this conclusion.

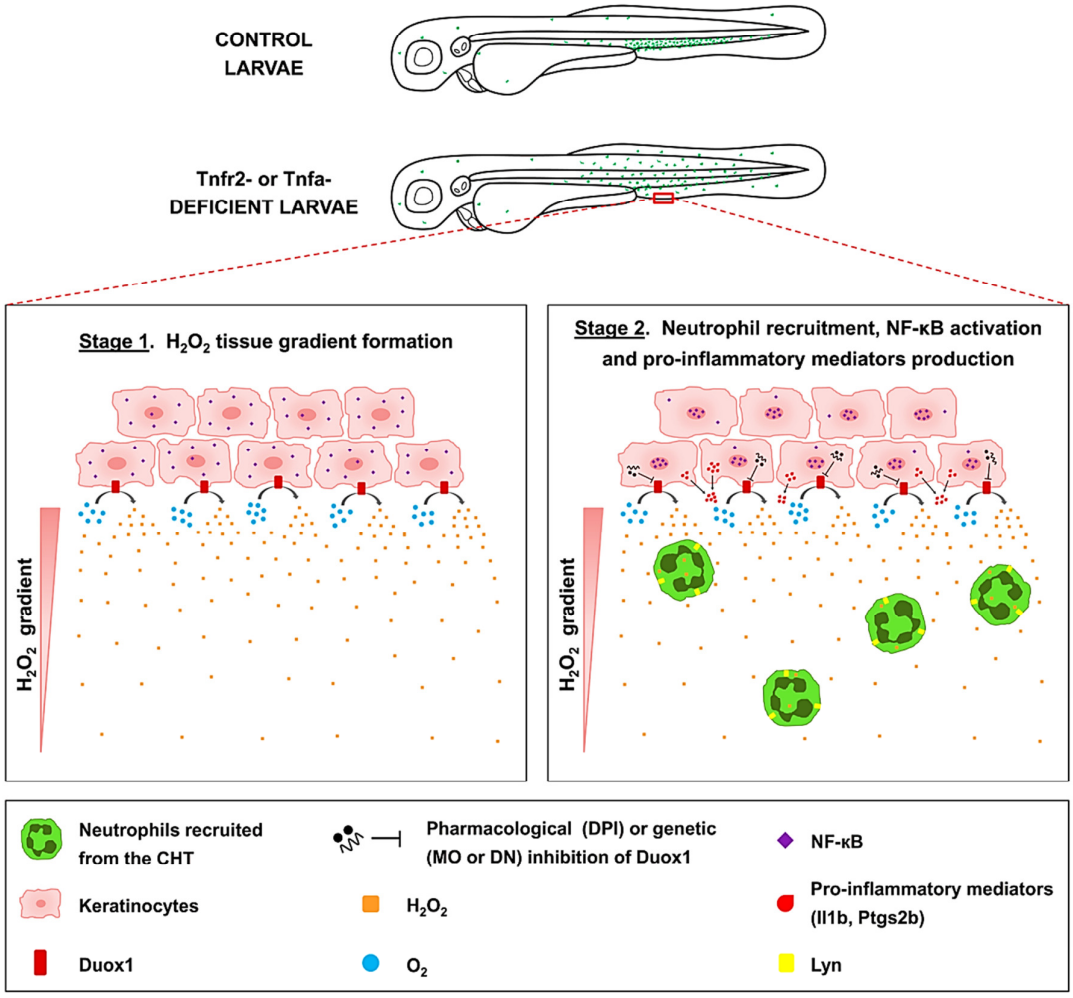

Figure 8: Proposed model illustrating the H_2O_2/NF-κB/Duox1 positive feedback inflammatory loop triggers in the skin of Tnfa- or Tnfr2-deficient zebrafish. Stage 1 (left panel): Tnfa or Tnfr2 deficiency triggers Duox1-dependent release of H_2O_2, which, in turn, promotes Lyn-mediated neutrophil infiltration. Stage 2 (right panel): H_2O_2 induces the activation of NF-κB which is then translocated to the nucleus and induces the activation of genes encoding pro-inflammatory mediators (Il1b, Ptgs2, and probably Duox1). Pharmacological or genetic inhibition of Duox1 restores skin homeostasis

It is known that different reactive oxygen species (ROS) act as second messengers, influencing various cellular signal transduction pathways, including NF-κB. However, there are still many inconsistencies concerning the influence of oxidative stress on NF-κB activity [43] and, unfortunately, most studies have been performed in vitro using H2O2 and cultured cells [44,45]. Such studies have shown that H2O2 can act as an activator of IκB kinases (IKKs) [46] or can inactivate these proteins [47], probably depending on the cell-type. More recently, it has been found that the same prolyl hydroxylases which confer oxygen sensitivity to the HIF pathway, namely PHD1 and PHD2, seem to act as repressors of the canonical NF-κB pathway through

mechanisms which could include direct hydroxylation of IKKβ [48]. Our epistasis study in zebrafish demonstrates for the first time that the absence of Tnfa/Tnfr2 signaling led to the production of H2O2 by keratinocytes which, in turn, resulted in NF-κB activation and the induction of genes encoding pro-inflammatory mediators. This self-perpetuating cycle may be of clinical importance in view of the presumably key role played by oxidative stress [40-42], HIF [49,50] and NF-κB in psoriasis and IBD. It is tempting to speculate that the Tnfa/Tnfr2 axis would be required to prevent skin oxidative stress through the regulation of ROS-detoxifying enzymes, as it has been reported for oligodendrocyte progenitor cells in vitro [51]. The model reported here might contribute to clarify the mechanisms involved in the regulation of oxidative stress by TNFα, the regulation of NF-κB activity by ROS, and the crosstalk between oxidative stress and inflammation in vivo.

The essential role played by NF-κB in the homeostasis of the skin is evidenced by the human X-linked genodermatosis incontinentia pigmenti (IP), which affects the regulatory subunit of IKK (IKKγ, NEMO) [17]. Humans suffering from this genetic disease exhibit severe skin inflammation, paradoxically due to impaired NF-κB activation and reduced resistance to TNFα/Tnfr1-mediated apoptosis [52,53]. Similarly, although NF-κB actively participates in the excessive inflammatory response observed in IBD patients [54,55], recent studies with mice defective in NF-κB activation have revealed that epithelial NF-κB activation is essential to preserve intestinal homeostasis [56,57]. Therefore, a critical NF-κB signaling balance is required for skin and gut homeostasis, since both excessive and defective epithelial NF-κB activation can result in inflammation. Similarly, while TNFα/TNFR1 axis was earlier appreciated to be involved in the apoptosis of both keratinocytes and enterocytes in the absence of NF-κB signaling [52,53,56,57], our results show that TNFα signaling through TNFR2 is also critically required for skin homeostasis. Whether the TNFα/TNFR2 axis is also required for gut homeostasis will require further investigation using germ free and gnotobiotic zebrafish larvae, since host-microbe interactions have a profound impact in gut physiology and are usually involved in IBD.

In conclusion, we have found that Tnfa signaling through Tnfr2 is indispensible required for the protection of the skin against oxidative stress-induced inflammation in the zebrafish. Thus, the absence of this signal triggers the local production of H2O2 by Duox1 which, in turn, activates NF-κB and result in the upregulation of genes encoding pro-inflammatory mediators and neutrophil infiltration. These results, together with the induction of DUOX1 in the skin lesions of psoriasis and lichen planus patients, reveal a crucial role of H2O2 and DUOX1 in skin inflammation and suggest that pharmacologic and genetic therapies that target these two key factors could provide innovative approaches to the management of psoriasis, lichen planus and other chronic inflammatory diseases.

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Supplementary Figures

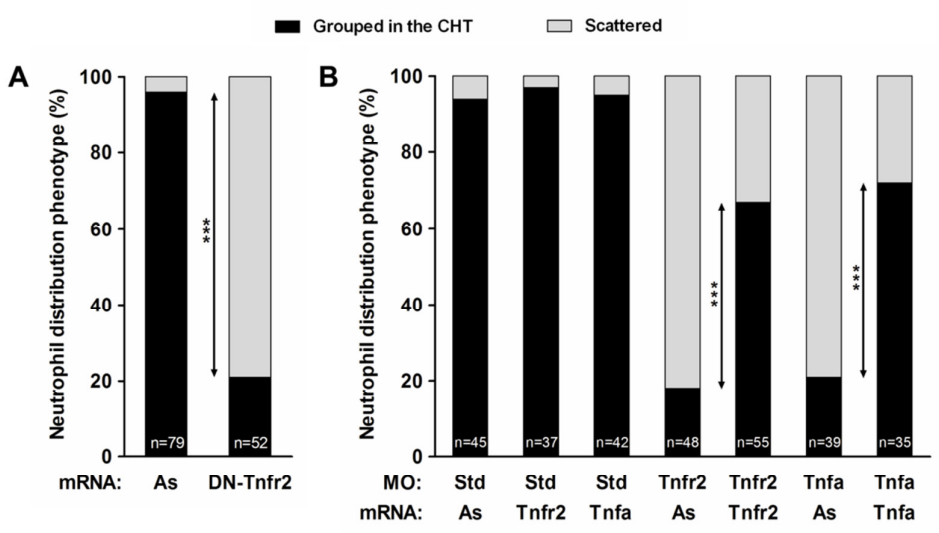

Figure S1. Tnfa and Tnfr2 deficiencies result in neutrophil mobilization. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Tnfr1+Tnfr2 morpholinos (MO) alone or combined with antisense (As), Tnfa, Tnfr2 or DN-Tnfr2 mRNAs. The phenotype of 3 dpf larvae was classified as neutrophil grouped in the CHT or scattered, as described in Figure 1. ***p<0.001.

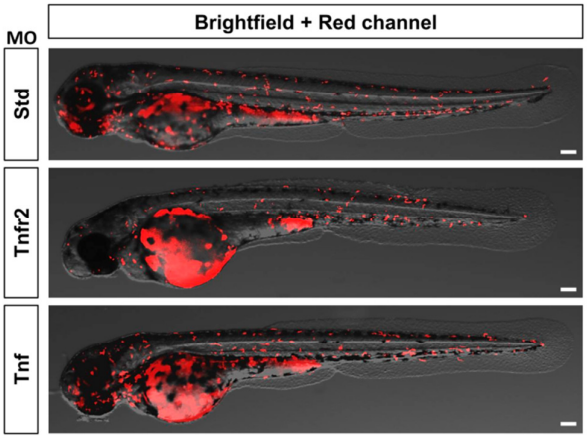

Figure S2. Macrophage distribution is not altered in Tnfa or Tnfr2-deficient larvae. Zebrafish one-cell *mpeg1*::mCherry embryos were injected with standard control (Std), Tnfr2 and Tnfa morpholinos (MO). Representative images showing macrophage distribution in 72 hpf larvae. Scale bars: 100 μm.

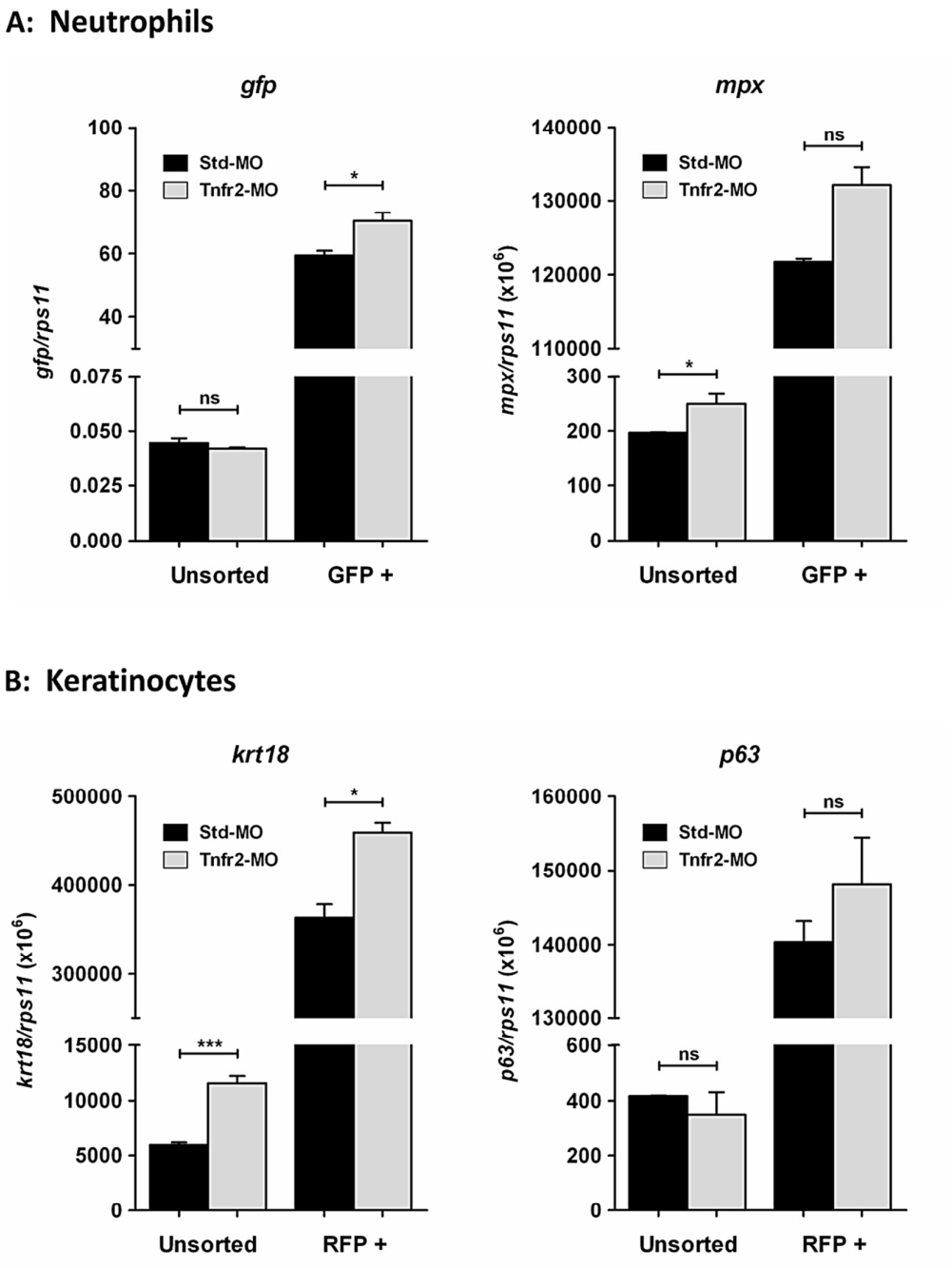

Figure S3. Efficiency of sorting of neutrophils and keratinocytes. Zebrafish one-cell *mpx*:eGFP (A) or *krt18*:RFP (B) embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). Neutrophils (A) and keratinocytes (B) were FACS-sorted from 72 hpf larvae and the expression of *gfp* (A) and *krt18* (B) genes was measured by RT-qPCR in unsorted and sorted cells. The data are shown as the mean ± S.E.M. ns: not significant. *p<0.05; ***p<0.001.

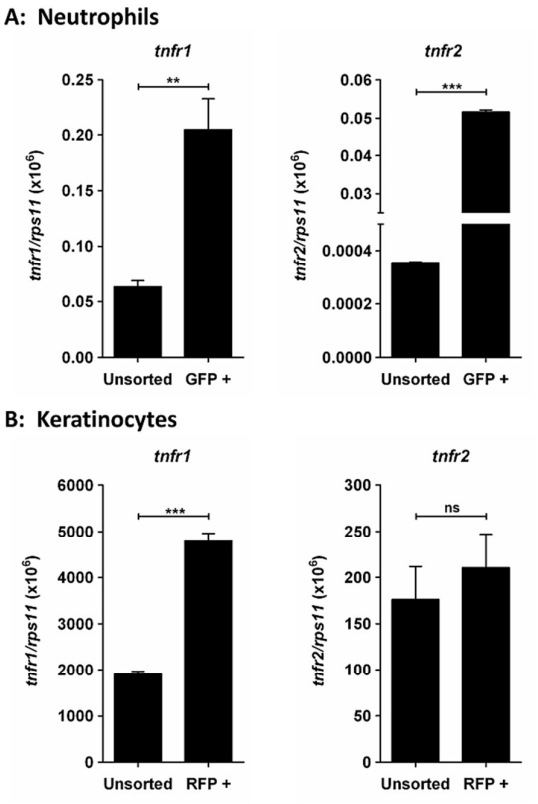

Figure S4. Neutrophils and keratinocytes expressed both Tnf receptors. Neutrophils (A) and keratinocytes (B) were FACS-sorted from 72 hpf *mpx:eGFP* and *krt18:RFP* larvae, respectively, and the expression of *tnfr1* and *tnfr2* genes was measured by RT-qPCR in unsorted and sorted cells. The data are shown as the mean ± S.E.M. ns: not significant. **p<0.01; ***p<0.001.

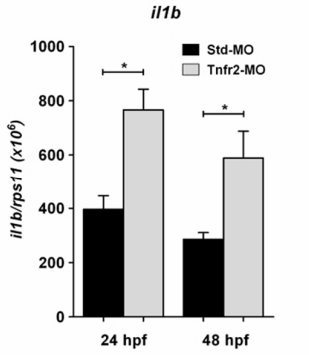

Figure S5. IL-1β is induced in Tnfr2-deficient embryos before the emergence of neutrophils. Zebrafish one-cell wild type embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). The expression of *il1b* gene was measured by RT-qPCR in whole embryos at 24 and 48 hpf. The data are shown as the mean ± S.E.M. *p<0.05.

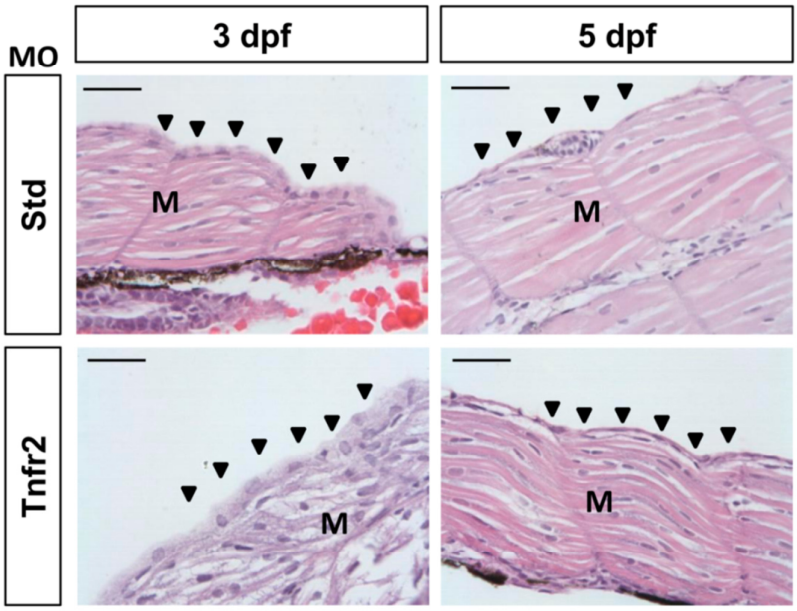

Figure S6. The skin of Tnfr2-deficient larvae do not show histopathological alterations. Zebrafish one-cell embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). At 3 (A) and 5 (B) dpf, the larvae were fixed, embedded in Paraplast Plus, sectioned at 5 μm and stained with H&E. M, muscle. Arrowheads, skin. Scale bars: 50 μm.

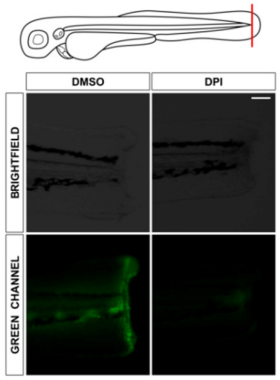

Figure S7. Pharmacological inhibition of Duox1 inhibits H_2O_2 production after wounding. Zebrafish one-cell wild type embryos were treated at 72 hpf by immersion in 100 μM DPI or vehicle alone (DMSO) in the presence of 50 μM acetyl-pentafluorobenzene sulphonyl fluorescein and tailfins were then transected. Representative images of the formation of the H_2O_2 gradient at 1 h post-wounding. Note that DPI treatment completely inhibits H_2O_2 formation at the wound. Scale bars: 100 μm.

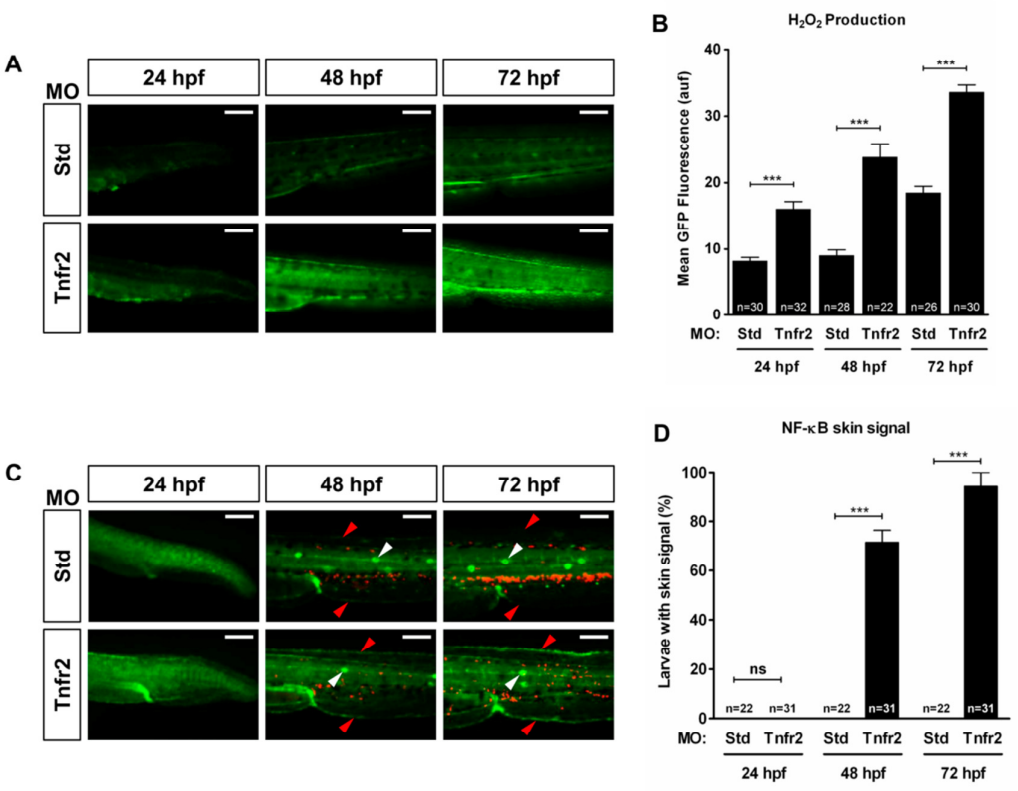

Figure S8. H_2O_2 production by Tnfr2-deficient keratinocytes preceded the activation of NF-κB. Zebrafish one-cell wild type (A, B) or *lyz:dsRED*; *NF-κB:eGFP* (C, D) embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). (A, B) larvae were dechorionated at 24 hpf and then labeled with 50 μM acetyl-pentafluorobenzene sulphonyl fluorescein at 24, 48 and 72 hpf. Representative images of green channels of Std and Tnfr2 morphants (A) and quantification of green fluorescence in the indicated number of larvae (B) are shown. Note that increased H_2O_2 production by skin keratinocytes is already observed at 24 hpf. (C) Representative pictures showing NF-κB activation levels in control and Tnfr2-deficient larvae at 24, 48 and 72 hpf. Note that NF-κB is induced in the skin (red arrowheads) of Tnfr2-deficient larvae at 48 h and that neutrophil dispersion is observed at 72 hpf and, to some extent, at 48 hpf. The neuromasts are indicated with white arrowheads. (D) Quantification of the percentage of larvae showing activation of the NF-κB in the skin. The results are shown as the mean ± S.E.M. The number of larvae analyzed is also indicated. Scale bars: 100 μm. ns, not significant. auf: arbitrary units of fluorescence. *p<0.05; **p<0.01; ***p<0.001.

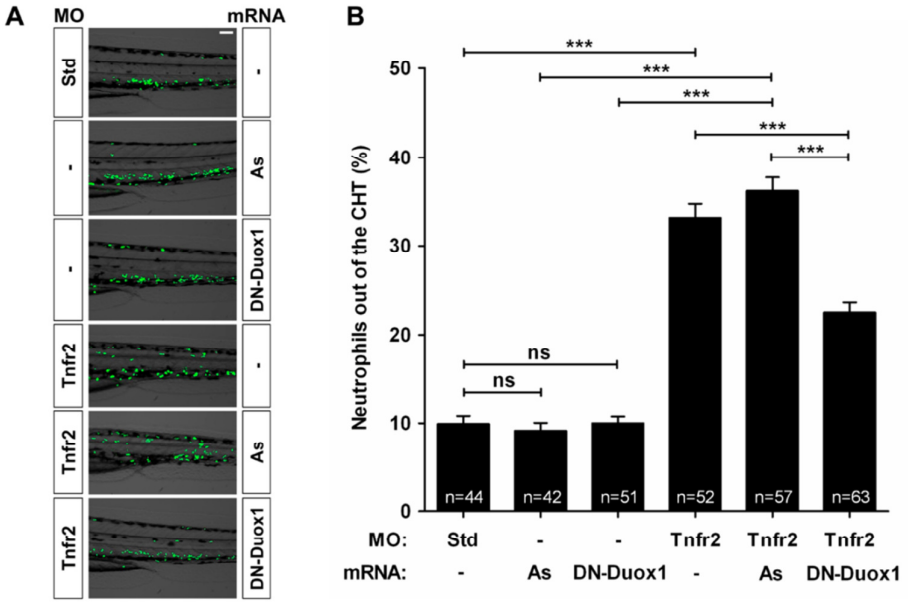

Figure S9. Genetic inactivation of Duox1 using a DN form partially prevents neutrophil infiltration into the skin of Tnfa- and Tnfr2-deficient zebrafish. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr2 or Tnfa morpholinos (MO) alone or combined with antisense (As) or DN-Duox1 mRNAs. Representative images of brightfield and green channels of morphants at 72 hpf showing the differences in the neutrophils distribution (A) and quantification of neutrophil mobilization from the CHT to the skin in the indicated number of larvae per group from 3 different experiments (B). The mean ± S.E.M. for each group is shown. Scale bars: 100 μm. ns, not significant. ***p<0.001.

Video S1. Neutrophils infiltrate the skin in Tnfr2-deficient larvae. Animations of tridimensional projections obtained by laser confocal microscopy of STD morphants showing neutrophils in green (GFP) and basal keratinocytes in red (p63). See Legend to Figure 1 for details.

Video S2. Neutrophils infiltrate the skin in Tnfr2-deficient larvae. Animations of tridimensional projections obtained by laser confocal microscopy of Tnfr2 morphants showing neutrophils in green (GFP) and basal keratinocytes in red (p63). See Legend to Figure 1 for details.

Video S3. NF-κB is induced in the skin of Tnfr2-deficient larvae. Animations of tridimensional projections obtained by laser confocal microscopy of STD morphants showing NF-κB activity in green and basal keratinocytes in blue (RFP). See Legend to Figure 3 for details.

Video S4. NF-κB is induced in the skin of Tnfr2-deficient larvae. Animations of tridimensional projections obtained by laser confocal microscopy of Tnfr2 morphants showing NF-κB activity in green and basal keratinocytes in blue (RFP). See Legend to Figure 3 for details.

Chapter 7

General Discussion

General discussion

All organisms face a constant challenge for surviving in their environment. Among other host responses, inflammation is an important process that occurs after tissue injury, infection or stress. The inflammatory response involves the cooperation of different tissues and cells in order to fulfill its essential goal: to restore host homeostasis. Neutrophils are one the major pathogen-fighting immune cells involved in this process and that are known for an outstanding recruitment ability and capacity to engulf and kill pathogens quickly through a combination of cytotoxic mechanisms. Several models were used throughout the years to better understand their mode of action but before the use of zebrafish, none of these was able to allow the study of neutrophil recruitment and function in vivo by using non-invasive procedures. Among other molecules chemokines and its receptors are important players in neutrophil response and behavior. In 2010, a study from the Crosier's lab reported the existence of a Cxcl8 homologue in zebrafish, along with two CXC chemokine receptors, that were expressed in several tissues and induced after immune stimulation (1). This prompted us to study the function of Cxcl8 in zebrafish inflammation, for which a true homologue is lacking in the mouse, the animal model which was then most prevalently used in the in vivo study of inflammation.

As mentioned before, zebrafish possesses two Cxcl8s, the Cxcl8-l1 and the Cxcl8-l2 (2). As both could play a role in inflammation, we initiated our work with an intensive and full characterization of their involvement in neutrophil recruitment in zebrafish inflammation elicited upon wounding (Chapter 2; (3)) or upon infection (short-report under final preparation included here as Chapter 3). We started our Cxcl8 study by confirming that Cxcl8-l1 and Cxcl8-l2 are up-regulated in tail fin tissue after wounding and that both chemokines are essential for normal neutrophil recruitment and resolution after wounding. Surprisingly, the analysis of neutrophil recruitment timelapse movies by an open-source MATLAB package, PhagoSight (4) allowed us to conclude that in the absence of Cxcl8s the migratory speed of the few neutrophils able to migrate towards the wound was significantly increased. This led us to propose the existence of different neutrophil subpopulations with probably distinct functions that could differentially respond to CXCL8 chemokines and other chemoattractants expressed locally during the inflammatory process and thus exhibit distinct migratory behaviors. Pase et al (5) reported a possible example of such by showing the existence of hydrogen peroxide (H_2O_2) consumers and producers among neutrophils at an early stage of the inflammatory response to wounds. At the same time, Sarris et al (6) reported a similar finding using a controlled cellular source of Cxcl8-l1. Their data showed that Cxcl8-l1 is guiding neutrophils by biasing cell speed and restricting cell motility near to the source. Besides this they showed in vivo that Cxcl8-l1 is able to establish robust tissue-bound gradients, via its binding to heparin sulphate proteoglycans (HSPGs), which are responsible for the recruitment and retention of leukocytes at sites of infection. Overall, it is very likely that the real situation may result from a combination of both models, in that Cxcl8s tissue-bound gradients and other

chemoattractants are able to guide neutrophil subpopulations, to bias cell speed and to restrict cell motility near to inflamed sites.

Having established a role for both Cxcl8s in tissue injury-elicited inflammation, we next addressed their role in the inflammatory process triggered upon infection (Chapter 3). We aimed here to understand whether these chemokines could play or not distinct functions in neutrophil recruitment under different inflammatory conditions - tissue damage versus infection. In the last years, several papers have reported data on the expression and/or role of zebrafish Cxcl8 in infection, but most of them have focused only on Cxcl8-l1 without any concern regarding Cxcl8-l2. So first we confirmed that Cxcl8-l1 and Cxcl8-l2 are induced after different infectious stimuli. Then, using the *S*. Typhimurium infection model, we observed that both cxcl8-l1 and cxcl8-l2 morphants presented lower survival rates upon infection as well as lower neutrophil recruitment and *S*. Typhimurium clearing in comparison to control conditions. Altogether these results place as well both chemokines as relevant mediators in infection-elicited inflammation in line with what was previously shown for the wound-associated inflammatory process. Interestingly, after the simultaneous knock down of both Cxcl8s we were not able to observe any further synergistic reduction on the larvae survival rates upon *S*. Typhimurium infection as well as on the recruitment of neutrophils to wounds (data not shown). Two hypotheses may explain these effects. On the one hand, Cxcl8s may be working as a heterodimer. Alternatively, Cxcl8 function in zebrafish may be a product of a cooperative action of both chemokines over tissues and cells. Regardless of this, our results and those from others strongly indicate that the basic principles of neutrophil recruitment seem to be shared by zebrafish and humans despite their divergence from a common ancestor occurred about 450 millions of years ago. This makes the zebrafish, an ideal and very promising animal model for the in vivo study of Cxcl8 and other chemokines. Several of these as well as their receptors have been shown to be key players in various cancers (7, 8) and autoimmune diseases (9). As such, understanding their mode of action in vivo will be central for the development of better and more efficient therapeutic approaches for these pathologies.

One of the most important findings regarding neutrophil recruitment mechanisms came with the discovery of a new function for an old player involved in the neutrophil response and function, the H_2O_2. Using zebrafish as a model, Niethammer et al (10) reported that dual oxidase 1 (DUOX1), a NADPH oxidase, is actually responsible for the formation of a H_2O_2 tissue gradient after wounding, that acts as a main trigger of early leukocyte recruitment. This report suddenly gave rise to a whole new line of study in neutrophil recruitment. Several major advances were made, including the identification of the redox sensors in leukocytes and epithelial cells, the SFKs Lyn (11) and Fynb(12) respectively, as well as the enzyme responsible for H_2O_2 clearing, the neutrophil-delivered myeloperoxidase, that controls further tissue damage that might be caused by excessive H_2O_2 presence (5). Despite the increased knowledge about H_2O_2 signaling, the mechanisms that regulate in vivo DUOX1 activation are poorly understood. We next focused our efforts on attempting to identify which early signaling molecules could be acting as Duox1 activity modulators, and thus controlling early neutrophil recruitment and inflammatory process (please refer to Chapter 4; (13)). In this regard, we concentrated our attention on two mediators, known to

be released upon wounding, namely, ATP and calcium. By combining the use of genetic tools with pharmacological drugs, we were able to show in vivo that the release of the early danger signal ATP following tissue injury, activates purinergic P2Y receptors, and modulates Duox1 activity through phospholipase C (PLC) and intracellular calcium signaling. Furthermore, we showed that Duox1-derived H_2O_2 is able to trigger the NF-κB inflammatory signaling pathway. Our findings clarify the role of these early signals in the modulation of wound inflammatory responses reinforcing Duox1/H_2O_2 as new potential therapeutic targets for inflammatory diseases. We believe that our results have importantly paved the way for future studies on the mechanisms orchestrating H_2O_2 oxidative regulation of complex biological processes, such as inflammation, regeneration and cancer.

After having clarified how early danger signals were able to modulate Duox1 activation, we next aimed to understand if these early H_2O_2 gradients could also affect neutrophil recruitment at later phases. Considering our previous results, we hypothesized and further investigated the existence of a crosstalk between two neutrophil chemoattractant molecules, H_2O_2 and Cxcl8s (Chapter 5; manuscript under final preparation). We verified that the early signal H_2O_2 is a key regulatory factor in the in vivo wound response that crosstalks with Cxcl8 signaling by modulating Cxcl8-l2 expression through a complex mechanism involving SFKs, p38 and Jnk/AP1 signaling pathways, as well as histone 3 modifications and AP1 binding to cxcl8-l2 proximal promoter. Importantly, these results raised several fundamental questions that will need further attention in the future, such for example how H_2O_2 is modulating p38 and Jnk signaling and ultimately, c-Jun phosphorylation levels, or which are the mechanisms involved in the modulation of histone 3 epigenetic markers. Altogether these results further suggest to us that H_2O_2 is probably able to modulate the expression and/or activity of several other pro-inflammatory and pro-resolution molecules, what may explain for example the results obtained by Yoo et al. (12). Again, these findings point out Duox1/H_2O_2 as new potential drug targets for the development of novel anti-inflammatory therapies for the clinical management of various inflammatory pathologies.

In this regard, we were then able to demonstrate the specific involvement of Duox1/H_2O_2 in skin inflammation in a translational study from the zebrafish to humans (Chapter 6; (14)). In this, we have first shown that Tnfa signaling through Tnfr2 is required for the protection of the skin against oxidative stress-induced inflammation in the zebrafish. Consequently, we showed that the absence of this signal triggers the local production of H_2O_2 by Duox1 which, in turn, activates NF-κB and results in the upregulation of genes encoding pro-inflammatory mediators (such as Il1β and Cxcl8s) and neutrophil infiltration. Additionally we found that DUOX1 is induced in the skin lesions of psoriasis and lichen planus patients, indicating an important role of H_2O_2 and DUOX1 in human skin inflammation. This H_2O_2-mediated mechanism may probably be present in several other chronic inflammatory diseases. As such, pharmacologic and genetic therapies that target these key factors could provide innovative approaches to the management not only of psoriasis and lichen planus but also of other main chronic inflammatory diseases.

To summarize, in this thesis we were able to unravel important and new findings concerning the mechanisms that modulate neutrophil recruitment and inflammatory response such as:

- Cxcl8s are key modulators of neutrophil recruitment and behavior both in wounding and infection;
- Cxcl8s modulate recruited neutrophil speed and possibly different neutrophil subpopulations;
- Cxcl8s are equally important for host survival upon infection;
- Early danger signal ATP activates purinergic P2Y receptors, and modulates Duox1 activity through phospholipase C (PLC) and intracellular calcium signaling in vivo;
- H_2O_2 is able to modulate NF-kB signaling pathway in vivo;
- H_2O_2 is a key regulatory factor in the in vivo wound response that crosstalks with Cxcl8-l2 signal by a complex mechanism involving SFKs, p38 and Jnk/AP1 signaling pathways, as well as histone 3 modifications and AP1 binding to cxcl8-l2 proximal promoter.
- Tnfa/Tnfr2 signaling is required for skin protection against oxidative stress-induced inflammation in the zebrafish;
- DUOX1 is induced in the skin lesions of psoriasis and lichen planus patients, revealing a potential crucial role of H_2O_2 and DUOX1 in human skin inflammation;
- H_2O_2/Duox1 are potential drug targets for the development of new anti-inflammatory treatments.

At the end all these finding bring new and interesting questions to the field like for example, i) how Cxcl8-l1 and Cxcl8-l2 exert their function in neutrophil recruitment, ii) whether these chemokines act as heterodimers, ii) how H_2O_2 is able to modulate distinct signaling pathways and epigenetic markers, and iii) whether this Duox1-derived H_2O_2 modulator effect is present or not in other chronic inflammatory diseases. Much more work will be needed to clarify these and other important questions in order to fully establish the exact chemotactic signals, inflammatory mediators and signaling proteins, besides the ones reported here, that are important throughout the different phases of the inflammatory response. Undoubtedly, zebrafish will be an ideal model to study these complex processes and to increase further our knowledge in the area so as to advance in the development of more effective treatments for inflammatory diseases by more efficiently targeting specific key components.

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Appendixes

Appendixes

Articles concerned with this thesis:

- "Cxcl8-l1 and Cxcl8-l2 are both required in the zebrafish defense against Salmonella Typhimurium"; **Sofia de Oliveira**, Azucena Lopez-Muñoz, Francisco Juan Martinez, Jorge Galindo-Villegas, Victoriano Mulero * and Ângelo Calado*; Manuscript under final preparation; (* These authors have contributed equally to the work);

- "Duox1-derived H2O2 modulates Cxcl8 expression after wounding via JNK/c-Jun/AP1 signaling and chromatin modifications"; **Sofia de Oliveira**, Pierre Boudinot, Ângelo Calado* and Victoriano Mulero*; Manuscript submited; (*These authors contributed equally to this work).

- "ATP modulates acute inflammation in Vivo through Dual Oxidase 1-derived H_2O_2 production and NF-κB activation"; **Sofia de Oliveira**, Azucena López-Muñoz, Sergio Candel Pablo Pelegrin, Ângelo Calado and Victoriano Mulero J Immunol. 2014 May 19. pii: 1302902. [Epub ahead of print]

- "Tnfr2 deficiency triggers skin inflammation in the zebrafish via the production of H2O2 and the activation of the NF-κB signaling pathway"; **Sergio Candel*, Sofia de Oliveira***, Azucena Lopez-Muñoz, Diana García-Moreno, Raquel Espín-Palazón, Sylwia D. Tyrkalska, María L. Cayuela, Stephen A. Renshaw, Huai-Jen Tsai, José Meseguer, María P. Sepulcre, Victoriano Mulero; PLoS Biol. 2014 May 6;12(5):e1001855. doi: 10.1371/journal.pbio.1001855. eCollection 2014 May; (* These authors have contributed equally to the work);

- "Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response"; **de Oliveira S**, Reyes-Aldasoro CC, Candel S, Renshaw SA, Mulero V, Calado A.; J Immunol. 2013 Apr 15;190(8):4349-59. doi: 10.4049/jimmunol.1203266. Epub 2013 Mar 18;

Articles not concerned with this thesis

- "Regulation of Immunity and Disease Resistance by Commensal Microbes and Chromatin Modifications During Zebrafish Development"; Jorge Galindo-Villegas; Diana García-Moreno; **Sofia de Oliveira**; José Meseguer; Victoriano Mulero; PNAS 2012 109 (39) E2605–E2614; published ahead of print September 4, 2012, doi:10.1073/pnas.1209920109;

Regulation of immunity and disease resistance by commensal microbes and chromatin modifications during zebrafish development

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How fish larvae are protected from infection before the maturation of adaptive immunity, a process which may take up to several weeks in most species, has long been a matter of speculation. Using a germ-free model, we show that colonization by commensals in newly hatched zebrafish primes neutrophils and induces several genes encoding proinflammatory and antiviral mediators, increasing the resistance of larvae to viral infection. Commensal microbe recognition was found to be mediated mainly through a TLR/MyD88 signaling pathway, and professional phagocytes were identified as the source of these immune mediators. However, the induction of proinflammatory and antiviral genes, but not of antimicrobial effector genes, also required the covalent modification of histone H3 at gene promoters. Interestingly, chromatin modifications were not altered by commensal microbes or hatching. Taken together, our results demonstrate that gene-specific chromatin modifications are associated with the protection of zebrafish larvae against infectious agents before adaptive immunity has developed and prevent pathologies associated with excessive inflammation during development.

epigenetic | cytokines | evolution | gene regulation | live imaging

Our understanding of the development of the vertebrate immune system has increased greatly during recent decades. Findings so far strongly suggest that events that occur in the early life stages of life have a profound impact on the organism's later development (1), structure (2), and function (3). Traditionally, the study of animal development has focused on understanding how interactions among animal cells trigger developmental pathways. A frequent assumption is that all the steps involved in the development of a complex multicellular organism are genetically preordained (4). Only now, however, the field of ecological developmental biology begun to focus on the idea that some developmental triggers may come from the environment (5). Particularly interesting are the events that happen around the time of birth. In this early developmental stage, all vertebrates are subjected to an imminent colonization by a diverse microbiota inhabiting the surrounding environment. Among humans of developed countries, such microbial influence has tended to be avoided, or, at least diminished, through high standards of hygiene. In contrast, researchers into host–microbe relationships recently have established that the first contact between the two entities is essential for the maturation of immunity. This phenomenon has been called "developmental immunologic programming" (DIP) (6). DIP is a process whereby an environmental factor acting during a sensitive or vulnerable developmental period exerts effects that impact the structure and function of organs in ways that, in some cases, persist throughout life (7). This process is not exclusive to higher vertebrates; indeed, in lower taxa, such as fish, it has been known as "bacterial priming" (8). Therefore, DIP seems to be a conserved feature that has been preserved throughout the evolutionary process.

Immune-competent cells recognize microbial components that are not present in any of their structures but are conserved among pathogens. Recognition occurs mainly via receptors that are expressed in all cells of a given type (9). Receptors of the innate immune system, called "pattern-recognition receptors," form two well-studied families with huge recognition capacities: the transmembrane Toll-like receptors (TLRs) and the intracytoplasmic Nod-like receptors (10). Their role in sensing is indispensable, and in mammals it is well recognized that all TLRs discovered to date, with the exception of TLR3, signal via their associated adaptor molecule, myeloid differentiation primary response protein 88 (MyD88) (11). Similarly, an almost complete set of TLRs has been described in the pufferfish *Fugu rubripes* and in the zebrafish *Danio rerio* (12), and, with the exception of TLR4 (13, 14), these TLRs seem to be functional orthologs of mammalian TLRs. Thus, they are able to sense the same ligands (15–17), use similar adaptor molecules for signaling (15), and activate the transcription factor NF-κB (13, 15, 18). In addition, it has been shown that in zebrafish MyD88 modulates innate immune responses to microbes (19–22).

Apart from the recognition mechanisms, vertebrates have developed means to support large societies of microbial partners during their life cycles. However, one puzzling issue is that the supported microbial partners have the same conformational, molecular, or locomotive structures as closely related pathogens, indicating that microbe-associated molecular patterns (MAMPs) are not limited to pathogens. Therefore, herein we use the term "MAMPs" instead of the term "pathogen-associated molecular patterns" (PAMPs).

This recognition feature is particularly conspicuous in teleost fish, because, although this phylogenetic group has developed a completely and fully functional immune system, it lives in one of the most aggressive habitats, the aquatic ecosystem. In this unique habitat an impressive number and diversity of microorganisms coexist (23–26). Therefore, fish might have specific functional mechanisms for discriminating between the threats of a pathogenic origin and the signals that come from commensals. The mechanisms controlling this fragile equilibrium are largely unknown but are thought to be mediated by specialized receptors, such as the TLRs. Although beneficial, this response could threaten the host integrity when uncontrolled and directed toward the host. In an effort to understand how microbial commensals are host-supported, powerful germ-free (GF) experimental approaches have been developed. For example, it has been noticed that intestines of GF mice can initiate but cannot complete their differentiation

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The authors declare no conflict of interest.

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when particular associations between host and microbes are lacking (4). Using a similar model, Hrncir et al. (27) found that bacterial LPS, which is present as a contaminant in mouse food, is needed to develop a healthy phenotype. Unfortunately, available model species are scarce, and most findings under GF conditions are derived largely from a few mammalian species, mainly rodents (28) and swine (29). Only recently, another species, the zebrafish, has emerged as a key and powerful model organism to widen basic and biomedical animal research (30–35). To date, the study of host–microbe interactions in GF conditions in fish has proven effective for studying developmental processes, including immunity (36, 37). Growing evidence in GF zebrafish larvae throws light on the profound impact that bacteria have in the gut when it first is internally colonized. As in other vertebrate models, the main changes involved rapid epidermal degeneration and altered enterocyte morphology and proliferation (36, 38–40). In addition, a recent study showed that the presence not only of commensals but also of multiple bacterial secretion factors is essential for influencing intestinal epithelial cell proliferation, suggesting that normal gut microbiota exert a direct modulation of the β-cathenin pathway, independent of inflammation (22). In the present study, we used zebrafish larvae as a vertebrate model of innate immunity to test the hypothesis that the contact with commensal microbiota after hatching is an essential external environmental triggering factor to shape the development of immunity and disease resistance.

Results

Zebrafish Innate Immune Development Is Regulated by Microbial Presence. To characterize the host response of zebrafish embryos after their first exposure to commensal microorganisms in rearing water, wild-type zebrafish embryos were raised under GF or conventional (CONR) conditions until 96 h postfertilization (hpf). Under standard culture conditions, the animals started to hatch at 48 hpf. Thus, to study the effect of the contact of CONR animals with commensal microbes after hatching, analyses were conducted at 48, 72, and 96 hpf. At 48 hpf, animals in both groups presented similar levels of IL-1β transcripts. Differences between CONR and GF fish became more evident at 72 hpf, returning to basal levels at 96 hpf (Fig. 1*A*). To clarify whether this response increased gradually during the time of continuous exposure to commensal microbes, a time-series analysis was performed. Zebrafish embryos were allowed to hatch naturally from their eggs at 48 hpf, and 30 individual samples from each group were collected and pooled at 0, 3, 8, and 24 h posthatching (hph). Datasets revealed a time-dependant response of CONR hatched embryos compared with the GF embryos (Fig. 1*B*). The response of the CONR group was characterized by the gradually

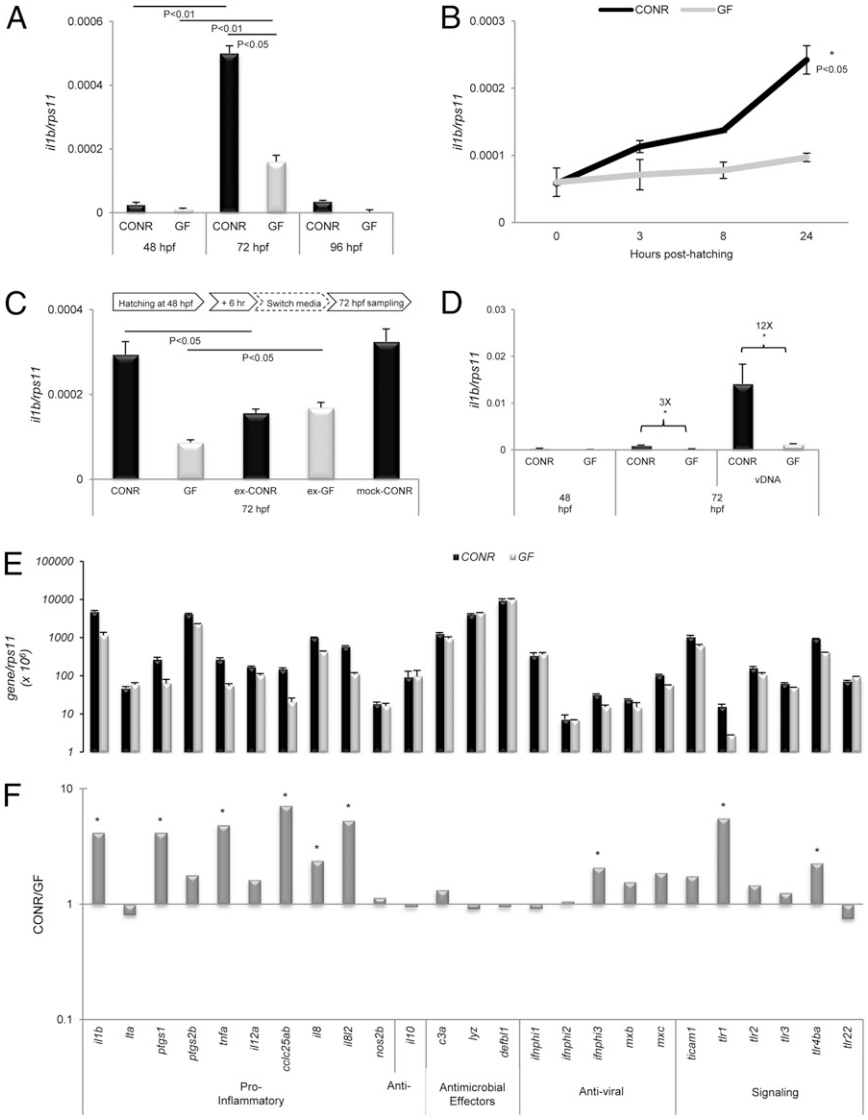

Fig. 1. Bacterial stimulation in the newly hatched zebrafish results in the activation of a mild inflammatory reaction followed by immunological tolerance. (*A*) qRT-PCR assay (IL-1β) of whole 48-, 72-, and 96-hpf naturally hatched larvae reared CONR or GF. (*B*) Starting 3 h after hatching (48 hpf), significantly higher IL-1β transcripts were found in CONR than in GF larvae. (*C*) Animals in both groups were raised following standard CONR or GF techniques and conventionalized at 48 hpf, 6 h after hatching. A mock switch (Mock-CONR) with CONR larvae handled identically but maintained in the same condition was included also. A switch in IL-1β transcript levels was observed in 72-hpf ex-CONR and ex-GF larvae. (*D*) Proinflammatory activity in zebrafish larvae raised in CONR or GF conditions revealed a threefold change between groups starting at 72 hpf. At this time, we tested the microbial priming effect using 50 larvae of each group that were incubated with 50 μg/mL vDNA for 4 h. (*E*) Commensals up-regulate genes intimately associated with innate immunity (identified in *F*) to significantly higher levels in CONR than in GF animals at the time of hatching. (*F*) Fold increases of statistical significance (denoted by asterisks) were recorded in several genes of the CONR group. Data are representative of two repeated trials in which all samples were run in triplicate (Student's *t* test, $P < 0.05$). Error bars indicate SD.

increasing expression of IL-1β gene transcripts, with a maximum at 72 hpf, i.e., 24 hph. However, no significant changes were observed in the GF group in the same time period.

Next we investigated whether the observed differences in IL-1β expression in CONR and GF zebrafish could be attributed only to the fish sensing of commensal microbes. This hypothesis was tested through a short reversal experiment in which fish raised as CONR or GF were switched to the opposite medium at 54 hph (Fig. 1*C*). As expected, a significant shift occurred in the IL-1β expression of ex-CONR and -GF groups as compared with the normal basal response at 72 hpf. However, IL-1β expression was unaffected in a mock swap control in which the CONR larvae were handled identically but maintained in the same condition (Fig. 1*C*). This reversal process suggests that the IL-1β induction previously recorded in CONR and GF conditions was triggered as a response to a major environmental pressure which could be exerted only by commensal microbes. Interestingly, these observations also confirmed that only a short period of contact with commensal microbes is needed to start the induction of the proinflammatory IL-1β gene. To analyze the innate immune response further, *Vibrio anguillarum* DNA (vDNA) was selected as immunogenic MAMP in addition to commensal microbes in the CONR or as a first signal in GF fish. The larvae of both groups were treated with a bath of vDNA for 4 h at 48 and 72 hpf. At 48 hpf, only a slight, nonsignificant increase was observed in the CONR group, indicating that zebrafish embryos in both groups respond similarly if they have not been exposed to microbes for prolonged periods of time. At 72 hpf, two major changes in the mRNA levels of the IL-1β gene could be distinguished. In the groups without vDNA pretreatment, a significant but moderate increase in the response was recorded in the CONR group as compared with the GF group (Fig. 1*D*). Nevertheless, both groups pretreated with vDNA for 4 h exhibited a strong induction of IL-1β expression. In the CONR group, particularly, IL-1β gene expression increased significantly, by more than 12-fold as compared with the treated GF group or more than 30-fold compared with the CONR group without vDNA pretreatment.

Quantitative real-time RT-PCR (qPCR) analysis of several gene markers of innate immunity in both CONR and GF zebrafish at 72 hpf revealed several shared features of the host response and reinforced the hypothesis that the presence of commensal microbes triggers the overall inflammatory immune response in CONR fish, although not all inflammatory genes responded to the microbial presence (Fig. 1*E*). The group of induced genes included the pro-inflammatory effectors IL-1β, prostaglandin-endoperoxidase synthase 1 (PTGS1, formerly COX1), and TNFα; the chemokines C-C motif chemokine C25ab (CCL-C25ab), IL-8, and IL-8–like 2; and the antiviral mediator IFNΦ3. It became particularly evident that the presence of commensal microbiota failed to trigger a significant induction of genes encoding antimicrobial effectors, such as lysozyme (LYZ), defensin β-like 1 (DEFBL1), complement component c3a (C3a), and anti-inflammatory cytokines, such as IL-10 (Fig. 1*F*). Intriguingly, a few genes encoding TLRs, such as TLR1 and TLR4ba, also were induced drastically in the CONR group.

Zebrafish Neutrophil Functions, but Not Myelopoiesis, Are Affected by the Microbiota. We next investigated the impact of the presence of commensal microbes on myelopoiesis and neutrophil activity using Tg(*mpx::eGFP*^{i114}) zebrafish larvae, whose neutrophils express eGFP (32). The results showed that the numbers of eGFP-expressing neutrophils within total cell suspensions from CONR and GF zebrafish did not differ significantly (Fig. 2 *A* and *B*). However, a strong difference between the CONR and GF groups was observed in response to phorbol-myristate 13-acetate (PMA) (Fig. 2*C*). Thus, although PMA was able to trigger H_2O_2 production in cells from the CONR group, no response was observed in GF cells. The specificity of the reaction was confirmed using the NADPH oxidase inhibitor diphenyleneiodonium (DPI), which inhibited H_2O_2 production almost completely. These observations suggest that, even if microbial signals do not promote a higher rate of myelopoiesis, their presence primes professional phagocytes.

Commensal Microbes Modulate Neutrophil Recruitment and Activation. So far, we have shown that immediately after hatching CONR embryos recognize the presence of microbes and that such recognition is translated into microbial priming. Our next goal was to explore whether this priming affects neutrophil kinetics after the wounding of Tg(mpx*::eGFP*^{i114}) zebrafish raised in CONR and GF conditions. Following sterile protocols, the tip of the tail fin in each larva was transected at 72 hpf. Wounding induced a faster and more robust recruitment of neutrophils to the injury site in the CONR group (Fig. 3*A*), although the injury took longer to resolve than in GF embryos (Fig. 3*B*). To explore

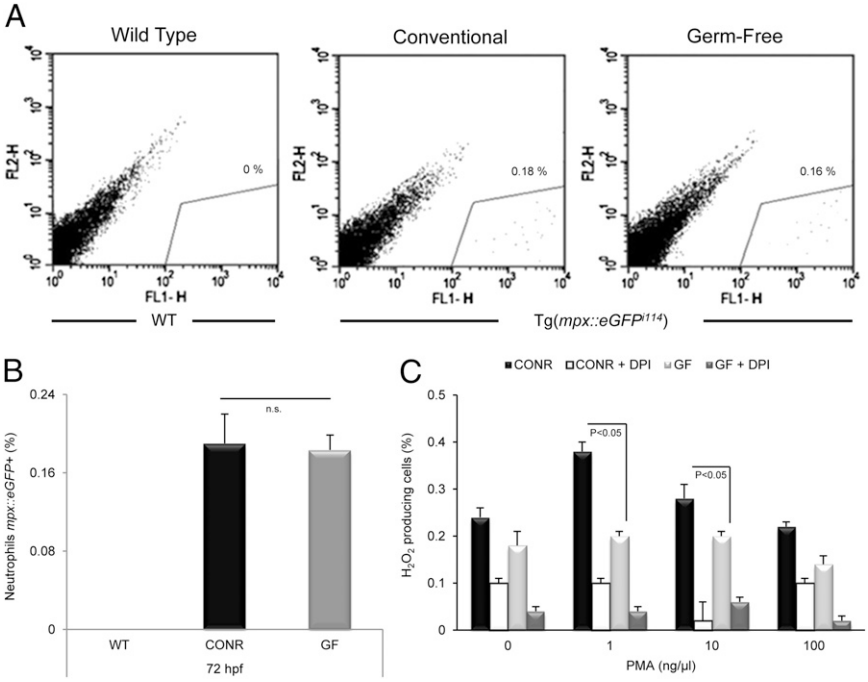

Fig. 2. Microbial presence primes neutrophils but does not affect myelopoiesis. Single-cell suspensions were prepared from wild-type whole larva raised in CONR conditions and Tg(*mpx*::eGFP^{i114}) larvae with positive eGFP neutrophils raised in CONR and GF conditions (n = 200 per group). (*A*) Representative dot-plots of FL1 vs. FL2 of the three groups showing as percentages the relative contribution of the gated compartment. (*B*) Average of three independent replicas of *mpx*::eGFP flow cytometric detection analysis demonstrating that the cell population labeled eGFP-positive was not significantly different in CONR and GF larvae. (*C*) Microbial presence primed the respiratory burst of total cell suspensions of 72-hpf zebrafish larvae (n = 3). To achieve this response, control cells were incubated for 1 h with the NADPH oxidase inhibitor DPI to avoid spontaneous respiratory burst activity. The respiratory burst was measured as the total DHR-fluorescence triggered by PMA. Data are presented as fold increase relative to cells incubated with medium alone. Statistically significant differences between the CONR and GF groups are shown (Student's t test; $P < 0.05$). Error bars indicate SD. n.s., nonsignificant.

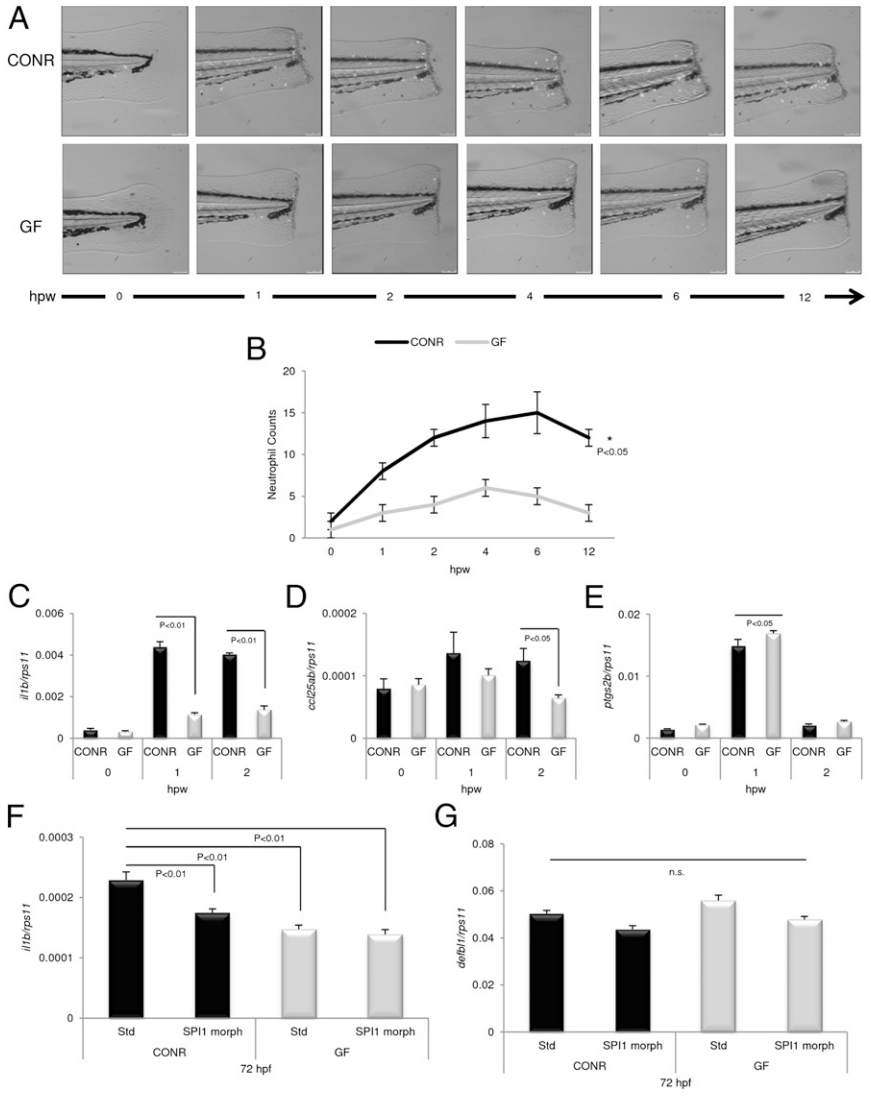

Fig. 3. Microbial presence increases neutrophil recruitment in vivo and induces the expression of proinflammatory mediators. Tail fins of Tg(*mpx*::eGFP^{i114}) CONR and GF zebrafish were transected at 72 hpf, and the number of fluorescent neutrophils visible in the tail was assessed by fluorescence microscopy. (*A*) Representative images of positive neutrophils expressing eGFP recruited to wounding site in a CONR and a GF zebrafish larva. hpw, hours postwounding. (*B*) Positive cells in animals from both groups were quantified visually from high-quality pictures from a 12-h time series. Inflammatory events were significantly faster, stronger, and took more time to resolve in the CONR group than in GF embryos (*$P < 0.05$). $n = 50$ larvae per group and sampling point. (*C–E*) At the time points indicated, 80 individual fish were anesthetized and then a sterile scalpel was used to incise the body between the anus and the wounded tail tip. Individual samples of the same batch were pooled and were immersed immediately in TRIzol for quantification of IL-1β (*C*), CCL-C25ab (*D*), and PTGS2B (*E*) mRNA levels by qPCR. (*F* and *G*) Zebrafish eggs were microinjected at the one-cell stage with 8 ng STD or SPI morpholinos per egg and then were divided into two batches. One batch was raised as CONR, and the other was derived as GF. IL-1β (*F*) and DEFBL1 (G) transcript levels, assayed by qPCR, of 72-hpf SPI morphants and their STD morphant siblings, raised CONR or GF. Error bars indicate the SD of three independent experiments, each using 30 pooled larvae per treatment. n.s., nonsignificant.

the mechanisms related to this response, we collected the last segment of the fish, from anus to tail tip, and used qPCR to quantify the gene expression of the inflammatory mediators after wounding, namely two proinflammatory genes (IL-1β and PTGS2B) and the gene encoding chemokine CCL-C25ab. We found that exposure to microbes before and during wounding was sufficient to cause the rapid induction of both IL-1β (Fig. 3*C*) and CCL-C25ab (Fig. 3*D*) genes, beginning just 1 h after injury. In contrast, PTGS2b transcript levels increased similarly in both CONR and GF groups after wounding (Fig. 3*E*). The impaired induction of IL-1β and CCL-C25ab after wounding in the GF group may explain the reduced neutrophil recruitment observed in this group. In addition, the results also confirm that not all proinflammatory genes are affected by commensal priming.

These results, together with the recent observation that neutrophils and macrophages mediate the proinflammatory effects of IFN-γ in zebrafish larvae (41), led us to hypothesize that these cells also might be involved in the priming by commensal microbes in newly hatched larvae. To test this hypothesis, neutrophils and macrophages were depleted by silencing the master myeloid transcription factor SPI1 (also known as "PU.1") with a translation-blocking morpholino (42). Strikingly, an impaired induction of IL-1β gene expression was observed in SPI1-deficient larvae of the CONR group (Fig. 3*F*), but the expression of the antimicrobial effector DEFBL1 was unaffected (Fig. 3*G*).

Microbial Priming by Commensal Microbes Is Essential for Protecting Zebrafish Larvae from Viral Infection. We next evaluated the disease resistance of CONR and GF zebrafish larvae at 72 hpf using a spring viremia of carp virus (SVCV) infection model (43) in which the virus is cleared by the IFN system (43, 44). Interestingly, the naturally primed CONR group showed increased resistance to SVCV as compared with GF larvae (Fig. 4*A*). Differences in mortality were not statistically significant under basal conditions. To look for direct evidence of enhanced transcriptional regulation as a result of the microbial priming, we analyzed the expression profile of several genes encoding proinflammatory and antiviral effectors in CONR and GF groups at 0 and 24 h postinfection (hpi) with SVCV. The results showed that, although the infection resulted in increased mRNA levels of proinflammatory (Fig. 4*B*) and antiviral (Fig. 4*C*) genes at 24 hpi in both groups, the induction of all genes was more robust in CONR than in GF larvae. These results strongly suggest that microbial priming at hatching is essential for further fortifying the innate immune response of fish and that the lack of such priming is detrimental for the resolution of infection.

Commensal Microbes Are Recognized Through a TLR/MyD88 Signaling Pathway in Zebrafish Larvae. At this stage, we hypothesized that commensal microbes could influence the observed inflammatory response of newly hatched zebrafish embryos through direct

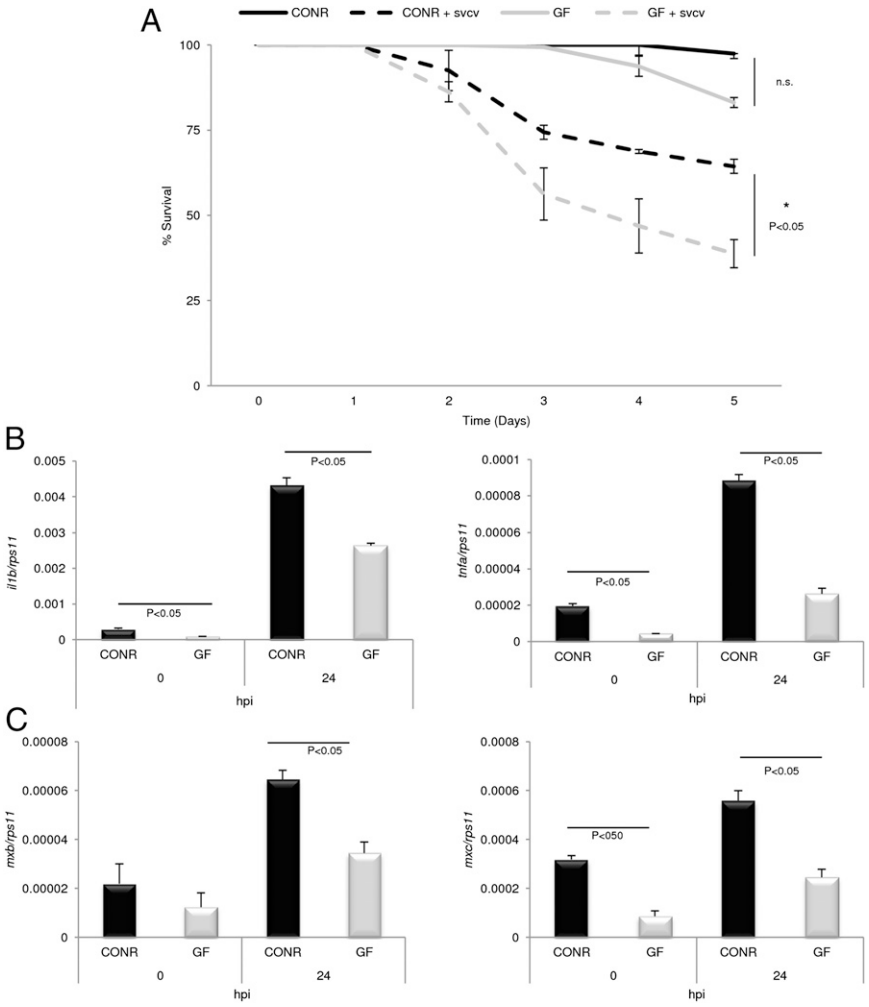

Fig. 4. Commensal priming after hatching potentiates zebrafish disease resistance. (*A*) Condition-dependent killing of CONR vs. GF 72-hpf zebrafish larvae exposed to 10^8 $TCID_{50}$/mL SVCV by immersion. Survival curves differ among groups (log-rank test, $P < 0.05$). n = 30 larvae in duplicate experiments. (*B* and *C*) The transcript levels of proinflammatory IL-1β and TNFα (*B*) and antiviral MxB and MxC (*C*), measured by qRT-PCR, were significantly lower in GF larvae than in CONR at 24 hpi ($P < 0.05$). Data are representative of two separate trials in which all samples were run in triplicate. Error bars indicate SD. n.s., nonsignificant.

transmembrane TLR signaling. To test this hypothesis, MyD88 was inhibited genetically using a translation-blocking morpholino (20). mRNA levels of IL-1β were significantly lower in MyD88-deficient fish than in their normal siblings (Fig. 5*A*). Strikingly, these levels correlated with those observed in their wild-type siblings raised under GF conditions. In sharp contrast, however, the mRNA levels of the LYZ (Fig. 5*B*) and DEFBL1 (Fig. 5*C*) genes in response to commensals was similar in MyD88-deficient fish and their wild-type siblings.

Histone Modifications Regulate Innate Immune Development. The induction of IL-1β expression in GF zebrafish after hatching, although at much lower levels than in CONR animals, suggests the existence of another microbe-independent mechanism that would activate a transient immune response when the embryos are devoid of their protective chorion. Furthermore, we previously observed the poor induction of genes encoding proinflammatory and antiviral molecules in larvae upon injection of MAMPs (13) or after viral infection (44), even though NF-κB was strongly induced. We then asked whether the expression of the immune genes was regulated at the level of chromatin remodeling via the covalent modification of histones, as has been observed in murine macrophages (45). Because acetylation of histone H3 at lysine 9 (H3K9ac) and trimethylation at lysine 4 (H3K4me3) mark transcriptionally active genes (46, 47), we treated zebrafish embryos microinjected with vDNA at the one-cell stage with trichostatin A (TSA), a histone deacetylase inhibitor, or pargyline, an inhibitor of the H3K4 demethylase LSD1 (48), and measured the induction of genes encoding IL-1β, IL-12α, TNFα, IFNφ1, LYZ, and DEFBL1. We confirmed that TSA and pargyline increased H3K9 acetylation and H3K4 trimethylation, respectively, at the IL-1β promoter (Fig. S1). In addition, vDNA injection significantly increased the mRNA levels of IL-1β and IFNφ1 (Fig. S2), as expected from its ability to activate NF-κB robustly in zebrafish embryos (13, 18). More interestingly, both TSA and pargyline facilitated the vDNA-mediated induction of IL-1β, IL-12α, and TNFα genes in embryos at 24 hpf but had a weak effect on the expression of IFNφ1, LYZ, and DEFBL1 (Fig. 6*A*). In addition, at 72 hpf zebrafish showed a marked increase in H3K9 acetylation (Fig. 6*B*) and H3K4 trimethylation (Fig. 6*C*) at the IL-1β and IFNφ1 promoters. Although the trimethylation of H3K4 at the IL-12α promoter showed a tendency similar to that of IL-1β and IFNφ1 (Fig. 6*C*), we did not observe H3K9 acetylation at this promoter, suggesting that other residues of this or other histones, most probably histone H4 (49), were acetylated. Importantly, these chromatin modifications were specific for the genes encoding these proinflammatory and antiviral factors, because the level of H3K9 acetylation (Fig. 6*B*) and H3K4 trimethylation (Fig. 6*C*) at the housekeeping genes β-actin2 and telomerase reverse transcriptase was similar in embryos (30 hpf) and larvae (72 hpf). In addition, neither of these histone modifications was observed at the promoters of LYZ and DEFBL1. Finally, we examined H3K4me3 at the IL-1β and β-actin2 promoters at a later time point, when inflammatory gene expression has dropped back down, to determine whether transient induction of inflammatory genes is accompanied by stable chromatin remodeling. The results showed that although H3K4me3 remained stable at the β-actin 2 promoter, it declined significantly at the IL-1β promoter (Fig. S3).

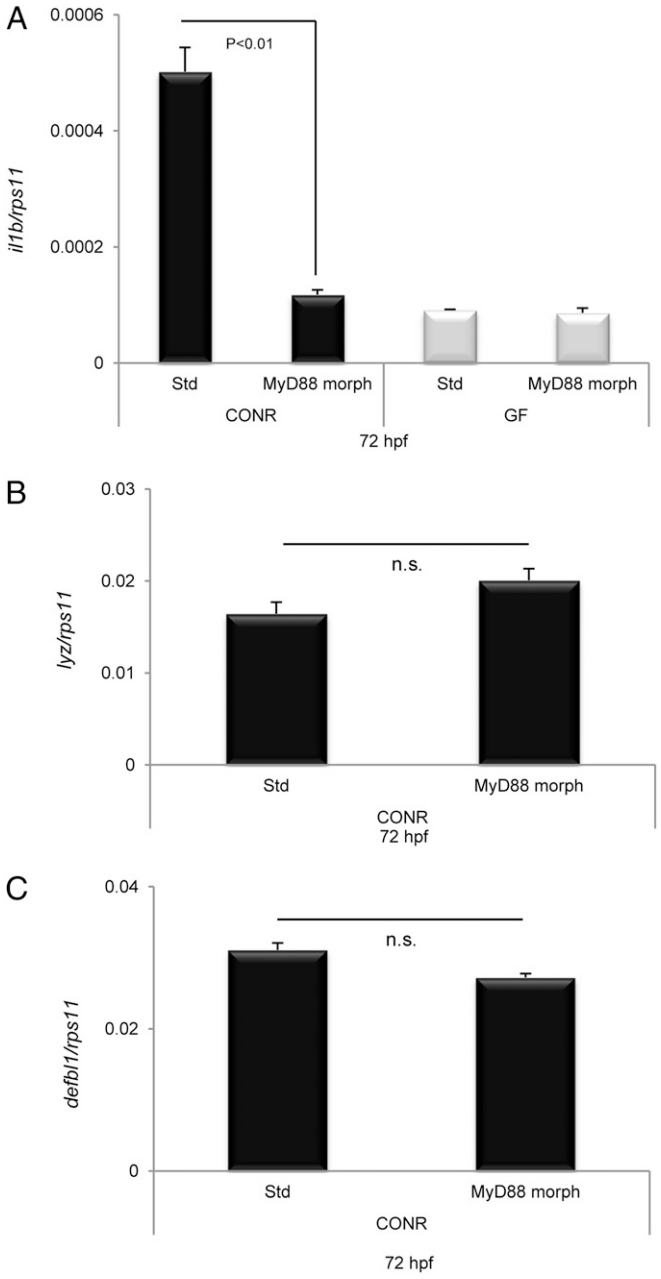

Fig. 5. MAMPs signal through a TLR/MyD88 signaling pathway. Zebrafish eggs were microinjected at the one-cell stage with STD or MyD88 morpholinos, 4 ng per egg, and then were divided into two batches. One batch was raised as CONR, and the other was derived as GF. IL-1β (*A*), LYZ (*B*), and DEFBL1 (*C*) transcript levels, assayed by qPCR, of 72-hpf MyD88 morphant zebrafish and their STD morphant siblings raised CONR or GF. Error bars indicate the SD of two independent experiments, each using 30 pooled larvae per treatment. n.s., nonsignificant.

We next examined whether the level of H3K9ac and H3K4me3 at the promoter of IL-1β was regulated by the presence of microbes during development. The results pointed to a similar degree of acetylation and trimethylation of these residues in CONR and GF larvae (Fig. 7 *A* and *B*). However, injection of vDNA was able to increase H3K9ac and H3K4me3 robustly at the IL-1β promoter (Fig. S4), suggesting that a stronger inflammatory stimulus may alter these histone covalent modifications. Furthermore, we also found that the level of trimethylation of H3K4 was not triggered by hatching, because it was similar at IL-1β and β-actin2 promoters in manually dechorionated and nondechorionated CONR embryos at 32 hpf (Fig. 7 *C* and *D*). In sharp contrast, however, 8 h later manually dechorionated CONR and GF embryos showed higher IL-1β and IL-12α transcript levels than nondechorionated embryos (Fig. 7 *C* and *E*), suggesting that a third factor, in addition to the presence of commensal microbes and chromatin modifications, also orchestrates the induction of immune genes after hatching.

Discussion

In the present study commensal microbes were found to have the capacity to prime the innate immunity of zebrafish larvae by inducing a transitory inflammatory response that, together with constitutively expressed antimicrobial effectors, acts to increase the resistance of zebrafish larvae to infectious disease soon after hatching. Commensals immediately surround cells, tissues, and indeed, the whole organism in vivo, colonizing the external surfaces of the vertebrate body and soon assembling into dense gastrointestinal communities (50). Vertebrate exposure to indigenous gut microbiota and their microbial products has been recognized widely to be a key element for the development and maturation of the mammalian immune system (4, 36, 51). Recently, Rawls' team (40) found that the zebrafish digestive tract is robustly responsive to microbial colonization because of the impressive number and diversity of bacteria that assemble in the organ compared with the amount present in the microenvironment of the larvae. Thus, the majority of previous host–microbe studies targeted the gastrointestinal tract (40). However, our present data in CONR- and GF-raised zebrafish larvae revealed increased transcripts of major proinflammatory markers, chemokines, and some antiviral effectors and TLRs as early as 54 hpf, when definitive hematopoiesis already has begun (52); the levels of these transcripts increased until 72 hpf, when maximum activity was recorded. These differences, together with the priming of neutrophils observed in CONR larvae and the inability of larvae depleted of neutrophils and macrophages to induce proinflammatory genes after hatching, demonstrate that the CONR group is able to respond to several environmental pressures at hatching. Comparison with the GF group shows that commensal microbes are mainly responsible for this response. Intriguingly, several studies with zebrafish larvae have suggested that commensal microbiota colonize the larvae as soon as they hatch, but all assign great importance to the role played by gut microbiota in modifying innate immunity, nutrient metabolism, impaired intestinal differentiation, and function when microbes invade the internal organs (19, 22, 36–38, 40, 53). Also, in contrast to our work, it previously was proposed that host defense mechanisms, such as proinflammatory cytokines, are inducible mostly upon recognition of virulence factors of pathogenic bacteria (9, 52, 54–56). However, it should be noted that spontaneous infection was not recorded in the present study; thus the "inflammatory state" observed in CONR fish was transient, rapidly returning to basal levels, marked here by the levels of IL-1β expression similar to those observed in the GF fish 24 h later (i.e., at 96 hpf). It is possible that the immunological tolerance observed here could be a direct response to microbial burden on the skin. In vitro studies in mammals have suggested that apical contact of commensals with the epithelium attenuates NF-κB activation via the regulation of PPARδ and inhibition of the cellular proteosome activity required for IκB degradation (57, 58). Further studies are necessary to clarify these issues. In any case, these results again indicate that the ability of immune-competent cells is strikingly reinforced by the mere presence of commensal microbes at hatching. However, we observed no difference between the two groups in the number of neutrophils, suggesting that commensal microbes do not regulate neutrophil differentiation or apoptosis. In contrast, it has been reported recently that LPS is able to promote granulopoiesis in zebrafish embryos through the induction of granulocyte-colony stimulating factor (59). These differences might be explained by the nature, duration, and potency of the stimuli provided.

GF animals provide a healthy but susceptible host for experimental colonization with defined microbial populations (60). Here,

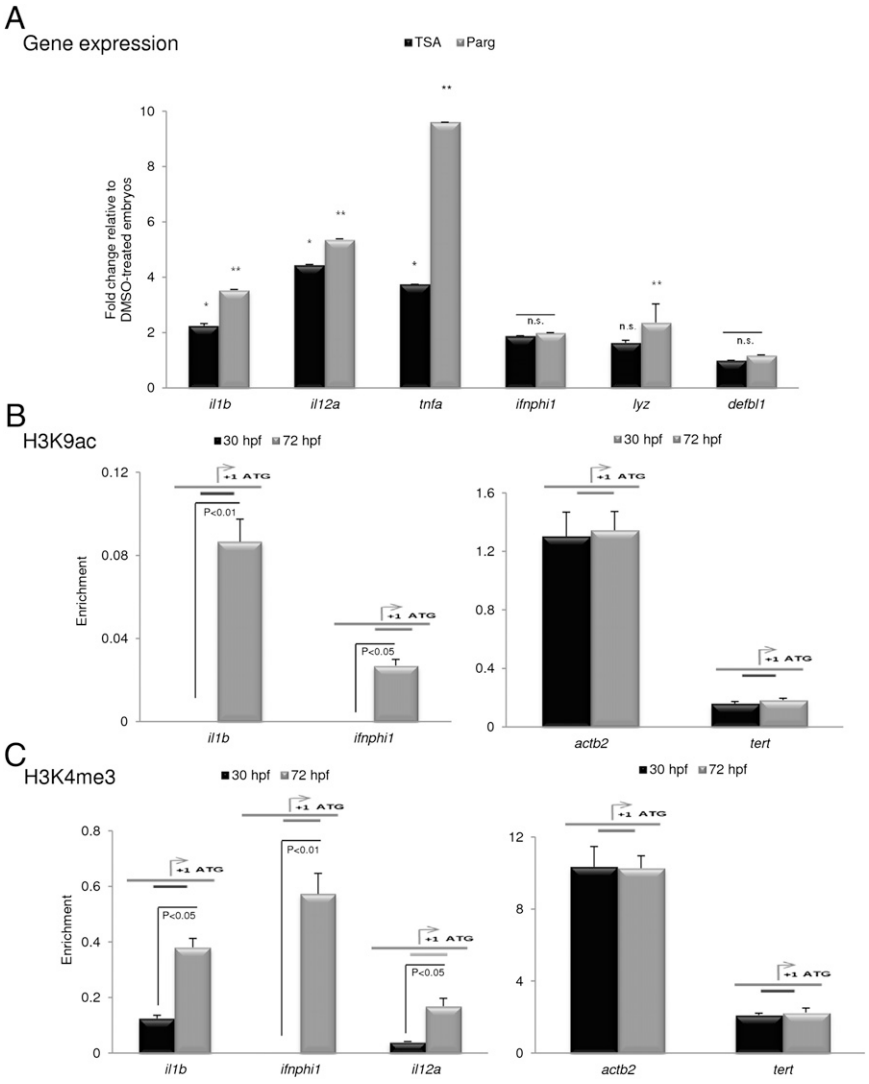

Fig. 6. Histone modifications are differentially regulated in proinflammatory and antimicrobial effector genes. (*A*) Zebrafish eggs microinjected at the one-cell stage with 5–10 ng vDNA per egg were dechorionated manually at 20 hpf and were treated with 3 μM pargyline (Parg) or 100 nM TSA for 5 h. IL-1β, IL-12a, TNFα, IFNφ1, LYZ, and DEFBL1 transcript levels then were assayed by qPCR. (*B* and *C*) CONR embryos/larvae were analyzed by ChIP (H3K9ac and H3K4me3, respectively, at the indicated times. The amplicon used for each promoter is indicated by a diagram above the bars. Error bars indicate the SD of two independent experiments, each using 30 pooled larvae per treatment. $*P < 0.05$ vs. DMSO-treated embryos. $**P < 0.01$ vs. DMSO-treated embryos. n.s., nonsignificant.

we have established that the transient inflammatory response of young larvae could be reversed partially in both directions, as seen at 72 hpf, from the increased or decreased mRNA IL-1β levels in the respective experimental groups Previous studies in which the GF zebrafish phenotype was rescued by exposing animals to commensal microbiota 3 or 6 days postfertilization support these findings (36). However, studies with GF mice colonized with human or mouse fecal microflora observed only a partial protection to oral tolerance, suggesting that colonization of GF mice after birth is unlikely to recapitulate fully the colonization of newborn mice (61, 62) and that bacterial subproducts released during the egg stage may influence the development of the immune system. Indeed, the development of host immunity is a complex process that requires a series of coordinated events, including the functional differentiation of immune cells, expansion of immune populations, and the expression of other immune functions (52). In the present study, the administration of bacterial CpG motifs in zebrafish larvae at 72 hpf induced the expression of IL-1β in the CONR group, confirming that previous exposure to commensals increases immunocompetence in fish, similar to findings in mammals (63).

Consistent with our results, the presence of assembled commensal microbes in newborn mice has been identified as a potent stimulus for an increase in immune-related genes (61, 62). In addition, a transcriptomic analysis in zebrafish demonstrated that some immune genes were expressed specifically in response to certain microorganisms, indicating that the early colonization of the gastrointestinal tract by a particular microorganism may be responsible for the changed metabolism in the fish larvae (36). In a functional genomics analysis, the same authors observed induction of the complement factor component 3 (C3) after bacterial colonization of the gastrointestinal tract. Notably we observed that C3, like the other genes encoding antimicrobial effectors, showed similar expression levels in CONR and GF animals. This result was not unexpected, because fish surfaces comprise a large area of delicate epithelium, the major route of entry for pathogenic microorganisms; thus, healthy fish, as well as mammals, are capable of limiting infection effectively through the release of systemically expressed antimicrobial effectors that neutralize a broad range of microbes (64–67). Indeed we speculate that these mechanisms are shared features among several phyla and constitute a primitive immune defense mechanism among a wide range of eukaryotic organisms. Priming could be either an evolutionary selective pressure, in which the functionality of the neonate immune system is tested and members presenting any immunodeficiency are excluded from the group through pathogenic microorganisms, or, alternatively, priming simply may be a method of training the immune system to support the enormous number of bacteria that the gut will harbor throughout the life of the vertebrate.

Our results also present clear evidence that MAMPs are sensed via a TLR/MyD88 signaling pathway, which in turn leads to the transcriptional up-regulation of proinflammatory and antiviral genes, and suggest that TLRs are responsible for sensing

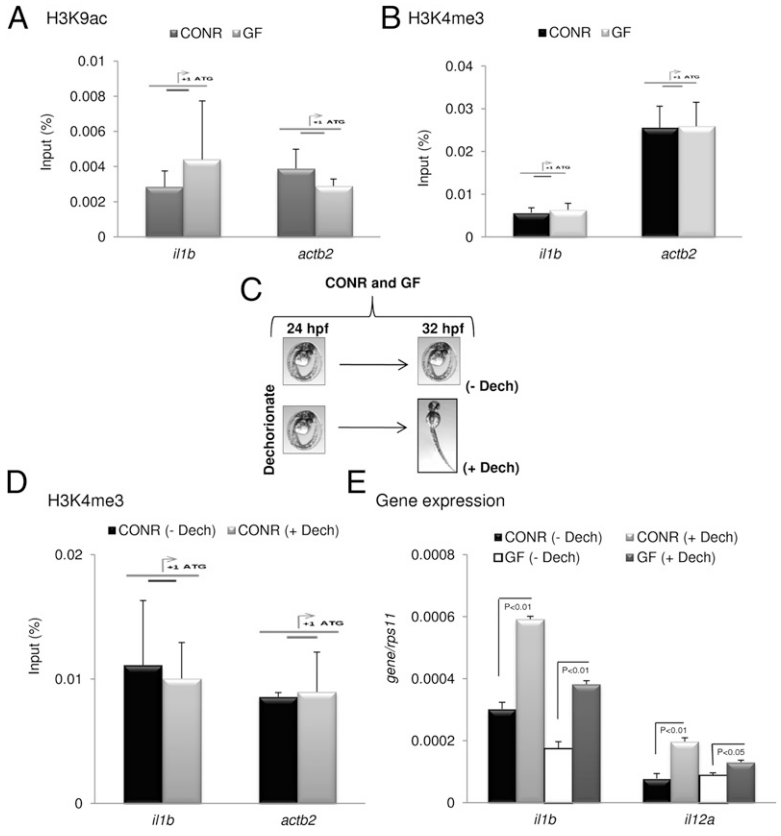

Fig. 7. Histone modifications are not regulated by commensals or hatching. (*A* and *B*) Naturally hatched CONR and GF zebrafish larvae were analyzed by ChIP (H3K9ac and H3K4me3) at 72 hpf. (*C*) Scheme showing the generation of CONR and GF embryos after manual dechorionation. These embryos were analyzed by ChIP (H3K4me3) (*D*) or gene expression (IL-1β and IL-12a) (*E*) at 32 hpf. The amplicon used for each promoter is indicated by a diagram above the bars. Error bars indicate the SD of two independent experiments, each using 30 pooled larvae per treatment.

MAMPs and the induction of inflammation in CONR animals. Strikingly, however, the expression of genes encoding antimicrobial effectors is independent of MyD88. Taken together, these results further suggest the importance of the TLR/MyD88 signaling pathway in the maturation of innate immunity triggered by commensal microbes and that the expression of gene-encoding antimicrobial effectors is independent of MyD88 or the presence of microbes, pathogenic or not. Similarly, it was found recently that the colonization of GF zebrafish by a commensal microbiota activated NF-κB via a MyD88 signaling pathway and led to the up-regulation of its target genes in intestinal and extraintestinal tissues of the digestive tract (40).

Our study also demostrates the involvement of chromatin modification in the gene-specific control of immunity during development. Although the genes encoding antimicrobial effectors, which are particularly essential for the defense of embryos/larvae in the absence of adaptive immunity and do not have the potential to cause tissue damage, are constitutively expressed at high levels during development, the genes encoding proinflammatory and antiviral mediators are activated transiently after hatching through the extensive methylation and acetylation of histone H3 at their promoters. Although these chromatin modifications were observed similarly in CONR and GF animals, our data cannot rule out an effect of commensals in their regulation at later developmental stages. In fact, injection of vDNA, which can induce NF-κB activation strongly (13, 18), robustly increased H3K9ac and H3K4me3 at the IL-1β promoter but not at the β-actin2 promoter, suggesting that a stronger inflammatory stimulus, which may be provided by pathogenic microbes, also may fine-tune the regulation of chromatin covalent modifications at proinflammatory gene promoters. In any case, because the major signaling molecules of TLRs, including NF-κB (68), MAPK (69), and PI3K (70), are required for the regulation of important developmental genes, we propose that nucleosome remodeling through covalent histone modification regulates the activation of proinflammatory and antiviral genes after hatching while preventing the pathology associated with excessive inflammation during early development. This mechanism is reminiscent of the one regulating the expression of proinflammatory (class T, tolerizeable) and antimicrobial (NT, nontolerizeable) genes in mouse macrophages (45), so we speculate that this mechanism is evolutionarily conserved in all vertebrate classes.

In summary, our results demonstrate that sensing of commensal microbes via both the TLR/MyD88/NF-κB signaling pathway and gene-specific chromatin modifications is associated with the protection of zebrafish larvae against infectious agents before adaptive immunity has developed and at the same time prevents pathologies associated with excessive inflammation during development.

Materials and Methods

Zebrafish Husbandry. All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU) and the Bioethical Committee of the University of Murcia (approval no, 333/2008). Zebrafish gametes were naturally expressed from wild-type (obtained from the Zebrafish International Resource Center), and the Tg(*mpx*::gfp)^{i114} (32) line held at our facilities following standard husbandry practices. Animals were constantly subjected to a 12/12-h light/dark cycle and maintained at 28.5 °C.

In each experiment, zebrafish embryos were produced through natural breeding conditions in clean breeding tanks with autoclaved system egg water containing 60 μg/mL sea salts in distilled water. As fish spawned, embryos were collected for 1 h and were transferred immediately to a sterile Petri dish. To generate and rear GF zebrafish embryos, one half of the total eggs collected was washed with antiseptics and derived as GF in a Telstar class II/B3 biological safety cabinet, following the protocol described in ref. 71 with slight modifications. The modifications were the use of glutaraldehyde (0.2 μg·mL^{-1}) for 2 min to disinfect egg surfaces (72) and the addition of antibiotics [penicillin and streptomycin (Sigma-Aldrich) at a final concentration of 10 μ· mL^{-1}] to the autoclaved egg water before filtration with a 0.22-μm membrane filter (gnotobiotic zebrafish medium with antibiotics; AB-GZM). After derivation, GF animals were kept in sterile, vented tissue-culture flasks at an average density of 10 individuals per milliliter of autoclaved and filtered egg water without antibiotics (GZM). On alternate days, GF animals were monitored for sterility using standard microbial culture methods as described else-

where (71), and 25% (vol/vol) of the total medium contained in each flask was replaced with fresh GZM. The remaining half of the total collected eggs, which did not receive any treatment, was raised conventionally (CONR) in culture vessels at the same densities as the GF group to standardize conditions. In both groups, dead eggs were removed aseptically twice each day.

Germ-Free Colonization Test. Most larvae in GF experiments started to hatch 48 hpf, and 6 h later all had lost their protective chorions. At this time point every single larva in each GF or CONR batch was recovered using a soft sieve and was transferred immediately to a new cell-culture flask. Each flask with GF larvae was filled with medium collected from tanks housing CONR zebrafish (71); CONR zebrafish simply were transferred to GF medium.

In Vivo MAMP Tests. Repeats of molecular CpG motifs obtained as phenol-extracted genomic vDNA (73) were used to stimulate whole zebrafish larvae by dip immersion at selected time points. Briefly, 50 $\mu g \cdot mL^{-1}$ vDNA was added to 25-cm^2 ventilated cell-culture flasks containing 50 larvae in 15 mL medium. Depending on the experiment, incubation was carried out for 4 and/or 24 h at 28.5 °C. Once incubation was finished, larvae from each flask were collected in 1.5-mL Eppendorf tubes containing 500 μL TRIzol reagent and were frozen immediately at −80 °C for further analysis.

Flow Cytometry Analysis of Zebrafish Neutrophils. Tg(mpx:eGFP)^{114i} zebrafish were reared under CONR or GF conditions. At 72 hpf larvae from each batch were killed with an overdose of MS-222 (Sigma). In each experiment, 100 animals per condition were pooled after killing and were dis-aggregated as described elsewhere (41). Single-cell suspensions were analyzed by a FACS bench-top cytometer (BD Biosciences). Live cells (10^6) were analyzed by flow-cytometry using the CellQuest software (Becton Dickinson Immunocytometry Systems).

Gene-Expression Analysis. Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's specifications and treated with amplification grade DNase I (1 U/μg RNA; Invitrogen). The SuperScript III RNase H^- reverse transcriptase (Invitrogen) was used to synthesize the first strand of cDNA with an oligo(dT) primer from 1 μg of total RNA for 50 min at 50 °C. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. For each mRNA, the gene expression was normalized to the ribosomal protein S11 content in each sample using the Pfaffl method (74). The primers used are shown in Table S1. In all cases, each PCR was performed with triplicate samples and was repeated at least twice.

Respiratory Burst Activity Using Dyhydrorodamine 123 (DHR). Total cells suspensions (5×10^6 cells$\cdot mL^{-1}$) of CONVR or GF 72-hpf zebrafish larvae diluted in RPMI-1640 culture medium (Life Technologies) containing 5% (vol/vol) fetal bovine serum (LIfe Technologies) were split into several aliquots for each treatment. Thereafter, one half of the aliquots was incubated with 100 μL of 10 μM DPI (an inhibitor of NADPH oxidase) for 1 h at room temperature. Then, all aliquots containing inhibited or naive cells were stained for 5 min at room temperature with 100 μL of 10 μM DHR solution in HBSS (75). The DHR-loaded cells were incubated further for 30 min at room temperature with 100 μL of PMA (Sigma-Aldrich) at 0, 1, 10, or 100 $ng \cdot ml^{-1}$ to trigger the respiratory burst. After incubation, samples were analyzed immediately by flow cytometry for total and mean fluorescence. Results were collected on the bright green fluorescence as FL1 and were compared with the FL2 signal.

Microinjection of Morpholino Nucleotides or RNA into Zebrafish Embryos. Morpholino antisense oligomers targeting MyD88 (TAGCAAAACCTCTGT-TATCCAGCGA) (20), the myeloid transcription factor SPI1 (GATATACTGA-TACTCCATTGGTGGT) (42), or a standard control (STD) CCTCTTACCTCAGTTA-CAATTTATA) were obtained from Gene Tools, LLC, solubilized in water to produce a stock solution (1 mM), and kept at −80 °C in small aliquots until use. Zebrafish embryos at the one-cell stage were microinjected with a mix of morpholino (4 ng per egg) in 0.5× Tango buffer and 0.05% phenol red as indicator using sterile egg supports consisting of 2% (wt/vol) low-melting-point agarose (Sigma-Aldrich) dissolved in sterile embryo medium (76) and a microinjector Narishige IM-300 with an attached glass capillary needle. After microinjection, eggs were removed aseptically from the agarose matrix and were left in egg water. CONR eggs were placed in tissue-culture flasks without any astringent solution, and GF eggs were derived immediately. After the derivation was verified, GF embryos were maintained in GZM until the end of each trial.

Imaging the Tail-Transection Wound. Tg(*mpx*::eGFP)^{114i} zebrafish were reared under CONR or GF conditions. At 72 hpf, larvae were anesthetized in tricaine and then were mounted in 1% (wt/vol) low-melting-point agarose dissolved in embryo medium. Complete transection of the tail was performed with a disposable sterile scalpel. The success of transection was confirmed immediately through a fluorescence stereomicroscope MZ16FA (Leica) equipped with green fluorescent filters. Each image was imaged at transection, which was established as time zero. Thereafter, images were captured at the selected times while animals were kept in their agar matrixes with the added medium at 28.5 °C. All images were acquired with the integrated camera on the stereomicroscope and were controlled under the Leica application suite using Windows operational system.

Infection Assays. The SVCV isolate 56/70 was obtained as previously reported (43). Briefly, the virus stock was propagated in EPC cells and titrated in 96-well plates. Thirty 72-hpf zebrafish larvae were challenged at 25 °C in disposable Petri dishes by immersion in SVCV [10^8 50% tissue culture infective dose ($TCID_{50}$)]. After challenge, the fish were monitored every 12 h over an 8-d period, and mortality was scored.

ChIP. vDNA (5–10 ng per egg) was microinjected into the yolk sac of embryos at the one-cell state (13). At the indicated times, embryos were dechorionated manually and treated with 3 μM pargyline or 100 nM TSA for 5 h and, if required, were raised under CONR or GF conditions. Embryos/larvae were processed for RNA extraction as described above or for ChIP using the MAGnify Chromatin Immunoprecipitation System (Life Technologies). Briefly, for each immunoprecipitation 200 embryos (30 hpf) or 80 larvae (72 hpf) were cross-linked with 1% formaldehyde for 8 min, and the chromatin was sheared by sonication to an average fragment size of 500–1,000 bp (77). Lysates were cleared by centrifugation and diluted 1:10 in the dilution buffer of the ChIP kit, and immunoprecipitation was performed with 2–4 μg of antibody. ChIP and input DNA were amplified by qPCR using specific primers for the 5′ upstream sequence of the different genes (Table S2). qChIP values are given as percent input or DNA recovery normalized to total immunoprecipitated histone H3. ChIP with control mouse and rabbit anti-IgG showed no enrichment of any target promoters. ChIPs were performed with at least two independent chromatin preparations. The antibodies used in this assay were anti-H3 (ab1791; Abcam), anti-H3K4me3 (ab1012; Abcam), anti-H3K9ac (06–942; Millipore), and control mouse and rabbit anti-IgG provided by the MAGnify kit. All these antibodies have been validated in zebrafish previously (77), and the epitopes that they recognize are identical in zebrafish and mammals.

Statistical Analysis. Statistics were generated with GraphPad Prism 5 software using Student's *t* tests for paired groups and ANOVA and post hoc Tukey's test for multiple comparisons. The log-rank test was used to calculate the differences in survival of the different experimental groups. Differences with P values <0.05 were considered statistically significant.

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Supporting Information

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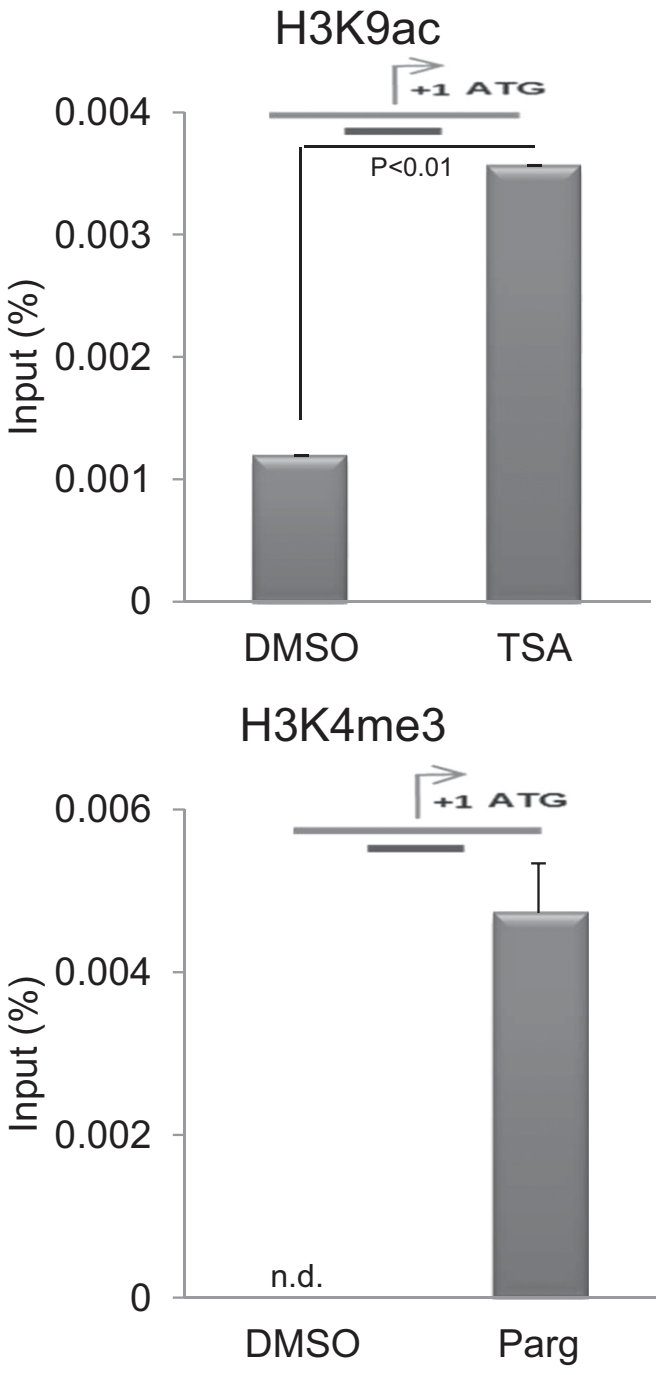

Fig. S1. Trichostatin A (TSA) and pargyline regulate chromatin modifications at the IL-1β promoter. Zebrafish eggs microinjected at the one-cell stage with 5–10 ng *Vibrio anguillarum* DNA (vDNA) per egg were dechorionated at 24 h post fertilization (hpf) and treated manually with 100 nM TSA or 3 μM pargyline for 5 h. Larvae then were processed and analyzed by ChIP (H3K9ac and H3K4me3). The amplicon used for each promoter is indicated by a diagram above the bars. Error bars indicate the SD of triplicate samples using 30 pooled larvae per treatment. n.d., not detected.

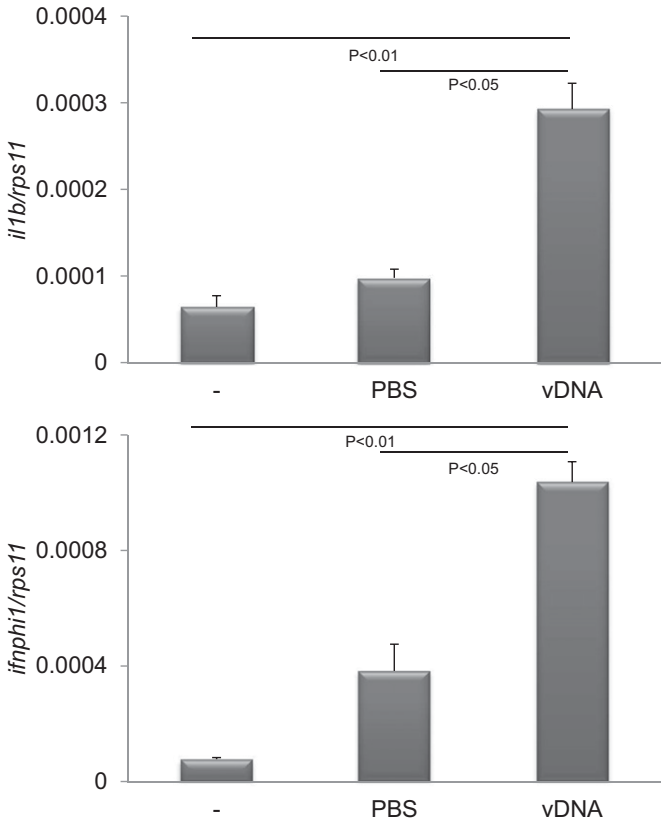

Fig. S2. vDNA induces IL-1β and IFNφ1 expression. Zebrafish eggs microinjected at the one-cell stage with vDNA or vehicle alone (PBS) (5–10 ng per egg) were processed at 30 hpf. IL-1β and IFNφ1 transcript levels then were analyzed by quantitative PCR. Error bars indicate the SD of triplicate samples using 30 pooled larvae per treatment.

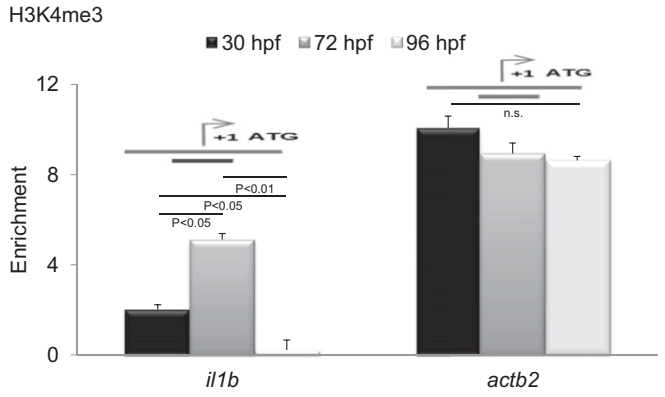

Fig. S3. Dynamics of chromatin modifications at the IL-1β gene promoter. Zebrafish embryos or naturally hatched larvae were processed and analyzed by ChIP (H3K4me3) at the indicated times. The amplicon used for each promoter is indicated by a diagram above the bars. Error bars indicate the SD of triplicate samples using 30 pooled larvae per sample point. n.s., nonsignificant.

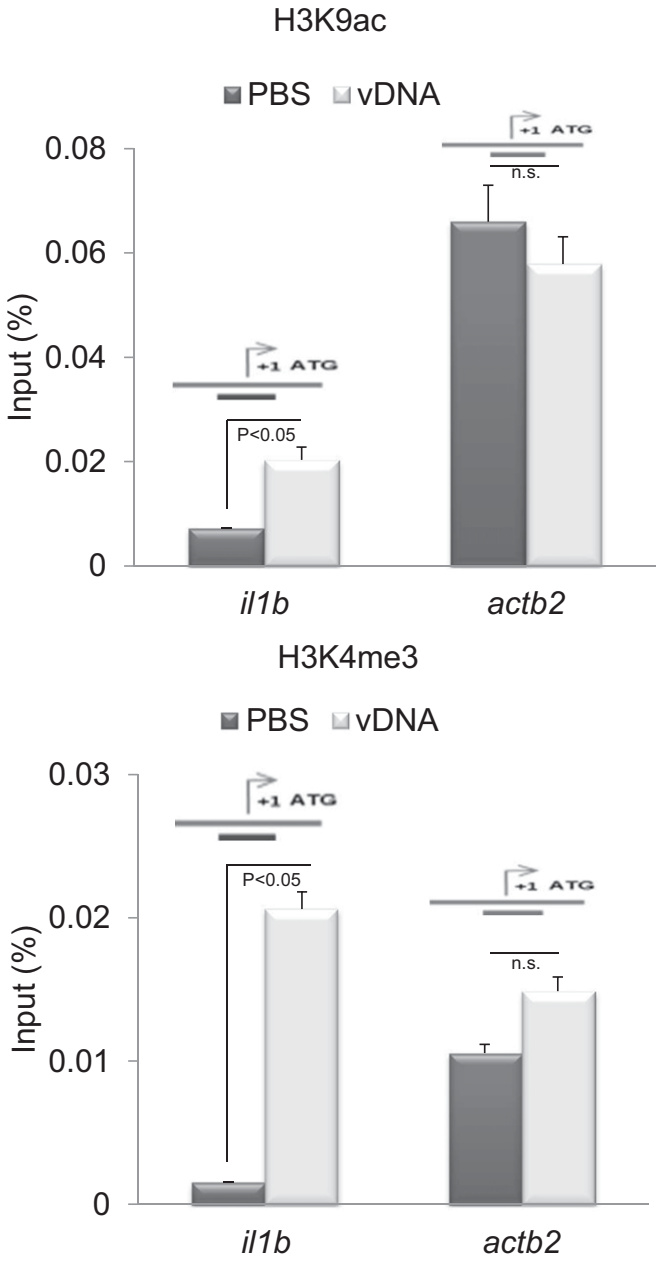

Fig. S4. vDNA injection increases chromatin modifications at the IL-1β gene promoter. Zebrafish eggs microinjected at the one-cell stage with 5–10 ng vDNA per egg were processed at 30 hpf and analyzed by ChIP (H3K9ac and H3K4me3). The amplicon used for each promoter is indicated by a diagram above the bars. Error bars indicate the SD of triplicate samples using 30 pooled larvae per sample. n.s., nonsignificant.

Table S1. Primers used to analyze gene expression in this study

Gene symbol	GenBank accession no.	Gene name	Primers	Sequence (5′ to 3′)
c3a	BC055564	*Complement component c3a*	F	ATGAGCTCCTGCAGAGGTGT
			R	AGTGGTTGTTGGAGGTCTGG
cclc25ab	NM_001129894	*C-C motif chemokine c25 ab*	F	AGCACCTCTCGCTTTGTGTT
			R	TGTTTGAAAGGCACTTGACG
defbl1	NM_001081553	*Defensin, beta-like 1*	F	CAGGACTGCCATCATCTGAA
			R	CTCCTTGTCTGCAAACACCA
ifnphi1	NM_207640	*IFN phi 1*	F3	GAGCACATGAACTCGGTGAA
			R3	TGCGTATCTTGCCACACATT
ifnphi2	NM_001111082	*IFN phi 2*	F1	CCTCTTTGCCAACGACAGTT
			R1	CGGTTCCTTGAGCTCTCATC
ifnphi3	NM_001111083	*IFN phi 3*	F1	TTCTGCTTTGTGCAGGTTTG
			R1	GGTATAGAAACGCGGTCGTC
il1b	NM_212844	*Interleukin 1 beta*	F5	GGCTGTGTGTTTGGGAATCT
			R5	TGATAAACCAACCGGGACA
il8	CT826376	*Interleukin 8*	F	GTCGCTGCATTGAAACAGAA
			R	CTTAACCCATGGAGCAGAGG
il8l2	EH441857	*Interleukin 8-like 2*	F	GCTGGATCACACTGCAGAAA
			R	TGCTGCAAACTTTTCCTTGA
il10	NM_001020785	*Interleukin 10*	F2	ATTTGTGGAGGGCTTTCCTT
			R2	AGAGCTGTTGGCAGAATGGT
il12a	AB183001	*Interleukin 12a*	F1	AGCAGGACTTGTTTGCTGGT
			R1	TCCACTGCGCTGAAGTTAGA
lta	NM_001024821.1	*Lymphotoxin alpha (TNF superfamily, member 1)*	F2	AAGCCAAACGAAGGTCA
			R2	AACCCATTTCAGCGATTGTC
lyz	NM_139180	*Lysozyme*	F	TGGCAGTGGTGTTTTTGTGT
			R	TCAAATCCATCAAGCCCTTC
mxb	NM_001128672	*Myxovirus (influenza) resistance B*	F1	AATGGTGATCCGCTATCTGC
			R	TCTGGCGGCTCAGTAAGTTT
mxc	NM_001007284	*Myxovirus (influenza virus) resistance C*	F	GAGGCTTCACTTGGCAACTC
			R	TTGTTCCAATAAGGCCAAGC
nos2b	NM_001113501	*Nitric oxide synthase 2b, inducible*	F1	GGCTTGCACTGCTTTTAAGG
			R1	TCCAGAGTGCAACTGTCCTG
ptgs1	NM_153656	*Prostaglandin-endoperoxide synthase 1*	F	TTTTGCTGCTGAGTGTGTCC
			R	CGAACACAGATCCCTTGGTT
ptgs2b	NM_001025504	*Prostaglandin-endoperoxide synthase 2b*	F1	TGGATCTTTCCTGGTGAAGG
			R1	GAAGCTCAGGGGTAGTGCAG
ticam1	NM_001044759	*Toll-like receptor adaptor molecule 1*	F1	ATGGAGAGCGCTTGAACTGT
			R1	TTGTCGCACAAACTCTCCTG
tlr3	NM_001013269	*Toll-like receptor 3*	F1	AAAGGGCTACGTTTGGTGTG
			R1	GTTGGTGGAGTTCAGCATT
tlr4ba	NM_001131051	*Toll-like receptor 4b, duplicate a*	F	CAATGGCTTGGGTACTTTGC
			R	GATTTGAGGAGTGCCGGATA
tlr22	NM_001128675	*Toll-like receptor 22*	F1	TGGGCCAAGAAGAATGAATC
			R1	ATGACAACAGGAGGGTGAGG
tnfa	NM_212859	*Tumor necrosis factor a (TNF superfamily, member 2)*	F2	GCGCTTTTCTGAATCCTACG
			R2	TGCCCAGTCTGTCTCCTTCT
rps11	NM_213377	*Ribosomal protein S11*	F	ACAGAAATGCCCCTTCACTG
			R	GCCTCTTCTCAAAACGGTTG

The gene symbols follow the Zebrafish Nomenclature Guidelines (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).

Table S2. ChIP-quantitative PCR primer sequences and position of amplicons of each gene used in this study

Gene symbol	Ensembl accession no.	Gene name	Primers	Sequence (5′ to 3′)	Position relative to transcription start site (nt)
actb2	ENSDARG00000037870	*Actin beta2*	F	ACTATGAACTGAACCGACTG	+919/+1072
			R	CTGCGATCAATTACACAACC	
ifnphi1	ENSDARG00000025607	*IFN phi 1*	F	CCCGAATAAATCCAGCACTC	−84/+100
			R	TTATCCTGTATCGGCCAAGC	
il1b	ENSDARG0000005419	*Interleukin 1 beta*	F	ATCAAGGAATTTCCCGCTTC	−62/+64
			R	TGCACTCCACATACCAGAGC	
il12a	ENSDARG00000038878	*Interleukin 12a*	F	GCGTGTGAGTGTGTGTGTGT	−91/+55
			R	GAGCCAGAGCCATGCTAATC	
tert	ENSDARG00000042637	*Telomerase reverse transcriptase*	F	GCGTGTCGTTATCTGGGAGT	−69/-10
			R	CGTGCTTGGGGAGTTCTATT	

The gene symbols follow the Zebrafish Nomenclature Guidelines (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).