UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Modelling the Impact of TNF Receptors in Inflammation Using the Zebrafish

Caracterización del Papel de los Receptores de TNF en Inflamación Usando el Pez Cebra Como Modelo

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2013

DEPARTAMENTO DE BIOLOGÍA CELULAR E HISTOLOGÍA

FACULTAD DE BIOLOGÍA

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MODELLING THE IMPACT OF TNF RECEPTORS IN INFLAMMATION USING THE ZEBRAFISH

Memoria que presenta

D. Sergio Candel Camacho

para optar al grado de Doctor

por la Universidad de Murcia.

Octubre de 2013.

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ABBREVIATIONS

µm	Micrometer
AGs	Acidophilic granulocytes
As	Antisense
auf	Arbitrary units of fluorescence
C+	Positive control
cDNA	Complementary deoxyribonucleic acid
CHT	Caudal hematopoietic tissue
COX2b	Cyclooxygenase 2b
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpf	Days post-fertilization
DPI	Dibenziodolium chloride
EDTA	Ethylenediaminetetraacetic acid
F	Forward oligonucleotide
FACS	Fluorescence activated cell system
GFP	Green fluorescent protein
hpf	Hours post-fertilization
hpw	Hours post-wound
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IL-1β	Interleukin 1 beta
Ly	Lymphocytes
LT	Lymphotoxin
MΦ	Macrophages
mAb	Monoclonal antibody

mem-TNFα	Membrane-anchored TNFα form
mM	Milimolar
MO	Morpholino
mRNA	Messenger ribonucleic acid
n	Sample size
NF-κB	Nuclear factor kappa beta
ns	Not significant
nt	Notochord
PBI	Posterior blood island
PRR	Pattern recognition receptor
PTGS2b	Prostaglandin-endoperoxide synthase 2b
R	Reverse primer
RFP	Red fluorescent protein
rps11	Ribosomal protein S11 gene
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
S.E.M	Standard error of the mean
Std	Standard control
sTNFα	Soluble tumor necrosis factor alpha
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
TNFRSF	Tumor necrosis factor receptor superfamily member
TNFSF	Tumor necrosis factor superfamily
TRADD	Tumor necrosis factor receptor associated death domain
TRAF	Tumor necrosis factor receptor-associated factor

TRAIL	Tumor necrosis factor-related apoptosis-induced ligand
*Va*DNA	*Vibrio anguillarum* genomic deoxyribonucleic acid
ZIRC	Zebrafish International Resource Center
ZFNs	Zinc finger nucleases

SUMMARY

TNFα overexpression has been associated with several chronic inflammatory diseases, including psoriasis and inflammatory bowel disease (IBD). Paradoxically, numerous studies have reported new-onset psoriasis following TNFα antagonist therapy in IBD patients. Here, we show that genetic inhibition of Tnfa and Tnfr2 in zebrafish results in the mobilization of neutrophils from the CHT to the skin. Using combinations of fluorescent reporter transgenes, fluorescence microscopy and flow cytometry, we identified local H_2O_2 production 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. Strikingly, DUOX1 was drastically induced in the skin lesions of psoriasis patients. These results reveal a crucial role of H_2O_2 in skin inflammation and could establish new therapeutic targets for psoriasis and IBD patients.

INTRODUCTION

1. Immunity

Immunity is a reaction to foreign substances including microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and macromolecules (proteins and polysaccharides), without involving the pathological result of such a reaction (Abbas *et al.*, 2001). The immune system is composed of cells and molecules that are responsible for immunity, and the collective and coordinated response against these foreign substances constitutes the immune response. Due to the wide variety of infectious agents, requires a variety of immune responses to combat each type of infection (Male and Roitt, 1996).

The immune response starts with the recognition of the pathogen or foreign material, and ends with the development of a mechanism able to remove it (Male and Roitt, 1996). The immune response can be divided into two branches: innate (natural or non-specific) and adaptive (acquired or specific). The fundamental difference between them is that the adaptive is highly specific for a particular pathogen and is most effective with each successive encounter with the same pathogen. Therefore, we can say that the two key features of the adaptive immune response are specificity and memory (Male and Roitt, 1996).

Innate immune response include physical barriers, phagocytic cells and eosinophils, natural killer cells and various blood molecules (complement and acute phase proteins) (Male and Roitt, 1996; Abbas *et al.*, 2001; Mollen *et al.*, 2006), acting as first line of defense against infection until the specific response is triggered.

Adaptive response including lymphocytes (Ly) and secreted antibodies and appears exclusively in vertebrates (Abbas *et al.*, 2001). The Ly are capable of specifically recognizing individual pathogens for which there are two main categories of cells: T Ly (T cells) and B Ly (B cells). B cells are responsible for recognizing and combating extracellular pathogens and their products through their secretions, antibodies, which have the property of binding to a target molecule called an antigen. T cells, in turn, have a large number of activities. T helper Ly (Th) are involved in the regulation of development and production of antibodies (Ab) by B cells or interacting with phagocytic cells helping them to destroy the pathogens that they have phagocytosed. Another type of T Ly, cytotoxic T cells (CTL) recognize and destroy virus-infected and tumor cells.

The innate and adaptive response acts in an integrated and coordinated form. There is considerable interaction between Ly and phagocytes (Male and Roitt, 1996). For example, some phagocytes capture and degrade antigens and present them to T cells attached to the surface major histocompatibility complex (MHC) in a form suitable for them to recognize. This process is called antigen presentation. In response, the Ly secrete soluble factors (cytokines) which activated phagocytes to destroy the pathogens that they have phagocytosed. The result of these interactions is that the majority of immune responses against pathogens consisting of a wide variety of innate and adaptive components. In the early stages of infection, the innate response predominates but lymphocytes subsequently begin to generate the adaptive response.

1.1. Immune system of teleost fish

In teleost fish, the immune system shows similar characteristics to those of birds and mammals, displaying cellular and humoral responses that have the characteristics of specificity and memory (Van Muiswinkel, 1995). Teleost are the first animal group that have an innate and adaptive immune system well structured and differenciated. Its innate response comprises physical barriers barriers (epithelium and mucosa), cellular effectors (phagocytic cells and nonspecific cytotoxic cells) and humoral factors (complement and other acute phase proteins) and adaptive response comprises a cellular (lymphocytes) and humoral (antibodies) components. However, despite their similarities with other vertebrate immune system, there are clear differences as fish depend more heavily on innate defense mechanisms, mainly in low temperature conditions (the fish are poikilothermic) since the adaptive immune response is dependent on the temperature (Cuchens and Clem, 1977; Avtalion, 1981; Abruzzini *et al.*, 1982; Clem *et al.*, 1984, 1985, 1991).

The organs and tissues of the immune system in teleosts have been classified, as in mammals, in primary and secondary organs (Zapata *et al.*, 1996). Fish lack of bone marrow, being the kidney, a primary organ, the par excellence hematopoietic organ. Kidney consists of two parts: the anterior or cephalic (head kidney, HK), with mainly hematopoietic function, and subsequent or posterior, basically with excretory function. In gilthead seabream, HK is formed by precursor cells and by several leukocytes populations such as macrophages (MΦ), Ly and AGs. AGs are the major cell type participating in innate host responses, while the HK is the central immune organ that provides a source for AGs (Sepulcre *et al.*, 2002; Chaves-Pozo *et al.*, 2007). Regarding

to secondary lymphoid organ, the spleen is the most important but presents few Ly although may increase in number by administration of an antigen.

1.1.1. Innate immune system

The first line of defense of fish against invasion of microorganisms consists by physical and chemical barriers such as scales, skin and its secretion, mucus. The most important function of the mucus is to prevent attachment of bacteria, fungi or parasites to epithelial surfaces and digest microorganisms, thanks to a battery of lytic enzymes such as lysozyme.

Innate cellular response of the fish includes a variety of leukocytes, they include phagocytes (monocytes/ MΦ and granulocytes) and nonspecific cytotoxic cells (Secombes, 1996). Phagocytes are more important in innate immunity by its capacity to eliminate viruses, bacteria and parasites (Rowley et al., 1988; Secombes and Fletcher. 1992; Sepulcre et al., 2002) and, moreover, can be the initiator of activation and regulation of the specific immune response (Clem et al., 1985, 1991; Vallejo et al., 1992). The process of phagocytosis in fish has the same steps as described for mammalian leucocytes, ending with two mechanisms responsible for the killing of phagocytized microorganisms: (i) production of reactive oxygen intermediates (ROIs) with a rapid and abrupt increase in the rate of oxygen consumption is known as respiratory burst and is independent of mitochondrial respiration, and (ii) the production of nitric oxide (NO) and other nitrogen reactive intermediates (RNIs). It is further known that ROIs produced by phagocytes of fish have bactericidal activity (Sharp and Secombes, 1993; Skarmeta *et al.,* 1995).

Specifically, studies in gilthead seabream describe AGs as the more active and abundant phagocytic cell of the species (Sepulcre *et al.,* 2002; Chaves-Pozo *et al.,* 2004). Moreover, AGs might be considered as functionally equivalent to mammalian neutrophils, since they are the most abundant circulating granulocytes (Sepulcre *et al.,* 2002), show strong phagocytic and ROIs production capabilities (Sepulcre *et al.,* 2002, 2007), produce cytokines (see below) in response to several immunological stimuli (Chaves-Pozo *et al.,* 2004; Sepulcre *et al.,* 2007) and express a broad range of Toll like receptors (TLRs), with the exception of TLR3 (Sepulcre *et al.,* 2007).

Beside cellular effectors described above, there are a wide variety of substances (humoral effectors) that act on the innate defense of fish (Alexander and Ingram, 1992).

These may be classified functionally into: (i) bacterial growth inhibitors such as transferrin, antiproteases and ceruloplasmin, (ii) viral replication inhibitors such as interferon, (iii) inhibitors of bacterial toxins, (iv) lysines such as lysozyme and chitinase, (v) agglutinins and precipitins such as lectins and C-reactive protein, and finally (vi) complement components that perform several functions, among which leukocyte chemotactic activity (Lamas and Ellis, 1994), opsonisation (Sakai, 1984a), inactivation of certain toxins (Von Eschen and Rudbach, 1974; Ellis, 1980; Sakai, 1984b), the bactericidal activity (Sakai, 1983), cytotoxicity and viral inactivation (Sakai, 1992), are the most remarkable.

1.1.2. Adaptive immune system

Antibodies are key mediators of the adaptive immune response, together with its producing cells, Ly. *In vitro* studies have shown that fish have two cell populations which are equivalent to B and T cells of mammals. These studies using conjugated monoclonal antibodies (mAbs) against specific antigenic determinants on the surface of Ly and functional immunological assays. mAbs against immunoglobulin M (IgM) of teleost serum are capable of reacting with only one of the Ly populations (Lobb and Clem, 1982; DeLuca *et al.,* 1983; Secombes *et al.,* 1983; Navarro *et al.,* 1993), suggesting that the surface Igs may be a marker for B cell-like cells and allowing the isolation of the two cell populations of lymphocytes of teleost: Ig+ e Ig-. Further, performing some functional studies, it have been shown that these two populations for fish (Ig+ and Ig- Ly) have the functional characteristics of the B and T Ly of mammals, respectively (DeLuca *et al.,* 1983; Sizemore *et al.,* 1984; Miller *et al.,* 1986; Marsden *et al.,* 1995). Interestingly, IgD (Edholm *et al.,* 2011) and IgT (Hansen *et al.,* 2005; Danilova *et al.,* 2005) have been also identified in fish. Although the functional relevance of IgD remains to be determined, as in mammals, the teleost-specific IgT seems to be especialized in mucosal immunity and, therefore, would be the functional equivalent to mammalian IgA (Zhang *et al.,* 2010). It has been more recently confirmed the existence of T cells in the teleost by the identification of T cell receptor (TCR) (Haire *et al.,* 2000; Wang *et al.,* 2001; Wermenstam and Pilstrom, 2001; Nam *et al.,* 2003).

Likely mammalian, adaptive immune system of teleost presents memory (Van Muiswinkel, 1995). Thus, after a first contact with the antigen (primary response) produces a specific titer of antibodies in the serum, that is increased in a subsequent

contact with the same antigen (secondary response), being this response dependent on temperature. The most common form of Ig in the serum of the teleost is a tetrameric form usually called IgM due to its high molecular weight and its polymeric structure. This Ig has several functions, including activation of the complement cascade that ends lysing the invading pathogen and binding to the surface antigens of the invading cells or virus-infected cells, thus indicating the target for subsequent action of cytotoxic and phagocytic cells.

As has been previously mentioned, the initiation of the adaptive immune response is controlled by MΦ which functions as antigen presenting cells (APC), degrading and presenting antigens together with MHC proteins to Ly so that they can recognize (Clem *et al.,* 1985; Vallejo *et al.,* 1992). Meanwhile, Ly may produce cytokines that activate MΦ after stimulation with an antigen (Graham and Secombes, 1988), showing, therefore, a coordinated and mutual control between the innate and adaptive response.

1.2. Regulatory molecules of the immune response

1.2.1. Cytokines

Cytokines are proteins (usually glycoproteins) with a low molecular weight (usually no more than 8-25kDa) that regulate all the important biological processes, including cell growth and activation, inflammation, tissue repair, fibrosis and morphogenesis. They are considered as a protein family from a functional point of view, since not all of them are chemically related (Feldmann, 1996). However, some cytokines share a high homology (about 30%), like interleukin (IL)-1β and IL-1α, or tumoral necrosis factor (TNF)-α and TNFβ. In addition, there are subfamilies with a really high structural homology (about 80%), like the interferon α (IFNα) subfamily with about 20 members.

Cytokines mediate effector phases in both innate and adaptive immunity (Abbas *et al.,* 2001). In the innate immunity, cytokines are produced mainly by mononuclear phagocytes and so are usually called monokines. Monokines are produced by mononuclear phagocytes in response to microorganisms and upon T-cell antigen stimulation as part of adaptive immunity. However, most of the cytokines involved in adaptive immunity are produced by activated T Ly and these molecules are referred to as lymphokines. Lymphokines present a double function, either regulating the

proliferation and differentiation of different Ly populations or participating in the activation and regulation of inflammatory cells (mononuclear phagocytes, neutrophils and eosinophils). Both Ly and mononuclear phagocytes produce other cytokines known as colony stimulating factors (CSFs), which stimulate the proliferation and differentiation of immature leukocytes in the bone marrow. Some other cytokines known as chemokines are chemotactic for specific cell types.

Although cytokines are made up of a diverse group of proteins, they share some features (Abbas *et al.*, 2001):

- They are produced during the effector stages of the innate and adaptive immunity, and regulate the inflammatory and immune response.
- Their secretion is brief and auto-limited. In general, cytokines are not stored as pre-formed molecules, and their synthesis is initiated by a new genetic transcription.
- A particular cytokine may be produced by many different cellular types.
- A particular cytokine may act on different cell types.
- Cytokines usually produce different effects on the same target cell, simultaneously or not.
- Different cytokines may produce similar effects.
- Cytokines are usually involved in the synthesis and activity of other cytokines.
- Cytokines perform their action by binding to specific and high affinity receptors present on the target cell surface. This action can be autocrine, paracrine or endocrine.
- The expression of cytokine receptors is regulated by specific signals (other cytokines or even the same one).
- For many target cells, cytokines act as proliferation factors.

In fish, cytokines are grouped into growth factors (Grondel and Harmsen, 1984; Lawrence, 1996; Yin *et al.*, 1997), pro-inflammatory cytokines (Jang *et al.*, 1995a, b; Zou *et al.*, 1999a, b; Fujiki *et al.*, 2000), chemokines (Daniels *et al.*, 1999; Fujiki *et al.*, 1999; Laing *et al.*, 2002), immunosuppressive or anti-inflammatory cytokines (Sumathy

et al., 1997; Laing *et al.,* 1999; Harms *et al.,* 2000) and IFNs (Congleton and Sun, 1996; Collet and Secombes, 2002; Hansen and La Patra, 2002).

1.2.2. Lipid mediators

Lipids, in addition to functioning as an energy source and as structural components of the cell membrane act as effectors and second messengers in a variety of biological processes (Cabral, 2005). These lipid mediators differ in the structural composition and exert multiple effects on cellular functions associated with homeostasis, immune response and inflammation.

Bioefectores lipids produced as a result of cutting the cell membrane phospholipids by the action of phospholipases. These enzymes are activated in response to a variety of extracellular stimuli such as bacterial peptides, cytokines, growth factors and mechanical trauma. Lipid mediators may act as second messengers intracellularly or extracellularly by signaling through G-protein coupled receptors. For their ability as immune effector molecules, lipids bioefectores complement the activities of proinflammatory and anti-inflammatory non-lipid immune modulators.

Arachidonic acid is the precursor of the immune active lipids, collectively called eicosanoids, which include prostanoids, leukotrienes lipoxins and endocannabinoids. Major producer of eicosanoids bioefectores are cells or derived from myeloid lineage such as platelets, monocytes, macrophages, neutrophils and mast cells (Harizi and Gualde, 2002), with the exception of leukotrienes that are produced by other non-immune cells. Several enzymes regulate cellular levels of arachidonic acid and kept in esterified form until it is mobilized by phospholipases. Inflammatory stimuli induce the translocation of cytosolic phospholipase to the endoplasmic reticulum and nuclear membrane thereby releasing arachidonic acid from the lipid membrane of these organelles.

An important group of these lipid mediators are prostanoids which includes prostaglandins (PTG) and thromboxanes (TXA). These molecules are synthesized *de novo* by the action of two cyclooxygenase isoforms, COX-1 (PTGS1) or COX-2 (PTGS2) from arachidonic acid released from cell membrane. These enzymes act upstream of a variety of isomerases whose action culminates in the production of PTGA2, PTGD, PTGE2, PTGI2 and TXA. COX-2 is involved in the synthesis of pro-inflammatory prostaglandins. Its expression is very low or undetectable in the majority

of cells and its expression increases significantly after stimulation, particularly in immune cells (Smith *et al.,* 1996).

The mode of action of these prostaglandins depends on their receptors. Thus, the receptors of pro-inflammatory prostaglandins can activate opposite signaling pathways. In this way, they can act as anti-inflammatory or pro-inflammatory mediators depending on the context.

1.3. Innate immune system receptors

The innate immune system uses a wide spectrum of receptors that recognize pathogen-associated molecular patterns (PAMPs) and have been called pattern recognition receptors (PRRs). These receptors may be present on the cell surface, into intracellular compartments, in the cytosol or may be soluble in blood or tissue fluids (Mendzhitoz and Janeway, 2000). The main functions of these receptors include opsonization, activation of complement cascades and coagulation, phagocytosis, activation of pro-inflammatory signaling pathways and the induction of apoptosis (Mendzhitoz and Janeway, 2000). The main group of membrane receptor PRRs are receptors similar to *Toll* proteins of *Drosophila melanogaster* (Toll-Like Receptors, TLRs). These receptors differ among themselves in their specificity for the ligands. In gilthead seabream, the animal model of this study, AGs express a broad range of TLRs, although not TLR3 (Sepulcre *et al.,* 2007), while TLR9 is the only one which is expressed in the gonad (Chaves-Pozo *et al.,* 2008).

2. Inflammation

Inflammation can be simply and concisely defined as the reaction of vascularised living tissue to local injury (Rippey, 1994). It is part of the biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants (Ferrero-Miliani *et al.*, 2007), supposing the first line of defense and starting the reparation of the injured tissues. The four cardinal signs of inflammation were already described in the 1st Century AD by the Roman medical writer Aulus Cornelius Celsus: redness (Latin *rubor*), heat (*calor*), swelling (*tumor*), and pain (*dolor*). A fifth sign, loss of function (*function laesa*), was added by the German doctor Rudolph Carl Virchow (13 October 1821 – 5 September 1902) in the 19th Century (Lawrence *et al.*, 2002; Kumar *et al.*, 2005; Martini and Nath, 2009) **(Figure 1)**.

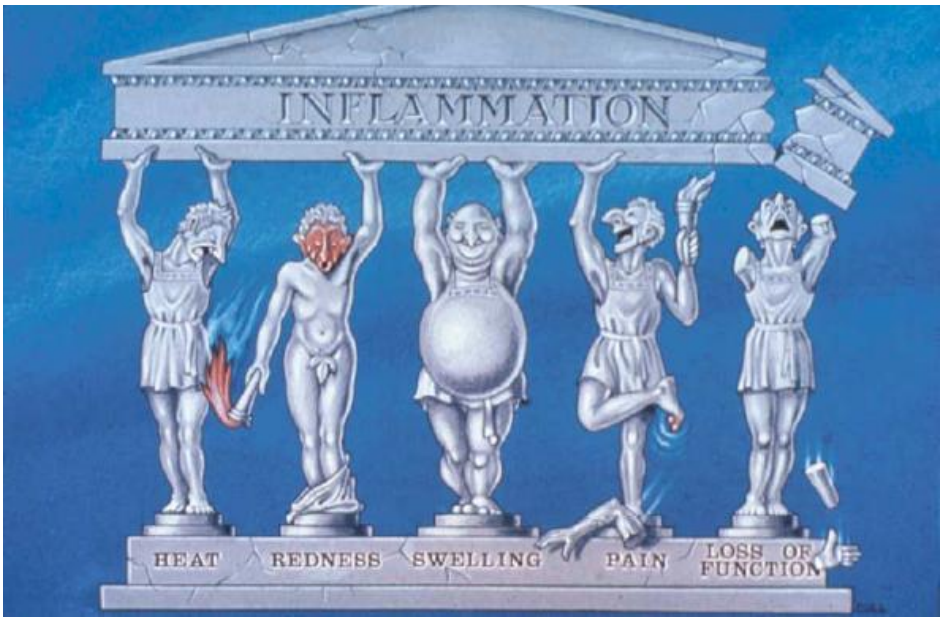


Figure 1. The five cardinal signs of inflammation. Cartoon representing the cardinal signs of inflammation (heat, redness, swelling, pain and loss of function) which are as appropriate today as they were when first described by Celsus more than 2000 years ago. (Lawrence *et al.*, 2002).

Inflammation is divided into acute inflammation, which occurs over seconds, minutes, hours, and days, and chronic inflammation, which occurs over longer times.

2.1. Acute inflammation

Acute inflammation begins within seconds to minutes following the injury of tissues. The damage may be exclusively physical, or it may unleash an immune response. Three main processes occur:

- **Increased blood flow** due to dilation of blood vessels (arterioles) supplying the region.
- **Increased permeability of the capillaries**, allowing fluid and blood proteins to move into the interstitial spaces.
- **Migration of neutrophils** (and perhaps a few macrophages) out of the capillaries and venules and into interstitial spaces.

A well-controlled acute inflammatory response has several protective roles (Medzhitov, 2008):

- It prevents the spread of infectious agents and damage to nearby tissues.
- It helps to remove damaged tissue and pathogens.
- It assists the body's repair processes.

2.2. Chronic inflammation

If the condition causing acute inflammation is not resolved, the inflammation may pass to a longer term chronic phase. Also, some pathologies by their nature tend to directly provoke chronic rather than acute inflammation. Many of the features of acute inflammation continue as the inflammation becomes chronic, including increased blood flow and increased capillary permeability. Accumulation of white blood cells also continues, but the composition of the cells changes **(Figure 2)**.

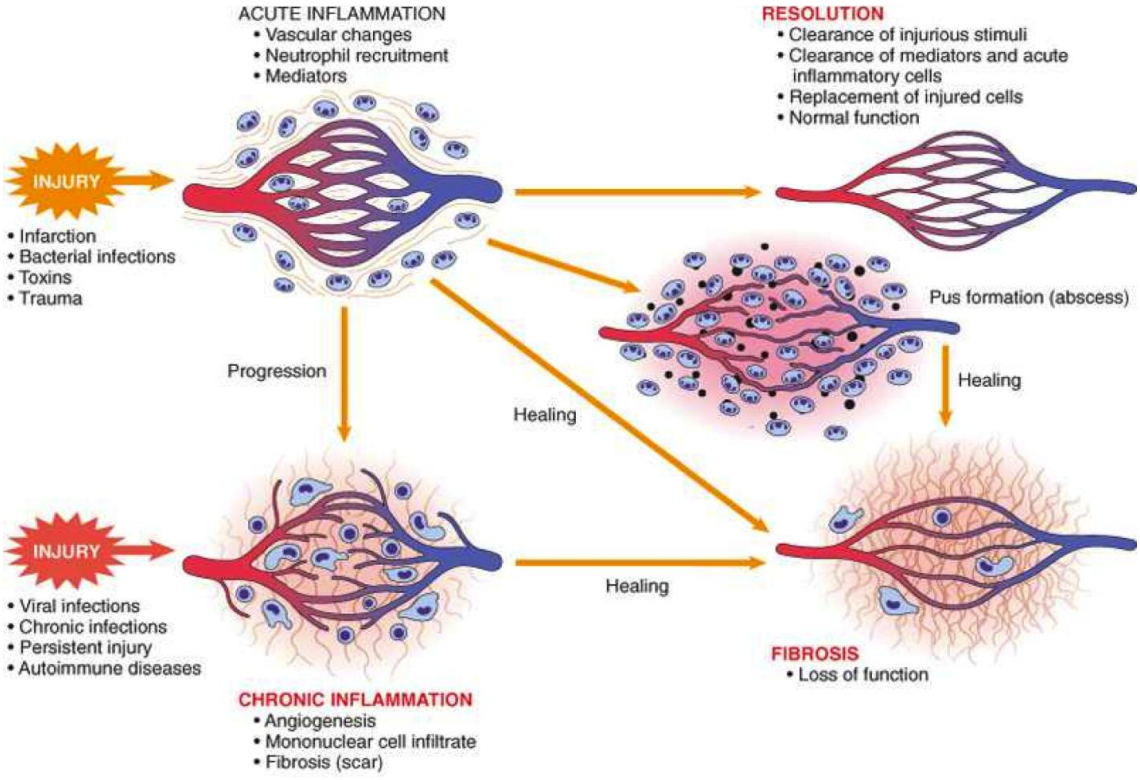


Figure 2. Differences between acute and chronic inflammation at tissue level. Scheme showing the main causes and events in acute and chronic inflammation. Acute inflammation may go on to develop into chronic inflammation for many different reasons. Chronic inflammation is a circular progression of the inflammatory response which is prolonged and where destruction and inflammation are proceeding at the same time as attempts at healing (Rippey, 1994). (Adapted from Kumar *et al.*, 2013).

After the unleashing of the pro-inflammatory program, neutrophils quickly enter the infected or damaged tissue, and these short-lived cells predominate initially. However, soon macrophages and lymphocytes begin to be recruited. The sequence by which they bind to cell adhesion molecules and pass through the endothelium is the same as for neutrophils. Thus, the primary cells of chronic inflammation are macrophages and lymphocytes.

Macrophages, which live far longer than neutrophils, phagocytize pathogens and other material at the site of the inflammation. Because they are long-lived, indigestible material may remain inside macrophages in vesicles for long periods. Moreover, macrophages are important secretory cells releasing inflammatory paracrines, growth factors, and a variety of other proteins.

Lymphocytes entering the inflamed tissue can serve several roles. Most notable, perhaps, are the T cells that activate macrophages. This is important for dealing with difficult pathogens. But this issue will arise most frequently in the context of autoimmune diseases, in which activated macrophages often are a major factor causing the damage. B cells making antibodies also can be present in inflamed tissue, adding antibodies locally to those that enter from the blood.

In certain cases of chronic inflammation, macrophages collect in layers surrounding the problematical material. Sometimes the macrophages will fuse, forming giant cells. The structure so formed, with layers of macrophages surrounding a central core, is called a granuloma. Granulomas are a characteristic feature of tuberculosis, in which macrophages can't destroy the phagocytized bacteria, apparently because the bacteria somehow prevent lysosomes from fusing with the phagocytic vesicles.

2.3. Chronic inflammatory diseases

The establishment of a chronic inflammation can be the starting point for a multitude of different chronic inflammation diseases **(Figure 3)**, which are defined by long-term inflammatory processes directed at a particular endogenous or exogenous antigen (Heap GA and van Heel DA, 2009).

The incidence of these diseases is being rapidly increased worldwide, mainly in the most developed countries, supposing a great impact for their national health systems. Taking only one example, of the ten leading causes of mortality in the United States of America in 2011, chronic inflammation contributes to the pathogenesis of at least seven. These include heart disease, cancer, chronic lower respiratory disease, stroke, Alzheimer's disease, diabetes, and nephritis (Centers for Disease Control and Prevention 2011; Bastard *et al.* 2006; Ferrucci *et al.* 2010; Glorieux *et al.* 2009; Kundu *et al.* 2008). Therefore, the necessity of new treatments for the attenuation of chronic inflammation is essential.

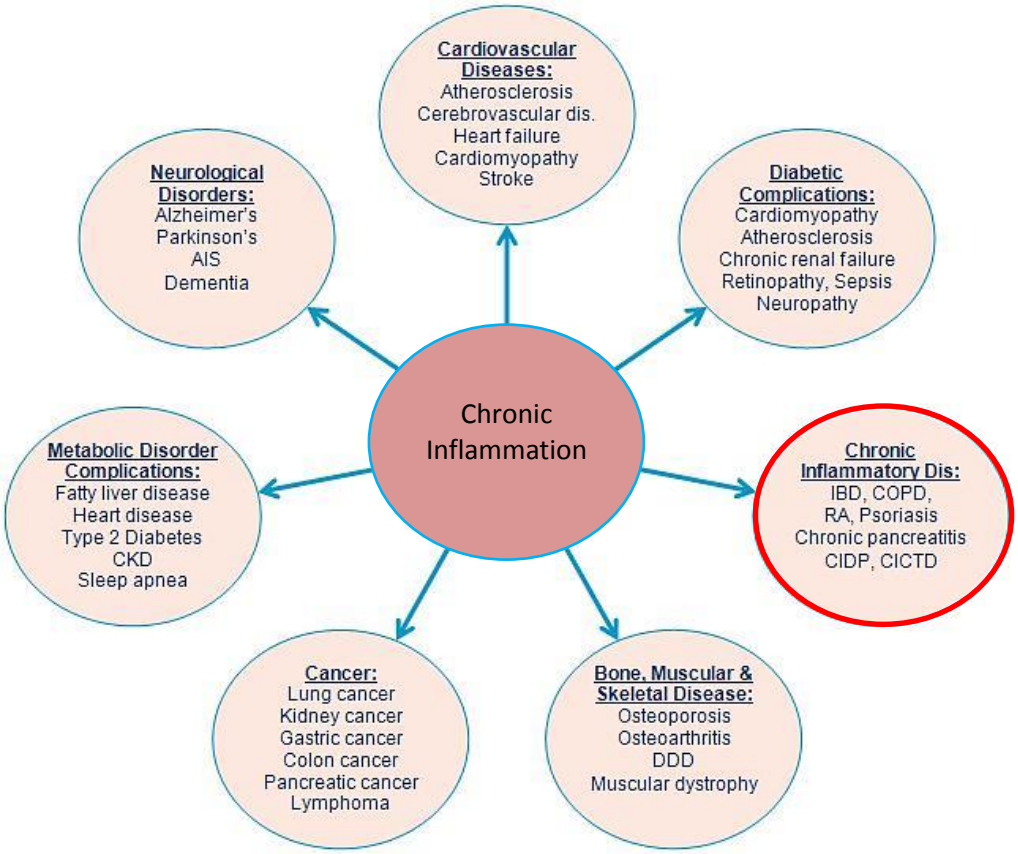


Figure 3. Many important diseases are caused as consequence of chronic inflammation. Scheme showing the main groups of disorders that can be caused by chronic inflammation and the most representative diseases within each group. The group composed by the chronic inflammatory diseases, where psoriasis is included, has been highlighted with a red circle due to its relevance for this thesis (adapted from http://www.automation-drive.com/chronic-ibd).

In chronic inflammatory diseases, several cytokines recruit activated immune and inflammatory cells to the site of lesions, thereby amplifying and perpetuating the inflammatory state. These activated cells produce many other mediators of inflammation. What causes these diseases is still not completely known, but the disease process results from an interplay of genetic and environmental factors. Genes, such as those for atopy in asthma and for HLA antigens in rheumatoid arthritis and IBD, may determine a patient's susceptibility to the disease and the disease's severity, but environmental factors, often unknown, may determine its course. Once established, a chronic inflammatory process appears to take on a momentum of its own. The vicious circle may be suppressed by glucocorticoid or immunosuppressive therapy, but until now there is no curative treatment for any chronic inflammatory disease (Barnes and Karin, 1997).

Chronic inflammation is characterized by the dominating presence of macrophages in the injured tissue. These cells are powerful defensive agents of the body, but the toxins they release (including reactive oxygen species) are injurious to the organism's own tissues as well as invading agents. Consequently, chronic inflammation is almost always accompanied by tissue destruction (Kumar *et al.,* 2005).

2.3.1. Relevance of NF-κB in chronic inflammatory diseases. A potential target for therapeutics

To understand the molecular mechanisms behind chronic inflammatory diseases it is essential to take into consideration the activity of the transcription factors, which are responsible for the modulation of the gene expression. Many of these transcription factors are cell-specific and are crucial in cell differentiation and the regulation of specific cellular processes such as proliferation. Other transcription factors are ubiquitous, and their activity may be modulated by environmental signals. It is these latter transcription factors that may have a key role in immune and inflammatory responses. One ubiquitous transcription factor of particular importance in immune and inflammatory responses is nuclear factor-κB (NF-κB) (Barnes and Karin, 1997; Kumar *et al.*, 2005).

The extensive involvement of NF-kB transcription factor in human inflammation and disease establishes it as target for therapeutics. Indeed, many common synthetic (e.g., aspirin), and traditional (e.g., green tea, curcumin) remedies target, at least in part, the NF-kB signaling pathway. However, there are over 800 compounds that have been shown to inhibit NF-kB signaling, and thus, the physiological or pharmacological utility of using any single compound for inhibition of NF-kB activity is a bit muddled. Nevertheless, our knowledge of the molecular details of this pathway is enabling the development of more specific and potent inhibitors of NF-kB signaling, and indeed, some NF-kB signaling inhibitors are entering clinical trials (Perkins, 2007; Gilmore and Wolenski, 2012).

2.3.2. Psoriasis, a chronic inflammatory skin disease

Psoriasis is a chronic, genetically influenced, remitting and relapsing scaly and inflammatory skin disorder (Greaves and Weinstein, 1995), characterized by the appearance of red plaques covered with silvery scale that flakes away from the skin **(Figure 4)**. Psoriatic plaques are often found on the elbows, scalp and knees but can

also affect other parts of the body such as the face, feet and mucous membranes. It affects approximately 1-3% of the world's population (Greaves and Weinstein, 1995; Schön and Boehncke, 2005), and it is not contagious nor is it caused by an allergy. In the 1990s, it was proved that psoriasis is multifactorial and linked to genetic, immunological and environmental factors (Crissey and Parish, 1998).

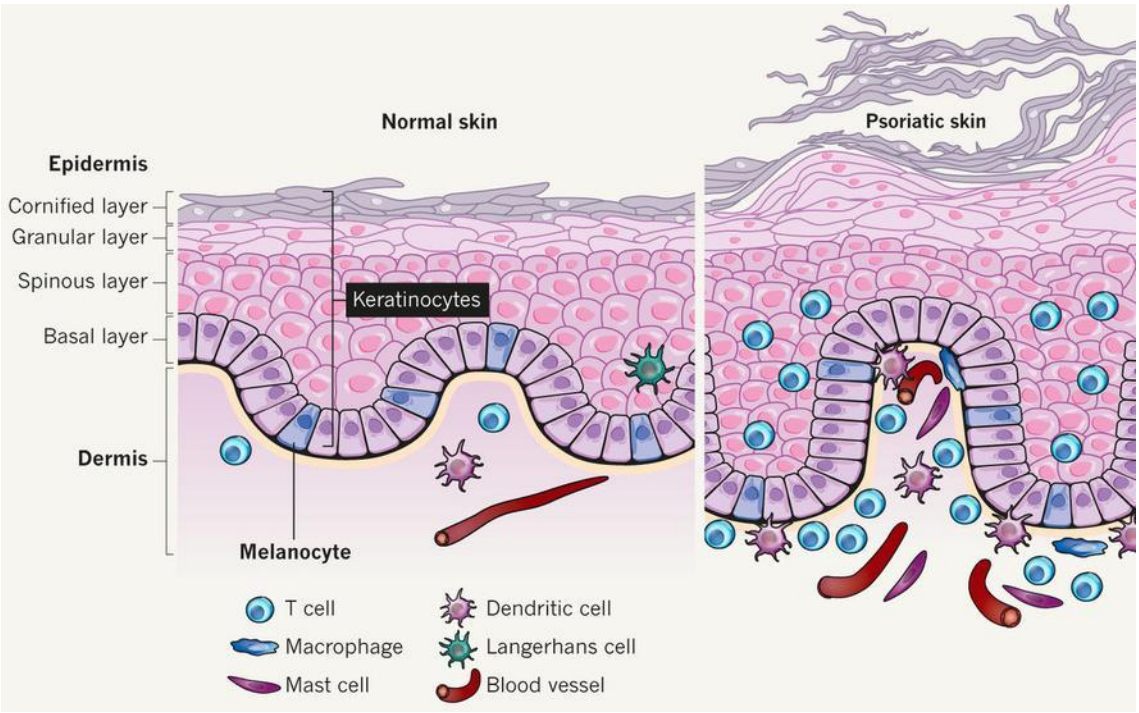


Figure 4. Differences between normal and psoriatic skin. Scheme showing the aspect of psoriatic compared to normal skin. Note the recruitment of immune cells into the skin and the scales production on their surface in psoriatic patients. Psoriatic skin exhibits pathological changes in most, if not all, cutaneous cell types. The typical erythematosquamous plaque contains histopathological hallmark features that include hyperproliferation of epidermal keratinocytes and hyperkeratosis, as well as infiltration of immunocytes along with angiogenesis, with resultant typical thickening and scaling of the erythematous skin. Mitotic activity of basal keratinocytes is increased by as much as a factor of 50 in psoriatic skin, so keratinocytes need only 3 to 5 days in order to move from the basal layer to the cornified layer (instead of the normal 28 to 30 days). This dramatically shortened maturation time is accompanied by altered differentiation, reflected by the focal absence of the granular layer of the epidermis and parakeratosis, or nuclei still present in the thickened cornified layer (Schön and Boehncke, 2005) (adapted from cassock.rssing.com).

Psoriasis is a disabling, though rarely life-threatening, disease with a high social and economic impact. Recently, progress has been made in understanding the

pathogenesis of psoriasis, and therapeutic advances are improving the care of even severely affected patients (Greaves and Weinstein, 1995). Although most established treatment regimens are reasonably effective as short-term therapy for psoriasis, extended disease control is difficult to achieve because the safety profile of most therapeutic agents limits their long-term use (Boehncke, 2003). Another unmet medical need is for agents that can be applied easily, since application of various currently available agents is difficult and thus compliance may be problematic. The most promising compounds are monoclonal antibodies, cytokines, and fusion proteins. Three fundamental modes of action are being explored: decreasing the number of pathogenic T cells, blocking T-cell migration and adhesion, and antagonizing effector cytokines (Schön and Boehncke, 2005).

2.3.3. Inhibition of TNFα as treatment for chronic inflammatory diseases, including psoriasis

Enhanced TNFα synthesis has been associated to the development of chronic inflammatory diseases, including psoriasis, and the inhibition of its activities in these diseases has been remarkably successful (Palladino *et al.*, 2003; Faustman *et al.*, 2010). TNFα can be functionally inhibited by the chimeric antibody infliximab or by the recombinant human TNF-receptor fusion protein etanercept. Both agents competitively inhibit interactions of TNF-α with cell-surface receptors and show convincing efficacy in treating psoriasis (Chaudhari *et al.*, 2001; Leonardi *et al.*, 2003). Shifting the immunologic microenvironment in psoriatic skin, dominated by Th1-type cytokines through substitution of type 2 helper T-cell–type cytokines, such as interleukin-10 (Asadullah *et al.* 1998) and interleukin-4 (Ghoreschi *et al.*, 2003), has been reported as effective in some cases of psoriasis.

Paradoxically, however, numerous studies have reported new-onset psoriasis, or worsening of existing psoriasis, following TNFα antagonist therapy in adult IBD patients (Denadai *et al.*, 2012; Sherlock *et al.*, 2012). Despite these clinical data pointing to an ambiguous function of TNFα in psoriasis, the role of TNFα, and in particular the contribution of each TNFR, in the regulation of skin inflammation has scarcely been 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 TNFR-deficient siblings responded normally (Kondo and Sauder, 2012). 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 (Pasparakis *et al.*, 2002; Gugasyan *et al.*, 2004; van Hogerlinden *et al.*, 2004; Omori *et al.*, 2006; Sayama *et al.*, 2006). The characteristics of this complex disorder are strikingly similar to those associated with the human X-linked genodermatosis incontinentia pigmenti (IP) (Smahi *et al.*, 2000). To the best of our knowledge, however, the role played by TNFα in the homeostasis of healthy skin has never been deeply studied before this thesis.

2.3.4. Role of neutrophils in chronic inflammatory diseases

Neutrophils represent the body's primary line of defence against invading pathogens such as bacteria **(Figure 5)**, and constitute 40–60% of the white blood cell population.

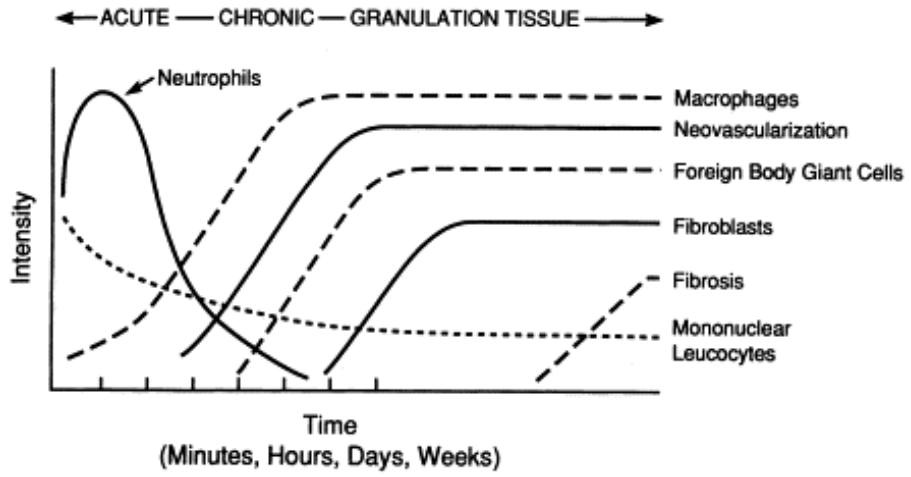


Figure 5. Sequence of events after inflammation unleashing. Picture showing the appearance order followed by the different cells implicated in the inflammatory process (Anderson and Shive, 1997).

In the circulation of healthy adults, neutrophils exist in a resting state, which ensures that their toxic intracellular contents are not accidentally released to damage host tissue. Neutrophils become activated via a two-stage process. Resting neutrophils

can become primed by agents that include bacterial products and cytokines or chemokines, e.g. TNFα, GM-CSF, IL-8 and IFNγ and primed neutrophils are then mobilized to the site of infection or inflammation, where they encounter activating signals to trigger bacterial killing (Hallett and Lloyds, 1995).

In the last years, the perception of the neutrophil playing a passive role and simply responding to external signals has been replaced by an appreciation that activated neutrophils can perform most (if not all) of the functions of macrophages. It is now recognized that appropriately activated neutrophils secrete a variety of pro-inflammatory cytokines and express MHC Class II (MHCII) in a manner that allows presentation of antigen to, and activation of, T cells. It is also recognized that neutrophils contribute to the pathogenesis of a number of human inflammatory diseases such as chronic obstructive pulmonary disease, psoriasis and inflammatory arthritis. In some of these conditions, neutrophils appear to have been inappropriately activated to release tissue-damaging molecules (such as proteases) or, alternatively, molecules that can promote inflammation such as chemoattractants (eicosanoids and chemokines) or cytokines (Wright *et al.,* 2010).

It was known that neutrophils are abundantly present in psoriatic infiltrates, being recruited by the neutrophil-attracting chemokine interleukin-8 (CXCL8). However, this pathway is probably not the exclusive means of neutrophil recruitment, since an interleukin-8–blocking monoclonal antibody had only modest efficacy in a clinical study (Homey, 2004).

Hydrogen peroxide gradients have been recently shown to contribute to the early influx of neutrophils in wound (Niethammer *et al.,* 2009) and tumor (Feng *et al.,* 2010). Interestingly, however, H_2O_2 is not required for neutrophil detection of localized infection (Deng *et al.*, 2012). These gradients are created by the dual oxidase 1 (Duox1) (Niethammer *et al.,* 2009) and sensed by neutrophils through the tyrosine kinase Lyn (Yoo *et al.,* 2011). Although identified and best studied in the zebrafish, H_2O_2 is likely to play the same function in human neutrophils (Yoo *et al.,* 2011).

2.3.5. Modulation of neutrophil function in inflammation by TNFα

TNFα has a dynamic effect on neutrophils: at low concentrations its effect is biphasic, promoting early apoptosis in a sub-population of cells, but delaying apoptosis in the remaining cells (Van den Berg *et al.,* 2001). It is thought that this mechanism is

controlled through stimulation of different signaling pathways via each TNF receptor. While both TNF receptors promote early cell death, only TNFR1 can delay apoptosis via NF-kB-controlled expression of pro-survival genes such as Bfl-1 and TRAF-1 (Murray *et al.,* 1997; Nolan *et al.,* 2000; Cross *et al.,* 2008). At high concentrations, TNFα induces neutrophil apoptosis via death receptor signaling through both TNF receptors, leading to caspase-8 activation and the loss of anti-apoptotic proteins such as Mcl-1 via caspase cleavage (Cross *et al.,* 2008). Thus, local concentrations of TNF can have opposing effects on neutrophil function in inflammation (Wright *et al.,* 2010).

3. Tumor necrosis factor alpha (TNFα)

3.1. Tumor necrosis factor superfamily (TNFSF)

Tumor necrosis factor alpha (TNFα) is a powerful pro-inflammatory cytokine which exerts its function by binding two specific receptors: TNFR1 and TNFR2. Since TNFα was cloned in 1984, many other proteins have been showed to have high sequence homology with this cytokine, including TNFα receptors. Thus, Tumor necrosis factor superfamily (TNFSF) was created to include all these sequence-related proteins. The TNFSF is nowadays composed by 19 ligands (TNF superfamily ligands: TNFSFs) and 29 receptors (TNF superfamily receptors: TNFSFRs) (Aggarwal *et al.*, 2012), and most of them are produced by immune cells such as natural killer (NK), T and B cells, macrophages, dendritic cells, mast cells, neutrophils and monocytes. However, other types of cells like hematopoietic cells, endothelial cells, skin cells and smooth muscle cells have been also reported to produce some of this proteins belonging to the TNFSF. Among TNFSF proteins, the following members can be emphasised: tumor necrosis factor beta, TNFβ (also called LTα); lymphotoxin beta, LTβ; fibroblast-associated ligand, FASL; LIGHT; LIGHTR; TNF-related apoptosis-induced ligand, TRAIL; proliferation-inducing ligand, APRIL; and vascular endothelial cell-growth inhibitor, VEGI.

All members of the TNF superfamily have been shown to have pro-inflammatory activity, yet some of them also exhibit proliferative activity on hematopoietic cells and play a role in morphogenetic changes and differentiation. Therefore, TNFSF members play roles as contradictory as cell apoptosis and survival, proliferation or differentiation **(Figure 6)**.

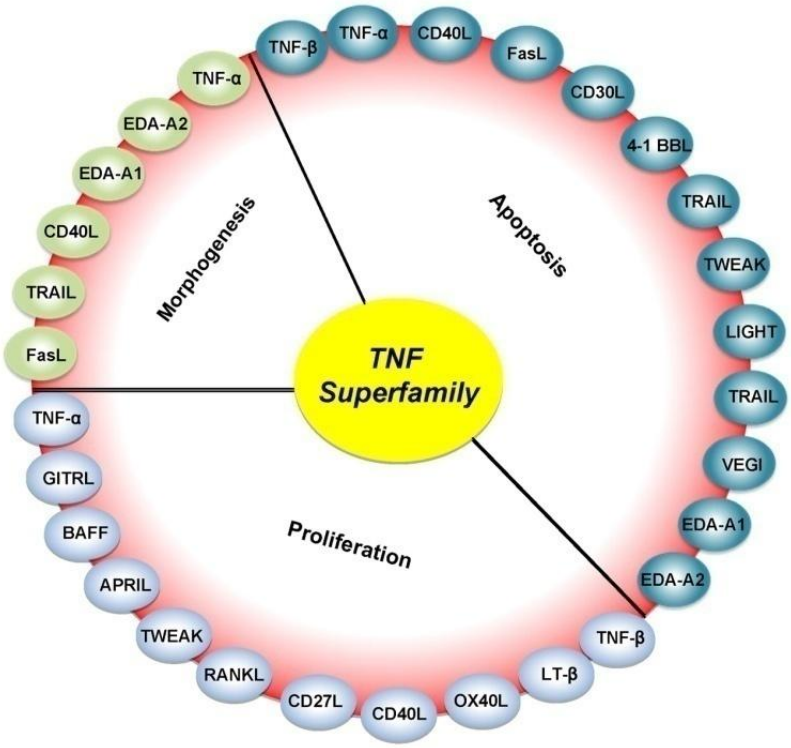


Figure 6. Roles of some members of TNFSF in apoptosis, morphogenesis and proliferation. All members of the TNF superfamily exhibit pro-inflammatory activity, in part through activation of the transcription factor NF-κB (full red circle); OX40L, CD40L, CD27L, APRIL, and BAFF exhibit proliferative activity in part through activation of various mitogen-activated kinases (sky blue); TNFα, TNFβ, FasL, and TRAIL control apoptosis (bluish-green); and EDA-A1, EDA-A2, TNFα, FasL, and TRAIL regulate morphogenesis (green). Adapted from Aggarwal *et al.,* 2005.

3.2. TNFα structure, nomenclature and function

TNFα presents an extracellular C- and an intracellular N- terminus when this cytokine is anchored to the plasma membrane (named pro-TNFα or membrane TNF-α, mem-TNFα) and it is composed of three identical TNFα units. Pro-TNFα can be processed by a TNFα converting enzyme (TACE), transforming the membrane precursor in a soluble C-terminal form (sTNFα), which exerts its biological actions as a 51 kDa soluble homotrimer (Wajant *et al.,* 2003) **(Figure 7)**.

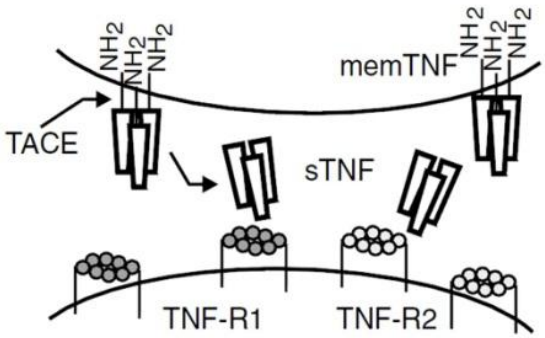


Figure 7. Mem-TNFα can be processed by TACE, releasing the extracellular C-terminal of pro-TNFα. Adapted from Wajant *et al.*, 2003.

TNFα nomenclature can be confusing since it has changed along the years. In 1998, the name for TNFα was changed upon recommendation by the TNF Congress to TNF. However, the term TNFα is still widely used and it is used in this thesis as a term which refers indistinguishable to both, pro-TNFα and mem-TNFα.

TNFα is mainly produced by macrophages, but is also produced by a broad variety of cell types including lymphoid cells, mast cells, T and B cells, NK cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons. TNFα is known to affect the growth, differentiation, survival and physiological functions of a variety of different cells, including cells outside of the immune system. Thus, TNFα is a true pleiotropic factor which plays an important role in the immune response as well as in other physiological processes, such as metabolism and reproduction. Perhaps one of the best known actions of TNFα is in macrophage-mediated cytotoxicity due to the proapoptotic effects of TNFα. However, TNFα is increasingly recognized as a key regulator of lipid metabolism in adipose tissue and protein catabolism in muscle. Also, TNFα has been shown to be linked to an array of pathophysiologies, including cancer, neurologic diseases, cardiovascular diseases, pulmonary diseases, autoimmune diseases and metabolic diseases (Aggarwal *et al.*, 2012) **(Figure 8)**.

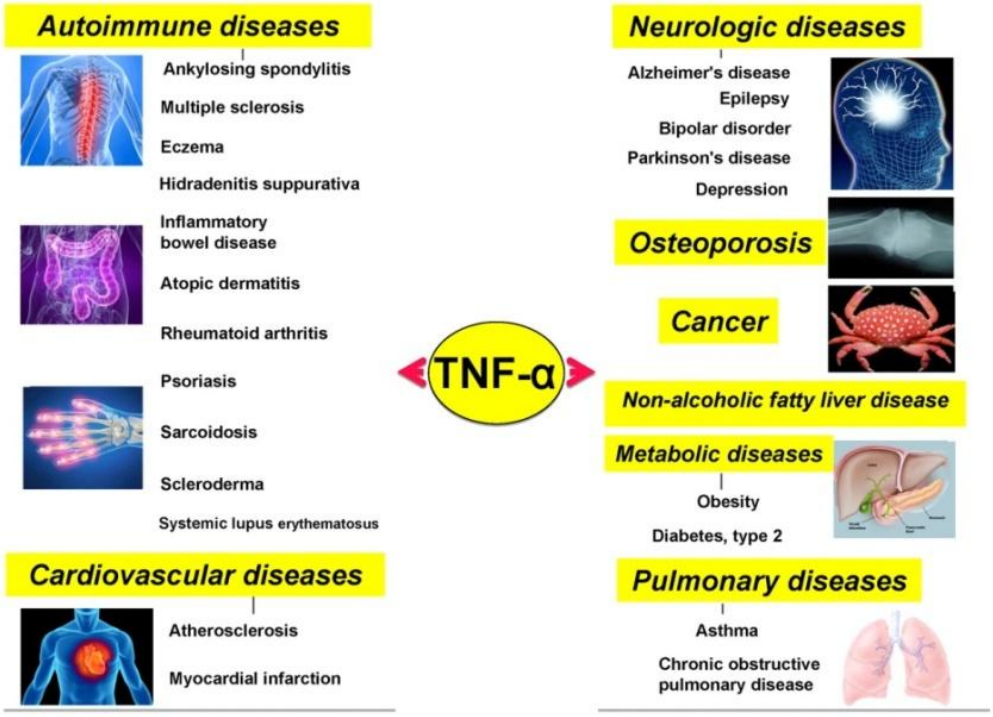


Figure 8. Various diseases that have been closely linked to TNFα. Adapted from Aggarwal *et al.*, 2012.

3.3. TNFα receptors: TNFR1 and TNFR2

Both forms, soluble and membrane-bound TNFα, can bind two transmembrane receptors: TNFR1 (also called TNFRSF1A, TNF receptor type 1, p55/60, CD120a) and TNFR2 (TNFRSF1B, TNF receptor type 2, p75/80, CD120b) **(Figure 9)**. Both receptors belong to the TNFSFR family, sharing with all their members the peculiarity of having the named cystein-rich domains (CRDs) in their extracellular domain, which is involved in the ligand binding. Even though TNFR1 is activated by both TNFα forms, TNFR2 is mainly activated by the membrane TNFα (Naudé *et al.*, 2011).

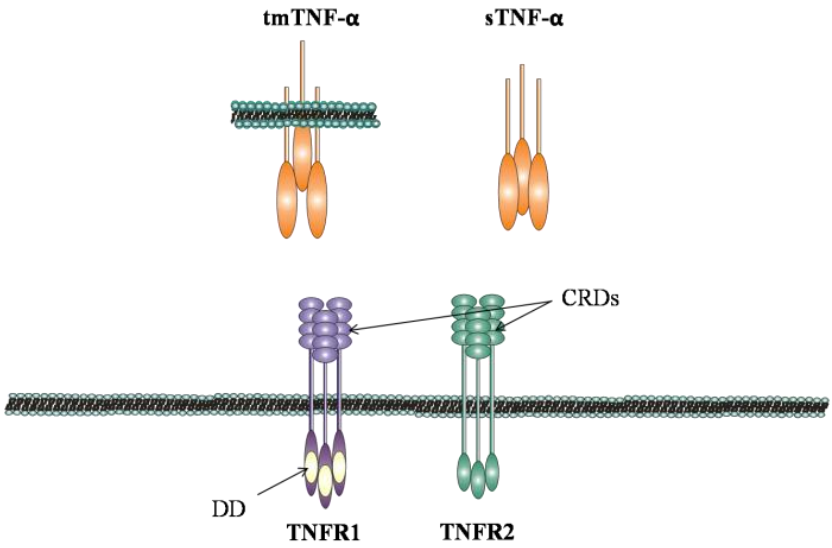


Figure 9. Schematic representation of the pro-TNFα, sTNFα and their receptors, TNFR1 and TNFR2. DD: Death domains; CRDs: Cystein-rich domains.

3.3.1. Structure and function

TNFα receptors have been reported to have marked differences in structure, expression patterns, signaling and function **(Table 1)**. While TNFR1 has been found in all cell types, TNFR2 seems to have a more restricted expression to certain cells such as CD4 and CD8 T lymphocytes, thymocytes, endothelial cells, microglia, oligodendrocytes, neuron subtypes and human mesenchymal stem cells (Faustman and Davis, 2010). This means that each cell types bearing TNFR2 also have TNFR1. The ratio of expression of TNFR1 to TNFR2 and the signaling behind these receptors typically fluctuates in relation to the cell type and its functional roles. Moreover, for immune cells, the state of prior activation of the cell is also a key variable.

Both receptors comprise four domains: a hydrophobic signal peptide, an intracellular domain, a transmembrane segment and a soluble extracellular cysteine-rich domain which functions as the receptor locus (Tuma *et al.*, 1995). The soluble extracellular domains of TNFR1 and TNFR2 have been purified, sequenced, and cloned (Hale *et al.*, 1995) and they exhibit 28% of sequence homology (Dembic *et al.* 1990), being capable of binding either TNFα or TNFβ. However, TNFR1 alone possesses a cytoplasmic death domain, an 80 amino-acid sequence that rapidly engages the apoptotic signaling pathway of the cells. The cytoplasmic domain of TNFR2 bears no

structural or functional resemblance to that of TNFR1 (Tartaglia *et al.*, 1993; Hsu *et al.*, 1995).

STRUCTURE	EXPRESSION	SIGNALING	FUNCTION
TNFR1			
-Extracellular domain: cystein-rich and similar to TNFR2 -Intracellular domain: death domain; no resemblance to TNFR2	Nearly all cells of the body	Adapter protein FADD and TRADD, which engage caspases	-Pro-apoptosis -Some pro-survival functions, depending on crosstalk with TNFR2, cell type, activation state, age and other factors.
TNFR2			
-Extracellular domain: cystein-rich and similar to TNFR1 -Intracellular domain: no death domain; no resemblance to TNFR1	Limited expression: CD4 and CD8 T lymphocytes, endothelial cells, microglia, oligodendrocytes, neuron subtypes, cardiac myocytes, thymocytes and human mesenchimal stem cells	One pathway involves adapter proteins TRAF1 and TRAF2 and the eventual cleavage of transcription factor NF-κB from its inhibitor molecule in the cytoplasm IκB	-Pro-survival functions in immunity to infection, injury, cancer and autoimmune disease via proliferation of T cell differentiation and recruitment of naive immune cells. -Some pro-apoptotic functions to limit immune response after injury or inflammation resolves. -Other pro-apoptotic functions depend on crosstalk with TNFR1, cell type, activation state, age and other factors

Table 1. TNFR1 and TNFR2 expression, signaling and function. FADD, Fas-associated death domain; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; NF-κB, nuclear factor-κB; TNFR, tumour necrosis factor receptor; TRADD, TNFR1-associated death domain; TRAF, TNF receptor-associated factor. Adapted from Faustman and Davis, 2010.

The DD of TNFR1 is capable of rapidly triggering apoptosis in the cell. Thus, TNFR1 has been described to mainly produce apoptosis, whereas proliferation is associated to TNFR2. TNFR2 can also induce apoptosis in certain circumstances, having been described that there is crosstalk between both receptors (Naudé *et al.*,

2011). Therefore, both TNFR1 and TNFR2 can mediate proliferation, differentiation and inflammation (Faustman and Davis, 2010).

3.3.2. Signaling pathways

TNFα signaling through TNFR1 and TNFR2 receptors is complex; mainly due to that neither TNFR1 nor TNFR2 possesses enzymatic activity *per se*, and different adaptor proteins need to be recruited. Moreover, some of these adaptor proteins are able to participate in both TNFR1 and TNFR2 pathways, making difficult to discriminate the receptor which TNFα is signaling through.

TNFα induces at least 5 different types of signals that include activation of NF-κB, apoptosis pathways, extracellular signal regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK), and c-Jun N-terminal kinase (JNK) **(Figure 10)**. When TNFα binds to TNFR1, it recruits a protein called TNFR associated death domain (TRADD) through its DD (Hsu *et al.,* 1995). TRADD then recruits a protein called Fas-associated protein with death domain (FADD), which then sequentially activates caspase-8 and caspase-3, leading to apoptosis (Hsu *et al.,* 1996). Alternatively, TNFα can activate mitochondria to sequentially release reactive oxygen species (ROS), cytochrome C, and Bax, leading to activation of caspase-9 and caspase-3 and thus apoptosis. Paradoxically, TNFα has also been shown to activate NF-κB, which in turn regulates the expression of proteins associated with cell survival and proliferation (Aggarwal *et al.,* 2004). NF-κB activation by TNFα is mediated through sequential recruitment of TNFR1, TRADD, TNFR-associated factor 2 (TRAF2/TRAF5) and receptor interacting protein (RIP), resulting in activation of TGF-β-activated kinase 1 (TAK1) and IκB kinase (IKK) complex, the phosphorylation, ubiquitination, and degradation of inhibitor of nuclear factor-κBα (IκBα), and, finally, the nuclear translocation of p50 and p65 NF-κB subunits and DNA binding (Devin *et al.,* 2000). The proinflammatory effect of TNF is mediated through NF-κB–induced transcriptions of genes encoding IL-6, IL-18, chemokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX), all major mediators of inflammation. Indeed, TNFα can induce expression of TNFα itself through activation of NF-κB (Aggarwal *et al.,* 2003). TNFα can also activate cellular proliferation through activation of another transcription factor, activator protein-1 (AP-1) (Natoli *et al.,* 1997), which is activated by TNFα through sequential recruitment of TNFR1, TRADD, TRAF2, MAP/ERK kinase kinase 1 (MEKK1), MAP kinase kinase 7 (MKK7), and

JNK. The activation of p38MAPK by TNFα is mediated through TRADD-TRAF2-MKK3. How TNFR2, which lacks a DD, activates cell signaling is much less clear than how TNFR1 activates cell signaling. Because TNFR2 can directly bind to TRAF2, it can activate both NF-κB and MAPK signaling quite well. Interestingly, TRADD has been reported recently to mediate cell signaling by TOLL-like receptors 3 and 4 (Ermolaeva *et al.*, 2008).

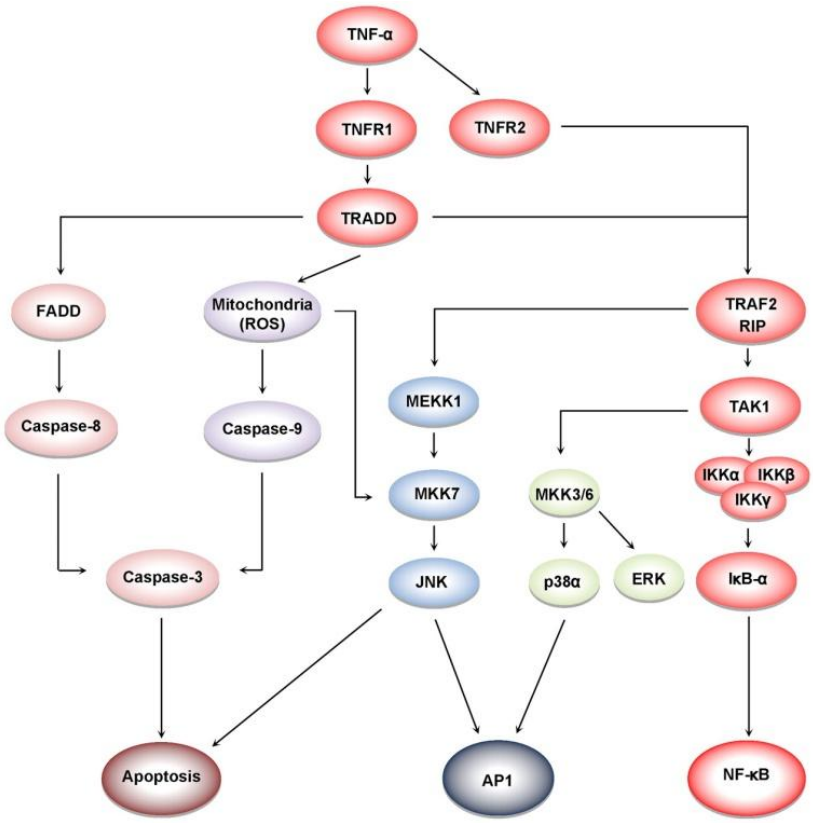


Figure 10. **Cell signaling pathways activated by TNFα**. TNFR1 activation leads to recruitment of intracellular adaptor proteins (TRADD, FADD, TRAF, and RIP), which activate multiple signal transduction pathways. TNFR sequentially recruits TRADD, TRAF2, RIP, TAK1, and IKK, leading to the activation of NF-κB; and the recruitment of TRADD, FADD, and caspase-8, leads to the activation of caspase-3, which in turn induces apoptosis. JNK is activated through the sequential recruitment of TRAF2, RIP, MEKK1, and MKK7. Exposure of cells to TNFα in most cases results in the generation of ROS, leading to activation of MKK7 and JNK. The activation of ERK and p38MAPK is via TRADD, TRAF2, RIP, TAK1, and MKK3/6. Adapted from Aggarwal *et al.*, 2012.

4. The zebrafish

4.1. Description, distribution, taxonomy, ecology and reproduction

Zebrafish (*Danio rerio*) are small (maximum size of 60 mm) shoaling cyprinid fish **(Figure 11)**. Although details of the distribution are unclear, *D. rerio* may be widely distributed in shallow, slow-flowing waters on the Indian subcontinent. Their natural range is centered around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, and Nepal. They are most commonly encountered in shallow ponds and standing water bodies with visibility to a depth of approximately 30 cm, often connected to rice cultivation (Spence *et al.*, 2008).

Figure 11. Adult zebrafish. Adapted from http://www.renalgenes.org/zebrafish.html.

Taxonomically, the zebrafish (*Danio rerio*) is a derived member of the genus *Danio*, of the family Cyprinidae, order Cypriniformes. For many years it was referred to in scientific literature as *Brachydanio rerio*, until its reassignment to the genus *Danio* (Mayden *et al.*, 2007).

Zebrafish are omnivorous, feeding primarily on zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales, arachnids, detritus, sand, and mud have also been reported from gut content analyses (Spence *et al.*, 2008).

Zebrafish are promiscuous and breed seasonally during monsoon season, which occur from April to August (spawning has also been recorded outside wet season, suggesting that breeding may be seasonal as a result of food availability). Mating behavior is also heavily influenced by photoperiod, as spawning begins immediately at

first light during breeding season and continues for about an hour. In order to initiate courtship about 3 to 7 males chase females and try to lead female towards a spawning site by nudging her and/or swimming around her in a tight circle or figure eight. Spawning sites consists of bare substrate that tends to be well vegetated. In captivity, gravel spawning sites are preferred to silt spawning sites. In the wild, zebrafish breed in silt-bottomed habitats. When a breeding pair reaches the spawning site, the male aligns his genital pore with the female's and begins to quiver, which causes the female to release her eggs and the male to release his sperm. The female releases 5 to 20 eggs at a time. This cycle repeats for about an hour. While the presence of female pheromones is required for initiation of courtship behavior in the male, male gonadal pheromones are required by the female for ovulation to occur. There is limited evidence for male-male competition and female mate preference (Spence *et al.,* 2006).

Zebrafish lay non-adhesive eggs without preparing a nest, and are considered to be group spawners and egg scatterers. Although time to hatching depends on water temperature, most eggs hatch between 48 and 72 hours after fertilization. Chorion thickness and embryo activity also impact incubation time. Zebrafish are approximately 3 mm upon hatching and are immediately independent. They are able to swim, feed, and exhibit active avoidance behaviors within 72 hours of fertilization (Engeszer *et al.*, 2004, 2007a, 2007b).

4.2. The zebrafish as a vertebrate research model

For many decades, zebrafish has been both a very popular aquarium fish and an important research model in several fields of biology (notably, toxicology and developmental biology). Since it was first used in a scientific laboratory 30 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect other vertebrate models. The development of zebrafish as a model organism for modern biological investigation began with the pioneering work of George Streisinger and colleagues at the University of Oregon (Streisinger *et al.,* 1981; Briggs, 2002).

The use and importance of zebrafish in biological research has exploded and diversified to the point that these fish are extremely important vertebrate models in an extraordinary array of research fields (Vascotto *et al.,* 1997), due to multiple advantages:

- Small size. Low maintenance cost and small space needed.
- Robust fish. High resistance to pathogens.
- High fecundity and large production of embryos (around 200 eggs/female/week) makes phenotype-based forward genetics doable.
- Short generation time (for a vertebrate). Typically 3 to 4 months, making it suitable for selection experiments.
- Zebrafish eggs are large relative to other fish (0.7 mm in diameter at fertilization time), optically transparent and externally developed following fertilization, making them easily accessible to embryonic manipulation and imaging.
- Transparency of zebrafish embryos, together with the large availability of transgenic lines, let *in vivo* tracking of cells easy **(Figure 12)**.
- Rapid development, with precursors to all major organs developing within 36 hours, and larvae display food seeking and active avoidance behaviors within five days after fertilization (2 to 3 days after hatching).
- As a vertebrate, zebrafish has special value as a model of human disease and for the screening of therapeutic drugs (Chakraborty *et al.* 2009) and is often more tractable for genetic and embryological manipulation and cost effective than other vertebrate models such as mice (Trede *et al.,* 2004).
- Easy to transfer among different labs by transporting their eggs.
- The zebrafish genome has now been completely sequenced, making it an even more valuable research organism.
- As a vertebrate, zebrafish shares considerable genetic sequence similarity with humans.
- It is relatively easy to knockdown specific genes by using morpholinos and overexpressing proteins by mRNA or plasmids.
- Use of reverse genetics approaches using zinc finger nucleases (ZFNs) (Meng *et al.,* 2008) and a transposon strategy (Kawakami, 2004) for generating transgenic zebrafish, which help in analyzing new roles of additional genes in larval and adult zebrafish.

- Existence of a centralized online resource for the zebrafish research community (http://zfin.org), making easier the work with this model.

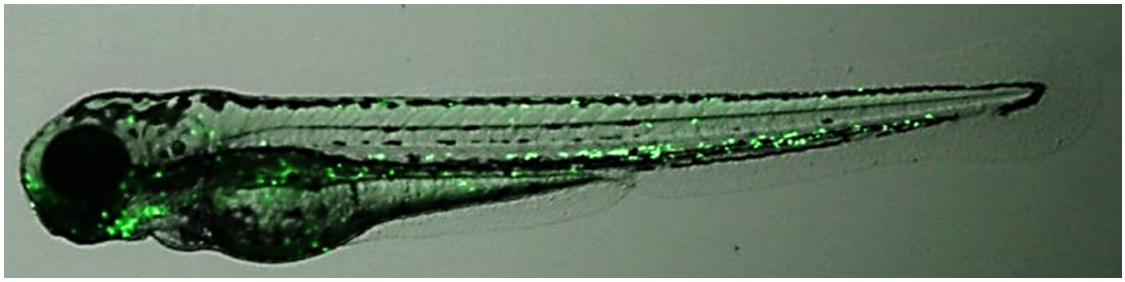


Figure 12. Transgenic *mpx:eGFP* larva at 72 hpf. Notice the transparency of the larva, which easily allows the in vivo tracking of the GFP-labeled neutrophils by using fluorescence microscopy.

All these advantages have lead to the increased interest of scientists using zebrafish as an animal model research in the last years and, nowadays, zebrafish has been proposed as an excellent vertebrate model for the study of the immune system (Renshaw and Trede, 2012), hematopoiesis (Martin *et al.*, 2011), vascular development (Isogai *et al.*, 2009; Quaife *et al.*, 2012; Gore *et al.*, 2012), neurogenesis (Schmidt *et al.*, 2013) and cancer research (Mione and Trede, 2010), among others. Some researchers have even used zebrafish to investigate the genetic basis of vertebrate behavior (Miklósi and Andrew, 2006; Spence *et al.*, 2008; Norton and Bally-Cuif, 2010).

4.3. The zebrafish as a model of inflammation

In the last years, the zebrafish has been established as a versatile model organism for the investigation of inflammatory immune responses. The immune system of zebrafish closely resembles that of mammals, but with the advantage that the adaptive immune system is not morphologically and functionally mature until 4-6 weeks post-fertilization, while the innate immune system is fully competent at early embryological stages before the emergence of lymphocytes. This temporal separation provides a suitable system to study the vertebrate innate immune response in vivo, independently from the adaptive immune response, allowing the various roles of different components of the innate and adaptive immune system in inflammation, infection and disease to be explored (Trede *et al.*, 2004; Novoa and Figueras, 2012).

The zebrafish has been established as an in vivo model to analyze the cellular innate inflammatory response by generating transgenic zebrafish lines that express fluorescent proteins under specific promoters, e.g. neutrophil- (Renshaw *et al.,* 2006) or macrophage-specific (Ellet *et al.,* 2011) promoters. Inflammation can be induced in different manners for these studies, including the transection of the tail fin of zebrafish larvae (Renshaw *et al.,* 2006; de Oliveira *et al.,* 2013), and the recruitment of myeloid cells to the injury site after tail fin transection can be monitored in vivo **(Figure 13)**.

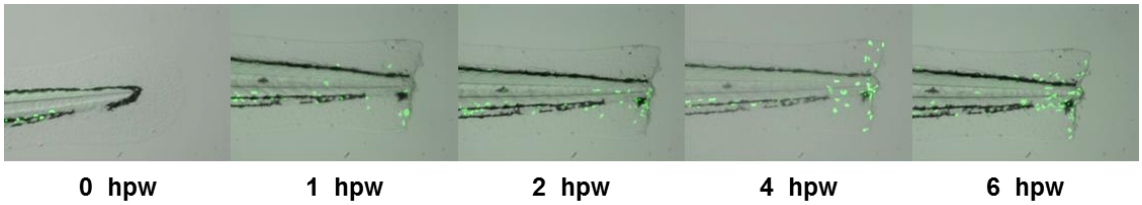


Figure 13. Transection of the tail fin of zebrafish larvae induces inflammation. Representative images showing the recruitment of GFP-labeled neutrophils to the inflammation site at different times post-wound. It supposes a powerful model system to study the cellular response to inflammation.

In addition, the utility of zebrafish as new model system to study chronic inflammation and to visualize the immune responses implicated with high resolution in vivo has been also demonstrated (Mathias *et al.*, 2007).

OBJECTIVES

The specific objectives of the present work are:

1. Characterization of the role played by Tnfa and its receptors (Tnfr1 and Tnfr2) in the neutrophil function and distribution patterns in zebrafish larvae.

2. Characterization of the Tnfr1 and Tnfr2 signaling pathways involved in skin homeostasis in zebrafish larvae.

3. Characterization of the role played by Tnfa and its receptors in chronic inflammation in the skin in zebrafish larvae.

4. Evaluation of the zebrafish larvae as a potencial model for the study of human chronic inflammatory diseases.

MATERIALS AND METHODS

1. Animals

Wild-type zebrafish (*Danio rerio* H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC) and mated, staged, raised and processed as described in the zebrafish handbook (Westerfield, 2000). The transgenic zebrafish line that expresses enhanced RFP driven by the keratinocyte cell-specific promoter *krt18* gene *Tg(krt18:RFP)* was previously described (Wang *et al.*, 2006) and provided by Dr. María Luísa Cayuela. The transgenic lines *Tg(mpx:eGFP)i114*, with green fluorescent neutrophils (Renshaw *et al.*, 2006), and *Tg(NFκB-RE*:eGFP) (*NF-κB:eGFP* for simplicity) (Kanther *et al.*, 2011) were provided by Prof. Stephen A. Renshaw. The transgenic line Tg(*mpeg1:eGFP*), with green fluorescent macrophages (Ellett *et al.*, 2011), was provided by Prof. Graham J. Lieschke.

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and IACUC. Experiments and procedures were performed as approved by the Bioethical Committee of the University of Murcia (approval number #537/2011).

2. Morpholinos and mRNA injection

Specific splice- or translation-blocking morpholinos were designed by and purchased from Gene Tools and resuspended in nuclease-free water to 1 mM **(Table 2)**.

Full-length TNFR2 (ENSDARG00000070165), IL-1β (NM_212844), TNFα (ENSDARG00000009511) and DN-TNFR2 (amino acids 1-162) were subcloned into the pCS2+ or pBluescript II KS+ vectors. *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.

Gene	ENA or Ensembl ID	Target	Sequence (5'→3')	Concentration (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	ei/i1	CCCACAAACTGCAAAATATCAGCTT	0.6	López-Muñoz *et al.*, 2011

Table 2. 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/).

3. Chemical treatments

In some experiments, 24 hpf embryos were manually dechorionated and 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.

4. Live imaging of zebrafish larvae

At 72 hpf, larvae were anesthetized in tricaine (200 μg/ml) and mounted in 1% (wt/vol) low-melting-point agarose (Sigma-Aldrich) dissolved in egg water. Images were captured using an epifluorescence LEICA MZ16FA 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 NF-κB activation was visualized and quantified using the transgenic zebrafish 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). The next step was to choose a threshold fluorescence value, corresponding to the background for our fluorescence measurements. Then, the first 15 pixels with fluorescence values higher than the previously established threshold were considered as the limits of the larvae.

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 (c). 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 (Feng *et al.,* 2010). 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 (de Oliveira *et al.,* 2013).

5. Flow cytometry

At 72 hpf, approximately 300 to 500 *Tg(mpx:eGFP) and Tg(krt18:RFP)* larvae were anesthetized in tricaine (200 µg/ml), incubated at 28°C for 90 min with 0.077 mg/ml Liberase (Roche) and the resulting cell suspension passed through a 30 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).

6. 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 (Pfaffl, 2001). The primers used are shown in **Table 3**. In all cases, each PCR was performed with triplicate samples and repeated at least with two independent samples.

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	
duox1	AB255050	F	ACACATGTGACTTCATATCCAG	
		R	ATTATTAACTCATCCACATCCAG	
ptgs2b	NM_001025504	F2	CCCCAGAGTACTGGAAACCA	
		R2	ACATGGCCCGTTGACATTAT	
gfp	EF591490	F1	ACGTAAACGGCCACAAGTTC	
		R1	**AAGTCGTGCTGCTTCATGTG**	
krt18	NM_178437	F2	AGAACCTGAAAGGGTCACTGG	
		R2	GTAGGTGGCGATTTCTGCCT	
Il1b	NM_212844	F5	GGCTGTGTGTTTGGGAATCT	
		R5	TGATAAACCAACCGGGACA	

Table 3. 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/).

7. Human skin samples

Skin biopsies from healthy donors (n=10) 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 ºC 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) followed by ImmunoCruz™ goat ABC Staining System (sc-2023) (both from 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 4ºC. 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.

8. Statistical analysis

All experiments were performed at least three times, unless otherwise indicated. The total number of used animals is indicated in each bar. Data were analyzed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups, while 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. Statistical significance was defined as $p < 0.05$.

RESULTS

1. Tnfa or Tnfr2 deficiency results in neutrophil mobilization to the skin

It has been described that most of neutrophils are located in the caudal hematopoietic tissue (CHT) **(Figure 14A)** in wild type zebrafish larvae by 72 hpf (Murayama *et al*., 2006). This area has been shown as the main transient site of hematopoiesis during these early stages, and it is also known as the posterior blood island (PBI) when we refer to the earlier transient wave in the region (Chen and Zon, 2009).

In order to elucidate how the signaling via Tnfa receptors (Tnfr1 and Tnfr2) modulates the myeloid cells function and distribution patterns, morpholinos previously tested in our lab **(Table 2)** were used for the genetic depletion of both receptors in the *lyz:DsRed* **(Figure 14B)**, *mpx:eGFP* **(Figure 14C)** and *mpeg1:eGFP* **(Figure 14D)** zebrafish transgenic lines at 72 hpf.

While no differences were observed in Tnfr1-deficient larvae in any case, the analysis of the images from *lyz:DsRed* **(Figure 14B)** as well as *mpx:eGFP* **(Figure 14C)** larvae deficient in Tnfr2 revealed dramatic differences in the neutrophil distribution patterns comparing to controls animals. However, nothing similar occurred with macrophages (mpeg1), since a similar scattered phenotype was observed in control and Tnfr2-deficient fish **(Figure 14D)**. Moreover, the macrophage distribution seemed to be much more variable comparing different larvae belonging to the same treatment, making highly complicated to detect and quantify the possible differences unless they are very obvious.

These findings led us to characterize and quantify the neutrophil altered phenotype, focusing on the caudal region where the CHT is located **(Figure 2A)**, as well as to go further in our study by extending it to the Tnfa- and Tnfr1 + Tnfr2-deficient larvae.

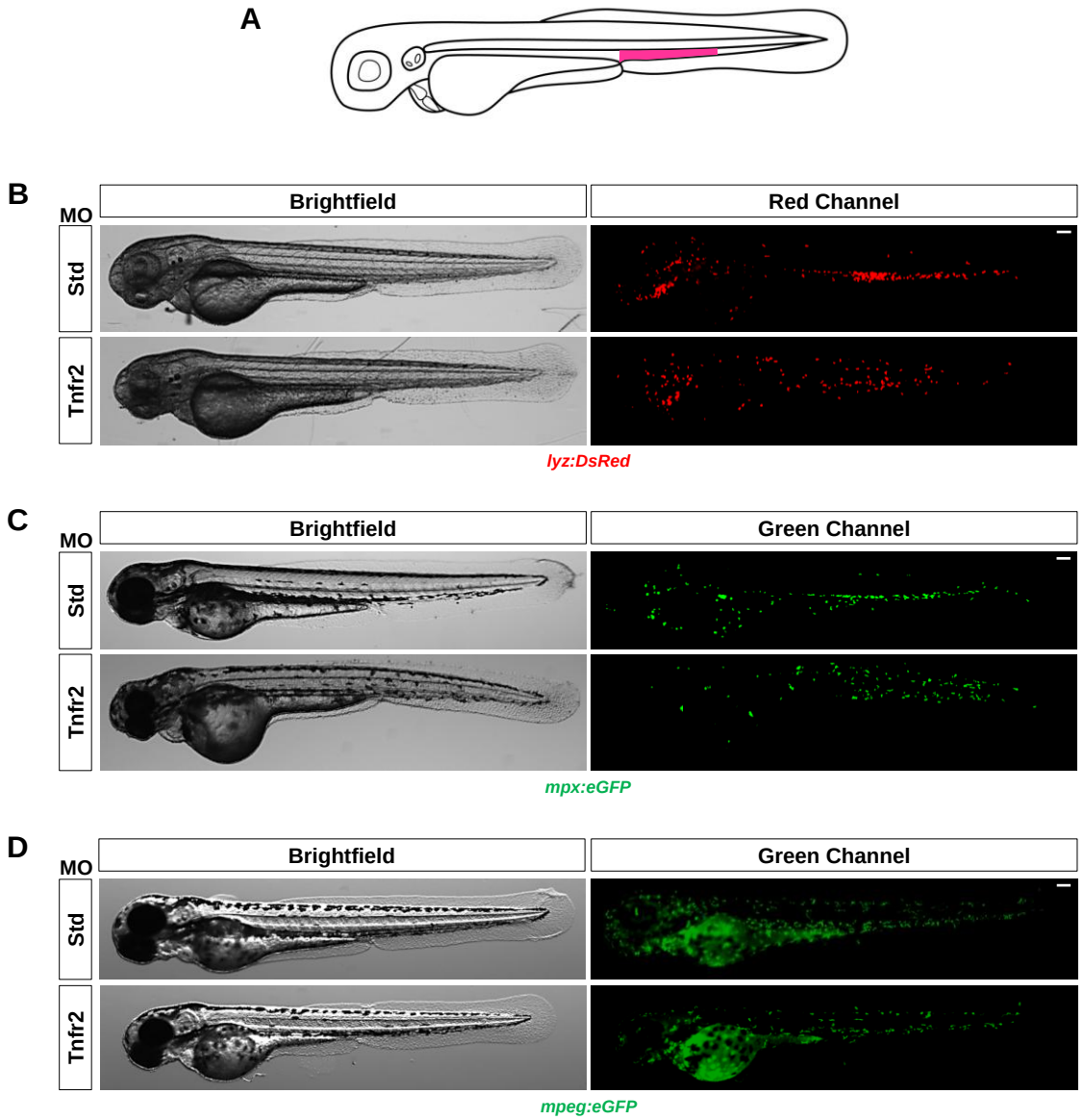


Figure 14. Genetic depletion of Tnfr2 results in altered neutrophil, but not macrophage, distribution patterns. Scheme highlighting (pink area) the location of the caudal hematopoietic tissue (CHT) in a zebrafish larvae at 72 hpf (A). Zebrafish one-cell *lyz:Dsred* (B), *mpx:eGFP* (C) and *mpeg:eGFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). Representative images, brightfield on the left panels and green channel on the right ones, showing the neutrophil (B,C) and macrophage (D) distribution patterns at 72 hpf. Scale bars: 100 µm.

Tnfa-deficient larvae presented a neutrophil mobilization phenotype identical to that one previously observed in larvae deficient in Tnfr2, while, surprisingly, the genetic depletion of both Tnfa receptors resulted in an intermediate phenotype **(Figure 15A)**. In addition, pixel by pixel transversal measurements of the mean GFP fluorescence from the ventral to the dorsal region, showed that most of the fluorescence was located in a

region corresponding to the CHT in control and Tnfr1-deficient larvae (the peaks in that area indicate a much higher fluorescence intensity compared to the fluorescence in the rest of the animal). However, the fluorescence intensity was uniformly distributed in larvae deficient in Tnfa or Tnfr2, not presenting any peaks, which would indicate that neutrophils were not grouped at any concrete region. Curiously, the fluorescence profile for the double morphants deficient in both Tnfr's, confirmed the previous observation that the phenotype induced by the Tnfr2 deficiency is partially rescued by the combined genetic depletion of Tnfr1 at the same time **(Figure 15B)**.

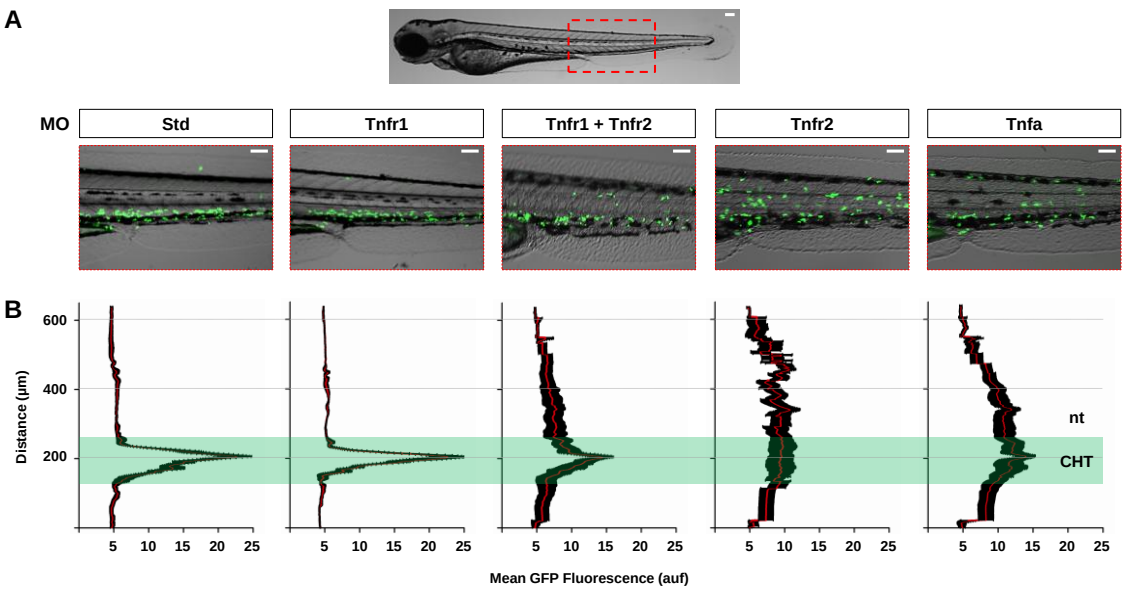


Figure 15. Tnfa or Tnfr2 deficiencies induce neutrophil mobilization from the CHT. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Tnfr1+Tnfr2 morpholinos (MO). (A) Representative images, brightfield and green channels, of the indicated region of morphants at 72 hpf showing the differences in the neutrophils distribution. Scale bars: 100 µm. (B) Fluorescence intensity was measured for all the groups in the area indicated in A, which includes the caudal hematopoietic tissue (CHT), where most neutrophils are located in wild type larvae at 72 hpf. The images were converted to a fluorescence value matrix where the value obtained for each pixel transversally was the mean (red dots) ± S.E.M. (white bars) 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. Scale bars: 100 µm.

The observed phenotype was quantified by counting the number of neutrophil inside and outside the CHT in each larva from the different treatments, taking the lower

limit of the notochord as the upper limit of the CHT. As it is shown in the **Figure 16**, about a 90% of neutrophils were located into the CHT in control or Tnfr1-deficient larvae, while almost a 40% are outside that region in Tnfa- or Tnfr2-deficient fish. Therefore, this quantification confirmed the phenotypes previously observed in the imaging experiments, including the existence of an intermediate phenotype in the double morphants deficient in both Tnfr's.

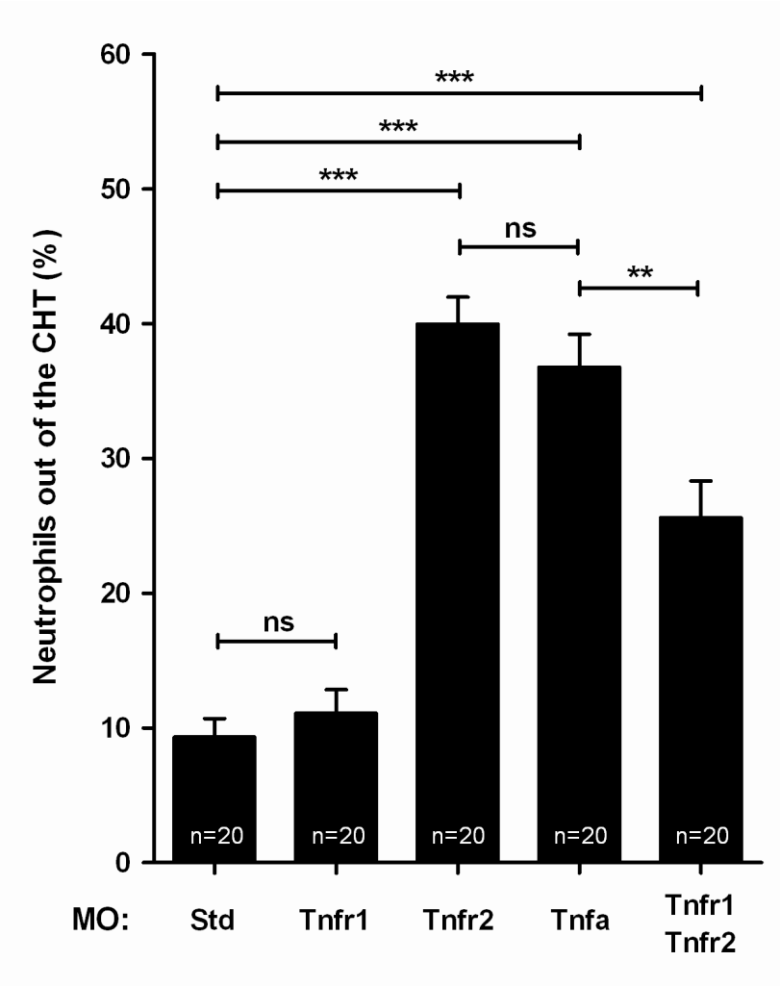


Figure 16. Quantification of the percentage of neutrophils outside the CHT in control, Tnfa-, Tnfr1-, Tnfr2- and Tnfr1+Tnfr2-deficient larvae. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Tnfr1+Tnfr2 morpholinos (MO). The neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT in 20 larvae per group from 3 different experiments, taking the lower limit of the notochord as upper limit of the CHT. The mean ± S.E.M. for each group is shown. ns, not significant. *p<0.05; **p<0.01; ***p<0.001.

Going further, the quantitative differences in the neutrophil distribution patterns among the different morphants, were also confirmed for the Tnfr2-deficient larvae using a completely different technique, consisting of the measurement of the distance between each neutrophil and its nearest neighbour for each individual larva at 72 hpf. This analysis revealed that the mean distance between each neutrophil and its nearest neighbour was significantly bigger in Tnfr2-morphant larvae compared to control ones, as expected for the distribution phenotype in which the cells are not grouped and close to each other, but scattered in the whole larvae **(Figure 17)**.

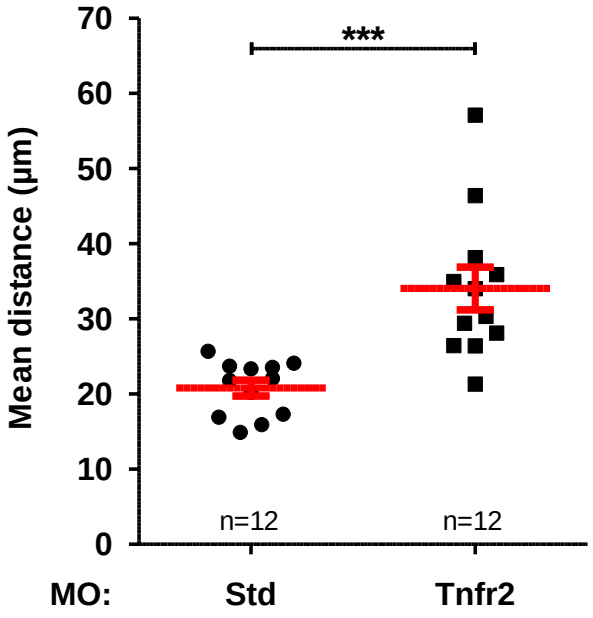


Figure 17. Measurement of the mean distance between each neutrophil and its neirest neighbour. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std) or TnfR2 morpholinos (MO). Each point in the graph represents the mean of all the distances between each neutrophil and its neirest neighbour for one larva. The mean ± S.E.M. for each group is shown ns, not significant. ***p<0.001.

It has been described that some different side effects can appear associated to the use of morpholinos (Eisen and Smith, 2008), existing the possibility that the morpholino inhibits the function of an irrelevant gene instead of, or in addition to, the intended gene. Sometimes, the problems derived of those "off-target" effects can be more dramatic, since the observed phenotype could be partially or completely caused by the

silencing of an irrelevant gene which might be affected by the morpholino (Eisen and Smith, 2008). Taking that into consideration, we designed two different strategies in order to confirm that we were working with a completely specific phenotype, as the consequence of the concrete genetic depletion of our target genes:

- On the one hand, a rescue assay by injecting the Tnfa or Tnfr2 mRNAs to larvae deficient in Tnfa or Tnfr2 respectively was performed, finding that, in both cases, the scattered distribution of neutrophils previously described was partially rescued **(Figure 18)**.

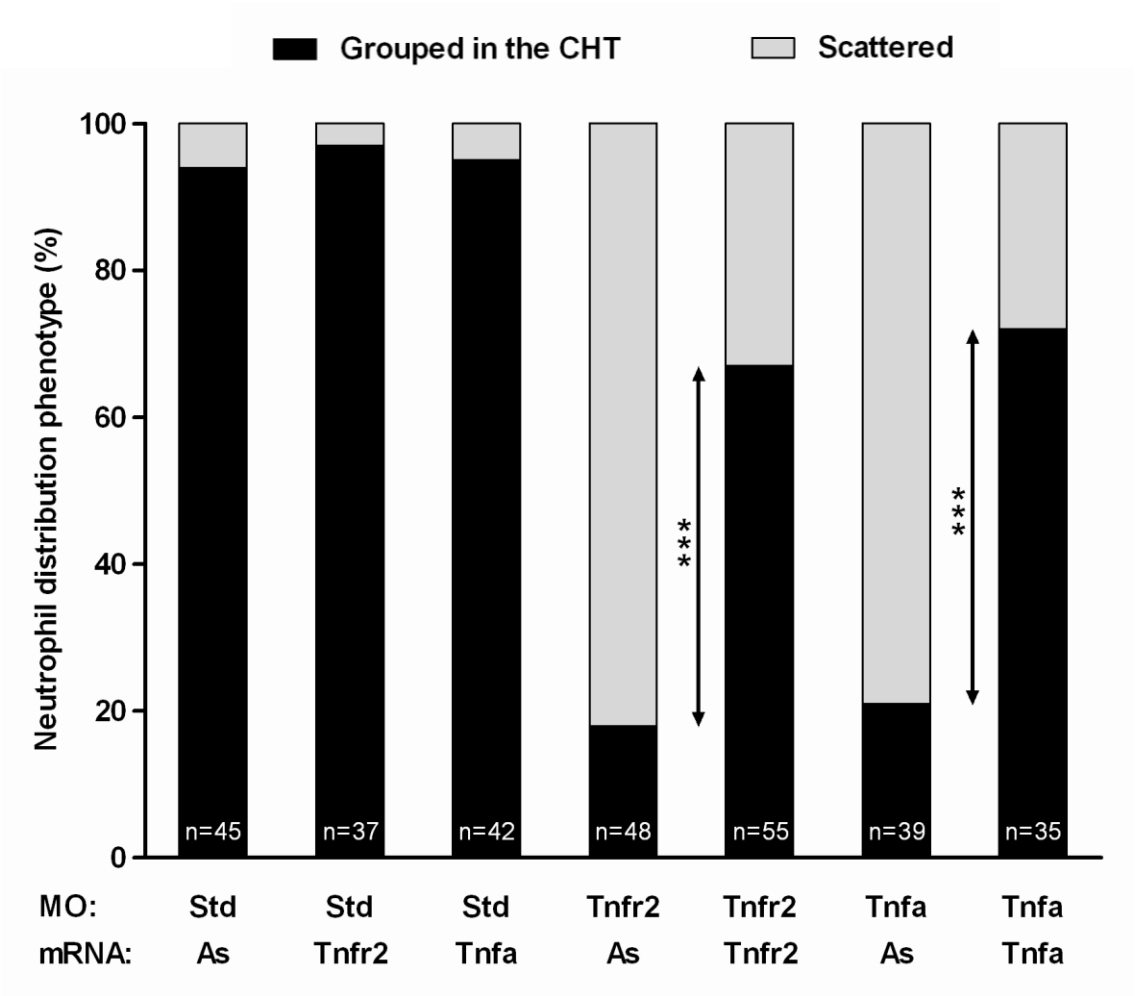


Figure 18. Rescue of the altered neutrophil distribution phenotype observed in Tnfa- or Tnfr2-deficient larvae by injection of the respective mRNAs. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr1, Tnfr2 or Tnfa morpholinos (MO) alone or combined with antisense (As), Tnfr2 or Tnfa mRNAs. The phenotype of 72 hpf larvae was classified as neutrophil grouped in the CHT or scattered, as described before. ***p<0.001.

- On the other hand, we also confirmed the specificity of the phenotype with a dominant negative (DN) Tnfr2 form, 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 (Espín *et al.*, 2013) **(Figure 19)**.

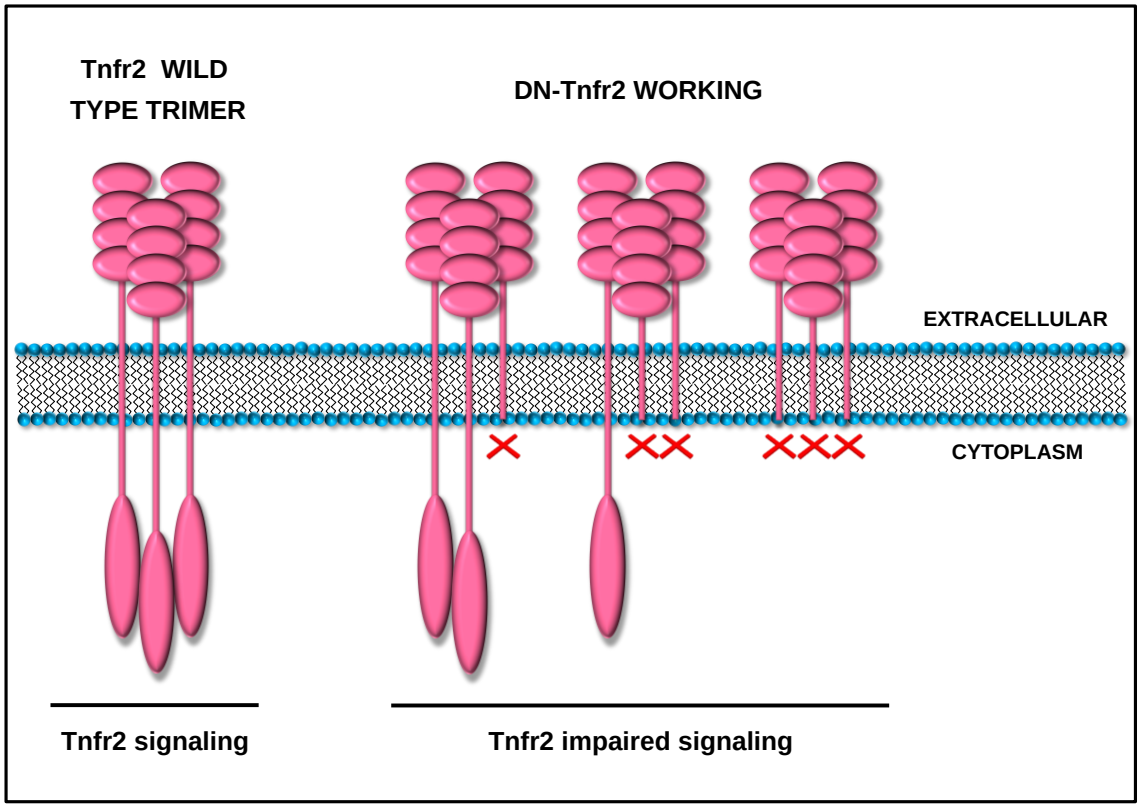


Figure 19. DN-Tnfr2 working. The scheme shows the different possibilities in which the DN-Tnfr2 can act to impare the Tnfr2 signaling. The DN lacks the complete intracellular signaling domain, and the presence of only one DN form in the Tnfr2 trimer would be enough to avoid its correct working although the other two elements are wild type.

The results showed that the altered neutrophil distribution of Tnfr2 morphants was phenocopied by overexpression of DN-Tnfr2 **(Figure 20)**.

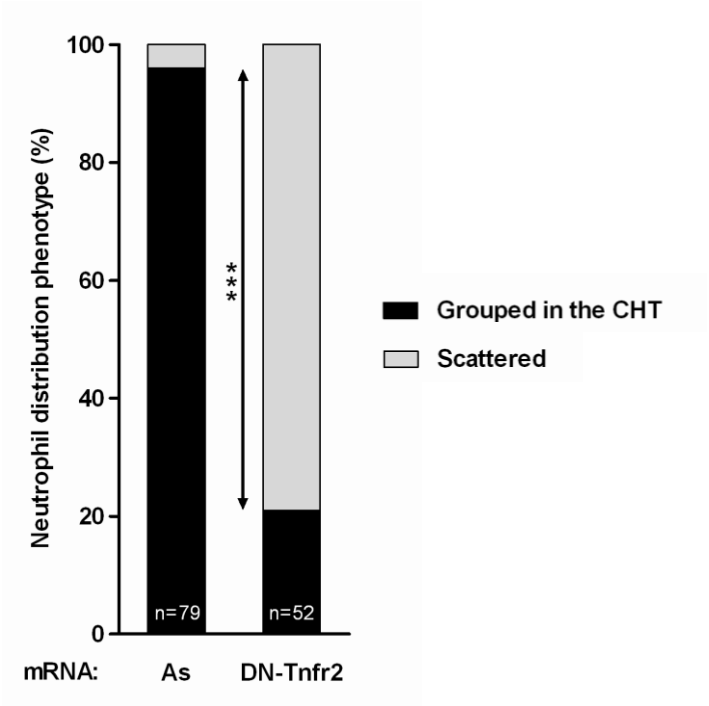


Figure 20. Neutrophil mobilization phenotype induced by DN-Tnfr2 overexpression. Zebrafish one-cell *mpx:eGFP* embryos were injected with antisense (As) or DN-Tnfr2 mRNAs. The phenotype of 72 hpf larvae was classified as neutrophil grouped in the CHT or scattered, as described before. ***p<0.001.

Considering that neutrophils were completely scattered through the whole larvae in Tnfa- or Tnfr2-deficient larvae and the consistency of this phenotype, we wanted to go further by studying the precise localization of neutrophils in those deficient larvae. To ascertain that, we knocked-down Tnfr2 in double transgenic *mpx:eGFP*; *krt18:RFP* animals in order to visualize neutrophils (GFP^+) and skin keratinocytes (RFP^+) at the same time in whole larvae. The results revealed that while neutrophils from control animals were mainly located in the CHT as expected, a high proportion of the scattered neutrophils from the Tnfr2-deficient larvae were seen in close contact with keratinocytes in the skin **(Figure 21)**.

Collectively, these results indicate that deficiency of either Tnfa or Tnfr2 specifically promotes neutrophil infiltration into the skin of zebrafish during early development.

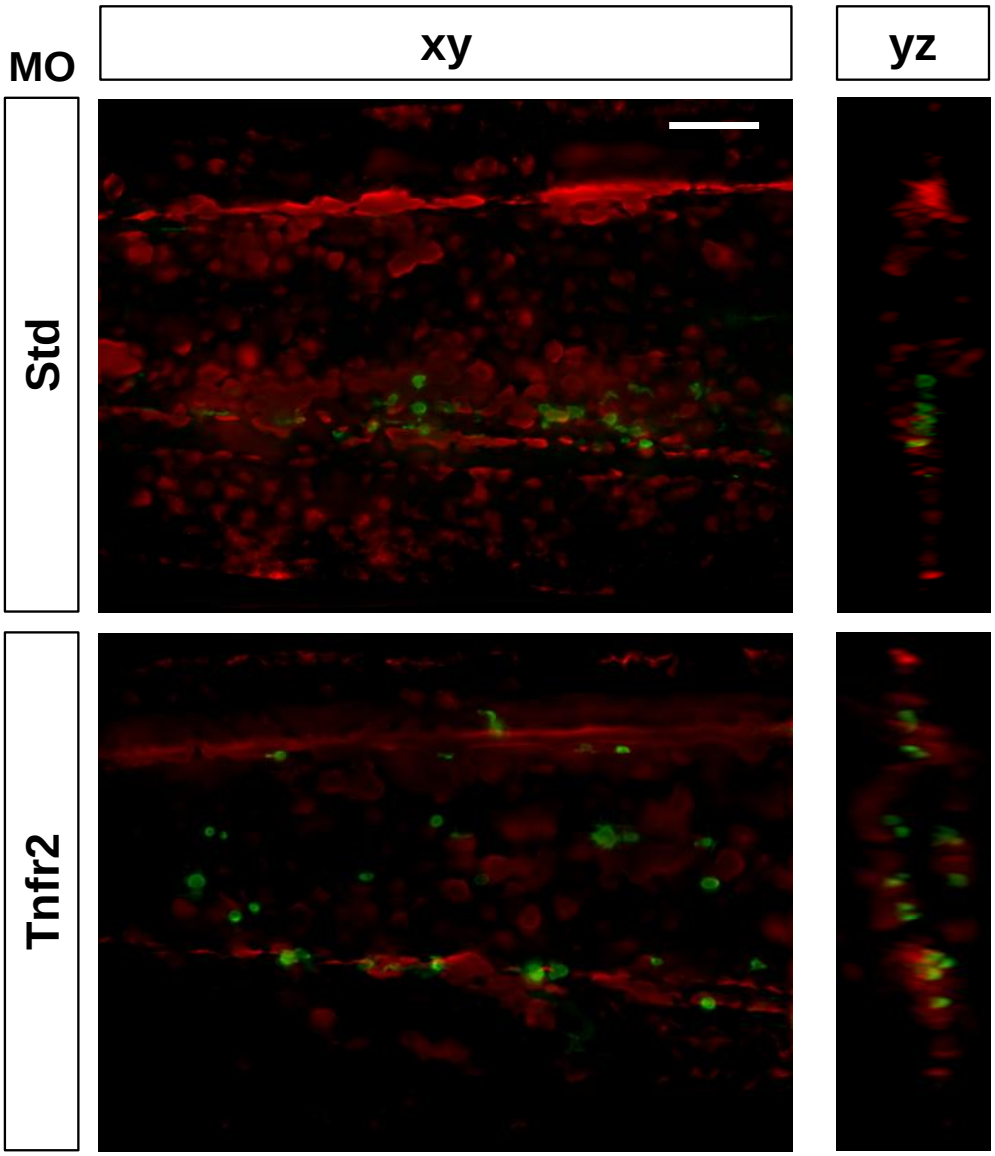


Figure 21. Most of neutrophils from Tnfr2-deficient larvae are located close to keratinocytes in the skin. Zebrafish one-cell *mpx:eGFP* and *krt18:RFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). Representative images showing the neutrophils distribution in the CHT area of control and Tnfr2-deficient larvae at 3 dpf (xy) and the tridimensional reconstructions (yz) of stacked images captured using 1 µm increments showing that neutrophils (green) in Tnfr2-deficient larvae are in close contact with keratinocytes (red), while they are mainly located in the CHT in their wild type siblings. Scale bar: 100 µm.

2. Tnfa or Tnfr2 deficiency triggers skin inflammation

2.1. Characterization and cell contribution

The phenotype observed in larvae deficient in Tnfa or Tnfr2 was 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 (Mathias *et al*., 2007; Carney *et al*., 2007; Dodd *et al.*, 2009). Therefore, in order to elucidate if Tnfr2-deficient fish presented a general inflammation which could be responsible of the neutrophil mobilization from the CHT to the skin, we examined the expression of genes encoding major pro-inflammatory molecules in several contexts, including the principal actors which would be involved in a possible relationship between neutrophils and skin: A) whole control and Tnfr2-deficient larvae at 72 hpf, B) sorted neutrophils (*mpx:eGFP*$^{+}$ cells) from control and Tnfr2-deficient larvae at 72 hpf and C) sorted keratinocytes (*krt18:RFP*$^{+}$ cells) from control and Tnfr2-deficient larvae at 72 hpf.

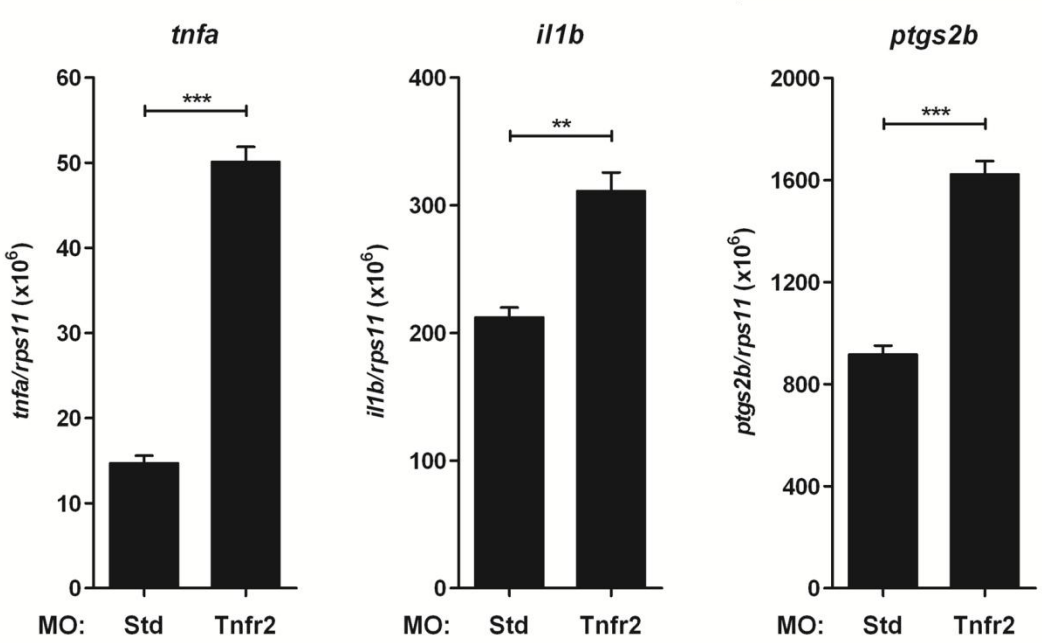


Figure 22. Tnfr2 deficiency triggers inflammation in whole larvae. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). The expression of *tnfa*, *il1b* and *ptgs2b* genes was measured by RT-qPCR in whole body. **p<0.01; ***p<0.001.

2.1.1. Whole larvae

The mRNA levels for Tnfa, Il1b and prostaglandin-endoperoxide synthase 2b (PTGS2b, also known as COX2b) were measured in whole control and Tnfr2-deficient larvae at 72 hpf. The results showed that Tnfr2 deficiency triggers the expression of *tnfa*, *il1b* and *ptgs2b* genes, indicating that a general inflammatory process was in progress **(Figure 22)**.

2.1.2. Contribution of neutrophils to the inflammatory process in progress

To determine the contribution of neutrophils in the inflammatory process in progress, these cells were sorted from control and Tnfr2-deficient fish. After checking the efficiency of the technique by measuring the GFP expression **(Figure 23A)**, it was verified that both Tnfa receptors were expressed in neutrophils, mainly Tnfr2 of which mRNA levels are 125 times higher in isolated neutrophils than in unsorted cells **(Figure 23B)**.

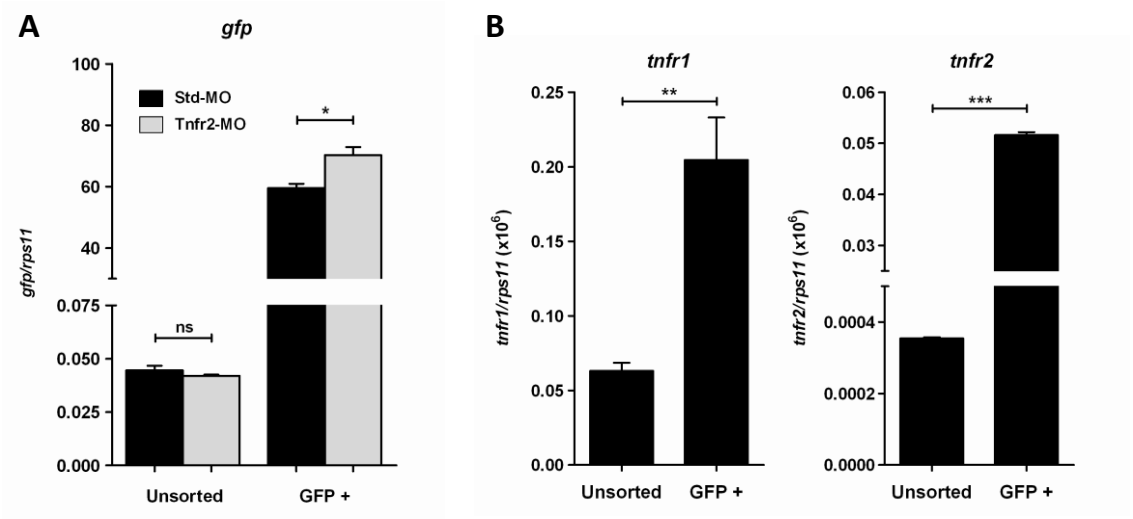


Figure 23. Efficiency of neutrophil sorting and expression of both Tnfr's in sorted cells. Zebrafish one-cell *mpx*:eGFP embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). Neutrophils were FACS-sorted from 72 hpf larvae and the expression of *gfp* was measured by RT-qPCR in unsorted and sorted cells (A). The expression of *tnfr1* and *tnfr2* genes was measured by RT-qPCR in unsorted and sorted cells from control larvae (B). The data are shown as the mean ± S.E.M. ns: not significant. *p<0.05; **p<0.01; ***p<0.001.

The analysis of the expression of pro-inflammatory molecules showed that, although neutrophils highly expressed the genes encoding Tnfa and Il1b **(Figure 24)**, they did not mediate the induction of *il1b* observed in Tnfr2-deficient whole larvae **(Figure 22)**. Nevertheless, the transcript levels of *tnfa* were higher in neutrophils from Tnfr2-deficient fish than in neutrophils from their wild type siblings, but this might reflect a positive feedback loop in response to Tnfr2 deficiency (Espín *et al.*, 2013) **(Figure 24)**.

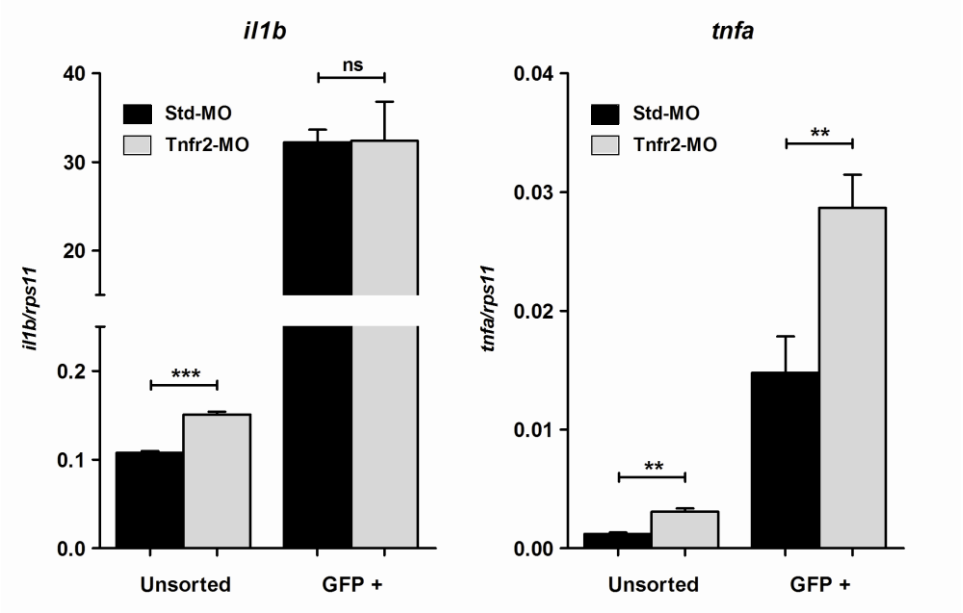


Figure 24. Expression levels of pro-inflammatory molecules in sorted neutrophils. Zebrafish one-cell *mpx*:eGFP embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). The expression of *il1b* and *tnfa* genes was measured by RT-qPCR in FACS-sorted neutrophils at 72 hpf. ns, not significant. **p<0.01; ***p<0.001.

The next step was to check if neutrophils were playing a key role in the inflammation unleashing or if, on the contrary, it was something pre-existent to the neutrophils emerging. For that, knowing that Il1b is over-expressed in whole Tnfr2-deficient larvae compared to control fish at 72 hpf, the expression of this pro-inflammatory cytokine was analyzed in whole control and Tnfr2-deficient larvae at 24 hpf (soon after the first neutrophil appearance and before hatching) and 48 hpf (when neutrophils are established in the CHT in wild type larvae) (Bennett *et al*., 2001; Le Guyader *et al*., 2008; Lieschke *et al*., 2001). The results showed that Il1b was induced

in Tnfr2-deficient embryos before the emergence of neutrophils, since the expression of that cytokine was even higher at 24 hpf **(Figure 25)**. Although neutrophils could play an important role in the development of the inflammation present in Tnfr2-deficient larvae in later stages, these findings suggest that these cells are not the direct responsible of the inflammation unleashing, but it is still established when they emerge.

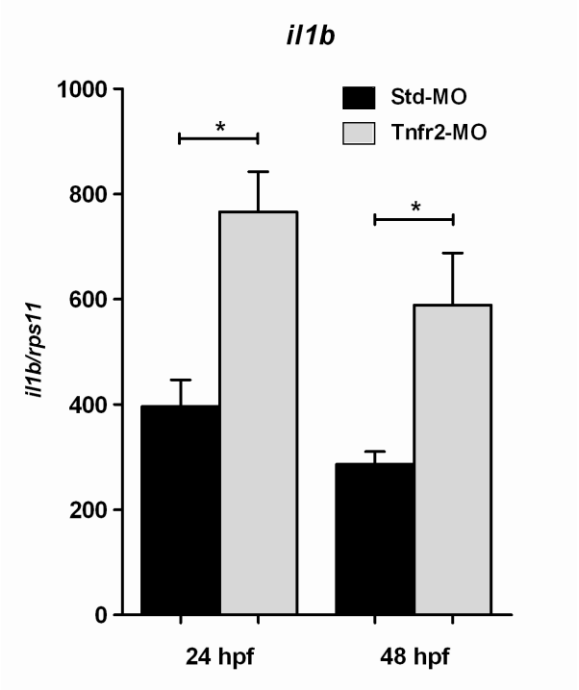


Figure 25. IL-1β is induced in Tnfr2-deficient embryos before the emergence of neutrophils. Zebrafish one-cell wild type embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). The expression of *il1b* gene was measured by RT-qPCR in whole embryos at 24 and 48 hpf. The data are shown as the mean ± S.E.M. *p<0.05.

2.1.3. Contribution of keratinocytes to the inflammatory process in progress

Taking into account that scattered neutrophils from Tnfr2-deficient fish were closed to keratinocytes, we wanted to clarify if those cells from the skin were contributing to the inflammation observed in whole Tnfr2-deficient larvae. Therefore, keratinocytes from control and Tnfr2-deficient animals were sorted using the transgenic zebrafish line *krt18:RFP* at 72 hpf. In the same way as it has been described before for sorted neutrophils, the efficienfy of the cell sorting was firstly checked by measuring RFP expression **(Figure 26A)** and it was verified that both Tnfr's were expressed in those cells **(Figure 26B)**.

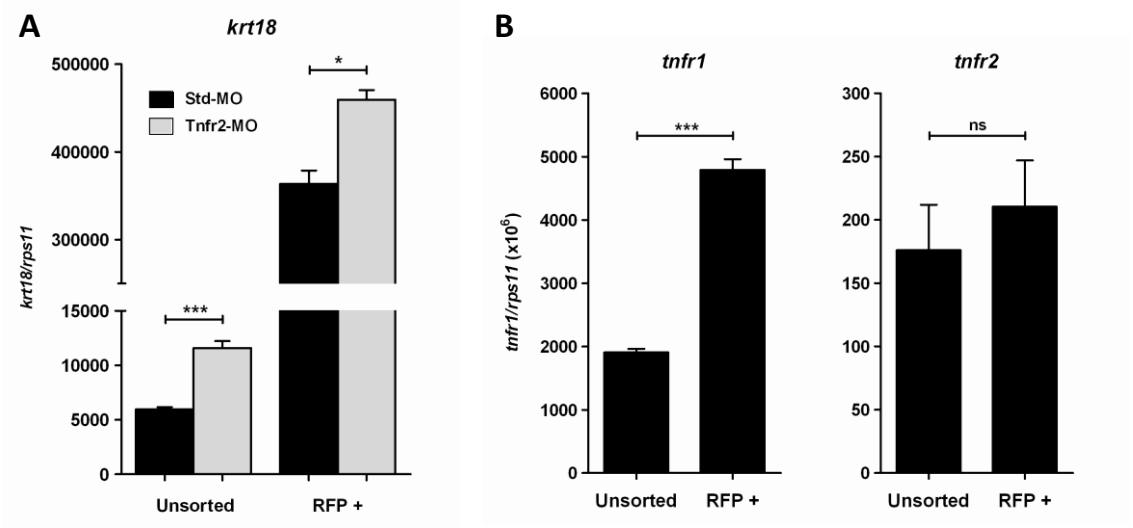


Figure 26. Efficiency of keratinocyte sorting and expression of both Tnfr's in sorted cells. Zebrafish one-cell *krt18*:RFP embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). Keratinocytes were FACS-sorted from 72 hpf larvae and the expression of *krt18* was measured by RT-qPCR in unsorted and sorted cells (A). The expression of *tnfr1* and *tnfr2* genes was measured by RT-qPCR in unsorted and sorted cells from control larvae (B). The data are shown as the mean ± S.E.M. ns: not significant. *p<0.05; ***p<0.001.

The study of the expression of *il1b* and *ptgs2b* genes revealed that their transcription levels were much higher in keratinocytes from Tnfr2-deficient larvae than in keratinocytes from control animals **(Figure 27)**, suggesting that keratinocytes may play an important role in the inflammatory process in progress in larvae deficient in Tnfr2.

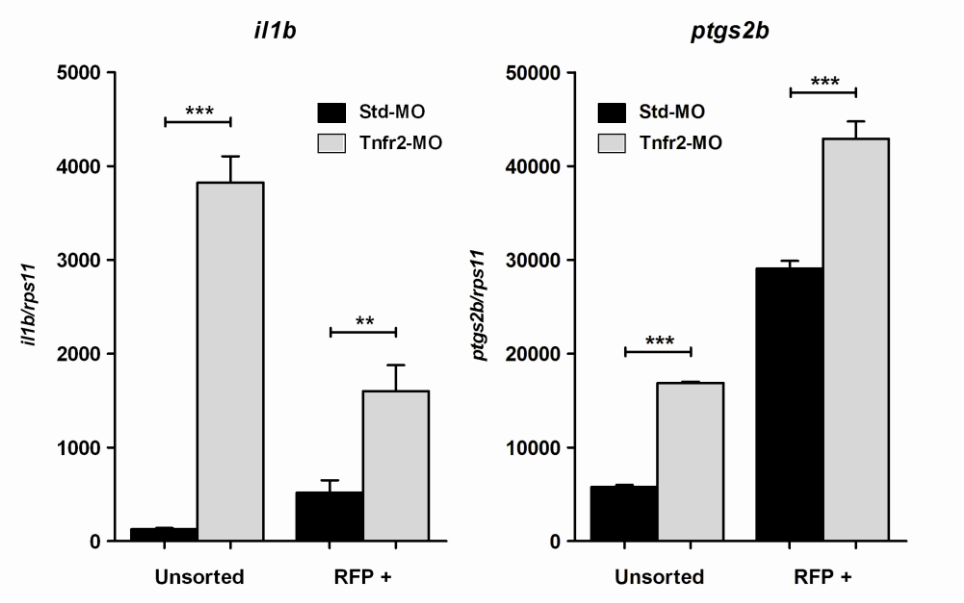


Figure 27. Expression levels of pro-inflammatory molecules in sorted keratinocytes. Zebrafish one-cell *krt18*:RFP embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). The expression of *il1b* and *ptgs2b* genes was measured by RT-qPCR in FACS-sorted keratinocytes at 72 hpf. **p<0.01; ***p<0.001.

2.2. Contribution of Il1b

We next wondered whether knockdown of Il1b using a specific morpholino (López-Muñoz *et al*., 2011) might rescue the skin inflammation and neutrophil dispersion observed in Tnfr2-deficient animals. As shown in **(Figure 28)**, genetic inhibition of Il1b failed to rescue the neutrophil dispersion observed in Tnfr2 morphants.

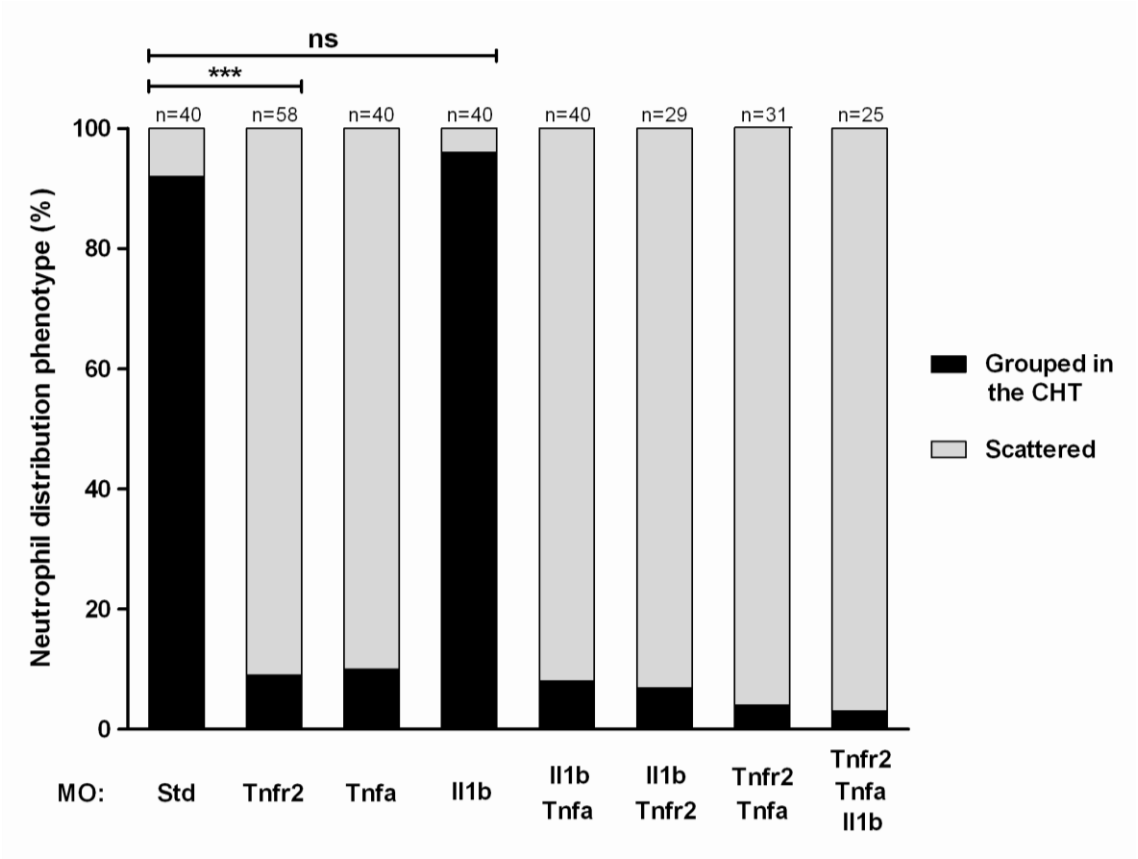


Figure 28. Rescue of the neutrophil mobilization in Tnfa- or Tnfr2-deficient larvae by IL-1β knockdown. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr2, Tnfa, Il1b+Tnfa, Il1b+Tnfr2, Tnfr2+Tnfa or Tnfr2+Tnfa+Il1b morpholinos (MO). The phenotype of 72 hpf morphant larvae was classified as neutrophils grouped in the CHT or scattered, as described before. Note that IL-1β knockdown failed to rescue the neutrophil mobilization in Tnfr2-deficient larvae. ns, not significant; ***$p<0.001$.

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 into the skin.

3. Tnfa and Tnfr2 deficiencies induce NF-κB activation in the skin

It has been described that 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 TNFα and TNFR1 depletion (Pasparakis *et al.*, 2002; Gugasyan *et al.*, 2004; van Hogerlinden *et al.*, 2004; Omori *et al.*, 2006; Sayama *et al.*, 2006). Taking that into consideration, together with the fact that our larvae deficient in Tnfa or Tnfr2 seemed to have an inflammation in the skin where neutrophils were being recruited, we, therefore, used the NF-κB reporter line *NF-κB:eGFP* (Kanther *et al.*, 2011) to visualize the dynamics of NF-κB in Tnfr2-deficient larvae at 72 hpf.

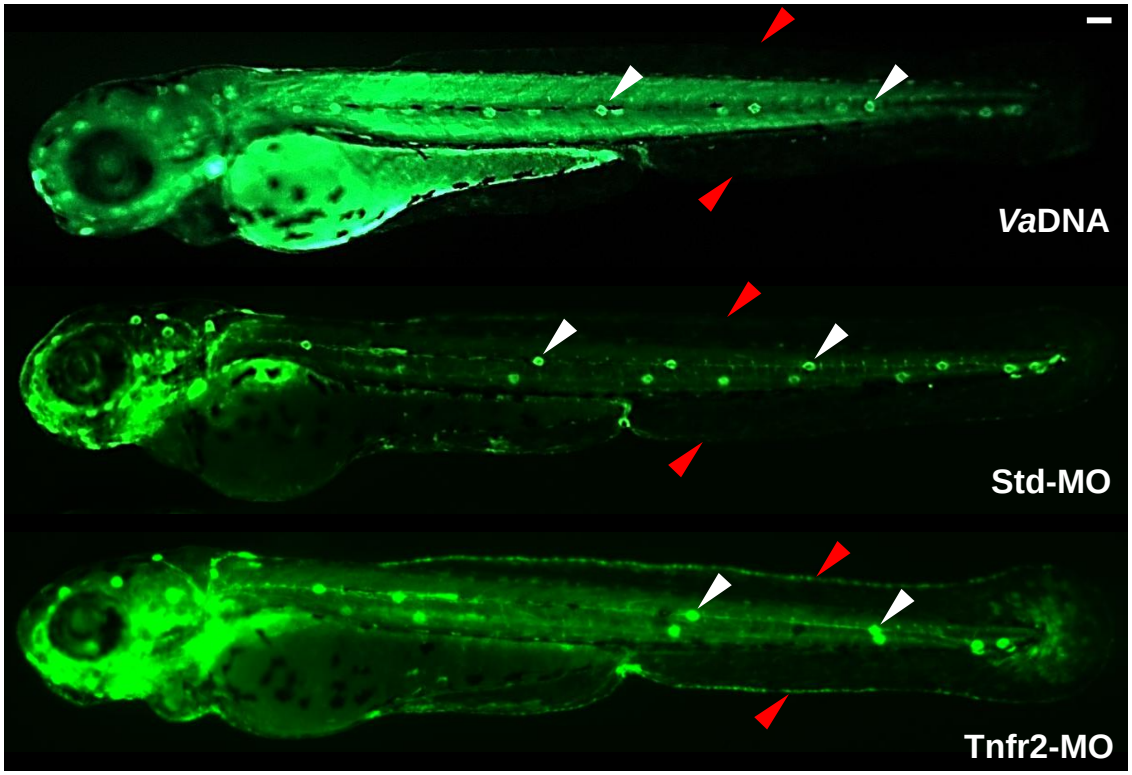


Figure 29. NF-κB activation in whole control and Tnfr2-deficient larvae. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO) alone or in the presence of 2.3 ng/egg of *V. anguillarum* genomic DNA (VaDNA), as a positive control for NF-κB activation. These representative pictures show 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). Scale bar: 100 μm.

Firstly, the proper working of this transgenic line was demonstrated by the injection of bacterial DNA from *Vibrio anguillarum* (*Va*DNA) as a positive control, which activates TLR9 resulting in a drastic activation of NF-κB in the whole larvae, as expected from previous results (Alcaraz-Pérez *et al.*, 2008; Sepulcre *et al.*, 2009) **(Figure 29A)**.

Working with whole larvae, the genetic depletion of Tnfr2 in *NF-κB:eGFP* larvae at 72 hpf resulted in clear qualitative differences in NF-κB activation **(Figure 29B)** comparing to control larvae **(Figure 29C)**, since the fluorescence was concentrated in the larvae outlines in Tnfr2-deficient larvae but not in control ones. However, the quantification of the mean GFP fluorescence for the whole larvae (a single value per larva, corresponding to the average of the fluorescence measurements for each pixel in

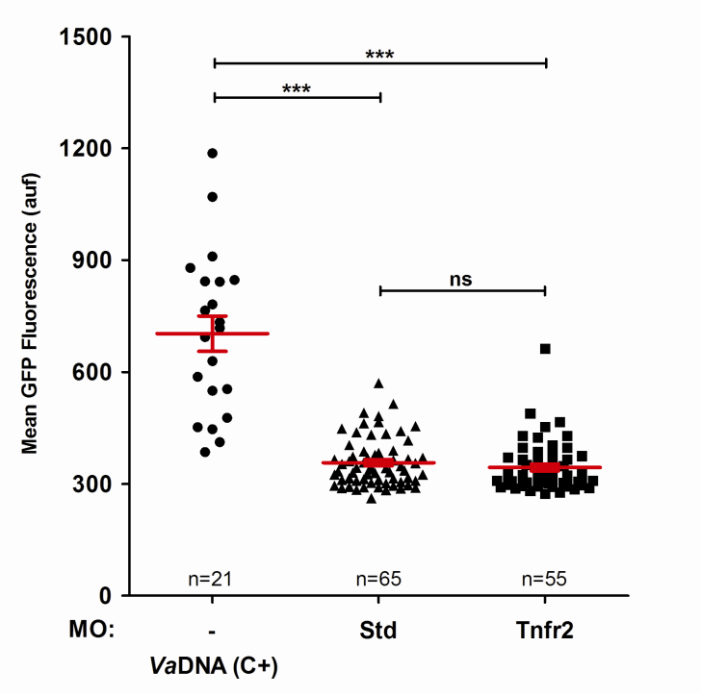


Figure 30. Quantification of NF-κB activation in whole control and Tnfr2-deficient larvae. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO) alone or in the presence of 2.3 ng/egg of *V. anguillarum* genomic DNA (VaDNA), as a positive control for NF-κB activation. 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. ns, not significant; *p<0.05; **p<0.01; ***p<0.001. auf: arbitrary units of fluorescence.

the whole larva) did not show significant differences between control and Tnfr2-deficient larvae, while the NF-κB activation was much higher in larvae injected with *Va*DNA as expected **(Figure 30)**.

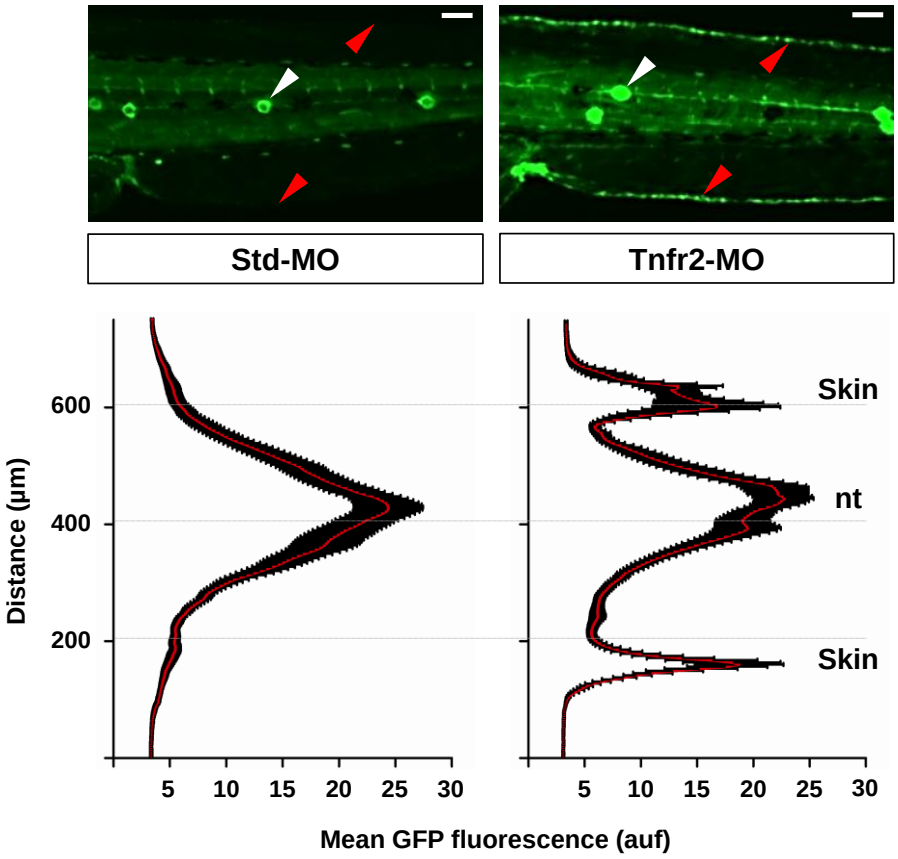
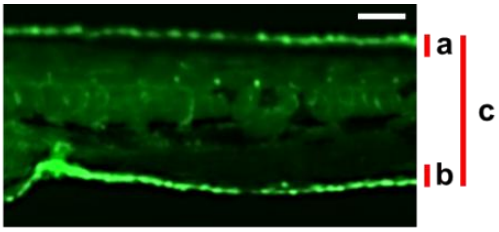


Figure 31. NF-κB activation patterns in the CHT of control and Tnfr2-deficient larvae. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std-MO) or Tnfr2 (Tnfr2-MO) morpholinos. (A) Representative pictures showing the induction of NF-κB activation in the skin (red arrowheads) in the CHT of Tnfr2-deficient larvae at 72 hpf. Note the strong expression of NF-κB in neuromasts (white arrowheads). (B) Mean GFP Fluorescence intensity was measured in the area indicated in A of wild type and Tnfr2-deficient larvae, where the CHT is included. The images were converted to a fluorescence value matrix where the value obtained for each pixel transversally was the mean (red dots) ± S.E.M. (white bars) for all the pixels for each row (15 larvae per treatment from 3 different experiments). The areas corresponding to the notochord (nt) as well as to both limits of the larvae (skin), ventral and dorsal, have been labeled to facilitate the larval orientation. Note the activation of NF-κB in the skin of Tnfr2-deficient larvae. auf: arbitrary units of fluorescence. Scale bars: 100 µm.

Given the qualitative but not quantitative differences between control and Tnfr2-deficient larvae when the mean GFP fluorescence is measured in whole larvae, the analysis was then focused on the area we were interested in, the CHT. The analysis of the images from that area reveled that, as we were seen before in the whole larvae, there were important qualitative differences comparing controls and Tnfr2-deficient larvae **(Figure 31A)**. Moreover, it was also confirmed by the fluorescence profiles for controls and Tnfr2-morphants, which were made using pixel by pixel transversal measurements of the mean GFP fluorescence, from the ventral to the dorsal regions. As shown in the **Figure 31B**, Tnfr2-deficient larvae present three peaks corresponding to the areas with a higher mean GFP fluorescence and, therefore, with a higher NF-κB activation. Those peaks overlap with the notochord (nt), in the centre, and with both limits of the larvae (skin), the dorsal limit on top and the ventral one on the bottom. However, control larvae only present a fluorescence peak, which overlaps with the notochord (nt).



$$\textbf{Skin NF-}\kappa\textbf{B activation index} = \frac{a+b}{c}$$

Figure 32. Definition of the Skin NF-κB activation index, used for the quantification of NF-κB activation in skin. A representative image of the CHT of a Tnfr2-deficient larva at 72 hpf is shown, and the different zones where we measure the GFP fluorescence intensity to calculate the index have been labeled. To obtain the GFP fluorescence intensity measurements, images from the same area as showed in this example of controls and Tnfr2-deficient larvae at 72 hpf were converted to fluorescence value matrix, where the value obtained for each pixel transversally was the mean ± S.E.M. for all the pixels for each row. The next step was to choose a threshold fluorescence value, corresponding to the background for our fluorescence measurements. Then, the first 15 pixels with fluorescence values higher than the previously established threshold were considered as the limits of the larvae (a, b). The skin NF-κB activation index was designed as the quotient between the mean GFP fluorescence intensity in the skin (a+b) divided by the mean GFP fluorescence intensity for all the width of the larvae (c). Therefore, this index indicates what proportion of the total GFP fluorescence intensity is located in the skin. Scale bar: 100 µm.

These results led us to design a method to quantify the differences in the NF-κB activation in the skin observed after comparing larvae deficient in Tnfr2 to control fish, resulting a parameter called skin NF-κB activation index. For that, the GFP fluorescence intensity was measured for both limits of the larvae (a and b), and then the quotient between those two values (a+b) divided by the measurement of the GFP fluorescence intensity for all the width of the larvae (c), which also includes both limits, was calculated **(Figure 32)**.

According to the phenotype previously observed in the images, the calculation of the skin NF-κB activation index for control and Tnfr2-deficient larvae showed significant differences between those two treatments **(Figure 33)**.

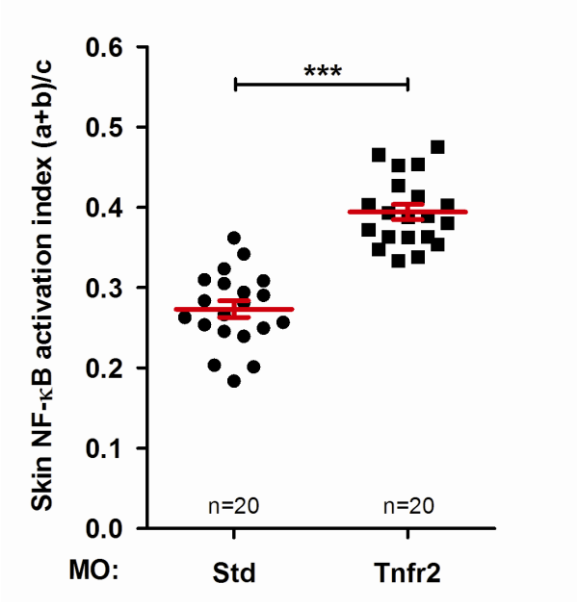


Figure 33. Skin NF-κB activation index for controls and Tnfr2-deficient larvae. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). The Skin NF-κB activation index was calculated for both treatments at 72 hpf, as we have previously described in the Figure 19. 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. ns, not significant. ***p<0.001.

4. Tnfa and Tnfr2 deficiencies trigger H_2O_2 production in the skin

The results obtained so far showed that the genetic depletion of Tnfr2 results in neutrophil recruitment from the CHT to the skin, due to an inflammatory process probably mediated by keratinocytes and NF-κB activation. Hydrogen peroxide gradients have been recently shown to contribute to the early influx of neutrophils in wound (Niethammer *et al.*, 2009) and tumor (Feng *et al.*, 2010). Interestingly, however, H_2O_2 is not required for neutrophil detection of localized infection (Deng *et al.*, 2012). These gradients are created by the dual oxidase 1 (Duox1) (Niethammer *et al.*, 2009) and sensed by neutrophils through the tyrosine kinase Lyn (Yoo *et al.*, 2011). Although identified and best studied in the zebrafish, H_2O_2 is likely to play the same function in human neutrophils (Yoo *et al.*, 2011).

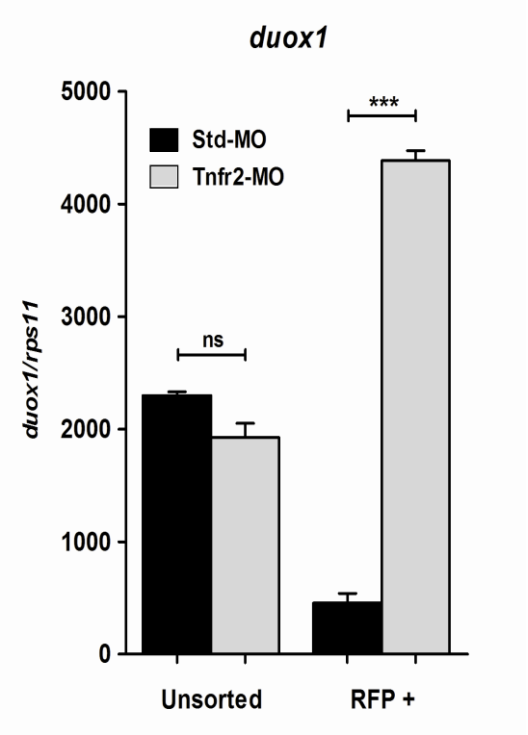


Figure 34. Expression levels of *duox1* in sorted keratinocytes. Zebrafish one-cell *krt18*:RFP embryos were injected with standard control (Std-MO) or Tnfr2 morpholinos (Tnfr2-MO). The expression of *duox1* was measured by RT-qPCR in FACS-sorted keratinocytes at 72 hpf. ns, not significant; ***p<0.001.

To get further insight into the signals responsible for neutrophil mobilization from the CHT to the skin, the expression of the gene encoding Duox1 was analyzed in

sorted control and Tnfr2-deficient keratinocytes. The results showed higher transcript levels of *duox1* in Tnfr2-morphants compared to control animals **(Figure 34)**.

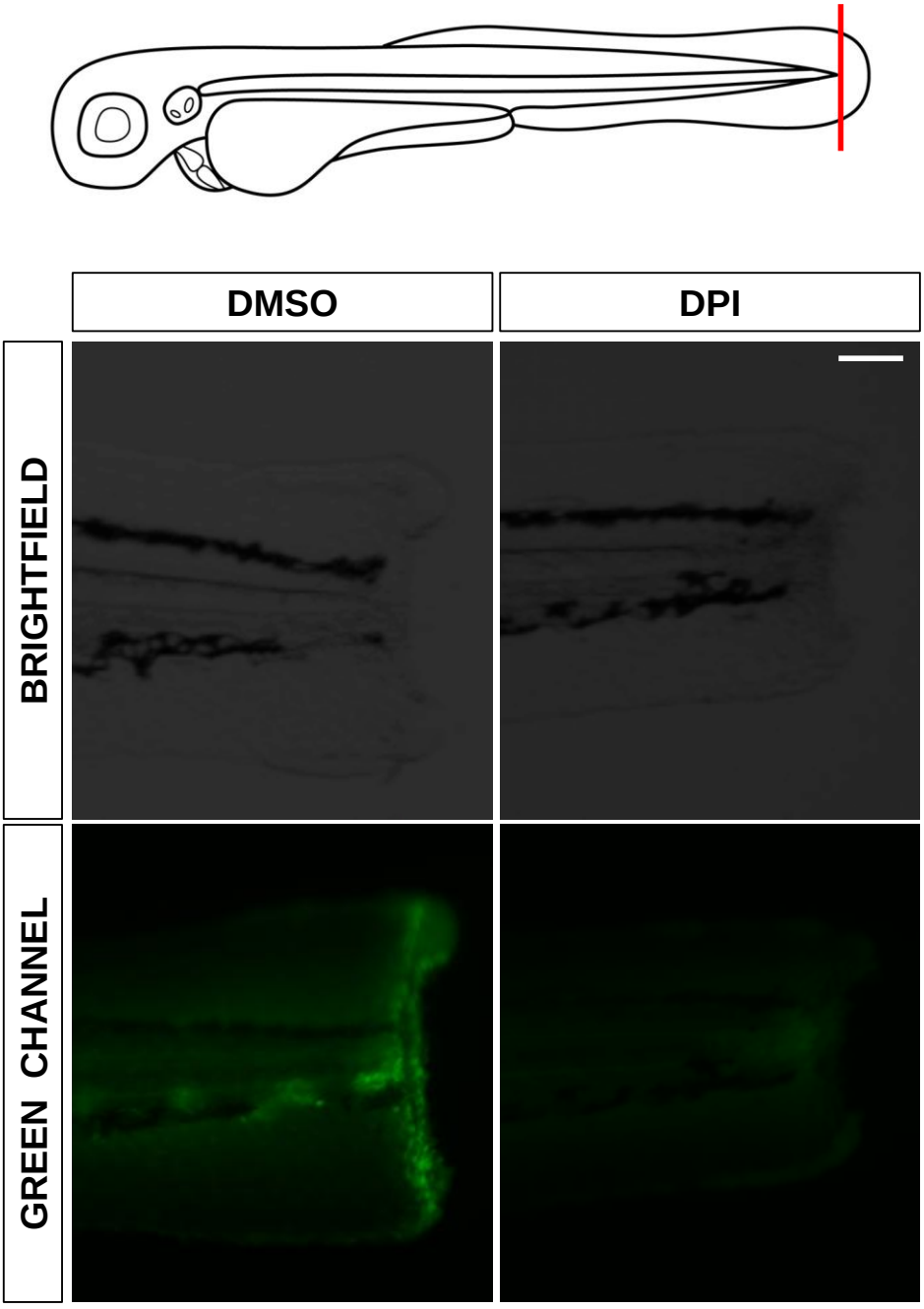


Figure 35. Validation of the H_2O_2 specific fluorescent probe. Zebrafish wild type larvae were treated at 72 hpf by immersion in 100 µM DPI or vehicle alone (DMSO) in the presence of 50 µM acetyl-pentafluorobenzene sulphonyl fluorescein, and tailfins were then transected. Scheme showing the transected area (on top) and 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 site. Scale bar: 100 µm.

The induction of Duox1 in keratinocytes from larvae deficient in Tnfr2 led us to hypothesize that the production of H_2O_2 could be involved in the recruitment of neutrophil to the skin in those larvae. To confirm this hypothesis, the production and release of H_2O_2 by the keratinocytes in the skin were measured by using a H_2O_2 specific fluorescent probe in control and Tnfr2-deficient larvae at 72 hpf. Firstly, the ability of the fluorescent probe to detect H_2O_2 was checked by cutting the tails of control and Tnfr2-deficient larvae at 72 hpf, and adding the NADPH oxidase inhibitor dibenziodolium chloride (DPI) as a negative control **(Figure 35)**.

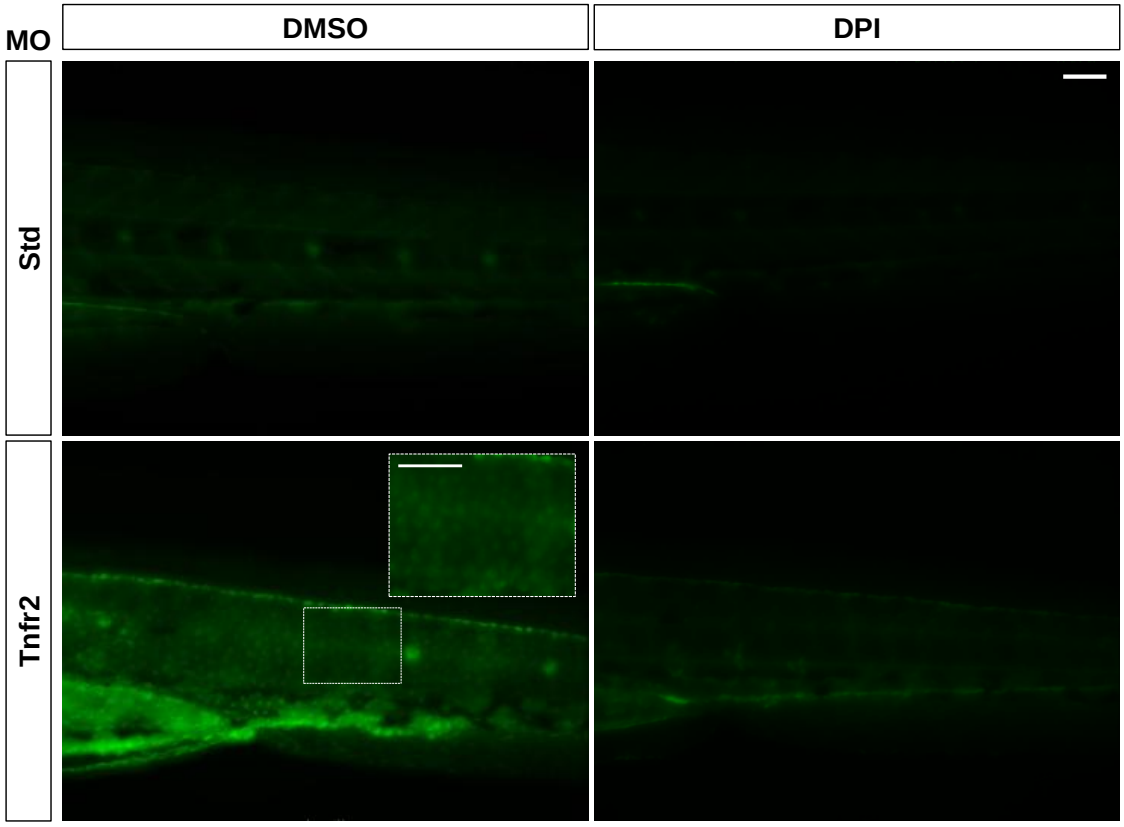


Figure 36. Tnfr2 deficiency results in the Duox1-derived H_2O_2 production by keratinocytes. Zebrafish one-cell wild type embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO). Larvae were dechorionated at 24 hpf and treated by immersion in 100 µM DPI or vehicle alone (DMSO) for 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). Scale bars: 100 µm.

The pictures showed the proper working of the specific probe and, in addition, that Tnfr2-deficient larvae produced H_2O_2 in the skin **(Figure 36)**, where indeed single

keratinocytes were labeled to similar levels than local keratinocytes following wounding **(Figure 35)**.

Despite the clear qualitative differences in H_2O_2 production comparing control to Tnfr2-deficient larvae, the mean GFP fluorescence was quantified in all conditions tested. The quantification confirmed that Tnfr2-morphants presented much higher GFP fluorescence levels than control larvae, and that the addition of the NADPH oxidase inhibitor (DPI) was able to reduce the fluorescence signal in controls as well as in Tnfr2-deficient larvae **(Figure 37)**.

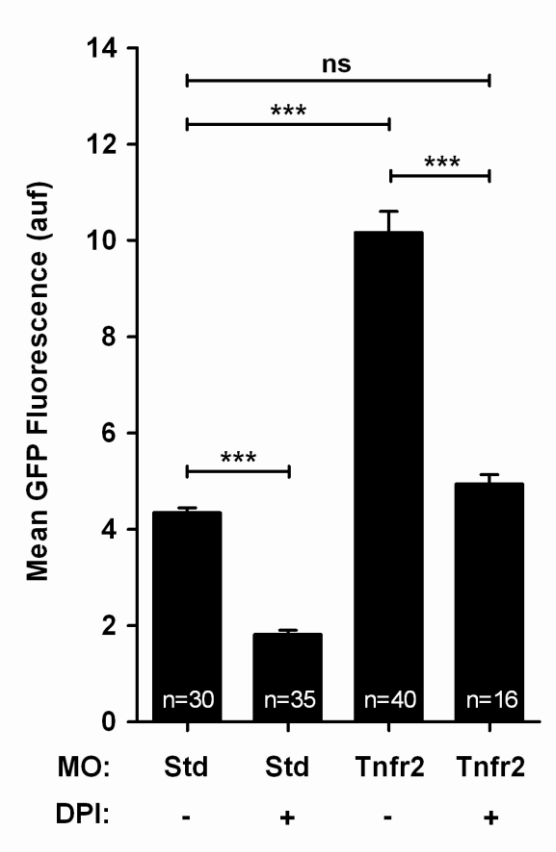


Figure 37. Quantification of the H_2O_2 production in control and Tnfr2-deficient larvae. Zebrafish one-cell wild type embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO) Larvae were dechorionated at 24 hpf and treated by immersion in 100 µM DPI or vehicle alone (DMSO) for 24 h, and then labeled with 50 µM acetyl-pentafluorobenzene sulphonyl fluorescein. The mean GFP fluorescence was quantified for all treatments. ns, not significant; ***$p<0.001$.

Interestingly, the genetic inhibition of Duox1 using an specific morpholino (Niethammer *et al.*, 2009) was able to partially rescue the neutrophil mobilization

phenotype in Tnfr2-deficient larvae **(Figure 38)**, supporting the idea that the H_2O_2 produced by the activity of that enzyme would be the signal responsible for the recruitment of neutrophils to the skin.

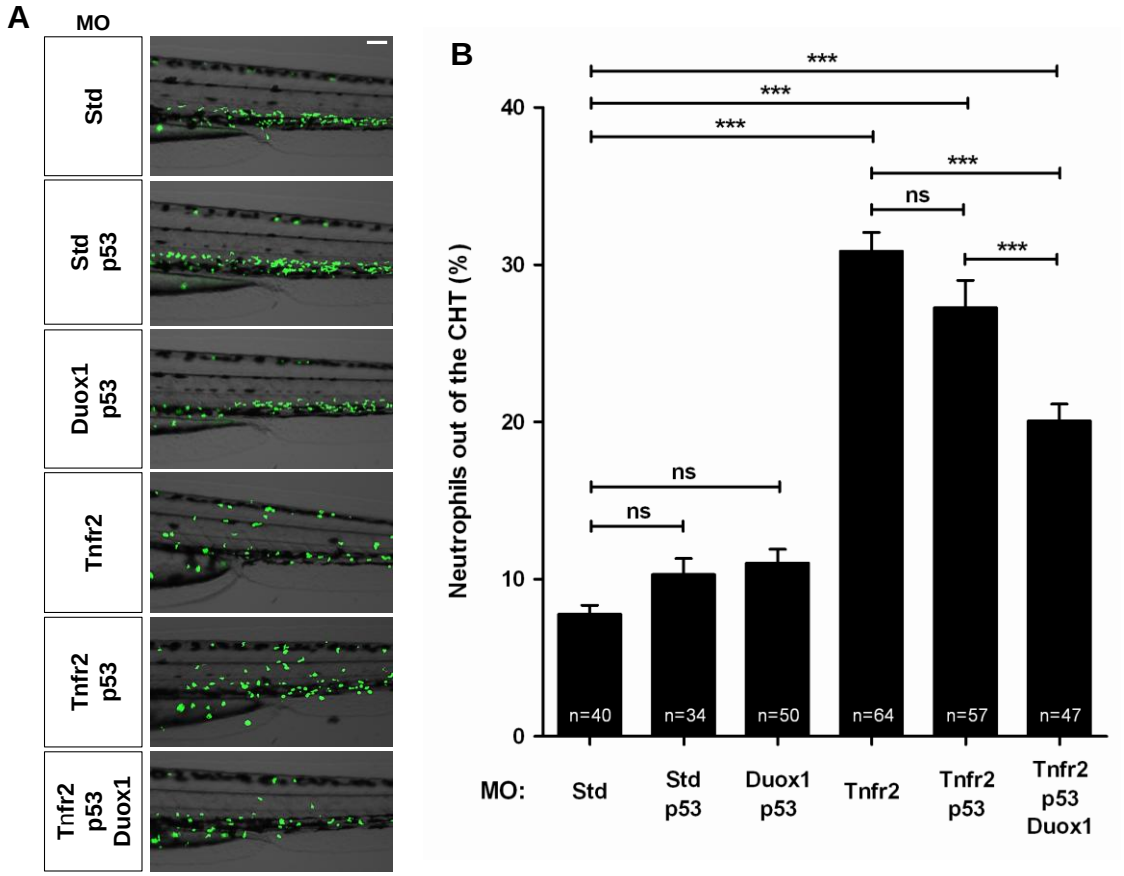


Figure 38. Genetic depletion of Duox1 using a specific morpholino rescues the neutrophil mobilization from the CHT to the skin. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Std+p53, Duox1+p53, Tnfr2, Tnfr2+p53 or Tnfr2+p53+Duox1 morpholinos (MO). (A) Representative images, brightfield and green channels, of the CHT of morphants at 72 hpf showing the differences in the neutrophils distribution. Scale bar: 100 µm. (B) The neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT. The mean ± S.E.M. for each group is shown. ns, not significant; ***p<0.001.

Since the Duox1 morpholino had strong side effects and had to be used in combination with a p53 morpholino to reduce generalized apoptosis and developmental defects (Niethammer *et al.*, 2009), a dominant negative (DN) form of Duox1 (manuscript under preparation) was designed in order to confirm the results previously obtained with the morpholino. The results showed that the overexpression of DN-

Duox1 was also able to partially rescue neutrophil infiltration in Tnfr2-deficient larvae **(Figure 39)**.

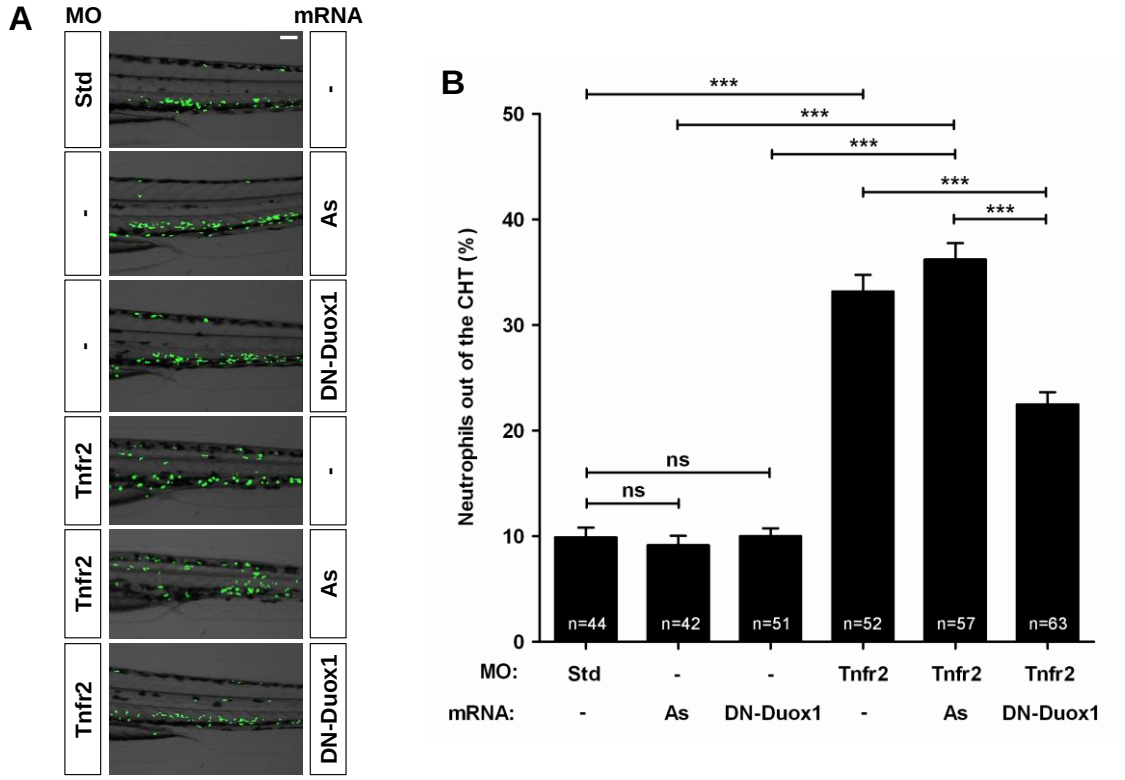


Figure 39. Overexpression of a dominant negative form of Duox1 rescues the neutrophil mobilization from the CHT to the skin. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std) or Tnfr2 morpholinos (MO) alone or combined with antisense (As) or DN-Duox1 mRNAs. (A) Representative images, brightfield and green channels, of the CHT of morphants at 72 hpf showing the differences in the neutrophils distribution. Scale bar: 100 µm. (B) The neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT. The mean ± S.E.M. for each group is shown. ns, not significant; ***p<0.001.

Furthermore, the genetic depletion of the H_2O_2 sensor of neutrophils, Lyn, using a specific morpholino (Yoo *et al.*, 2011), resulted in a full rescue of neutrophil infiltration in both Tnfr2- and Tnfa-deficient animals **(Figure 40)**.

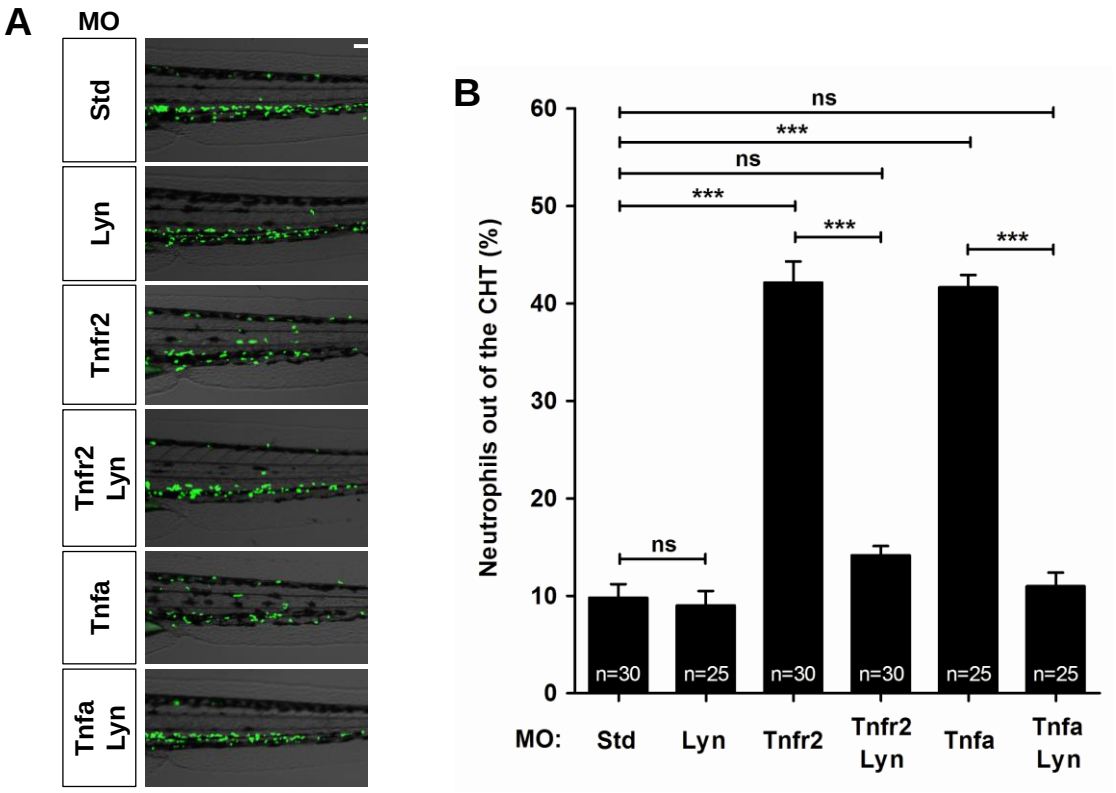


Figure 40. Genetic depletion of Lyn using a specific morpholino rescues the neutrophil mobilization from the CHT to the skin. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Lyn, Tnfr2, Tnfr2+Lyn, Tnfa or Tnfa+Lyn morpholinos (MO). (A) Representative images, brightfield and green channels, of the CHT of morphants at 72 hpf showing the differences in the neutrophils distribution. Scale bar: 100 µm. (B) The neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT. The mean ± S.E.M. for each group is shown. ns, not significant; ***p<0.001.

5. 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 (Niethammer *et al.*, 2009; Feng *et al.*, 2010; Yoo *et al.*, 2011; Deng *et al.*, 2012), may attenuate skin inflammation in Tnfa- and Tnfr2-deficient larvae.

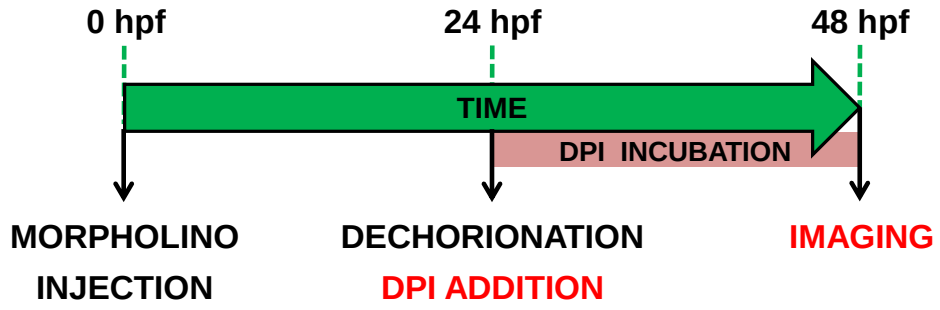


Figure 41. Experimental design of the pharmacological inhibition of Duox1 using the NADPH inhibitor dibenziodolium chloride (DPI). Zebrafish one-cell *mpx:eGFP* or *NF-κB:eGFP* embryos (0 hpf) were injected with standard control (Std), Tnfa or Tnfr2 morpholinos (MO). Embryos were manually dechorionated and then treated by immersion in 100 µM DPI or vehicle alone (DMSO) at 24 hpf. Imaging to analyze the neutrophil distribution or NF-κB activation patterns was performed after 24 hours of incubation with DPI or DMSO, at 48 h, at 48 hpf.

Following the experimental design detailed in the **Figure 41**, a complete rescue of the neutrophil mobilization phenotype was obtained using the pharmacological inhibitor **(Figure 42)**, exactly as we found with the genetic depletion of Duox1 using a morpholino **(Figure 38)** or a DN **(Figure 39)**, and with the genetic depletion of LYN **(Figure 40)**.

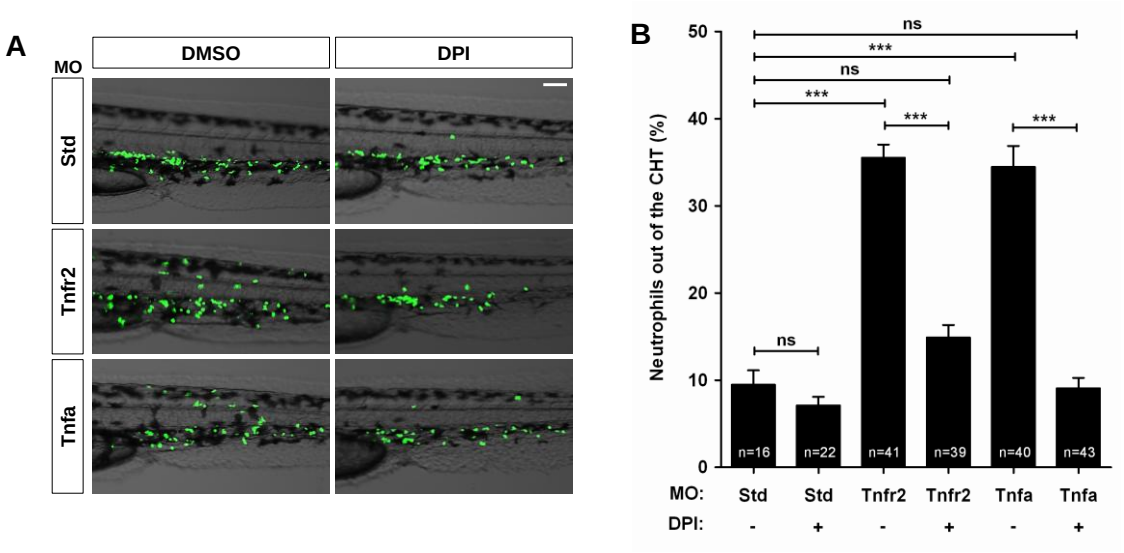


Figure 42. Pharmacological inhibition of Duox1 rescues the neutrophil mobilization from the CHT to the skin. Zebrafish one-cell *mpx:eGFP* embryos were injected with standard control (Std), Tnfr2 or Tnfra morpholinos (MO). (A) Representative images of brightfield and green channels of the morphants at 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. Scale bar: 100 µm. ns, not significant; ***p<0.001.

At this point, the results obtained demonstrated that:

1) Tnfa or Tnfr2 deficiencies resulted in neutrophil migration from the CHT to the skin.

2) Proinflammatory molecules were induced in keratinocytes sorted from Tnfr2-deficient larvae.

3) Neutrophil recruitment from the CHT to the skin was mediated by Duox1-dependent H_2O_2 produced and released by keratinocytes in Tnfr2-deficient larvae.

4) Neutrophil recruitment to the skin was mediated by Lyn, the H_2O_2 sensor present in neutrophils, in Tnfa- or Tnfr2-deficient larvae.

5) NF-κB was activated in the skin of Tnfa- or Tnfr2-deficient larvae.

In order to establish whether NF-κB activation was upstream or downstream the H_2O_2 production, we knocked-down Tnfa or Tnfr2 in the NF-κB reporter line *NF-κB:eGFP,* treated or not with DPI. The results clearly showed that NF-κB was active in

the skin of Tnfa- or Tnfr2-deficient larvae **(Figure 43)**, while in the presence of the NADPH inhibitor (DPI) it was impossible to differentiate them from control larvae. **(Figure 43)**. Therefore, these results position the NF-κB activation downstream the H_2O_2 production in Tnfa or Tnfr2-deficient larvae.

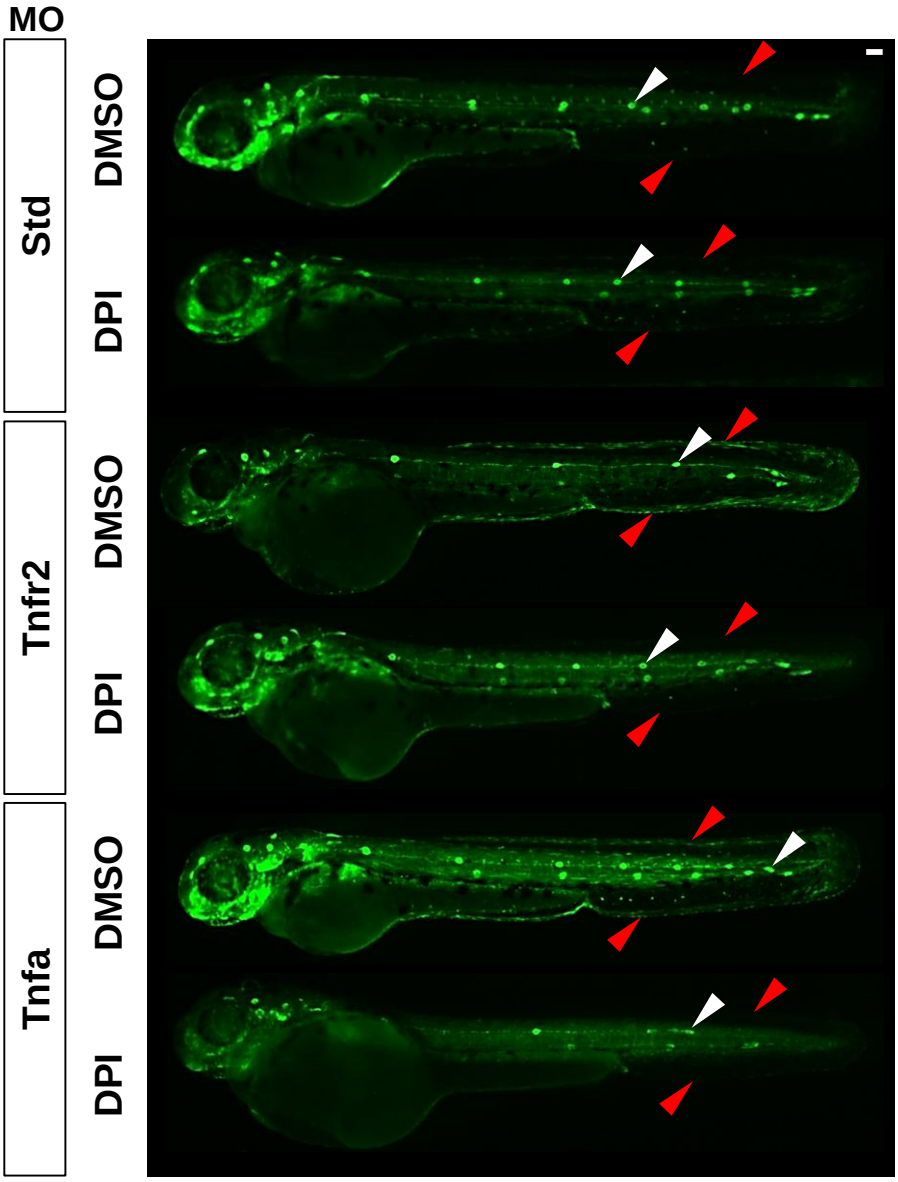


Figure 43. Modulation of the NF-κB activation patterns by the NADPH inhibitor DPI in control and Tnfr2- or Tnfa-deficient larvae. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std), Tnfa or Tnfr2 morpholinos (MO) and treated with DPI as described before **(Figure 41)**. Representative images showing the induction of NF-κB activation in the skin (red arrowheads) of Tnfr2- or Tnfa-deficient larvae at 72 hpf. Note the strong expression of NF-κB in neuromasts (white arrowheads). Scale bar: 100 μm.

The detailed analysis of the fluorescence profiles of control and Tnfa- or Tnfr2-deficient larvae, showed that in the presence of the pharmacological inhibitor DPI both GFP fluorescence peaks, corresponding to the NF-κB activation in the skin and typical in those deficient larvae, completely disappeared **(Figure 44)**.

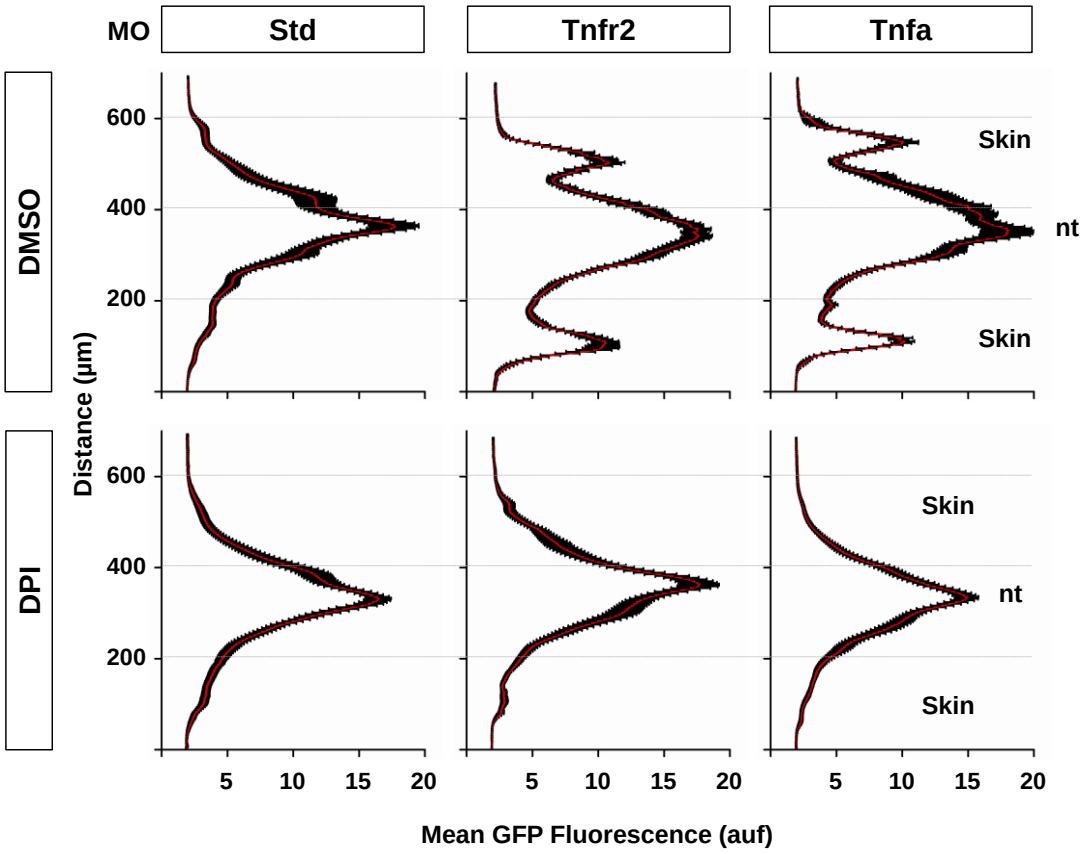


Figure 44. Fluorescence profiles of controls and larvae deficient in Tnfr2 or Tnfa confirm the differences in the NF-κB activation patterns. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std), Tnfr2 or Tnfa morpholinos (MO). Larvae were treated with DPI as described before **(Figure 41)**. Mean GFP Fluorescence intensity was measured in the area in which the CHT is included. The images were converted to a fluorescence value matrix where the value obtained for each pixel transversally was the mean (red dots) ± S.E.M. (white bars) for all the pixels for each row (15 larvae per treatment from 3 different experiments). The areas corresponding to the notochord (nt) as well as to both limits of the larvae (skin), ventral and dorsal, have been labeled to facilitate the larval orientation. Note the activation of NF-κB in the skin of Tnfr2-deficient larvae. auf: arbitrary units of fluorescence.

In addition, these results were confirmed by the calculation of the skin NF-κB activation index (as described in **Figure 32**) for control and Tnfa- or Tnfr2-deficient larvae before and after treatment with DPI **(Figure 45)**.

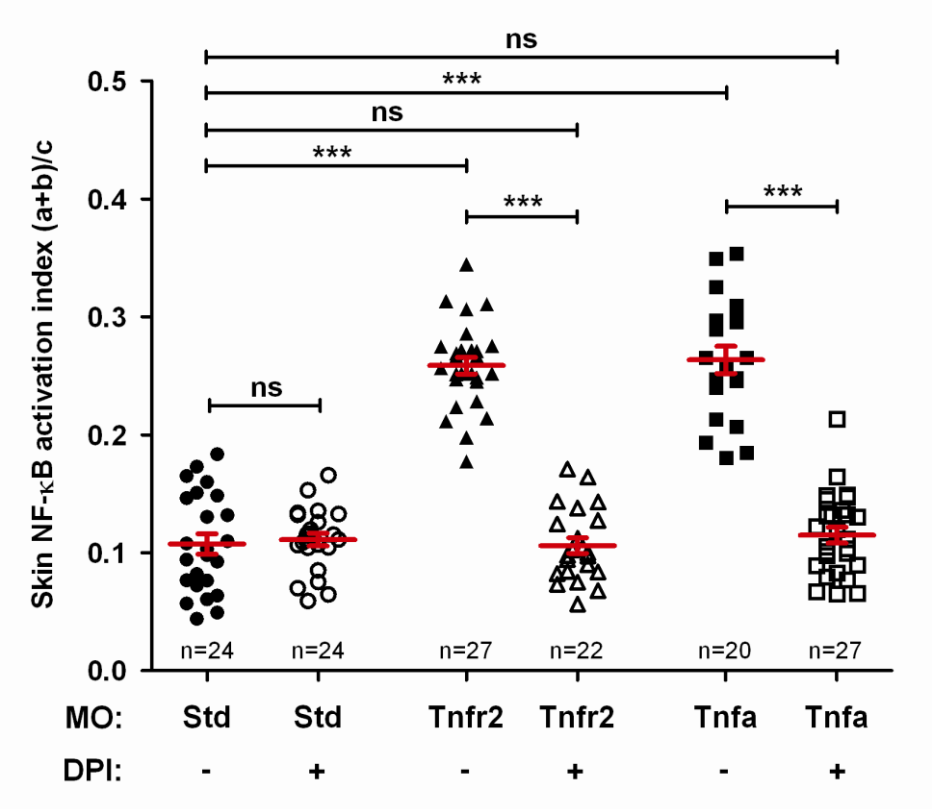


Figure 45. Skin NF-κB activation index for controls and larvae deficient in Tnfr2 or Tnfa, treated or not with DPI. Zebrafish one-cell *NF-κB:eGFP* embryos were injected with standard control (Std), Tnfr2 or Tnfa morpholinos (MO). Larvae were treated with DPI as described before **(Figure 41)**. The Skin NF-κB activation index was calculated for all treatments at 72 hpf, as we have previously described in the **Figure 32**. 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. ns, not significant. ***p<0.001.

These results obtained using the NADPH inhibitor demonstrated that i) the pharmacological inhibition of the Duox1 activity was able to rescue the neutrophil infiltration into the skin of Tnfa- or Tnfr2-deficient larvae, and ii) the use of DPI was also able to avoid the inflammation in the skin since the NF-κB activation (which is located downstreams the H2O2 production in the sequence of signals) disappears after the treatment. Both conclusions could be very interesting thinking of the design of possible potential treatments for the future.

Summarizing, we found that DPI treatment completely inhibited the generation of H_2O_2 in the skin **(Figures 35-37)**, the infiltration of neutrophils **(Figure 42)** into this tissue and, more importantly, skin NF-κB activation **(Figures 43-45)** in both Tnfa- and

Tnfr2-deficient animals. Collectively, these demonstrate that the Tnfa/Tnfr2 axis is indispensible 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 46)**.

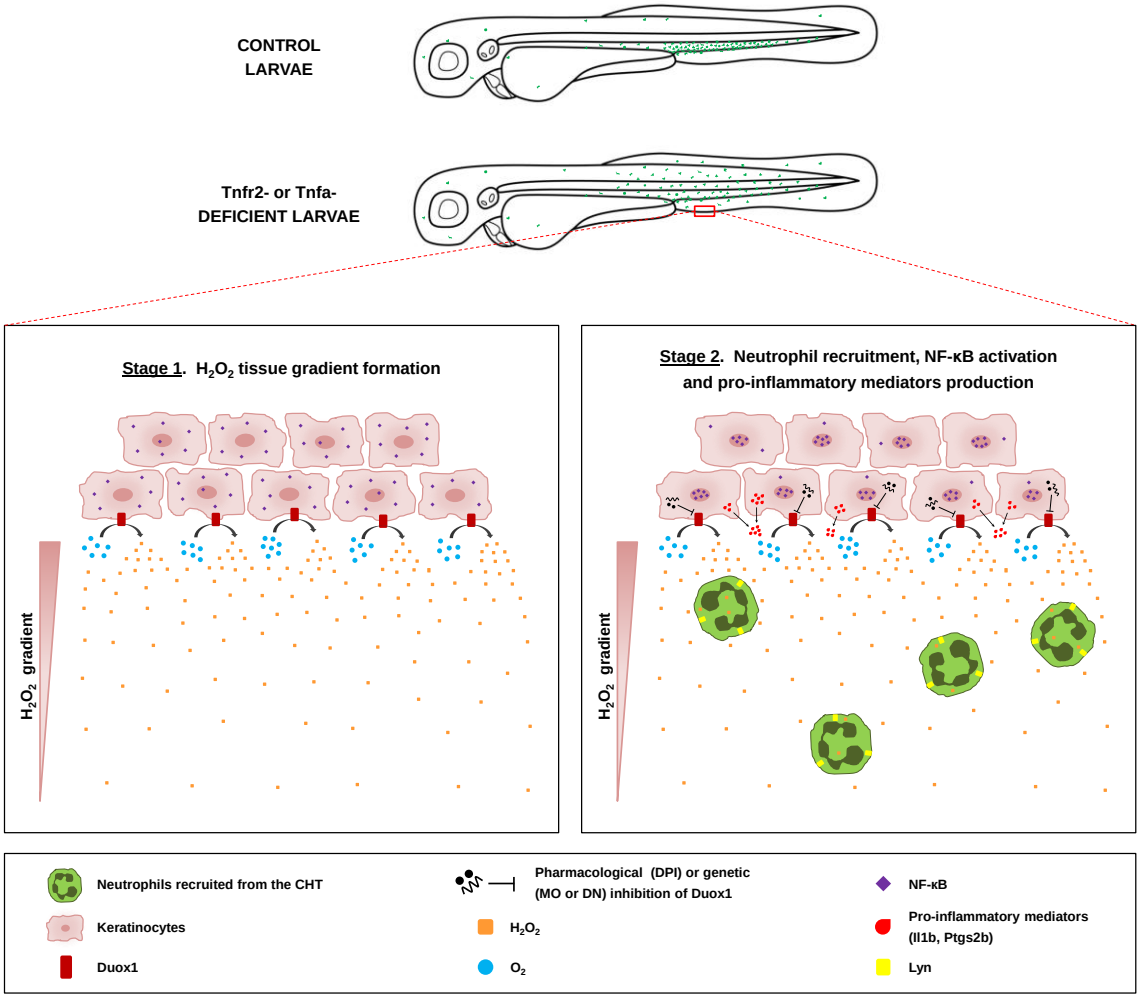


Figure 46. Model describing the sequence of signals studied in this work, which are responsible of the altered neutrophil phenotype observed in Tnfa- or Tnfr2-deficient larvae. In a first stage, a H2O2 gradient is established thanks to the production of that molecule by Duox1 from the membrane of the keratinocytes in the skin. In a second stage, neutrophils are able to sense the H2O2 gradient, due to the oxidation of Lyn, and are recruited to the skin from their original position in the CHT. NF-κB gets activated in keratinocytes and goes into the nucleus, and pro-inflammatory mediators are released from those cells. It has been also indicated the step we are blocking using the Duox1 morpholino, the DN-Duox1 as well as the pharmacological NADPH inhibitor DPI.

6. DUOX1 is induced in human psoriatic lesions

The crucial role of Duox1-generated H_2O_2 in the inflammation of the zebrafish skin prompted us to investigate if this inflammatory signal may also play a role in human psoriasis. We analyzed by immunohistochemistry 10 healthy skins and 15 psoriatic lesions using an antibody to human DUOX1 **(Figure 47)**. 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 psoriatic 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 psoriatic 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.

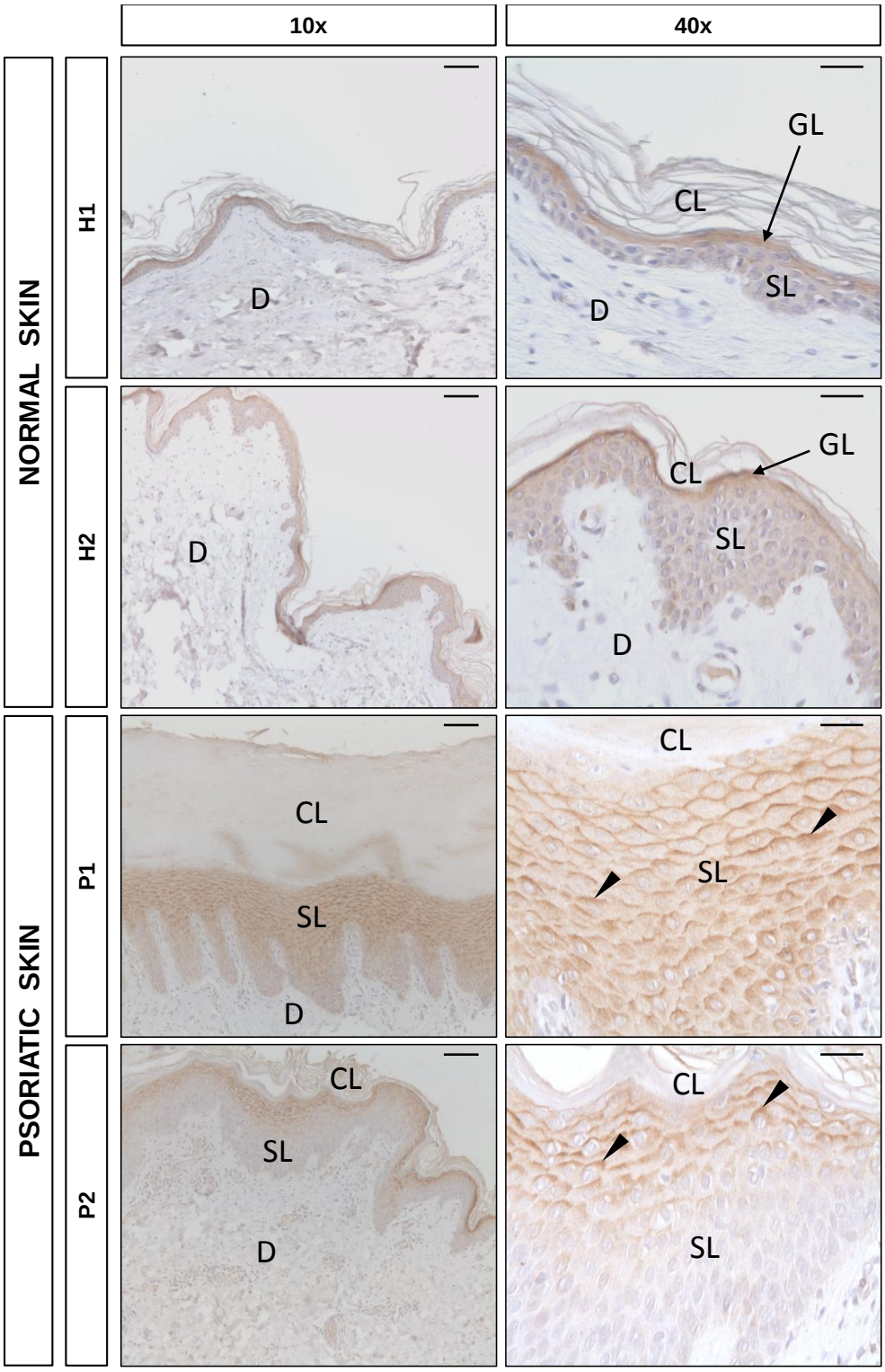


Figure 47. DUOX1 is induced in human psoriatic lesions. Representative images of sections from two healthy (H) and two psoriatic (P) skin biopsies that have been immunostained with an anti-DUOX1 goat polyclonal antibody and then slightly counterstained with hematoxilin. Note that DUOX1 is weakly expressed in healthy epidermis, mainly in the granular layer (GL), while it is strongly expressed (arrowheads) in the spinous layer (SL) of psoriatic 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, rheumatoid arthritis and IBD. We have used the unique advantage 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 skin inflammation through the activation of an H_2O_2/NF-κB/Duox1 positive feedback inflammatory loop. Strikingly, neutrophils are rapidly attracted to the inflamed skin but skin inflammation 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 patients. Collectively, these results (i) indicate a critical role of Tnfa/Tnfr2 signaling for the homeostasis of the skin, (ii) might explain the appearance of psoriasis in IBD patients treated with anti-TNFα therapies (Denadai *et al.,* 2012; Sherlock *et al.,* 2012), 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 (Van Hauwermeiren *et al.,* 2011; Espín *et al.,* 2013).

One of the most intriguing observations from this study is that impaired Tnfr2 signaling led to the induction of *duox1* and the production of H_2O_2 by keratinocytes. H_2O_2 gradient was recently shown to contribute to the early influx of neutrophils in wound (Niethammer *et al.,* 2009) and tumor (Feng *et al.,* 2010), although it seems to be dispensable for neutrophil detection of localized infection (Deng *et al.,* 2012). To the best of our knowledge, this is the first study showing a role for Duox1-derived H_2O_2 in the induction of skin inflammation *in vivo*, suggesting that H_2O_2 might play a critical role in the initiation and maintenance of chronic inflammatory diseases in both zebrafish and human. These observations suggest that both H_2O_2 and DUOX1 might be therapeutic targets for clinical treatment of patients suffering psoriasis and IBD. 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 (Zhou *et al.,* 2009; Zhu and Li, 2012)). 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 (Zhou *et al.,* 2009; Kim *et al.,* 2011; Zhu and Li, 2012). 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. The ability of Duox1 inhibition by genetic and pharmacological approaches, but not of IL-1β, to reduce skin inflammation in Tnfa- and Tnfr2-deficient zebrafish embryos further supports this conclusion.

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 (Siomek, 2012) and, unfortunately, most studies have been performed *in vitro* using H_2O_2 and cultured cells (Schreck *et al.,* 1991; Byun *et al.,* 2002;). Such studies have shown that H_2O_2 can act as an activator of IκB kinases (IKKs) (Kamata *et al.,* 2002) or can inactivate these proteins (Korn *et al.,* 2001), 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 (Cummins *et al.,* 2006). Our epistasis study in zebrafish demonstrates for the first time that the absence of Tnfa/Tnfr2 signaling led to the production of H_2O_2 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 (Zhou *et al.,* 2009; Kim *et al.,* 2011; Zhu and Li, 2012), HIF (Rosenberger *et al.,* 2007; Colgan and Taylor, 2010) and NF-κB in psoriasis and IBD.

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) (Smahi *et al.,* 2000). 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 (Makris *et al.,* 2000; Nenci *et al.,* 2006). Similarly, although NF-κB actively participates in the excessive inflammatory response observed in IBD patients (Ellis *et al.,* 1998; Schreiber *et al.,* 1998), recent studies with mice defective in NF-κB activation have revealed that epithelial NF-κB activation is essential to preserve intestinal homeostasis (Nenci *et al.,* 2007; Kajino-Sakamoto *et al.,* 2008). 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 (Makris *et al.*, 2000; Nenci *et al.*, 2006; Nenci *et al.*, 2007; Kajino-Sakamoto *et al.*, 2008), 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 indispensable required for skin homeostasis in the zebrafish and that the absence of this signal triggers the local production of H_2O_2 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 patients, reveal a crucial role of H_2O_2 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 and other chronic inflammatory diseases.

CONCLUSIONS

The results obtained in this work lead to the next conclusions:

1. Genetic inhibition of Tnfa or Tnfr2, but not of Tnfr1, results in neutrophil mobilization from the CHT to the skin, where they get infiltrated.

2. Target gene silencing of Tnfa or Tnfr2 results in the induction of the expression of genes encoding pro-inflammatory mediators in the skin.

3. The absence of Tnfa signaling through Tnfr2 triggers the local production of H_2O_2 by Duox1.

4. Genetic inhibition of Tnfa or Tnfr2 results in the activation of the master regulator of inflammation NF-κB in the skin, downstream the production of H_2O_2.

5. Tnfa signaling through Tnfr2 is critically required for skin homeostasis.

6. DUOX1 induction and/or the subsequent production of H_2O_2 in the skin of psoriasis patients, may be new targets for pharmacologic and genetic therapies for the treatment of psoriasis. These new strategies could be applicable to other chronic inflammatory diseases as well.

7. Zebrafish can be used as a model organism for the study of psoriasis and other inflammatory chronic diseases.

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RESUMEN EN CASTELLANO

1. Introducción

La inmunidad es la reacción de los organismos frente a sustancias extrañas, incluyendo todo tipo de microorganismos y macromoléculas (Abbas *et al.,* 2001). Tanto células como moléculas forman parte del sistema inmunitario, que es el responsable de la inmunidad, y sus respuestas colectivas y coordinadas constituyen la respuesta inmunitaria (Male and Roitt, 1996). La respuesta inmunitaria, que comienza con el reconocimiento del patógeno o sustancia extraña y termina con el desarrollo de mecanismos capaces de eliminarlos (Male and Roitt, 1996), se puede dividir en dos ramas: **respuesta inmunitaria innata, natural o no específica** (actúa rápidamente como primera línea de defensa antes de que se desencadene la respuesta adaptativa, y que fundamentalmente incluye barreras físicas, células fagocíticas, eosinófilos, células *natural killer* y varios tipos de moléculas circulantes (Male and Roitt, 1996; Abbas *et al.,* 2001; Mollen *et al.,* 2006)) y **respuesta inmunitaria adaptativa, adquirida o específica** (aparece más tarde y supone una respuesta altamente específica frente a un patógeno en particular, siendo especificidad y memoria sus dos características más destacables (Male and Roitt, 1996)). A pesar de que aparecen en momentos diferentes y de que presentan características distintas, las respuestas inmunitarias innata y adaptativa actúan de forma integrada y coordinada (Male and Roitt, 1996).

El sistema inmunitario de peces teleósteos presenta características similares al de otros vertebrados, incluyendo mamíferos (Van Muiswinkel, 1995), siendo el primer grupo animal que desarrolló ambos sistemas inmunitarios, innato y adaptativo, bien estructurados y diferenciados. Sin embargo, presenta algunas diferencias como su mayor dependencia de los mecanismos de defensa innatos, sobre todo a bajas temperaturas puesto que la respuesta inmunitaria adaptativa depende de ese parámetro (Cuchens and Clem, 1977; Avtalion, 1981; Abruzzini *et al.*, 1982; Clem *et al.*, 1984, 1985, 1991). Además, los peces carecen de médula ósea, actuando la parte anterior del riñón (riñón cefálico) como principal órgano hematopoyético.

El funcionamiento del sistema inmunitario está modulado por la acción de una serie de moléculas reguladoras, fundamentalmente citoquinas (Feldmann, 1996; Abbas *et al.,* 2001) y mediadores lipídicos (Smith *et al.*, 1996; Harizi and Gualde, 2002; Cabral, 2005). Además, la existencia de receptores capaces de reconocer específicamente patrones moleculares asociados a patógenos (PAMPs), es

particularmente importante para la respuesta inmunitaria innata, siendo los TLRs (Toll-Like Receptors) los más destacables (Mendzhitoz and Janeway, 2000).

La inflamación, definida como la reacción de los tejidos vivos vascularizados frente a daños locales causados por patógenos, daños físicos, agentes químicos irritantes, etc..., (Rippey, 1994; Ferrero-Miliani *et al.,* 2007), se caracteriza por una serie de síntomas bien caracterizados desde los comienzos de la medicina: enrojecimiento, calor, hinchazón, dolor y pérdida de función (Lawrence *et al.,* 2002; Kumar *et al.,* 2005; Martini and Nath, 2009). Se puede diferenciar entre dos tipos de inflamación: inflamación aguda, que comienza pocos segundos o minutos después de que se produzca el daño a los tejidos (Medzhitov, 2008), e inflamación crónica, a la que se llega si las causas que ocasionan la inflamación aguda no se resuelven y perduran en el tiempo (Rippey, 1994; Kumar *et al.,* 2013). El establecimiento de una inflamación crónica puede suponer el punto de partida de multitud de enfermedades inflamatorias crónicas (Heap GA and van Heel DA, 2009), cuya incidencia se está viendo rápidamente incrementada en todo el mundo en los últimos años, principalmente en los países más desarrollados (Bastard *et al.* 2006; Ferrucci *et al.* 2010; Glorieux *et al.* 2009; Kundu *et al.* 2008). Todavía no se conocen bien las causas de las enfermedades inflamatorias crónicas, pero parece claro que se trata del efecto conjunto de una serie de factores genéticos y ambientales. Esa complejidad en su origen hace que no existan tratamientos capaces de curar ninguna de estas enfermedades (Barnes and Karin, 1997) y, por tanto, se hace esencial el desarrollo de nuevas terapias capaces curarlas o, al menos, de atenuar sus efectos.

Entre las enfermedades inflamatorias crónicas, la psoriasis tiene un alto impacto tanto social como económico, ya que se trata de una enfermedad incapacitante en muchos casos. Se caracteriza por una inflamación crónica en la piel, donde suelen aparecer placas rojas cubiertas por escamas plateadas (Greaves and Weinstein, 1995). Afecta a entre el 1% y el 3% de la población mundial (Greaves and Weinstein, 1995; Schön and Boehncke, 2005), y no es contagiosa ni de origen alérgico, sino que su etiología es multifactorial (causas genéticas, inmunológicas y ambientales) (Crissey and Parish, 1998). El incremento de la síntesis de TNFα ha sido asociado con el desarrollo de enfermedades inflamatorias crónicas, incluyendo la psoriasis, y la inhibición de su actividad ha resultado ser un tratamiento efectivo en muchos casos (Palladino *et al.,* 2003; Faustman *et al.,* 2010). Sin embargo, paradójicamente, algunos estudios han

demostrado la aparición de nuevos casos de psoriasis, o el agravamiento de otros preexistentes, en pacientes tratados con antagonistas de TNFα para intentar paliar los efectos de otras enfermedades inflamatorias crónicas (Denadai *et al.,* 2012; Sherlock *et al.,* 2012). A pesar de que estos datos indican que TNFα desempeña funciones ambiguas en el desarrollo de la psoriasis, su papel, y en particular la contribución de cada uno de sus receptores a la inflamación crónica en la piel, apenas han sido estudiados.

Los neutrófilos representan la primera línea de defensa frente a patógenos invasores. En los últimos años ha sido descrito cómo el establecimiento de un gradiente de peróxido de hidrógeno contribuye al reclutamiento de los neutrófilos hasta el lugar donde se ha producido una herida (Niethammer *et al.,* 2009) o donde se encuentran células tumorales (Feng *et al.,* 2010). Dichos gradientes son creados por la enzima DUOX1 desde el lugar de la herida (Niethammer *et al.,* 2009), y detectados por los neutrófilos gracias a la tirosín quinasa LYN (Yoo *et al.,* 2011).

El Factor de Necrosis Tumoral α (TNFα) es una citoquina que ha sido muy bien caracterizada por su función en la inflamación. Sin embargo, el TNFα se caracteriza por tener un papel pleiotrópico, y se le ha atribuido un papel en procesos biológicos tan dispares como desarrollo embrionario, diferenciación, supervivencia celular, cáncer, hematopoyesis, metabolismo lipídico y reproducción.

El TNFα se produce principalmente por células del sistema inmunitario tales como macrófagos, linfocitos T y B, células linfoides y células NK. Sin embargo, otros tipos celulares también son capaces de producir TNFα, entre los que podemos destacar células endoteliales, miocitos, tejido adiposo, células hematopoyéticas, fibroblastos y neuronas. La producción de TNFα por esta gran variedad de tipos celulares es un indicativo de los múltiples procesos en los que esta citoquina está involucrada. Además, el efecto pleiotrópico del TNFα relaciona esta citoquina con una gran variedad de enfermedades humanas, tales como desórdenes inflamatorios y autoinmunes (psoriasis, artritis reumatoide y enfermedad inflamatoria intestinal), isquemia seguida de daño por reperfusión y cáncer (Aggarwal *et al.,* 2012).

El TNFα ejerce su acción a través de la unión a dos receptores de membrana denominados TNFR1, también llamado TNFRSF1A, y TNFR2 o TNFRSF1B (Shalaby *et al.,* 1990). Mientras que TNFR1 está presente en la mayoría de células, TNFR2 tiene una expresión más restrictiva, encontrándose fundamentalmente en células del sistema

inmunitario y células endoteliales (Aggarwal *et al.*, 2003). En cuanto al efecto que desencadena cada receptor de TNFα, se ha demostrado que TNFR1 provoca apoptosis o inflamación, pero TNFR2 promueve la reparación tisular y regeneración (Aggarwal *et al.*, 2003). Sin embargo, hay cierto solapamiento en sus funciones, ya que los receptores de TNFα no poseen actividad enzimática per sé, y es a través del reclutamiento de diferentes proteínas adaptadoras cómo ejercen su función, pudiendo compartirlas (Pimentel-Muinos *et al.*, 1999).

Son tres tipos de proteínas las que interaccionan con los dominios citoplasmáticos de los TNFRs: TAFs, factores asociados a TNFR; FADDs: dominios de muerte asociados a FAS y TRADDs, dominios de muerte asociados a TNFR. La unión de TNFα a TNFR1 provoca el reclutamiento de TRAF2 y TRADD, los cuales interaccionan con otras proteínas señalizadoras tales como BIRC2 (cIAP1) y BIRC3 (cIAP2). De esta forma se forma lo que se denomina complejo I que induce la degradación de IκB, que es el inhibidor de NF-κB. De esta forma, NF-κB se trasloca al núcleo, desencadenando la transcripción de genes pro-inflamatorios y de supervivencia (Locksley *et al.*, 2001; MacEwan, 2002). A partir de este complejo I se puede formar el complejo II. Este complejo II recluta FADD y caspasa-8, lo que resulta en la activación de ésta última que desencadenará muerte celular (Locksley *et al.*, 2001; MacEwan, 2002). Por el contrario, la unión de TNFα a TNFR2 desencadena el reclutamiento de TRAF1 y TRAF2, que interaccionan con BIRC2 y BIRC3 (Rothe *et al.*, 1995), llevando a la activación de NF-κB.

El pez cebra (*Danio rerio* H), perteneciente a la familia Cyprinidae, presenta una serie de características que le han convertido en un organismo modelo de vertebrados importante en investigación en multitud de áreas del conocimiento (Vascotto *et al.*, 1997). De entre sus principales ventajas destacan su pequeño tamaño, su elevada fecundidad (unos 200 huevos por hembra a la semana), su corto tiempo de generación, su rápido desarrollo, el conocimiento de la secuencia de su genoma, el desarrollo extrauterino de sus embriones (facilitando una manipulación sencilla) y, sobre todo, su transparencia, lo que permite el seguimiento y estudio de células individuales trabajando con líneas transgénicas. Todas esas ventajas han hecho posible que el pez cebra se establezca como un organismo modelo crucial en el estudio del sistema inmunitario (Renshaw and Trede, 2012), hematopoyesis (Martin *et al.*, 2011), desarrollo vascular (Isogai *et al.*, 2009; Quaife *et al.*, 2012; Gore *et al.*, 2012), neurogenesis (Schmidt *et al.*,

2013), cancer (Mione and Trede, 2010), comportamiento (Miklósi and Andrew, 2006; Spence *et al.*, 2008; Norton and Bally-Cuif, 2010) y, lo que es más importante para la presente tesis, inflamación (Trede *et al.*, 2004; Novoa and Figueras, 2012) e inflamación crónica (Mathias *et al.*, 2007).

2. Objetivos

Los objetivos específicos de este trabajo son:

5. Caracterización del papel desempeñado por Tnfa y sus receptors (Tnfr1 y Tnfr2) en la función y distribución de los neutrófilos en larvas de pez cebra.

6. Caracterización de las vías de señalización de Tnfr1 y Tnfr2 involucradas en la homeostasis de la piel en larvas de pez cebra.

7. Caracterización del papel jugado por Tnfa y sus receptors en la inflamación crónica de la piel en larvas de pez cebra.

8. Evaluación de las larvas de pez cebra como possible modelo para el studio de enfermedades inflamatorias crónicas humanas.

3. Resultados

3.1. Los neutrófilos son movilizados hacia la piel en larvas deficientes en Tnfa o Tnfr2

Nuestro objetivo inicial era estudiar cómo la señalización a través de los receptores de Tnfa era capaz de modular tanto la funcionalidad como los patrones de distribución de las células mieloides, macrófagos y neutrófilos, en larvas de pez cebra. La depleción genética de Tnfa y de ambos receptores (Tnfr1 y Tnfr2), por separado o al mismo tiempo, mediante el uso de morfolinos previamente validados en nuestro laboratorio, nos permitió observar grandes diferencias en los patrones de distribución de los neutrófilos entre los diferentes tratamientos, pero no así en macrófagos: el análisis de larvas de la línea transgénica *mpeg1:eGFP* (en cuyos macrófagos se expresa la proteína verde fluorescente GFP) deficientes en Tnfa o en sus receptores no nos mostró ninguna diferencia en cuanto al patrón de distribución de los macrófagos a 72 hpf. Sin embargo, cuando utilizamos larvas de las líneas transgénicas *mpx:eGFP* o *lyz:DsRed* (en cuyos neutrófilos se expresan la proteína verde fluorescente GFP o la proteína roja fluorescente DsRed respectivamente) también a 72 hpf, encontramos que los neutrófilos se encontraban dispersos por toda la larva en individuos deficientes en Tnfa o en Tnfr2 y no agrupados en la CHT, como si ocurría en larvas control y deficientes en Tnfr1 y como había sido descrito en la bibliografía (Murayama *et al*., 2006).

Puesto que existe la posibilidad de que los morfolinos presenten efectos inespecíficos (Eisen and Smith, 2008), la especificidad del fenotipo observado en larvas deficientes en Tnfa y Tnfr2 fue comprobada mediante experimentos de rescate, utilizando sus respectivos ARNs mensajeros por un lado, y una forma dominante negativa de Tnfr2 (que carece del dominio intracelular encargado de la transducción de la señal) por otro lado (Espín *et al.,* 2013).

Además, el análisis de larvas dobles transgénicas *krt18:RFP* y *mpx:eGFP* (que expresan la proteína roja fluorescente RPF en queratinocitos y la proteína verde fluorescente GFP en neutrófilos) deficientes en Tnfr2, nos mostró que los neutrófilos que son movilizados desde la CHT se encuentran en contacto con los queratinocitos en la piel.

3.2. Las larvas deficientes en Tnfa o Tnfr2 presentan inflamación en la piel

El fenotipo de dispersión de los neutrófilos era similar al que había sido descrito previamente para larvas mutantes *spint1a* and *clint1*, que desarrollaban inflamación crónica en la piel (Mathias *et al*., 2007; Carney *et al*., 2007; Dodd *et al.,* 2009). La expresión de moléculas pro-inflamatorias fue analizada mediante qPCR en larvas completas, neutrófilos aislados y queratinocitos aislados procedentes de larvas control y larvas deficientes en Tnfr2 a 72 hpf. Los resultados nos mostraron que existía una inducción de Tnfa, IL-1β y Ptgs2b en larvas completas deficientes en Tnfr2 comparadas con controles, y que lo mismo ocurría con IL-1β y Ptgs2b en queratinocitos aislados. Sin embargo, no se apreciaba una inducción de IL-1β en neutrófilos aislados morfantes para Tnfr2. Este hecho, junto con que la inflamación ya se había desencadenado en el momento en que aparecen los primeros neutrófilos, sugieren que estás células no parecen encontrarse directamente relacionadas con el inicio de la inflamación.

3.3. NF-κB se activa en la piel de larvas deficientes en Tnfa o Tnfr2

Ha sido descrito que el regulador de la inflamación NF-κB juega un papel fundamental en el mantenimiento de la homeostasis en la piel (Pasparakis *et al.,* 2002; Gugasyan *et al.,* 2004; van Hogerlinden *et al.,* 2004; Omori *et al.,* 2006; Sayama *et al.,* 2006). La depleción genética de Tnfr2 o Tnfa en larvas de la línea transgénica *NF-κB:eGFP*, que expresan la proteína verde fluorescente GFP en las zonas donde el regulador de la inflamación NF-κB se encuentra activo, nos permitió comprobar que NF-κB se activaba significativamente en la piel de larvas morfantes para Tnfr2 a 72 hpf, mientras que dicha activación era inexistente en la piel de larvas control.

3.4. La piel de larvas deficientes en Tnfa o Tnfr2 produce y libera H_2O_2

Puesto que ha sido descrito que los gradientes de H_2O_2 median el reclutamiento de neutrófilos a los lugares donde se ha producido una herida, y que dicho H_2O_2 es producido por la enzima DUOX1 (Niethammer *et al.,* 2009), comprobamos que existía una inducción de la expresión de dicha enzima en queratinocitos aislados a partir de larvas deficientes en Tnfr2 comparados con larvas control. A continuación, gracias al uso de una sonda que emite fluorescencia específicamente en presencia de H_2O_2 (acetyl-pentafluorobenzene sulphonyl fluorescein), pudimos observar que las larvas deficientes

en Tnfr2 producían H_2O_2 en la piel, donde incluso era posible ver a los queratinocitos marcados individualmente. Para confirmar la hipótesis de que el fenotipo de dispersión de los neutrófilos en larvas deficientes en Tnfa o Tnfr2 se debía a que seguían el gradiente de H_2O_2 producido por Duox1 en la piel, se llevaron a cabo experimentos de rescate de dicho fenotipo utilizando un morfolino para Duox1, un dominante negativo para Duox1 y un morfolino para Lyn (que es el sensor de H_2O_2 presente en los neutrófilos (Yoo *et al.,* 2011). En todos los casos se consiguió revertir el fenotipo, confirmándose así nuestra hipótesis.

3.5. La inhibición farmacológica de Duox1 restaura la homeostasis en la piel de larvas deficientes en Tnfa o Tnfr2

Los resultados anteriores nos llevaron a evaluar si la inhibición farmacológica de Duox1 con el inhibidor de NADPH oxidasa dibenziodolium chloride (DPI), cuya capacidad de inhibir Duox1 y la formación de gradientes de H_2O_2 ha sido demostrada (Niethammer *et al.,* 2009; Feng *et al.,* 2010; Yoo *et al.,* 2011; Deng *et al.,* 2012), era capaz de atenuar la inflamación presente en la piel de larvas deficientes en Tnfa o Tnfr2. El tratamiento con DPI de larvas de la línea transgénica *NF-κB:eGFP*, controles y deficientes en Tnfa o Tnfr2, mostró que el rescate del fenotipo de dispersión de los neutrófilos, así como de la activación de NF-κB en la piel, eran totales tras el tratamiento con el inhibidor de NADPH oxidasa. Además, estos resultados nos permitieron establecer que la activación del regulador de la inflamación NF-κB se encuentra aguas abajo de la producción de H_2O_2.

3.6. DUOX1 se encuentra inducido en las lesiones de la piel de pacientes con psoriasis

El papel fundamental en la inflamación en la piel de larvas de pez cebra jugado por el H_2O_2 producido por Duox1, nos llevó a analizar si esta señal inflamatoria también desempeñaba un papel importante en pacientes humanos con psoriasis. Para ello, analizamos mediante inmunohistoquímica muestras de piel de 10 pacientes sanos y 15 enfermos de psoriasis usando un anticuerpo anti-DUOX1 humana. Los resultados mostraron una inducción drástica de DUOX1 en las muestras de piel provenientes de lesiones psoriásicas, sugiriendo un papel importante para esta enzima en psoriasis.

4. Discusión

El incremento de la producción de TNFα se asocia con el desarrollo de enfermedades inflamatorias crónicas, incluyendo psoriasis, artritis reumatoide e inflamación intestinal crónica (IBD). En este trabajo hemos aprovechado las ventajas que presentan los embriones de pez cebra y que nos permiten estudiar células individuales *in vivo*, para demostrar que la deficiencia en Tnfa o Tnfr2, pero no en Tnfr1, causa inflamación en la piel a través de la activación de un bucle de retroalimentación positiva en el que participan H_2O_2/NF-κB/Duox1. Sorprendentemente, los neutrófilos son atraídos rápidamente hasta la piel inflamada, aunque dicha inflamación ya existía desde antes de la aparición de los primeros neutrófilos en el embrión en desarrollo. Además, DUOX1 se induce fuertemente en muestras humanas procedentes de lesiones psoriásicas. En conjunto, estos resultados indican que la señalización a través de Tnfa/Tnfr2 juega un papel clave en el mantenimiento de la homeostasis de la piel, podrían explicar la aparición de psoriasis en pacientes con IBD tratados con terapias anti- TNFα (Denadai *et al.,* 2012; Sherlock *et al.,* 2012), y apoyan la idea de que la inhibición específica de la señalización a través del eje TNFα /Tnf1 sin afectar el eje TNFα /Tnfr2 podría inhibir los efectos patológicos de TNFα pero reduciendo los efectos perjudiciales asociados a esta terapia (Van Hauwermeiren *et al.,* 2011; Espín *et al.,* 2013).

Una de las observaciones más interesantes de este estudio, es que cuando impedimos la señalización vía Tnfr2 se induce duox1 y los queratinocitos producen H_2O_2. Hasta donde nosotros sabemos, esta es la primera vez que se describe que el H_2O_2 producido por Duox1 induce inflamación en la piel *in vivo*, sugiriendo que el H_2O_2 podría jugar un papel importante en la iniciación y mantenimiento de enfermedades inflamatorias crónicas tanto en pez cebra como en humanos. Estas observaciones sugieren que H_2O_2 y DUOX1 podrían ser dianas terapeúticas para el tratamiento clínico de pacientes con psoriasis e IBD.

Nuestro estudio demuestra por primera vez que la ausencia de señalización a través del eje Tnfa/Tnfr2 conduce a la producción de H_2O_2 por los queratinocitos, dando lugar posteriormente a la activación de NF-κB y a la inducción de la expresión de mediadores pro-inflamatorios.

En conclusión, en este trabajo hemos encontrado que la señalización de Tnfa a través de Tnfr2 es imprescindible para mantener la homeostasis de la piel en pez cebra, y que la ausencia de dicha señalización conduce a la producción de H_2O_2 por la enzima Duox1, activándose seguidamente NF-κB e induciéndose a continuación la expresión de mediadores pro-inflamatorios y la infiltración de neutrófilos en la piel. Estos resultados, conjuntamente con la inducción de DUOX1 en muestras humanas de piel procedentes de lesiones psoriásicas, revelan un papel crucial de H_2O_2 y DUOX1 en la inflamación en la piel, sugiriendo que las terapias farmacológicas y genéticas que tengan como dianas esos dos factores podrían suponer aproximaciones innovadoras e interesantes para el tratamiento de la psoriasis y otras enfermedades inflamatorias crónicas.

4. Conclusiones

Los resultados obtenidos en este trabajo nos han permitido extraer las siguientes conclusiones:

1. La inhibición genética de Tnfa o Tnfr2, pero no de Tnfr1, da como resultado la movilización de los neutrófilos desde la CHT hasta piel, lugar donde se infiltran.

2. El silenciamiento de Tnfa o Tnfr2 provoca la inducción en la piel de la expresión de genes que codifican para moléculas pro-inflamatorias.

3. La ausencia de señalización de Tnfa a través del receptor Tnfr2 desencadena la producción local de H_2O_2 por la enzima Duox1 en la piel.

4. La inhibición genética de Tnfa o Tnfr2 da como resultado la activación del regulador maestro de la inflamación NF-κB en la piel, aguas abajo de la producción de H_2O_2.

5. La señalización de Tnfa a través del receptor Tnfr2 es indispensable para el mantenimiento de la homeostasis de la piel.

6. La inducción de DUOX1 y/o la consiguiente producción de H_2O_2 en la piel de pacientes con psoriasis, podrían ser nuevas dianas para terapias farmacológicas y genéticas para el tratamiento de la psoriasis. Estas nuevas estrategias podrían ser también aplicables para otras enfermedades inflamatorias crónicas.

7. El pez cebra puede ser utilizado como organismo modelo para el estudio de la psoriasis y otras enfermedades inflamatorias crónicas.

ANNEXE I

Participation in publications during the PhD

1. Espín R, Roca FJ, **Candel S**, Sepulcre MP, González-Rosa JM, Alcaraz-Pérez F, Meseguer J, Cayuela ML, Mercader N, Mulero V (2013). TNF receptors regulate vascular homeostasis through a caspase-8, caspase-2 and P53 apoptotic program that bypasses caspase-3. Dis Model Mech 6, 383-396

2. de Oliveira S, Reyes-Aldasoro CC, **Candel S**, Renshaw SA, Mulero V, Calado A (2013). Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. J Immunol 190, 4349-4359

3. Espín-Palazón R, Stachura DL, García-Moreno D, Campbell CA, **Candel S**, Meseguer J, Traver D, Mulero V (2013). TNF signaling is critical for hematopoietic stem cell survival and expansion in the developing embryo. PNAS (under review)

4. **Candel S**, de Oliveira S, García-Moreno D, Espín-Palazón R, Tyrkalska SD, Cayuela ML, Renshaw SA, Tsai H, Meseguer J, Sepulcre MP, Mulero V (2013). Tnfr2 deficiency triggers skin inflammation in the zebrafish via the production of H2O2 and the activation of the NF-κB signaling pathway. Immunity (under review)

ANNEXE II

Contributions to scientific conferences during the PhD

1. Espín R, Roca FJ, **Candel S**, Meseguer J, Mulero V. Desarrollo de un modelo de inflamación en el pez cebra y estudio del papel de la proteolisis intramembrana del TNFα en la regulación de la respuesta inmunitaria. XII Congreso de la SEBC, Pamplona (Spain). 2-5 July 2007. Poster.

2. **Candel S**, Sepulcre MP, Meseguer J, Mulero V. Negative regulation of toll-like receptor (TLR) signaling: molecular and functional characterization of zebrafish MD1 and RP105. 6th European Zebrafish Genetics and Development Meeting, Rome (Italy). 15-19 July 2009. Poster.

3. **Candel S**, Sepulcre MP, Meseguer J, Mulero V. Evolution of negative regulation of Toll-like receptor (TLR) signaling: zebrafish MD1 is a co-receptor of TLR4 and RP105. XIII Congreso de la SEBC, Murcia (Spain). 16-18 December 2009. Poster.

4. **Candel S**, Espín R, García-Moreno D, Mulero V. Transcriptomic profiles of zebrafish larvae deficient in tumor necrosis factor receptors. Zebrafish Disease Models 4 (ZDM4), Edimburg (Scotland). 9-11 July 2011. Poster.

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