UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

THE ROLE OF VITAMIN B6 IN PSORIASIS

ESTUDIO DEL PAPEL DE LA VITAMINA B6 EN LA PSORIASIS

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2019

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Grupo Inmunidad, inflamación y cáncer

Facultad de Biología

Universidad de Murcia/ IMIB

Memoria que presenta **Francsico Juan Martínez Navarro** para optar al grado de Doctor, con Mención Internacional, por la Universidad de Murcia.

Septiembre, 2019.

Thesis submitted by **Francisco Juan Martínez Navarro** for the degree of Doctor, with International Mention, of the University of Murcia.

September, 2019.

La realización del presente trabajo ha sido posible gracias a la adjudicación de un Contrato Predoctoral de Formación de Doctores (FPI) del Subprograma Estatal de Formación 2015 del Ministerio de Economía y Competitividad del Gobierno de España.

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LIST OF ABBREVIATIONS

µg	Microgram
µm	Micrometre
µM	Micromolar
ANOVA	Analysis of variance
AOX1	Aldehyde oxidase
AUF	Arbitrary units of fluorescence
BCR	B-cell receptor
CBS	Cystathionine beta-synthase
cDNA	Complementary deoxyribonucleic acid
cAMP	Cyclic adenosine monophosphate
CHT	Caudal hematopoietic tissue
Clint1a	Clathrin interactor 1a
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSE	Cystathionine gamma-lyase
CXCL8	CXC chemokine ligand 8, Interleukin 8
CXCR2	C-X-C chemokine receptor type 2
DAMPs	Damage-associated molecular patterns
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpf	Days post-fertilization
DsRed	Red fluorescent protein from *Discosoma sp.*
Duox1	Dual oxidase 1
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
Et al.	*Et alii*
F	Forward primer
G6PD	Glucose-6-phosphate dehydrogenase
GFP	Green fluorescent protein
GLUT1	Glucose transporter 1
H&E	Hematoxylin and eosin stain
h	Hours
hpf	Hours post-fertilization
IL	Interleukin
IP3	Inositol-3-phosphate
JNK	c-Jun N-terminal kinase
krt18	Keratin 18 gene
LTB4	Leukotriene B4
Lyn	Tyrosine-protein kinase Lyn
lyz	Lysozyme gene
MHC	Major histocompatibility complex
min	Minutes
ml	Mililitre

mM	Milimolar
MO	Morpholino
Mpeg1	Macrophage expressed protein 1
Mpx	Myeloperoxidase
mRNA	Messenger ribonucleic acid
Nrf2	Nuclear factor erythroid 2-related factor 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
nl	Nanolitre
p	p-value
P2ry	Purinergic receptors
PAMPs	Pathogen-associated molecular patterns
PA	Pyridoxic acid
PAr	Pyridoxic acid ratio
PASI	Psoriasis Area Severity Index
PCR	Polymerase chain reaction
pg	Picogram
PDXK	Pyridoxal kinase
PDXP	Pyridoxal phosphatase
PL	Pyridoxal
PLP	Pyridoxal phosphate
PM	Pyridoxamine
PMP	Pyridoxamine phosphate
PN	Pyridoxine
PNP	Pyridoxine phosphate
PNPO	Pyridoxamine 5'-phosphate oxidase
PRRs	Pattern recognition receptors
Ptgs2	Prostaglandin-endoperoxide synthase 2
PUVA	Psoralen-ultraviolet A light treatment
PYGL	Glycogen phosphorylase
R	Reverse primer
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
rps11	Ribosomal protein S11 gene
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
Spint1a	Serine peptidase inhibitor, Kunitz type 1a
STAT3	Signal transducer and activator of transcription 3
std	Standard control
TCR	T cell receptor
Tg	Transgenic
TGFB	Tumor growth factor beta
Th	Helper T cell

Th1	Type 1 helper T cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily member
TNFSF	Tumor necrosis factor superfamily
TNFSFRs	Tumor necrosis factor superfamily receptors
Treg	Regulatory T cell
UVA	Ultraviolet A light
UVB	Ultraviolet B light
ZFIN	The Zebrafish Information Network

SUMMARY

Psoriasis is a skin inflammatory disorders that affects 3 % of the human population. Although several therapies based in the neutralization of proinflammatory cytokines have been used with relative success, additional treatments are required. Here we report by *in silico* analysis of publicly available gene expression data of psoriasis lesional and non-lesional skin, together with the analysis of vitamin B6 metabolites in the sera of psoriasis patients before and after PUVA treatment, altered vitamin B6 metabolism at both local and systemic levels. Functional studies in the zebrafish embryo/larval model shows that different vitamin B6 vitamers were able to reduce in a dose-dependent manner skin neutrophil infiltration, oxidative stress and NFκB activity in three independent skin inflammation models, namely tumor necrosis factor α receptor 2 (Tnfr2, also known as Tnfrsf1b), serine peptidase inhibitor, Kunitz type 1 a (Spint1a) and Clathrin interactor 1a (Clint1a) deficient animals. Furthermore, glycogen phosphorylase L (PYGL) and glucose-6-phosphate 1-dehydrogenase (G6PD), two vitamin B6-dependent enzymes were revealed as potential targets for the treatment of psoriasis.

Keywords**:** skin, psoriasis, inflammation, neutrophils, vitamin B6, oxidative stress, NF-κB, hydrogen peroxide.

INTRODUCTION

Immunity

Immunity is the balance between protection against foreign elements, like microorganism, and the tolerance to avert allergy and autoimmune diseases (Waller and Sampson, 2018). A variety of molecules, cells and tissues participate in a coordinated response conforming the immune system. Depending on the reaction time and specificity of the response, immune system can be classified in two main branches, named innate and adaptive immunity (Parkin and Cohen, 2001).

Despite innate and adaptive responses play it role in different moments, there is a coordination and a regulation between them (Dempsey *et al.*, 2003). This requires control mechanisms to avoid self-immune responses that could develop disorders as lupus, diabetes, hypothyroidism, rheumatoid arthritis or psoriasis (Gregersen and Behrens, 2006).

Innate immune system

The innate immune system is the fastest response against infectious threats. Physical and chemical barriers like the epithelia and its secretions are the first protection layer against the environment and possible pathogens (MacPherson and Austyn, 2013). When these barriers have been vulnerated, soluble molecules, as complement and different kind of cells, as natural killer, mast, dendritic and myeloid cells, namely neutrophils and macrophages, are the main players in the innate immune response (Figure 1).

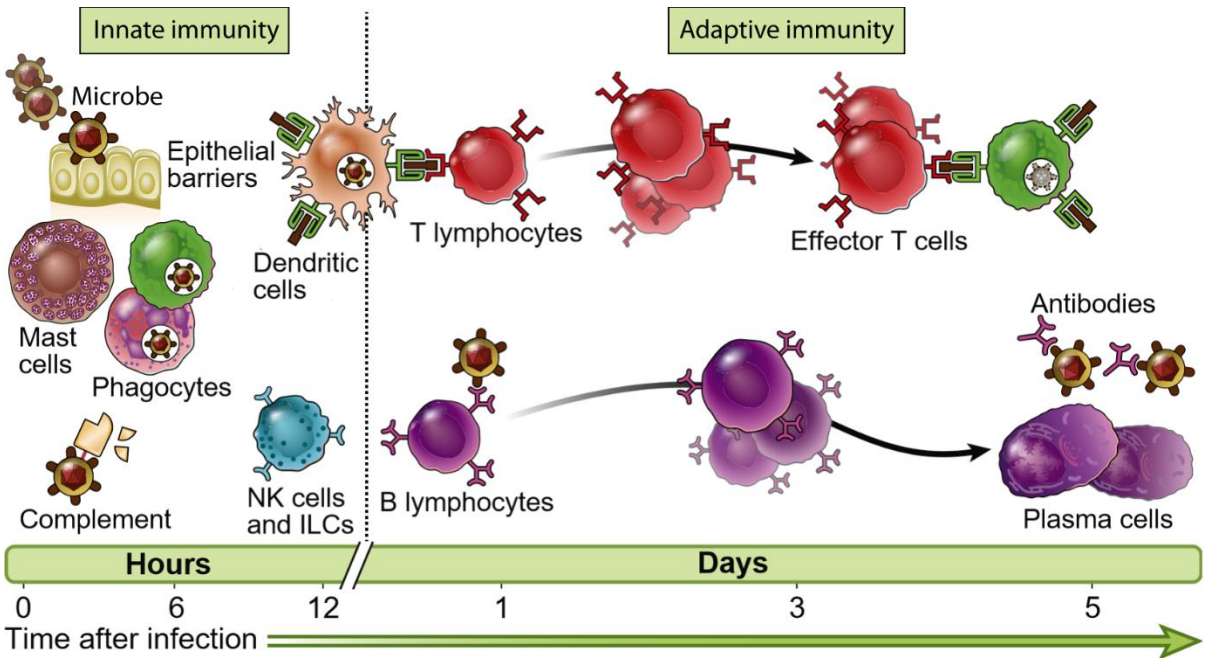


Figure 1. Main characteristics of innate and adaptive immunity. Adapted from (Abbas *et al.*, 2019).

Myeloid cells detect, phagocyte and clear the pathogen in the first hours after the first contact. They express pattern recognition receptors (PRRs) that can detect pathogen associated molecular patterns (PAMPs) and trigger the immune response (McComb *et al.*, 2013). These receptors can also detect damage associated patterns (DAMPs) released after tissue disruption or by necrotic cells (Venereau *et al.*, 2015). When PAMPs or DAMPs are detected, the release of chemokines and cytokines is induced to attract more immune cells (Medzhitov, 2007). Furthermore, dendritic cells can present the antigen to adaptive immune cells and lead to the activation of the adaptive immunity (Banchereau and Steinman, 1998).

Adaptive immune system

The adaptive immune system is specific and develops memory for future encounters with the same pathogen. While the innate immune system emerged 700 million years ago, adaptive immunity is more complex and appeared 450 million years ago in the first jawed vertebrates, fish (Schluter *et al.*, 1999).There are two main classes of adaptive immunity, humoral and cell-mediated immunity. Both are carried out by different lymphocytes (Figure 1).

Humoral immunity protects from extracellular pathogens and is based on the antibody production by B lymphocytes. To recognize these pathogens, they express B cell receptors (BCRs) capable to detect components of infectious agents present in the blood or extracellular compartments (Abbas *et al.*, 2019). On the other hand, the cell-mediated immunity can protect against intracellular pathogens and is based on the T lymphocytes. T cell receptors (TCRs) can recognize antigens presented by the major histocompatibility complexes (MHC) from the antigen-presenting cells (Neefjes *et al.*, 2011).

BCR and TCR are created through DNA reorganization generating specificity for every possible antigen. After finding the specific antigen the lymphocyte responsible of the receptor production proliferate and differentiate to attack the pathogen and rest in lymphoid organs until a next exposure to the same antigen. Lymphocytes expressing receptors that recognize self-body components are eliminated by tolerance mechanisms. However, if these mechanisms fails it can drive to develop autoimmune disorders (Gregersen and Behrens, 2006).

Inflammation

Inflammation is the pathophysiological response of the innate immune system against infections or tissue damage, malfunction or stress. Its purpose is to neutralize the agent that is producing the damage (Chovatiya and Medzhitov, 2014). This response is developed after the activation of PRRs.

Upon detection of a stimulus, tissue resident macrophages and epithelial cells start the inflammatory response. Then, neutrophils and monocytes, which are going to differentiates into macrophages, migrate on the way to inflammation site (Schmid-Schonbein, 2006). This migration is due to the production of proinflammatory cytokines and chemokines like tumor necrosis factor alfa (TNFA), interleukins, mainly interleukin-1 beta (IL1B) and interleukin-8 (CXCL8) by tissue residing phagocytic cells (Silva, 2010). These cytokines and chemokines together with other mediators as leukotriene B4 (LTB4) induce vasodilatation, increase the blood vessels permeability, causing swelling and the expression of the endothelial adhesion molecules and neutrophil recruitment are increased (Figure 2).

Depending on the duration and the severity of the inflammatory response, it can be classified into phases acute or chronic.

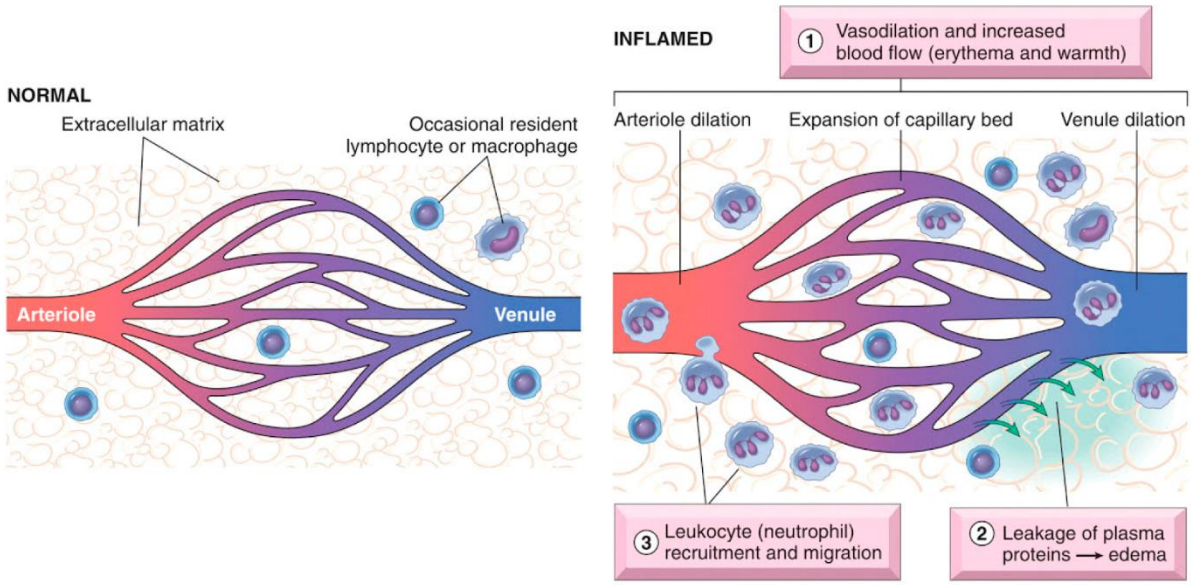


Figure 2. Local changes in the inflammation process. Adapted from (Huether and McCance, 2017).

Acute inflammation

The local acute inflammation manifestations are swelling, pain, heat (fever) and erythema. All of them derived from the vascular changes and the infiltration of immune components into the tissues (Huether and McCance, 2017). The main players are neutrophils and macrophages. After the successfully elimination of the damage, inflammation should end restoring the normal physiological conditions, this is the resolution of the inflammation (Kumar, 2018). If there is a progression of the response instead the resolution, it could derive to chronic inflammation.

Chronic inflammation

Chronic inflammation can arise when the pathogen is difficult to eliminate and persist in the time, when exist a hypersensitivity disease as autoimmune or allergic diseases, and when there is a long time of exposure to a toxic agent exogenous as silica or endogenous as cholesterol and other lipids that could induce atherosclerosis (Kumar, 2018).

The features like increased vascularity and immune cell accumulation of the acute inflammation remains when it becomes chronic. But there is another kind of cells involved, lymphocytes. The neutrophils start to degranulate, lymphocytes become activated and fibroblast release mediator that induce more infiltration of immune cells, mainly macrophages and lymphocytes (Figure 3).

The rising of the cases of chronic inflammatory diseases like rheumatoid arthritis, diabetes and psoriasis have become chronic inflammatory diseases as one of the major causes of morbidity and mortality in developed countries.

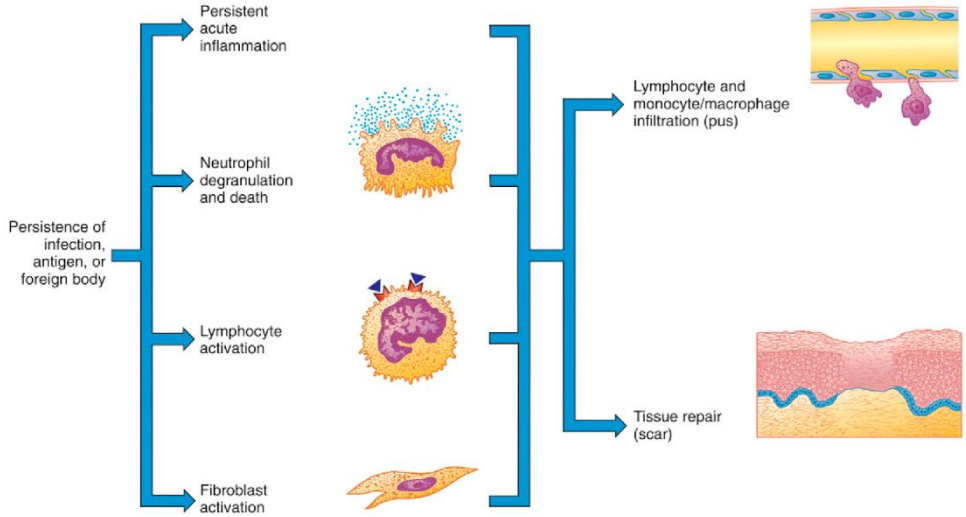


Figure 3. Chronic inflammation. Adapted from (Huether and McCance, 2017).

Psoriasis

Autoimmune and autoinflammatory disorders are a newly expanding concept in most medical areas, but with substantial relevance in the field of human dermatology (Murthy and Leslie, 2016). Among the several non-pathogenic skin disorders reported so far, one of the most recurrent is psoriasis. Psoriasis is a chronic, immune-mediated skin-disease with strong inflammatory and systemic manifestations, which possess a complex genetic architectural background (Greb *et al.*, 2016; Woo *et al.*, 2017).

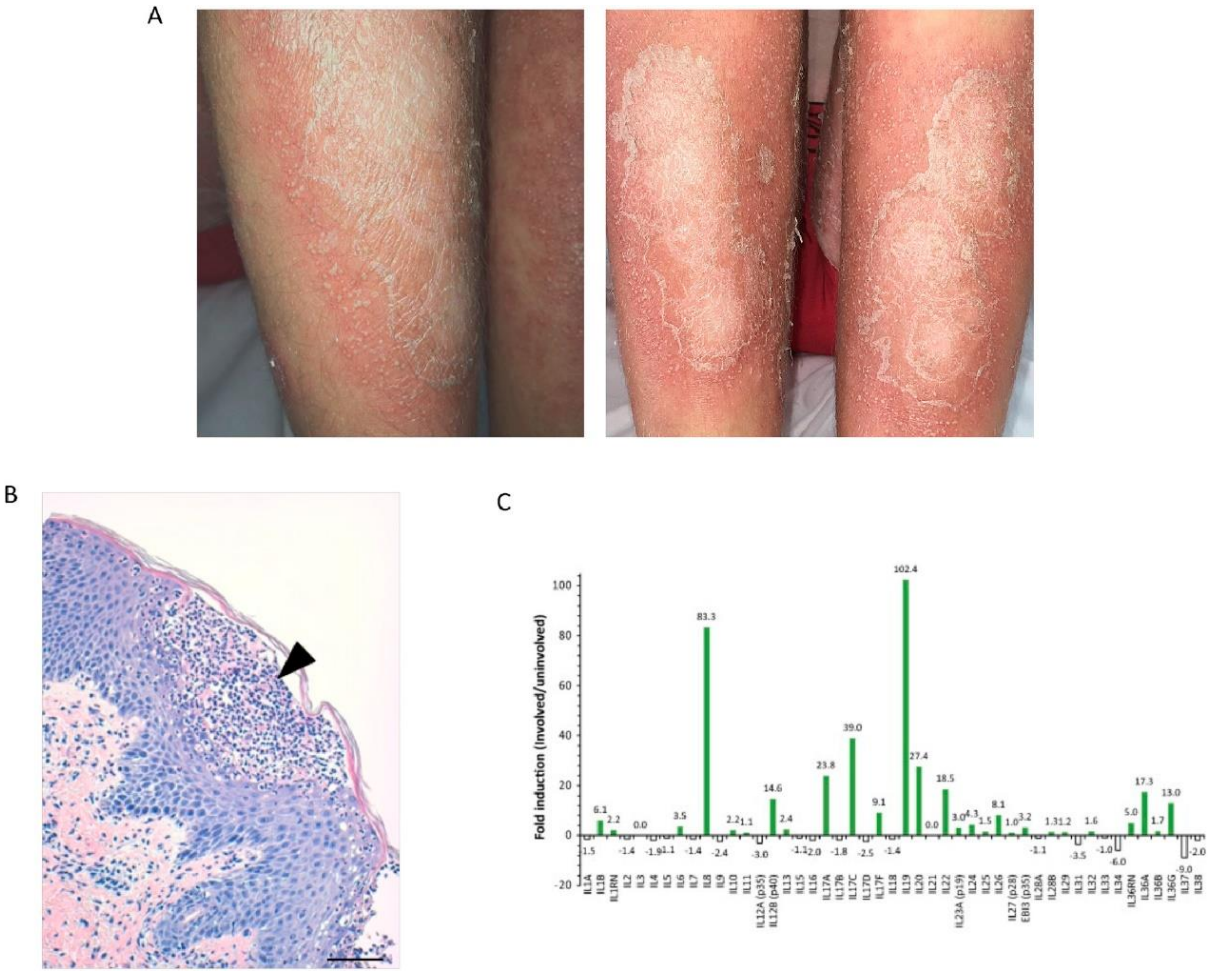


Figure 4. Cytokines in psoriasis. General characteristics of psoriasis in humans. A) The abnormal keratinocyte proliferation produces characteristic sterile pustules overlying erythematous skin in the extremities of an adult patient. Redness areas denote inflammation signs. B) Classical histologic biopsy of lesional pustular psoriasis. The black arrowhead is pointing to an intense infiltration of immune cells affecting the different skin layers. (H&E, 30×). Scale bar 80 μm. C) RNA-seq data fold change showing differential regulation of interleukins 1 to 36 in psoriatic lesions compared with healthy skin (Baliwag *et al.*, 2015).

Clinical manifestations of psoriasis vary and it can be classified in different type: psoriasis vulgaris, plaque psoriasis, scalp psoriasis, guttate psoriasis, inverse psoriasis, erythrodermic psoriasis, palmoplantar psoriasis, and pustular psoriasis (Lebwohl, 2018;

Raposo and Torres, 2016; Renton, 2014; Syed and Khachemoune, 2011). However, despite the particular phenotype expressed in each type, the pathophysiology in all psoriasis variants is characterized by an abnormal keratinocyte proliferation and strong immune cell infiltration among the different layers of the skin causing shared symptoms that include: intense itching, burning, and soreness (Ippagunta *et al.*, 2016) (Figure 4A). These symptoms mostly result from the recruitment of immune cells to specific skin sections, which together with keratinocytes release potent inflammatory mediators, like interleukins (Figure 4B, C).

All psoriatic phenotypes have been reported worldwide with a global prevalence within adult populations, regardless of sex, estimated at 3% of the total world population (Lebwohl, 2003). However, prevalence estimates are strongly affected by marked variations observed among ethnic groups and geographical locations. In fact, reported incidence of psoriasis in Taiwan (Chang *et al.*, 2009) and Japan is low (0.30%) (Kubota *et al.*, 2015), moderate in Spain (2.31%) (Ferrandiz *et al.*, 2014) or USA (2.50%) (Gelfand *et al.*, 2005), and impressively high in Norway (11.43%), indicating that different races had diverse genetic backgrounds that profoundly affect the disease output (Danielsen *et al.*, 2013).

Patients affected with psoriasis suffer not only the externally exposed physical condition but, given its systemic characteristic, additional severe pathologies expressed as comorbidities use to be present as well (Parisi *et al.*, 2013). Arthritis, autoimmune disease, cardiovascular disease, chronic obstructive pulmonary disease, inflammatory bowel disease, liver disease, metabolic syndrome, migraine, obesity, sleep apnea, psychiatric illness, sexual dysfunction, and addictive behavior have been repeatedly reported to promote a remarkably complicated disease loop in psoriatic patients (Capo *et al.*, 2018; Greb *et al.*, 2016; Molina-Leyva *et al.*, 2018). Moreover, patients suffering the combination of extreme visually exposed psoriatic lesions, linked to a complex comorbidity ultimately can trigger an uncontrolled, strong emotional stress burden, that in the worst scenario shall lead to suicidal behaviors (Wu *et al.*, 2017; Wu and Armstrong, 2019).

Pathogenesis of Psoriasis

In all vertebrates, the skin consists of two multi-layered regions, the dermis, and the epidermis that is in contact with the environmental factors. Keratinocytes form the epidermis but are differentially represented and stratified according to the taxonomic level of the host. However, despite the species represented, the basic morphology is always present and continuously renewing (Chermnykh *et al.*, 2018). Keratinocytes are versatile cells which

perform diverse essential mechanical protective functions while providing an immunological defense to the host (Johansen, 2017). Thus, understanding the regulation of keratinocyte proliferation, their complex functions, and the multiple interactions with immune cells is fundamental to get track of the pathogenesis of psoriasis.

Despite psoriasis disease results from an immune disorder, at the very early stage neither innate nor adaptive cells are present in high numbers to produce the cytokines and related chemokines required on site to activate an exacerbate immune response. Therefore, it has been proposed that environmental factors via innate immune effectors, like the Toll-like receptors (TLRs), activate keratinocytes to release potent inflammatory mediators and active signaling molecules to recruit further macrophages, neutrophils, and mast cells that will amplify the inflammatory network and trigger the disease (Albanesi *et al.*, 2018; Candel *et al.*, 2014; Schubert and Christophers, 1985). Indeed, psoriasis etiology is based on an increased epidermal keratinocyte turnover that produces a focal coalescing raised cutaneous plaque with consistent scaling and variable erythema in connection to the presence of excessive infiltrating immune cells (Buchau and Gallo, 2007). Using human biopsies and last generation microarray technology, recently it has been characterized the keratinocyte-specific transcriptome signature of lesional and non-lesional skin in psoriasis patients (Pasquali *et al.*, 2018). Results revealed that keratinocyte-specific gene expression in psoriatic disease is mainly enriched for genes related to the cell cycle, innate immunity, DNA repair/replication, and keratinocyte development and differentiation, unequivocally supporting the notion that keratinocytes are significant contributors to molecular changes in psoriasis skin.

Some causes of increased keratinocytes proliferation and disturbed cell maturation have been suggested. Among them, the excessive presence of cAMP, the altered metabolism of vitamins and calcium, or modifications in the arachidonic acid downstream signaling (Andres *et al.*, 2017; Cubillos and Norgauer, 2016; Kharaeva *et al.*, 2009; Setkowicz *et al.*, 2015). These theories may in part explain some of the events occurring in psoriasis pathogenesis. However, due to critical overlapping mechanisms and the different immune cellular elements occurring in the development of the disease, several aspects could be overlooked, and much more research, particularly at the innate level is still missing.

In contrast, identification of expanded infiltrates of T-cells in psoriatic lesions (Cai *et al.*, 2012), composed of polarized T helper (Th) cells populations, particularly Th1 and Th17 cells, and plenty pro-inflammatory mediators strongly put front the adaptive T-cells as crucial

effectors in psoriasis (Greb *et al.*, 2016; Yamaguchi *et al.*, 2018). However, the imbalance among Th cells subsets shown by the Th1 and Th17 cell increments, but not the regulatory Th2 and Treg is a matter of interest which deserves further clarification. Whatever the case, several excellent reviews published so far have analyzed in detail the role and particularities of the adaptive effectors in psoriasis pathogenesis under several scenarios (Karczewski *et al.*, 2016; Krueger and Bowcock, 2005; Singh *et al.*, 2019).

Nevertheless, despite the notorious advances on the contribution of T-cells in psoriasis, up to date the complete set of elements present in the pathogenesis of the disease is still unravelling and far of being completely understood. Our attention here faces the primary innate leukocytes present in the skin, namely neutrophils. On the sequence of inflammatory events triggered at the epidermis, keratinocytes initiate the response, and later myeloid cells, notably neutrophils take center stage in the pathogenesis of psoriasis (Ikeda *et al.*, 2013). This controversial notion has been extended support by the histologic changes observed in different animal models which resemble psoriasis lesions, with a high detection of the archetypal pro-inflammatory cytokine interleukin-1 (IL1), but with a limited presence of T-cell infiltrates.

To clarify it, Nakajima et al. (Nakajima *et al.*, 2010), following an elegant approach using IL1 receptor antagonist mutant (*Il1rn* -/-) mice, clearly demonstrate the development of cutaneous inflammation without the involvement of T-cell-mediated immunity, confirming that T cells are not required for the early pathogenesis of skin diseases. After that, many information supporting the role of innate immunity in the developmental pathogenesis of psoriasis has emerged (Sweeney *et al.*, 2011).

The clinical efficacy of compounds blocking TNFA, a critical cytokine that induces cell survival, apoptosis, and necrosis and contributes to both physiological and pathological process, highly suggests a vital role of the innate immune system in psoriasis (Boehncke and Schön, 2015; Zenz *et al.*, 2005). TNFA is considered a key messenger within the network of pro- and anti-inflammatory cytokines that have the capacity of triggering even its production, as well as that of other essential cytokines in inflammatory diseases (Kim and Moudgil, 2017). Consequently, anti-TNFA therapy has become a mainstay treatment for autoimmune diseases (Li *et al.*, 2017). Interestingly, despite the significant adaptive differences known between mice and human immune system, responses at the innate level are functionally fully comparable. Using relevant murine mutant lines to test the role of proinflammatory cytokines in the

pathogenesis of skin inflammation, was demonstrated that TNFA, but not IL6 or IL17, is crucial in this process (Nakajima *et al.*, 2010).

Likewise, clinical development of early lesions in psoriasis link to periodic waves of autoinflammation, represented by a burst of neutrophils and their associated cytokines related to the IL1 family and IL36, that along with TNFA all possess a full capacity of initiating the disease (Christophers *et al.*, 2014; Mahil *et al.*, 2017) using knockout individuals with IL1 receptor like 2 (IL1RL2) mutations validate IL36 as a viable psoriasis target, proposing the development of IL36 blockade as a therapeutic strategy. Interestingly, the IL36 dependent genes signature profile in keratinocytes was extensive but particularly attractive among them is the high production of IL17, IL8, and CXCL1, which are inflammatory elements closely related to neutrophils and were all three cytokines over expressed in psoriatic patients.

Besides, it was confirmed that increased production and activation of IL36 might act on neutrophils and further exacerbate neutrophilic inflammation.(Wang *et al.*, 2018a). To round the concept, using RNA-seq, the gene expression response of primary epidermal keratinocytes to stimulation by IL1B, IL36A, IL36B, and IL36G was evaluated (Swindell *et al.*, 2018). Strikingly, applying CRISPR/Cas9 mutagenesis, they demonstrate that shared IL1B/IL36 responses depend entirely upon MyD88 adaptor protein. Together, these results strongly emphasize the critical role of innate immunity in epidermal keratinocytes and neutrophils on triggering an exacerbated inflammatory response in psoriatic patients.

So far, using murine models and human psoriatic lesional biopsies with elevated levels of IL17 and IL22 was demonstrate that anti-IL17 treatment dramatically improves the psoriatic skin lesions, regulates IL23 and reduce inflammation by normalizing the levels of IL17 (Malakouti *et al.*, 2015; Paek *et al.*, 2018). However, the cell types related were not identified. To do so, it was. demonstrated that a single dose of the anti-IL17A antibody Secukinumab resulted in skin normalization as soon as two weeks after injection, a finding paralleled by the disappearance of $IL17^{+}$ neutrophils population, but not the T-cells (Reich *et al.*, 2015).

Meanwhile, several different immune cells, out of Th17 have been recognized as IL17 producers. Whether being still debated, granulocytes like neutrophils and mast cells appear to synthesize IL17 actively and release it through the formation of extracellular traps (Brembilla *et al.*, 2018). Strikingly, this granulocytes behavior seems to expand well beyond psoriasis, and it extends along most inflammatory diseases. As an example, searching after the mechanisms underlying asthma it was observed that experimentally exacerbated mice released high amounts

of several proinflammatory cytokines (Lunding *et al.*, 2016). Strikingly, this behaviour was further associated with increased IL17 and infiltration of variated $IL17^+$ immune cells in animals deficient either for IL23A or the transcription factor RORγt, suggesting the crucial role of neutrophils in the response. However, caution should apply when evaluating the interrelation of these cytokines and neutrophils due to several isoforms exist, and further functional analyses are warranted.

By the moment, the most extended treatment applied to psoriasis patients is the phototherapy. Mainly based in the ultraviolet A (UVA) radiation combined with oral intake of psoralen (PUVA) to sensitize the skin (Zhang and Wu, 2018). This treatment consists in at least 15 repeated seasons separated by 48 hours to avoid burns in the skin. Also, ultraviolet B (UVB) could be used without the intake of psoralen. But they only work temporarily, after some weeks the lesions could appear again, so more researches are needed to find new target and treatments.

Types of models for Psoriasis research

To understand the mechanisms associated with psoriasis, *in vitro, ex-vivo,* and *in vivo* preclinical models of the human disease have been described (Bochenska *et al.*, 2017; Danilenko, 2008; Hawkes *et al.*, 2018; Wcislo-Dziadecka *et al.*, 2018). Notably, the murine models have proven to be extremely valuable in investigating critical molecular mechanisms that underlie the complex interplay between epidermal keratinocytes, and the innate and adaptive immune system in human psoriasis (Bezdek *et al.*, 2018; Chuang *et al.*, 2018; Nakajima and Sano, 2018)

"In vitro"

Several studies have evaluated psoriatic keratinocytes to identify intrinsic defects, differentiation, proliferation, and gene expression profile in cell culture or transplant systems (Dombrowski *et al.*, 2011; Piskin *et al.*, 2006). Keratinocytes used *in vitro* are from diverse origins, from intact or lesional human skin to established cell lines, like the normal human epidermal keratinocytes (Buth *et al.*, 2007) and immortalized human keratinocytes line (HaCaT) (Borowiec *et al.*, 2013).

In vitro keratinocyte research has provided valuable information. By themselves, however, these studies do not match *in vivo* psoriatic lesions entirely, and high variability among studies is observed turning quite tricky to draw comparisons. So far, several factors have been proposed as the source of variation (e.g., culture temperature, serum quality and

quantity, calcium content, or lack of cell-cell interactions, and the intraspecies variability in the biological response due to cells commonly are isolated from individual members of the species).

Despite clarifying the controversy, recently a transcriptomic study was conducted applying RNA-seq technology in a primary confluent keratinocytes monolayer culture grown from full-thickness punch biopsies, and the full-thickness skin biopsies itself from psoriasis patients, and control subjects (Swindell *et al.*, 2017b). Results are puzzling, due to transcriptomic findings from the *in vitro* study, agreed only partially with results from full-thickness skin biopsies. These findings suggest that analysis of full-thickness skin biopsies may obscure functionally significant expression declines in psoriatic and normal patient keratinocytes which can, in contrast, be detected from *in vitro* analysis of patient-derived cells. Outcomes like this highlight the intrinsic constraints of the *in vitro* keratinocyte models of psoriasis produced at least due to the lack of blood and cell-cell interactions in the test system and suggest the use of direct physiological relevant models instead.

"Ex vivo"

Ex-vivo models are invaluable research tools and have been used to investigate psoriasis. In general, *ex vivo* human or rat skin is excised by abdominal surgery followed by removal of the adhering fat and visceral tissues. Eventually, (considered as a source of variation) the skin is rinsed thoroughly with NaCl solution before using it in the study of skin permeation and deposition. Hydrophobic or formulated compounds delivered by niosomes or liposomes containing gels are assayed for percutaneous absorption studies using Franz diffusion cells (Abu Hashim *et al.*, 2018).

Ex vivo skin is particularly suited to address topical treatments of psoriasis or sun protection with dedicated biomarkers, such as pyrimidine dimers, p53 activation, caspase activation, and sunburn cells, based in histology and immuno-labeling (Agarwal *et al.*, 2001; Bochenska *et al.*, 2017). However, the effect of topic treatments assayed for permeation *ex vivo* could be affected by numerous combined reasons like the adsorption and fusion efficiency of niosomes, the nanosized particles, penetration rate, the richness of lipid in the environment, solubility and stability of compounds, or some other factors.

Pre-clinical

Despite useful, *in vitro* and *ex vivo* systems are by far not capable of modelling whole-body physiology. As such, research into the pathogenesis of psoriasis has been severely hampered by the lack of a naturally occurring disorder in laboratory animals that mimic the complex phenotype and pathogenesis of the human disease. Throughout time, primates, dogs, pigs and several murine models related to the research process of psoriasis have been described (Bochenska *et al.*, 2017; Danilenko, 2008; Hawkes *et al.*, 2018; Wcislo-Dziadecka *et al.*, 2018; Yang and Wu, 2018). The approach clearly shows the longstanding practice of using animals for scientific purposes in biological research and medicine. Nevertheless, now this practice turns the issue an ordinary matter of debate by the radical supporters of the 3R's concept (MacArthur Clark, 2018) in our societies (Barre-Sinoussi and Montagutelli, 2015).

Nowadays, most current *in vivo* studies are conducted using one of the more than 40 unique mouse models of psoriasis described so far (Hawkes *et al.*, 2018). However, while many mouse models of psoriasis have been proposed, a standardized validation criterion encompassing most models is not widely applied. On the aim to do so, Swindell et al. (Swindell *et al.*, 2011), performed a whole-genome transcriptional profile study to compare gene expression pattern manifested by human psoriatic skin lesions with those that occur in five classical psoriasis mouse models displaying phenotypes associated to the TLR-imiquimod, transforming growth factor β (TGFB), endothelial tyrosine receptor, amphiregulin or signal transducer and activator of transcription 3 (STAT3).

Results revealed that while cutaneous gene expression profiles associated with each mouse phenotype exhibited statistically significant similarity to the expression profile of psoriasis in humans, each model displayed unique sets of similarities and differences in comparison to human psoriasis. Several many other studies show the double-edged pattern associated with mice research as a model of psoriasis. A recent study demonstrates that one of the most used mouse models of psoriasis, the TLR- imiquimod does not produce psoriasis only, but triggers a core set of pathways active in different skin diseases (Swindell *et al.*, 2017a). From these reports, we can conclude that mice are quite useful in the study of psoriasis and have been the predominant animal bridge between the bench and the bedside in the past. Nevertheless, now is time to look forward and open the door to a new complementary animal model of psoriasis, the zebrafish.

Nevertheless, this far, not any one of the systems mentioned above are neither homologous nor isomorphic and do not entirely phenocopy the human disease, suggesting the urgent necessity of searching for alternative relevant matching models. In this regard, a new player with particular characteristics has emerged in the last decade to complement the research efforts achieved so far in understanding intimate mechanisms of inflammatory skin diseases, such as, psoriasis. The zebrafish (*Danio rerio*) has striking similarities between cells, tissues, and physiological functions to those of humans (Galindo-Villegas, 2016). After its complete genome assembly, it was recognized that more than 70% of all human genes have at least one ortholog in zebrafish (Howe *et al.*, 2013). Thus, by using the latest mutagenesis resources diverse human disease models have been generated in this model animal (Santoriello and Zon, 2012).

The Zebrafish as a model of skin inflammation

The use of zebrafish to investigate the genetic causes and molecular mechanisms of psoriasis is rapidly gaining popularity. Zebrafish is a small freshwater fish taxonomically positioned in the Cyprinid family. Compared to rodent models, zebrafish exhibit much more efficient reproduction, rapid external development, and undefeatable optical transparency throughout the early larval stages that allows the traceability of different fluorescent molecules to evaluate, for example, inflammation status (Figure 5). Besides, zebrafish provides infinite possibilities of experimental reproducibility due to a daily high progeny availability (Meshalkina *et al.*, 2017). Also, zebrafish enables the characterization of gene function via overexpression, transient depletion, or genome editing by applying variated gene editing technologies, including the latest CRISPR/Cas-based method (Ablain *et al.*, 2018; Varshney *et al.*, 2015).

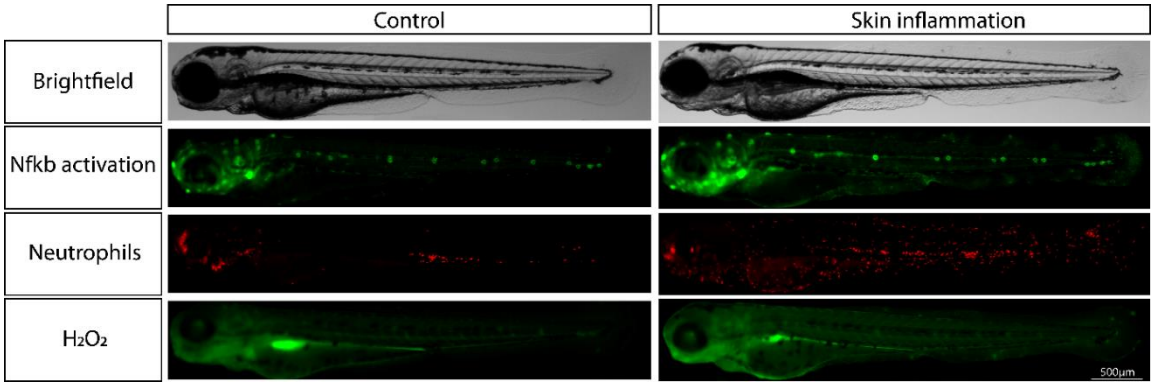


Figure 5. *In vivo* imaging of inflammation in zebrafish larvae. Skin inflammation can be easily monitored at real time in whole zebrafish larvae as activation of Nfkb (using the reporter line *NFKB:eGFP*) and neutrophil infiltration into the skin (using the line *lyz:dsRed*) (top panel). H_2O_2 release by skin keratinocytes can also be visualized suing the genetic or chemical fluorescent probes (low panel).

Zebrafish are not only embryo/larvae with robust phenotypes or genetically tractability, but they also present accessibility optically for intravital real-time *in vivo* imaging and display a fully functional innate immune system where myeloid cells are present as soon as 24 hours post fertilization (hpf) mimicking their mammalian counterparts (Gurevich *et al.*, 2018; Henry *et al.*, 2013; Torraca and Mostowy, 2018). Besides that, the zebrafish genome is fully sequenced, highlighting a remarkable similarity with humans, with at least 71.4% human coding genome having a direct ortholog in zebrafish (Howe *et al.*, 2013; Shim *et al.*, 2016).

The Zebrafish Model Organism Database (ZFIN https://zfin.org) is the central resource where genetic, genomic, and phenotypic data on zebrafish research is curated and run (Bradford *et al.*, 2017). Similar to human, the zebrafish epidermis is a multi-layered tissue composed by keratinocytes, separated from the dermis by a basal membrane. In fish, this structure is much simpler than the mammalian one, and in contrast to terrestrial vertebrates, the skin does not function to prevent dehydration due to the lack of stratum corneum (Webb *et al.*, 2008). However, several physical similarities exist. Among them, at 24 hpf different skin layers representing the epidermis and the dermis are separated from the underlying tissue stroma by a basal membrane, and at 2 days post fertilization (dpf) the lamina lucida densa can be identified (Le Guellec *et al.*, 2004).

Previous studies have shown the mechanism of H_2O_2 in acute and chronic inflammation in zebrafish. In acute inflammation was proposed that ATP released from damaged cells activates P2ry purinergic receptors, which promotes the activation of phospholipase C (Plc), the production of inositol-3-phosphate (IP3) and Ca^{2+} release from the endoplasmic reticulum.

Cytosolic Ca^{2+} activates Duox1, which produces H_2O_2 that then activates Nfkb, Jnk and Erk, promoting the phosphorylation of Jun and Fos and the expression of target pro-inflammatory genes, including *cxcl8*. The mechanism of how these molecules are activated by H_2O_2 is still unknown. Additionally, H_2O_2 is also able to modulate *cxcl8* expression via covalent chromatin modifications, such as acetylation of H3K9 and trimethylation of H3K4. Newly synthesized Cxcl8 is then secreted to the extracellular matrix, forms a gradient, and together with H_2O_2 gradient, induce Cxcr2- and Lyn-mediated neutrophil recruitment (de Oliveira *et al.*, 2014) (Figure 6).

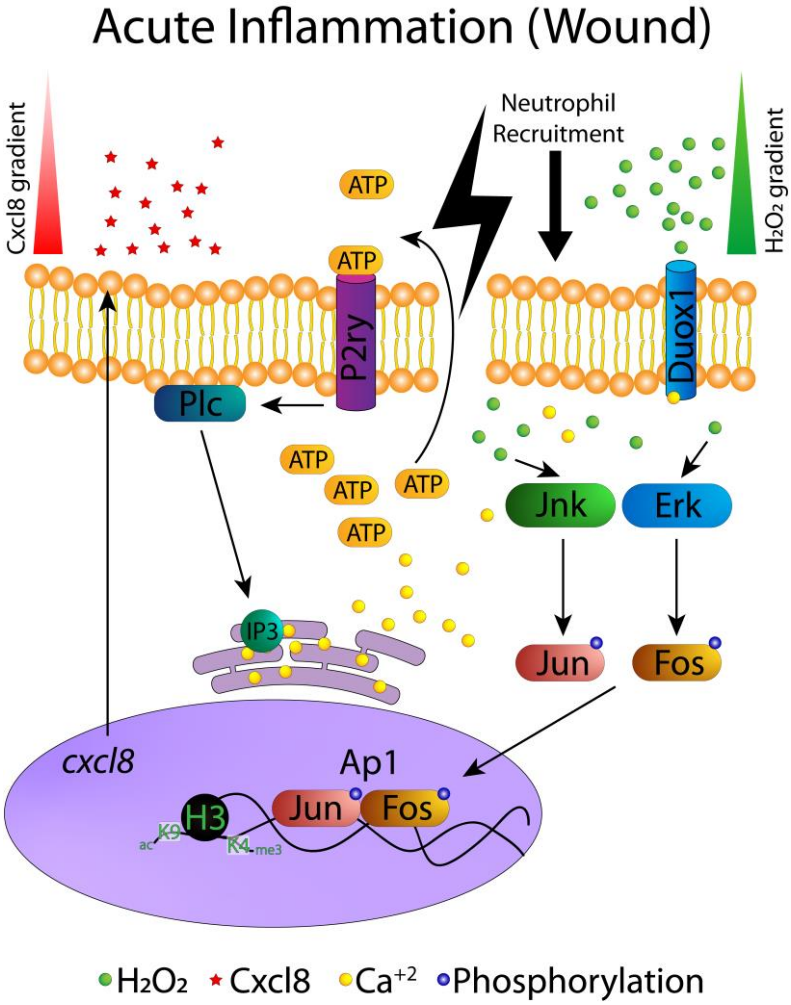


Figure 6. Proposed models showing the role of H_2O_2 in acute inflammation in zebrafish.

In chronic inflammation, alterations in skin homeostasis and barrier trigger Duox1-dependent release of H_2O_2, which promotes Lyn-mediated neutrophil infiltration and activation of Nfkb, which induces the expression of genes encoding pro-inflammatory mediators, including Il1b and Ptgs2, and Duox1. Probably, Ca^{2+} perturbation also activates Duox1 and H_2O_2 then activates Jnk and Erk leading to the production and release of Cxcl8, generating a positive feedback inflammatory loop (Candel *et al.*, 2014; de Oliveira *et al.*, 2015) (Figure 7).

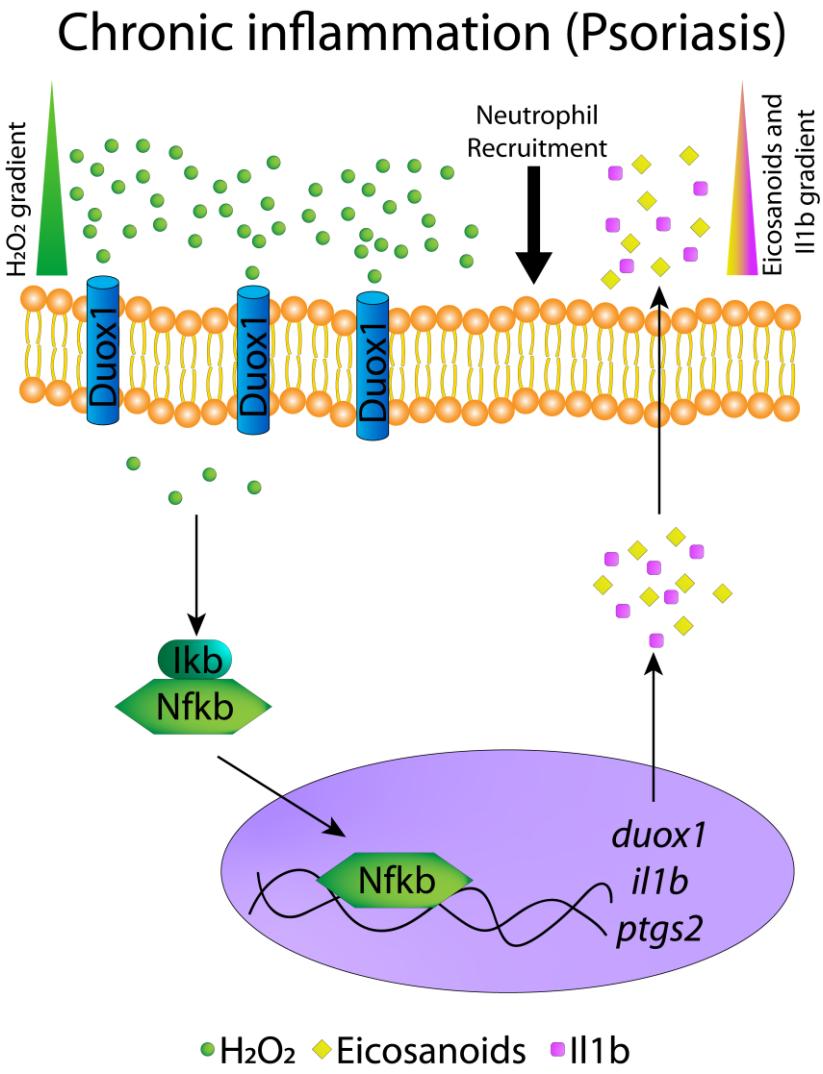


Figure 7. Proposed models showing the role of H_2O_2 in chronic inflammation in zebrafish.

Interestingly, it has been observed *in vitro* and *ex-vivo* that alteration of these elements forming paracellular barriers for solutes and inflammatory cells, together with proinflammatory cytokines are related to the early events in psoriasis (Kirschner *et al.*, 2009). These unique features present in the zebrafish model provides a unique platform to further understand *in vivo* their functionality in the psoriatic disease. Last but not least, in zebrafish, the lipid-rich cornified layer observed in higher mammals is not present, and epidermis remains metabolically active and readily available as a live substrate for experimentation throughout all life stages (Glover *et al.*, 2013). Consequently, relevant zebrafish disease models of psoriasis have been developed to aid in unravelling the complicated pathogenesis in the human disease (Table 1).

Name	Description	Advantage	Reference
Penner/lethal giant larvae 2	The lack of *pen/lgl2* disrupts the basal localization of keratin cytoskeletons on fish keratinocytes.	Target the process of hemidesmosome formation, maintenance of cytoskeletal elements, and cellular morphology in the basal epidermis.	(Sonawane *et al.*, 2005)
Hai1a/Spint1a	Keratinocytes acquire a mesenchymal-like characteristic, lose contact, become mobile and highly susceptible to apoptosis. Fish embryos exhibit inflammation in areas of epidermal hyperproliferation.	Useful to uncover crucial signaling players on defects caused by the loss of Hai1 and Matriptase 1a. Enable the study of chronic inflammation and visualization of immune responses with high resolution in real-time.	(Carney *et al.*, 2007) (Mathias *et al.*, 2007)
Psoriasis/m14	This fish exhibits widespread over proliferation of the epidermis and a defect in keratinocyte differentiation	Allow the study of epidermal growth regulation and may point further insights into skin development in fish.	(Webb *et al.*, 2008)
Tnfa-Tnfr2	Inhibition of the ligand *tnfa* and related receptor 2 results on neutrophil mobilization to the fish skin in response to H_2O_2 derived enzyme (DUOX1) released by keratinocytes	A robust platform to study the management of inflammatory molecular mechanisms resulting from oxidative stress.	(Candel *et al.*, 2014)

Table 1. Synopsis of relevant zebrafish models supporting the comparative study of human psoriatic disease.

Mutant models

Zebrafish with its extensive toolkit for genome modification and its capacity for recapitulating human disease has found a niche among the preclinical models of psoriasis to study and validating genetic variants, as well as to identifying previously unrecognized disease-associated genes. The first zebrafish model generated to study the development of the basal epidermis and the associated mechanisms were the mutant penner/ lethal giant larva 2 (*pen/lgl2*). The *pen/lgl2* zebrafish has a defect which disrupts the basal localization of keratin cytoskeletons on epidermal keratinocytes. This effect in humans is mediated by the lgl2 homolog, the *HUGL1* gene, which is a crucial regulator of epidermal polarity, and, in turn, keratinocyte proliferation (Zimmermann *et al.*, 2008).

The mutant pen/lgl2 zebrafish larvae shown overgrowth of epidermal cells leading to different morphological shapes and fail to form basal hemidesmosomes, which link the epidermis to the underlying basement membrane (Sonawane *et al.*, 2005; Sonawane *et al.*, 2013). Therefore, while in pen/lgl2-/- skin detaches from the basement membrane, epidermal cells hyper-proliferate and migrate ectopically, resulting in a psoriasis-like phenotype. Deletion

also removes an essential enhancer for keratinocyte differentiation, and the loss of this regulatory element allows for the study of psoriasis association. In connection with the same, the pen function is revealed as specifically required for the process of hemidesmosome formation. However, suggested caution should be applied with this model due to the disruption of lgl2 would lead to epidermal tumor formation.

Not long after the creation of the pen/lgl2 mutant, two more lines carrying mutations in the serine peptidase inhibitor, Kunitz type 1 a (Spint1a), also known as Hai1a, (Figure 8A) and clathrin interactor 1a (Clint1a) exhibited epidermal proliferation, and keratinocytes with mesenchymal-like characteristic (Carney *et al.*, 2007; Dodd *et al.*, 2009; Mathias *et al.*, 2007). In both models, loss of Spint1a produces neutrophil skin infiltration and losing of contact among keratinocytes which become mobile and highly susceptible to apoptosis (Figure 8B, B', C, C'). In the *spint1a* mutant, antagonistic roles between Hai1 and its target matriptase 1a (St1a) are produced. The mutation over the *spint1a* in zebrafish enable the study of chronic inflammation and visualization of immune responses with high resolution in real-time. These models demonstrate their utility in the identification of functional interactions between the keratinocytes and surrounding leukocytes (Figure 8D, D'). At 24 hpf, the *spint1a* -/- phenotype is characterized by an erratic localization of E-cadherin in epidermal cells, the fast expression of the damage phenotype, and a keratinocyte hyperproliferation in regions of cell aggregation.

An additional mutant model in this series is the *Psoriasis*. This zebrafish mutant model was identified resulting from a large-scale ethylmethanesulfonate (EMS) mutagenesis screen for genes required for zebrafish embryogenesis (Webb *et al.*, 2008). Mutants of this fish do not regulate cell proliferation in the epidermis late in embryogenesis and concomitantly accumulate aggregates of epidermal cells in the embryo surface, clearly resembling psoriasis. A loss-of-function mutation in *atp1b1a*, encoding the beta subunit of a Na, K-ATPase pump, has been responsible for this phenotype (Hatzold *et al.*, 2016). Blockade of the ensuing PI3K-AKT-mTORC1-NF-κB-MMP9 pathway activation in basal cells, as well as systemic isotonicity, prevents keratinocyte hyperproliferation and subsequent malignant transformation.

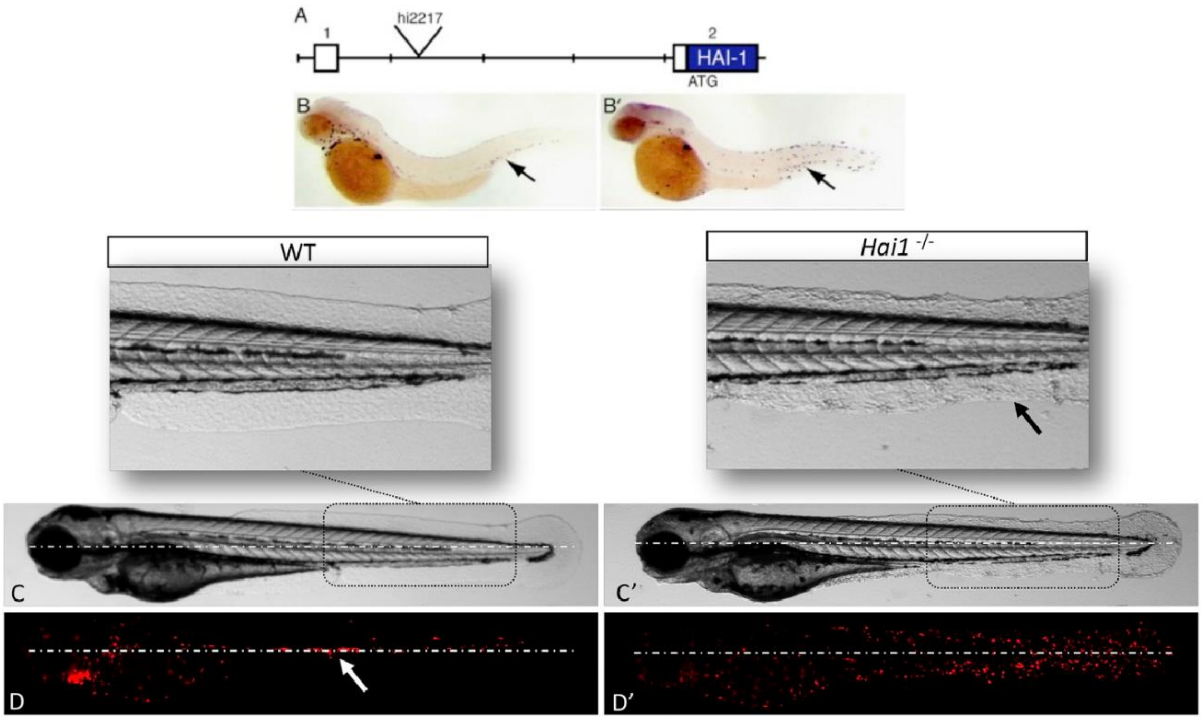


Figure 8. Development of a mutant zebrafish model of psoriasis disease. A) Genomic map of *hi2217* insertion and the start of the *hai1a* (*spint1a*) open reading frame (blue) is indicated (ATG); each section equals 500 bp. B, B′) *In situ* hybridization of zebrafish myeloperoxidase (*mpo*) at 2 dpf, arrows indicate the position of the intermediate cell mass (ICM), the location of neutrophil development in zebrafish embryos (Mathias *et al.*, 2007). C, C') Bright field and fluorescence images showing lateral views of (C) wild-type sibling (WT) and (C') a homozygous *hi2217 larvae (hai1a* $^{-/-}$*)* mutant at 3 dpf. Ectopic keratinocyte aggregates are pointed by the black arrow in the magnified area. (D, D') WT siblings or mutant *hi2217* were crossed with the transgenic zebrafish line Tg*(lyz:DsRed2)*^{nz50} to visualize neutrophil (in Red) behavior in the resulting larvae at 3 dpf. (D) In WT fish, the white arrow indicates the basal state of neutrophils in the caudal hematopoietic tissue (CHT). (D') While in *hi2217* mutants the presence of Hai1 affects the matriptase producing an exacerbated behavior of neutrophils resulting in high proliferation and massive recruitment in the affected epidermal tissue. White dash-dot line act only as a visual guide.

Tnfr2 morphant model

A recent study has approached the functionality of Tnfa, its receptors (Tnfr), and neutrophils behavior in the skin of zebrafish (Candel *et al.*, 2014). Morpholino technology producing a transient gene knock-down cannot be used to study gene function in mice because antisense oligonucleotides are rapidly diluted during mouse development (Flynt *et al.*, 2017). In a strict sense, morpholino is not a right genetic approach due to the possibility of producing unspecific reactions, but they have been used extensively in zebrafish research with a high degree of success (Kok *et al.*, 2015). Candel et al. (Candel *et al.*, 2014), following elegant approaches, including morpholinos, demonstrate that the knock-down of *tnfr1* have little effect on the neutrophil trafficking in the developing larvae at 3 dpf, while *tnfa* and *tnfr2* had a strong

effect in their mobilization (Figure 9A). Additionally, in this study the use of the transgenic zebrafish Tg(*mpx:GFP*) in which neutrophils express green fluorescence under the promoter of myeloperoxidase allow these researchers to determine the particular position of neutrophils.

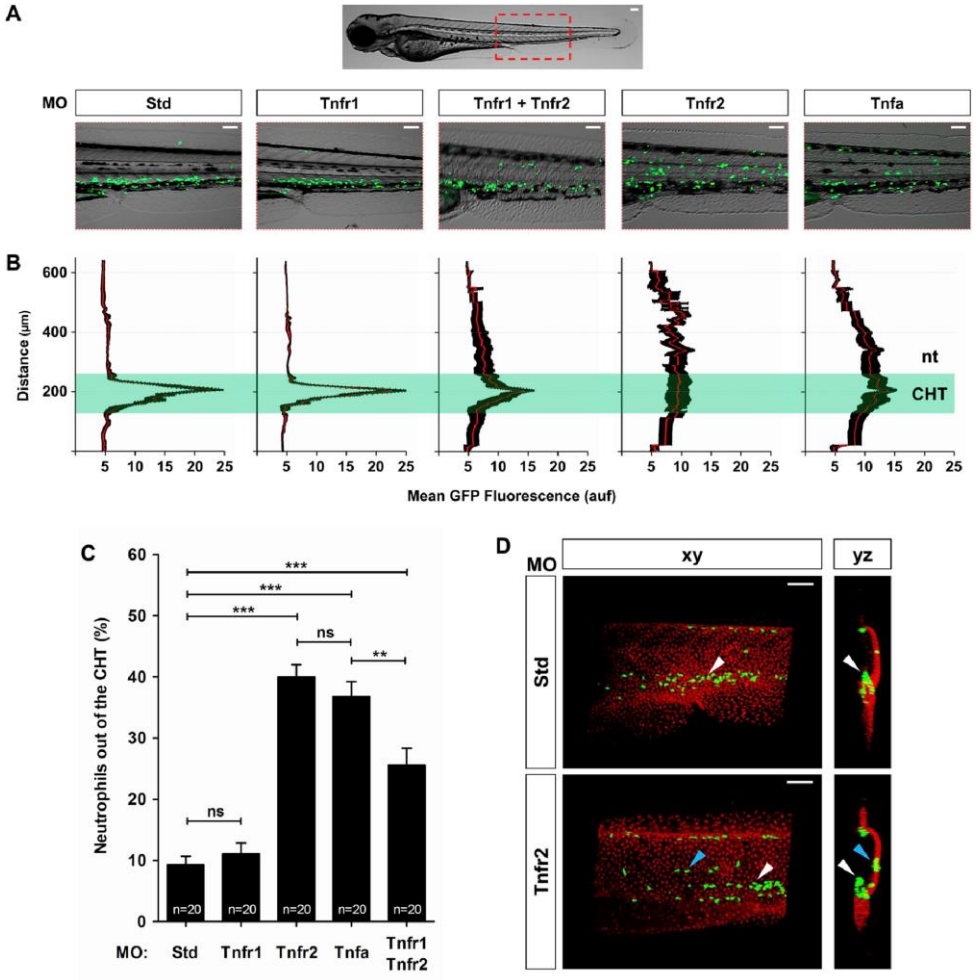


Figure 9. Zebrafish allows the detailed *in vivo* study effect of key molecules and cells related to psoriasis. Transgenic zebrafish embryos microinjected with standard control (Std), ligand/receptors of tumor necrosis factor (*tnfr1*, *tnfr2*, *tnfa*, or *tnfr1+tnfr2*) morpholinos (MO) were microscopically analyzed in real-time. A) Bright field and green channels, of the morphants at 3 dpf showing the differences in the neutrophil's distribution. B) Fluorescence intensity was measured for all the groups in the area indicated (A), the CHT. C) The neutrophil mobilization from the CHT in *tnfa*- and *tnfr2*-deficient larvae was quantified. D) Representative dorsal (*xy*) and lateral (*yz*) views of tridimensional reconstructions from confocal microscopy images of whole-mount immunohistochemistry of Tg(*mpx:GFP*) larvae stained at 3 dpf with anti-p63 antibodies (basal keratinocyte marker, red) showing the neutrophils' distribution in the CHT area of control and *tnfr2*-deficient larvae (Candel *et al.*, 2014). Scale bars, 100 µm. ns, not significant. *p≤0.05, **p≤0.01, ***p≤0.001.

Besides, they supported this result by applying a novel fluorescence quantification technique and contrasted by visual inspection (Figure 9B, C). The specificity of the observed phenotype for the *tnfr2* was confirmed by using a dominant-negative of Tnfr2 lacking the entire intracellular signaling domain, which phenocopy the results previously observed. To establish

other cell types, intimately related in the response whole mount immunohistochemistry against p63 (basal keratinocyte marker) was conducted in morphant fish with neutrophils expressing GFP. Results revealed that mobilized neutrophils in *tnfr2* deficient larvae were in close contact with skin keratinocytes.

In addition to neutrophils mobilization, *tnfr2* or *tnfa* morphants triggers a steady production of master proinflammatory molecules (*tnfa*, *il1b*, and *ptgs2b*) through the Nfkb pathway, resembling the phenotype of mutant *spint1a* and *clint1a* mutant zebrafish where chronic inflammation triggers *il1b* and neutrophil infiltration. To round their results, they sorted neutrophils from Tg*(mpx:GFP)* and keratinocytes from Tg*(-2.9krt18:RFP)* embryos revealing that both cell types overexpress inflammatory cytokines, reflecting a positive feedback loop between both cell types potentiating skin the inflammation. Strikingly, using an H_2O_2-detecting probe, it was observed that Tnfr2-deficient larvae keratinocytes activate dual oxidase 1 (DUOX1) (Candel *et al.*, 2014) enzyme creating H_2O_2 gradients which were sensed by neutrophils through the tyrosine kinase Lyn (Yoo *et al.*, 2011). Together, these results give strong support to the interplay between neutrophils and TNFA which defines the immune pathology in psoriasis.

The power of the model is solely based on the zebrafish simple skin structure, and the high similarity to the human epithelium and the potent immune mediators it produces in compromised conditions (Figure 10) On this perspective a myriad of environmental, genetic and drug screening tests can be conducted, and aid on enabling rapid discovery of possible chemical targets. Finally, in further research applying this model the identification and understanding of crucial aspects of epigenetic, post-translational modifications, host-microbe interactions, and trained immunity (all possible using zebrafish) could help on bridging the gap between genetic and environmental risk factors to understand the psoriasis disease.

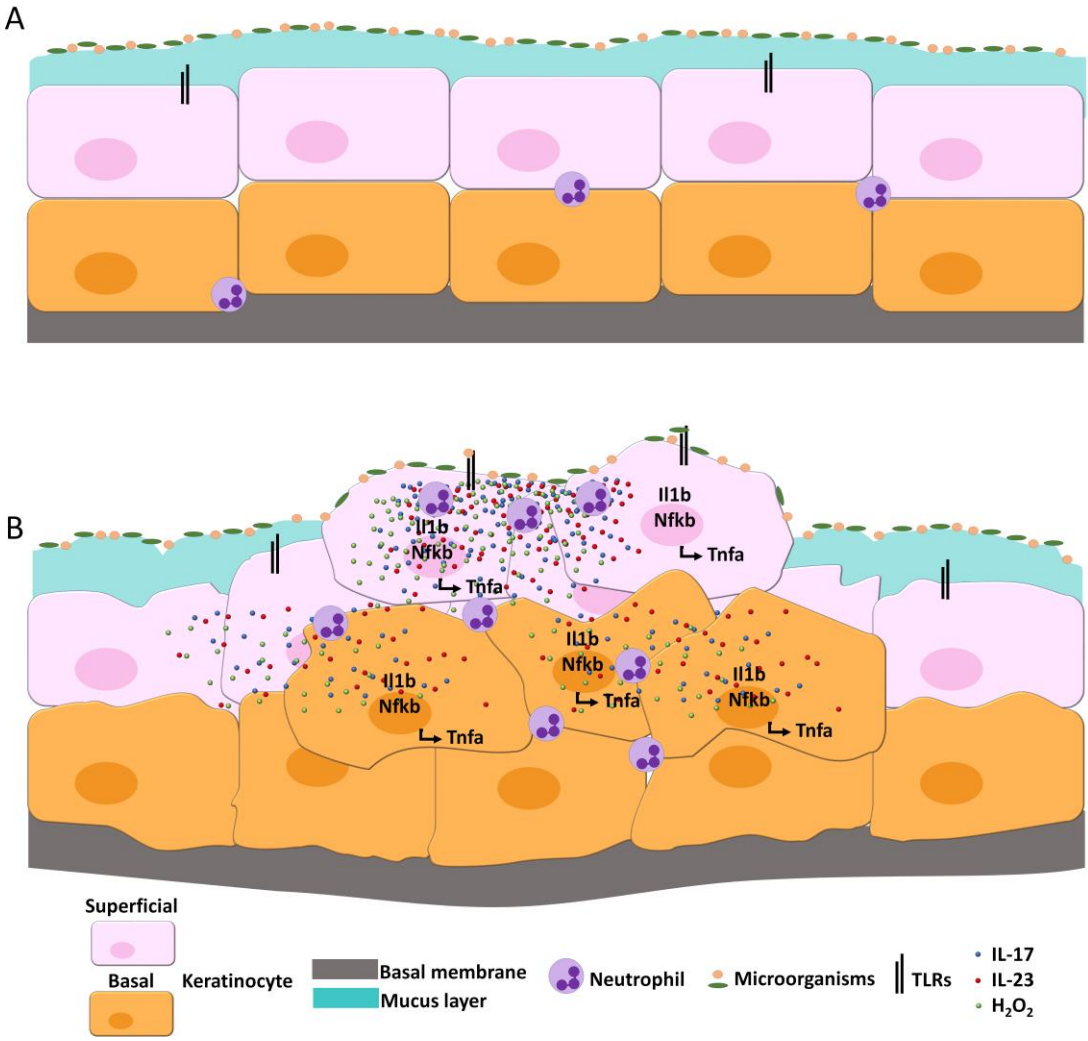


Figure 10. Inflammation model in zebrafish skin larvae. A) Normal epithelium. Superficial and basal keratinocytes are correctly organized forming a uniform layer. B) Swollen epithelium. Superficial and basal keratinocytes over-proliferate and lose their organization and skin integrity. This behavior induces the production of different types of chemoattractants that heavily recruit neutrophils to the skin, resembling the human psoriasis disease.

Vitamin B6 and inflammation

Vitamin B6 is a water-soluble vitamin that includes different vitamers like pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their phosphorylated forms (Coburn, 1996), being pyridoxal 5'-phosphate (PLP) its most active biological form. Humans obtain vitamin B6 from dietary and intestinal microbiota due to not be *de novo* synthesized in humans (Said, 2015).

The chemical structure of vitamin B6 vitamers is like pyridine, a benzene ring with a nitrogen atom instead one methine group, also has two -OH groups. Depending on the vitamer they may have another -OH group (PN), a $-NH_2$ group (PM) or a hydroxymethyl group (PL).

In the case of the phosphorylated forms they have a phosphate group instead of a -OH group (Figure 11).

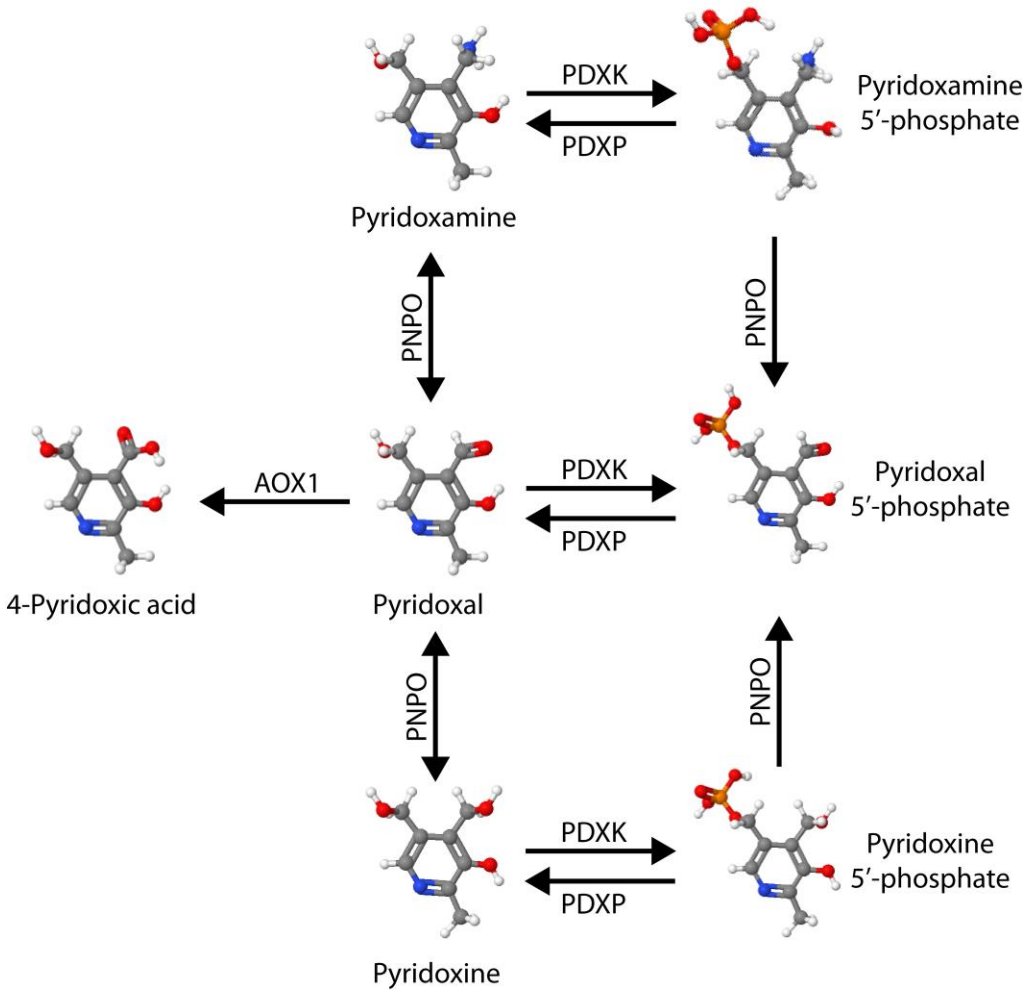


Figure 11. Vitamin B6 structure and metabolism. Atoms are Carbon (grey), Hydrogen (white), Nitrogen (blue), Oxygen (red) and Phosphate (orange).

The interconversion between the different vitamers is mainly occurs in the liver, where PN, PM and PL are delivered by circulation. Pyridoxal kinase (PDXK) rephosphorylated them, and pyridoxine phosphate oxidase (PNPO) converts pyridoxine 5'-phosphate (PNP) and pyridoxamine 5'-phosphate (PMP) to PLP (Albersen *et al.*, 2013; Coburn, 2015). Pyridoxal phosphatase (PDXP) is the enzyme in charge of dephosphorylate these forms. The preferred degradation route ends in pyridoxic acid (PA), which is the catabolic product from the vitamin B6 produced by aldehyde oxidase 1(AOX1) (Garattini *et al.*, 2009; Merrill *et al.*, 1984) (Figure 11).

Due to its characteristic chemical structure, vitamin B6 display different activities by itself. *In vitro* assays have shown that vitamin B6 by is able to diminish reactive oxygen species (ROS) and lipid peroxidation, two important oxidative stress markers, caused by H_2O_2 (Kannan and Jain, 2004). Vitamin B6 also acts as chaperone helping in the process of enzyme folding (Cellini *et al.*, 2014) and as a metal chelator (Wondrak and Jacobson, 2012).

Although the incidence of vitamin B6 deficiency is low in developed countries, low levels has been associated with a huge variety of diseases, like rheumatoid arthritis (Chiang *et al.*, 2005; Roubenoff *et al.*, 1995), inflammatory bowel disease (Selhub *et al.*, 2013), diabetes (Friedman *et al.*, 2004), risk of thrombosis (Hron *et al.*, 2007), stroke (Kelly *et al.*, 2003), cardiovascular disease (Cheng *et al.*, 2008; Dalery *et al.*, 1995; Verhoef *et al.*, 1996) and several types of cancers (Eussen *et al.*, 2010; Johansson *et al.*, 2014; Larsson *et al.*, 2010; Le Marchand *et al.*, 2011; Lurie *et al.*, 2012; Wu *et al.*, 2013).

PLP levels in plasma are usually inversely related to different inflammation markers (Abbenhardt *et al.*, 2014; Friso *et al.*, 2001; Morris *et al.*, 2010; Sakakeeny *et al.*, 2012). Plasma contains PL, PLP and PA. The ratio in blood levels $\frac{\mathrm{PA}}{\mathrm{PLP+PL}}$ is an indicator of vitamin B6 homeostasis, inflammation and a cancer incident predictor (Zuo *et al.*, 2015).

PLP acts as a cofactor of more than 100 enzymes (Percudani and Peracchi, 2009). These enzymes are involved in a variety of pathways. Most of them are related to amino acid synthesis and degradation (Eliot and Kirsch, 2004), but others are involved in one-carbon and lipid metabolism, neurotransmitter biosynthesis or gluconeogenesis (Percudani and Peracchi, 2009).

One of these other pathways is the hydrogen sulfide (H_2S) formation. H_2S is a gaseous messenger which has anti-inflammatory effects when is found at low concentrations, but at high concentration it has proinflammatory effects (Whiteman and Winyard, 2011). This molecule is involved in angiogenesis and vasodilation among other activities (Liu *et al.*, 2011). Two PLP dependent enzymes are responsible of the production of H_2S, cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS).

Another relevant pathway is the glycogen degradation. In this process two enzymes that use PLP as cofactor are involved, glycogen phosphorylase (PYGL) that releases glucose from glycogen and glucose-6-phosphate 1-dehydrogenase (G6PD) that produces nicotinamide adenine dinucleotide phosphate (NADPH) (Combs, 2008).

OBJECTIVES

This work has the following specific objectives:

1. To determine the expression of gene encoding the key enzymes involved in vitamin B6 metabolism and PLP-dependent enzymes in psoriasis lesional skin.

2. To determine vitamin B6 metabolites in the serum of psoriasis patients.

3. To determine the effects of exogenous addition of different vitamin B6 vitamers in zebrafish embryo/larval models of psoriasis.

4. To determine the impact of pharmacological or genetic inhibition of the PLP-dependent enzymes Pygl and G6pd in the Spint1a-deficient zebrafish model of psoriasis.

MATERIALS AND METHODS

Ethics statements

The experiments performed comply with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Experiments and procedures were performed as approved by the Bioethical Committees of the University of Murcia (approval numbers #75/2014, #216/2014 and 395/2017) and Ethical Clinical Research Committee of The University Hospital Virgen de la Arrixaca (approval number #8/13).

Animals

The lines Tg(*lyz:DsRed2*) (Hall et al., 2007), *spint1a*$^{hi2217Tg/hi2217Tg}$ (Mathias et al., 2007) and Tg(*NFKB:eGFP*) (Kanther et al., 2011) were previously described. *spint1a*$^{hi2217Tg/hi2217Tg}$ fish were crossed with Tg(*NFKB:eGFP*) and their offspring were incrossed to obtain *spint1a*$^{hi2217Tg/hi2217Tg}$;Tg(*NFKB:eGFP*) fish. *spint1a*$^{hi2217Tg/hi2217Tg}$ fish were crossed with Tg(*lyz:DsRed2*) and their offspring were incrossed to obtain *spint1a*$^{hi2217Tg/hi2217Tg}$; Tg(*lyz:DsRed2*) fish.

Morpholino and chemical treatments

The following splice blocking morpholinos (MOs) obtained from Gene Tools were used: MO *tnfr2* I1/E2: 5'-GGAATCTGTGAACACAAAGGGACAA-3' (2,5 pg/egg) (Candel *et al.*, 2014; Espin *et al.*, 2013; Lopez-Munoz *et al.*, 2011); MO *clint1a*-E1/I1: 5'-ACATCCAAAATACTCACGCTTTATC-3' (Dodd *et al.*, 2009); and MO std 5'-CCTCTTACCTCAGTTACAATTTATA-3' (used as a control). MOs were resuspended at 0.2 mM in microinjection buffer (0.05% phenol red and 0,5X Tango buffer) and microinjected in the yolk salk of zebrafish one-cell embryos using a using a microinjector (Narishige) (0.5-1 nl per embryo).

Vitamers of vitamin B6 (PN, PL and PLP, all from Sigma-Aldrich) and the selective glycogen phosphorylase inhibitor CP-91149 (Santa Cruz Biotechnology) were added to egg water in different concentrations together with a fixed amount of DMSO (1%) to facilitate embryo absorption. All treatment started 24 hpf, were replaced at 48 hpf and maintained until 72 hpf.

H_2O_2 imaging

H_2O_2 imaging using a live cell fluorescein dye was performed as previously described (Candel *et al.*, 2014; de Oliveira *et al.*, 2015; de Oliveira *et al.*, 2014). Briefly, 72 hpf larvae

were loaded for 60 min with 50 μM acetyl-pentafluorobenzene sulphonyl fluorescein (Cayman Chemical) in 1% DMSO. Larvae were left to recover in probe solution and imaging was made immediately.

Image acquisition and processing

At 72 hpf, larvae were anesthetized in buffered 0.16 mg/ml tricaine. Images were captured with a MZ16FA stereomicroscope (Leica) equipped with green and red fluorescent filters. All images were acquired with the integrated camera on the stereomicroscope, DFC350FX, and were used for subsequently counting the number of neutrophils (*lyz:DsRed2*), and examined their distribution. The activation of NF-κB was visualized and quantified using the line Tg(*NFKB:eGFP*). Images were processed using the free source software ImageJ (http://rsbweb.nih.gov/ij) to obtain the fluorescence intensity of each area of interest.

Human keratinocyte culture and gene expression analysis

The human keratinocyte cell line HaCaT (authenticated by Bioidentity S.L. and mycoplasma-free according to Hoechst DNA staining assays) was maintained in DMEM, high glucose supplemented with 10% fetal bovine serum (FBS), 2 mM glutamin, and 1% penicillin-streptomycin (Thermo Fisher Scientific). Cells were split before confluence every 72h and stimulated with human recombinant 40 ng/ml TNFA and 100 ng/ml IL17A (Peprotech) for 20 hours and incubated for the last 3 h with 10 μM CP-91149 in the presence of 0.1% DMSO. Cell were collected and RNA extracted with TRIzol reagent and purified with RNAquous Micro Kit, total RNA purification system (Ambion) and treated with DNase I, amplification grade (1 U/μg RNA; Thermo Fisher Scientific). The SuperScript IV RNase H- reverse transcriptase (Thermo Fisher Scientific) was the 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 (qPCR) was performed with a QuantStudio 5 (Thermo Fisher Scientific) using SYBR Green-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. Gene expression was normalized to the β-actin (*ACTB*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Pfaffl, 2001). The primers used are described in Table S2. In all cases, each PCR was performed in triplicate samples and repeated at least with two independent experiments.

Human microarray dataset analysis

Data from the microarray datasets GDS4602 were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) website and analyzed in R programming language (http://www.r-project.org) using the R environment Rstudio (http://www.rstudio.com). Gene expression plots and regression curves for correlation studies were obtained using GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA).

Determination of vitamin B6 metabolites in human serum samples

Blood samples were collected from control and psoriasis patients pre- and post-PUVA treatment. Serum were extracted, filtered with AMICON™ ULTRA 0.5 mL centrifugal filters 3 KDa cutoff (UFC500396; EMD Millipore) and supplemented with N-Acetyl-Glutamine at 1 mM as an internal standard. Samples were injected in a HPLC Agilent 1290 Infinity II system equipped with hybrid mass spec Agilent Q-TOF 6550 with JetStream electrospray + i-Funnel. Metabolites were analyzed in positive and negative polarity with scan fragmentation and the metabolites selected were analyzed in a HPLC/MS-MS.

Human skin immunohistochemistry

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 rabbit polyclonal to human PYGL (HPA000692, Sigma-Aldrich) or mouse monoclonal to human G6PD (sc-373886, Santa Cruz Biotechnology) antibodies followed by ImmunoCruz™ rabbit/mouse ABC Staining Systems (Santa Cruz Biotechnology) following the manufacturer's recommendations. Sections were examined under a Leica microscope equipped with a digital camera Leica DFC 280 and the photographs were processed with Leica QWin Pro software.

Statistical analysis

Data were analyzed by one- or two-way analysis of variance (ANOVA) followed by a Tukey post-test to determine differences among groups. Contingency graphs were analyzed by the Chi-square (and Fisher's exact) test. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$, ****$p \leq 0.0001$.

RESULTS

Vitamin B6 metabolism is altered in psoriasis patients

To determine possible alterations in vitamin B6 metabolism in psoriasis patients, publicly available transcriptomic data from psoriasis lesional and non-lesional skin were analyzed. It was found that the transcript levels of the genes encoding all the enzymes involved in vitamin B6 metabolism were induced in psoriasis lesional skin compared to non-lesional skin and skin of healthy individuals, but aldehyde oxidase 1 (*AOX1*), which encodes an enzyme that catabolizes vitamin B6 vitamers to PA, that was reduced (Figure 12).

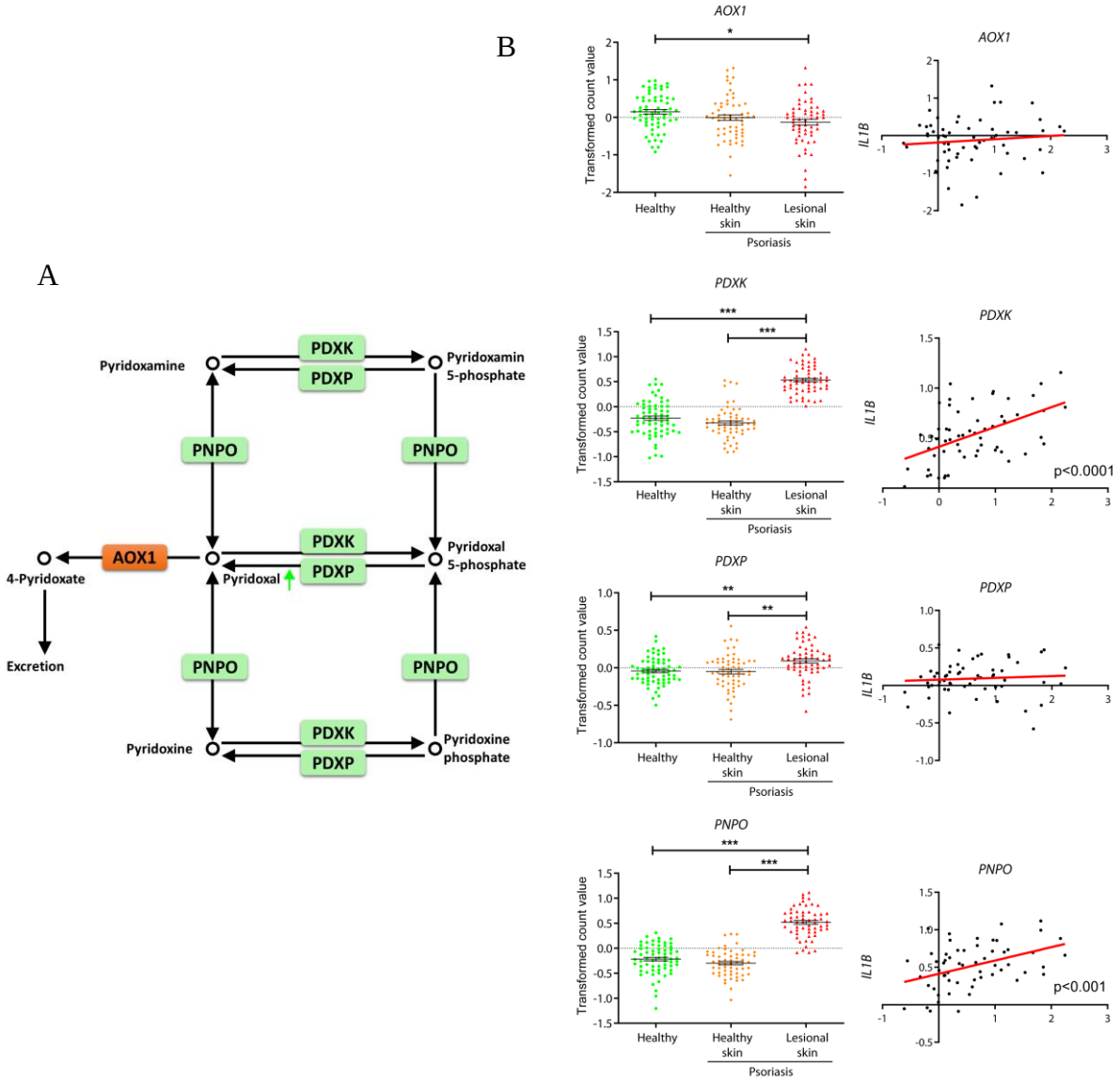


Figure 12. Vitamin B6 metabolism is altered in psoriasis. A) Main vitamin B6 metabolic pathway and representation of the mRNA levels of the genes encoding the involved enzymes in psoriatic lesional skin respect to sin of heathy controls. Green means overexpression and orange means underexpression. B) Expression levels of the genes encoding the enzymes involved in vitamin B6 metabolism and their correlation with the inflammation marker *IL1B*. The data were obtained from the dataset GDS4602 hosted in GEO database. Each dot represents a patient and the mean ± S.E.M. for each group is also shown. *p≤0.05; **p≤0.01; ***p≤0.001.

In addition, a statistically significant positive correlation between *PDXK* and *PNPO* with the inflammation marker *IL1B* was observed (Figure 12B), suggesting a high demand of PLP in psoriasis lesional skin.

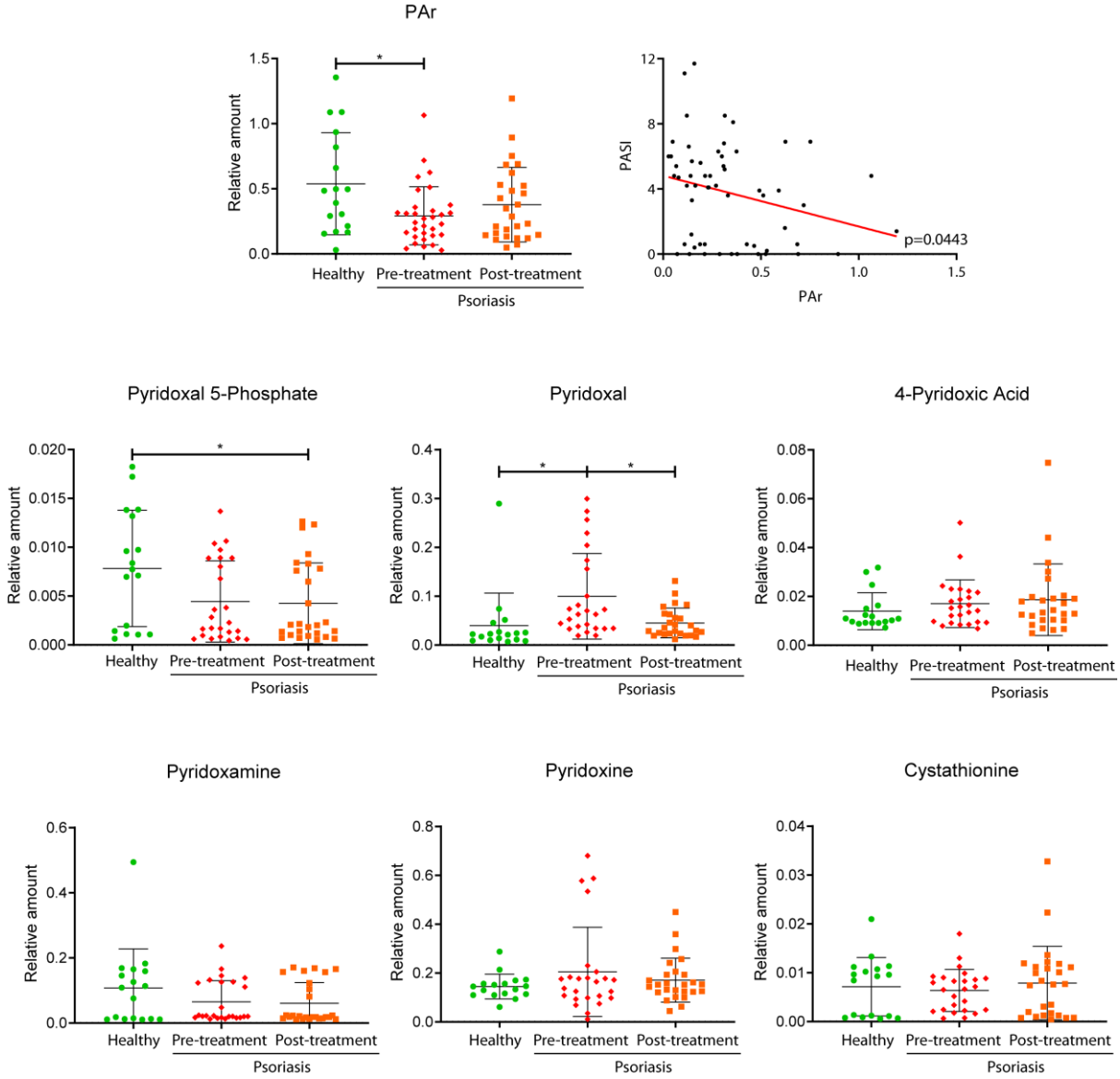


Figure 13. Vitamin B6 metabolites are altered in the serum of psoriasis patients. Levels of main vitamin B6 vitamers and their catabolic products in the serum of 64 samples from psoriasis patients before and after PUVA treatment and healthy donors analyzed using HPLC-MS. All the data obtained were corrected using the levels of an exogenous metabolite, N-acetyl glutamine, added before sample processing. Each dot represents a patient and the mean ± S.E.M. for each group is also shown. *p≤0.05.

To increase the knowledge about vitamin B6 metabolism in psoriasis patients, blood serum samples from healthy donors and psoriasis patients pre- and post-PUVA treatment were collected and the main vitamin B6 metabolites were analyzed. Interestingly, although weak alterations of vitamin B6 vitamers were observed in the sera of psoriasis patients before and after PUVA treatment, a reduced PA/(PLP+PL) ratio (PAr) was found in psoriasis patients

before PUVA treatment that recover to normality after the treatment (Figure 13). Importantly, PAr showed a negative correlation with the psoriasis area severity index (PASI) used for the classification of the severity and extension of psoriasis patient lesions.

Vitamin B6 alleviates skin inflammation in zebrafish psoriasis models

Vitamin B6 vitamers effects in Tnfr2-deficient larvae

To understand the role of vitamin B6 in chronic inflammation, we next tested the effects of vitamin B6 vitamers in a zebrafish model of psoriasis based in the transient genetic inactivation of the Tnfa/Tnfr2 signaling (Candel *et al.*, 2014). In this model, Duox1-derived H_2O_2 promotes neutrophil infiltration and Nfkb activation, perpetuating an inflammatory loop (Candel *et al.*, 2014). In the normal situation, most of the neutrophils remains in the CHT, but in Tnfr2-deficient larvae around a 40% are outside that region. We firstly tested several concentrations of PN directly added to the zebrafish water from 24 to 72 hpf and found a dose-dependent reduction of neutrophil skin infiltration in Tnfr2-deficient larvae (Figure 14). 10 µM was the dose selected for the following experiments.

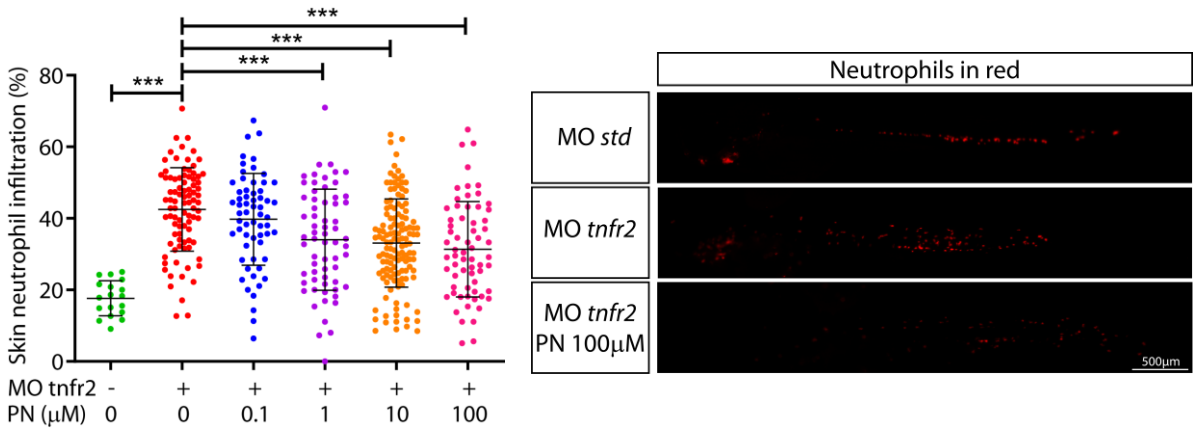


Figure 14. PN reduces in a dose dependent manner skin neutrophil infiltration in Tnfr2-deficient larvae. Zebrafish one-cell *lyz:DsRed* embryos were injected with standard control (*std*) or *tnfr2* MO. At 24 hpf, larvae were treated by bath immersion with different concentrations of PN that was refreshed at 48 hpf. Images were taken at 72 hpf and the neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. ***p≤0.001.

Next, the effect of different vitamin B6 vitamers were tested. Although PN, PL or PLP showed a similar tendency to reduce neutrophil skin infiltration in Tnfr2-deficient larvae, the vitamin B6 active form, PLP, was the most potent (Figure 15).

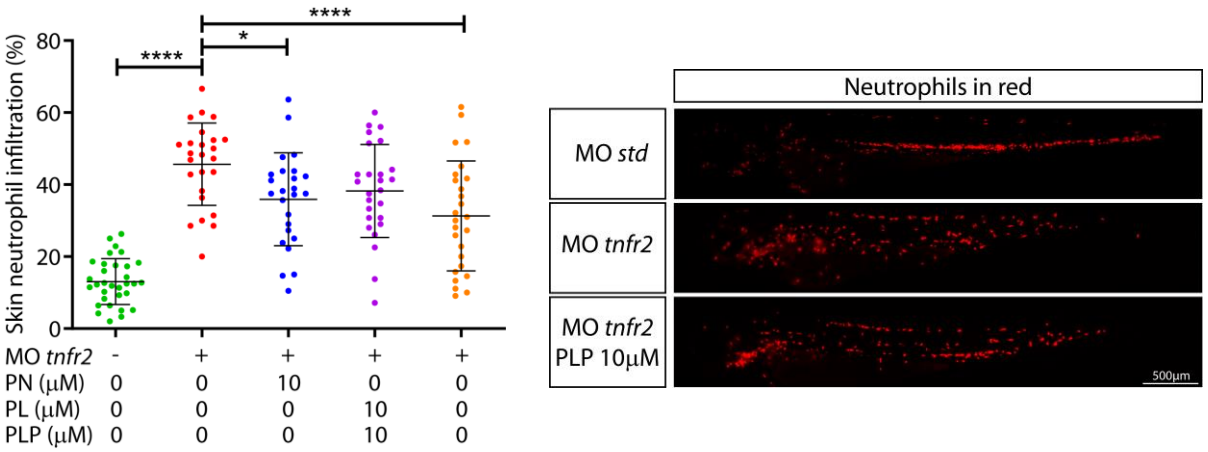


Figure 15. Vitamin B6 vitamers reduce skin neutrophil infiltration in Tnfr2 morphant larvae. Zebrafish one-cell *lyz:DsRed* embryos were injected with *std* or *tnfr2* MO. At 24 hpf larvae were treated by bath immersion with PN, PL and PLP that were refreshed at 48 hpf. Images were taken 72 hpf and the neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. *p≤0.05, ***p≤0.001.

It has been described the relevance of H_2O_2 to mobilize neutrophils (Candel *et al.*, 2014; de Oliveira *et al.*, 2015; de Oliveira *et al.*, 2014), so we decide to evaluate the effect of different vitamin B6 vitamer in H_2O_2 releasing using a specific probe with GFP fluorescence. PL and PLP were able to reduce skin H_2O_2 levels almost restoring control levels (Figure 16).

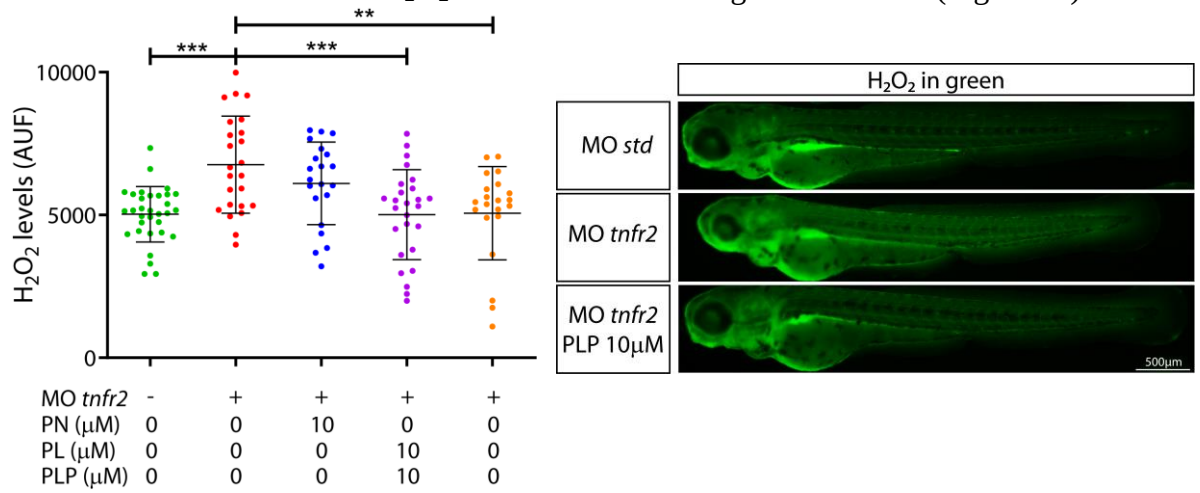


Figure 16. Vitamin B6 vitamers decrease hydrogen peroxide release in Tnfr2-deficient larvae. Zebrafish one-cell *lyz:DsRed* embryos were injected with *std* or *tnfr2* MO. At 24 hpf larvae were treated by bath immersion with PN, PL and PLP that were refreshed at 48 hpf. For 1 hour immediately before taking images at 72 hpf, larvae were incubated in 50 µM of the H_2O_2 probe acetyl-pentafluorobenzene sulphonyl fluorescein solution. GFP fluorescence was measured and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. **p≤0.01, ***p≤0.001.

Previous studies have shown the critical role of NF-kB in the inflammatory response (Candel *et al.*, 2014; de Oliveira *et al.*, 2015; de Oliveira *et al.*, 2014), so we decided to analyze it with different vitamers treatments using a zebrafish transgenic line which allow to measure the NFkB activation with GFP fluorescence. We found similar results as in the previous inflammatory markers, PN and PLP were able to reduce the NF-κB activation (Figure 17).

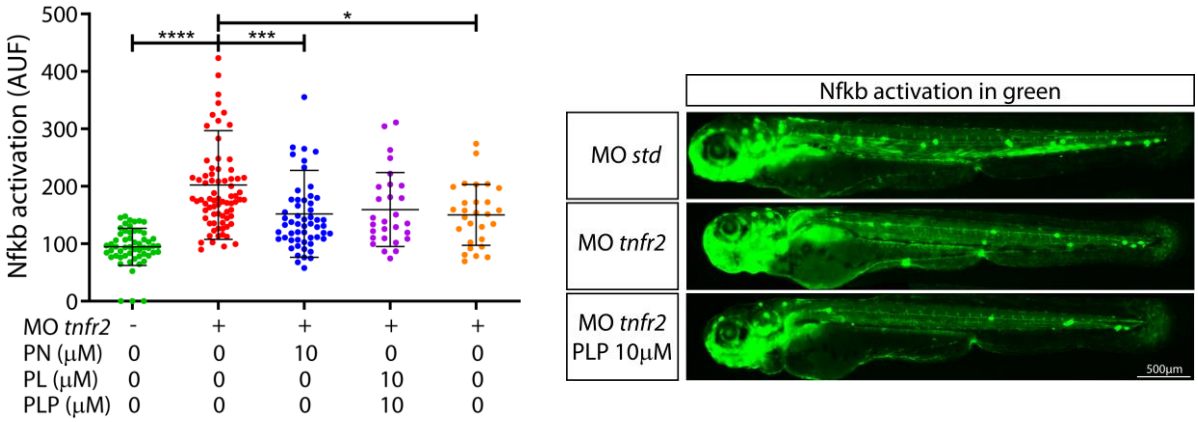


Figure 17. Vitamin B6 vitamers diminish Nfkb activation in Tnfr2-deficient larvae. Zebrafish one-cell *NFKB:eGFP* embryos were injected with *std* or *tnfr2* MO. At 24 hpf larvae were treated by bath immersion with PN, PL and PLP added to the water that were refreshed at 48 hpf. Images were taken 72 hpf and GFP fluorescence was measured in top skin and bottom skin and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. *p≤0.05, ***p≤0.001.

PLP effects in Spint1a- and Clint1a-deficient larvae

In order to confirm the results obtained in Tnfr2-deficient larvae, we decide to check the effects of exogenous addition of PLP in Spint1a-deficient larvae, another well-characterized model of psoriasis which shows keratinocyte hyperproliferation and cell death and robust neutrophil infiltration (Mathias *et al.*, 2007). Similar to the previous results, PLP was able to reduce neutrophil skin infiltration in Spint1a-deficient larvae (Figure 18).

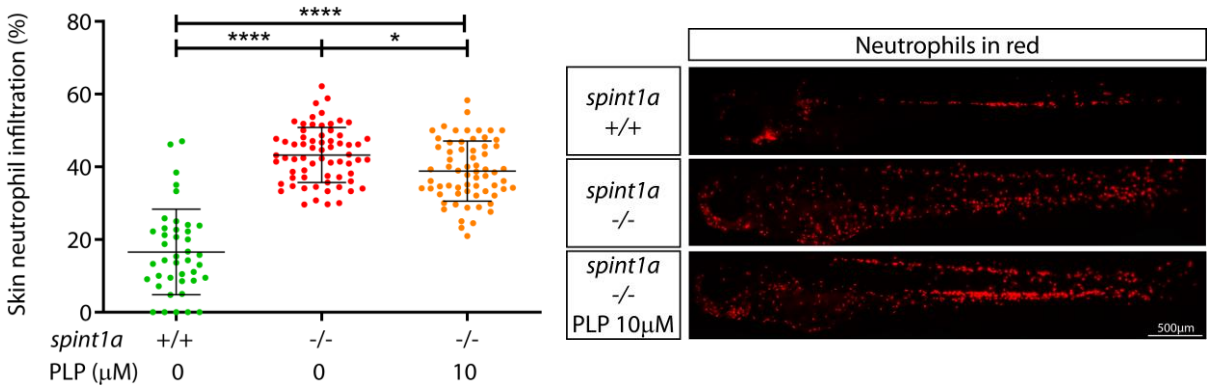


Figure 18. PLP reduces skin neutrophil infiltration in Spint1a-deficient larvae. Zebrafish Spint1a-deficient embryos with labeled neutrophils (*lyz:DsRed*) were treated by bath immersion at 24 hpf with PLP that was refreshed at 48 hpf. Images were taken 72 hpf and the neutrophil mobilization from the CHT was quantified as the percentage of neutrophils outside the CHT in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. *p≤0.05, ****p≤0.0001.

We then analyzed H_2O_2 release in this model and found overproduction of H_2O_2 by the inflamed skin and, notably, PN, PL and PLP were all able to reduce skin H_2O_2 (Figure 19).

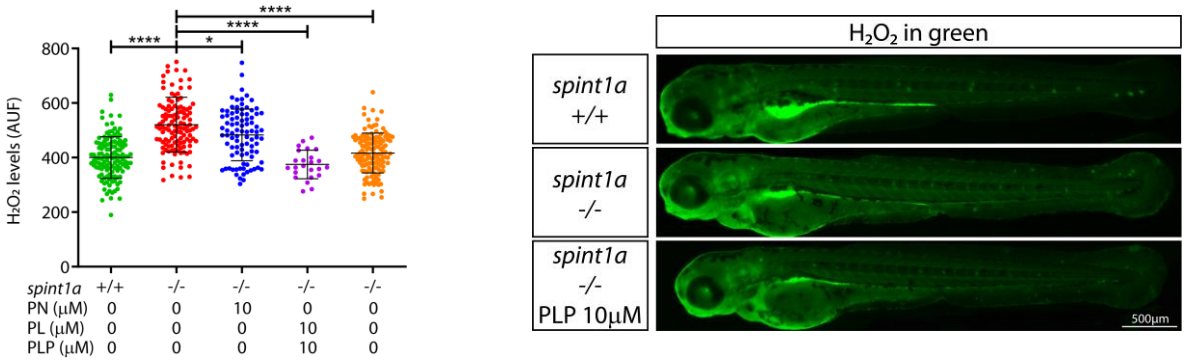


Figure 19. Vitamin B6 vitamers diminish hydrogen peroxide release in Spint1a-deficient larvae. Zebrafish Spint1a-deficient embryos were treated at 24 hpf by bath immersion with PN, PL or PLP that were refreshed at 48 hpf. One hour immediately before taking images at 72 hps, larvae were incubated with 50 µM of the H_2O_2 probe acetyl-pentafluorobenzene sulphonyl fluorescein solution. GFP fluorescence was measured and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. *p≤0.05, ****p≤0.0001 according to one-way.

In addition, PLP also diminishes skin Nfkb activation, but their levels were still remarkable higher than in wild type larvae (Figure 20).

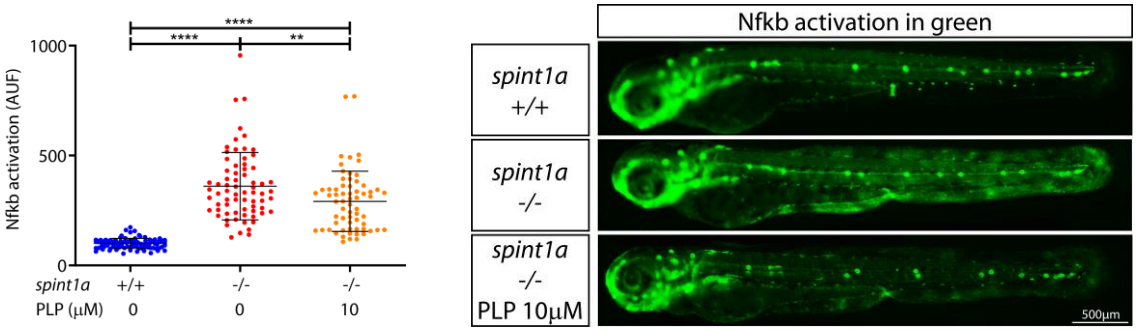


Figure 20. PLP diminishes Nfkb activation in Spint1a-deficient larvae. Zebrafish Spint1a-deficient embryos with labeled Nfkb activation (*NF-κB:eGFP*) were treated by bath immersion at 24 hpf with PLP that was refreshed at 48 hpf. Images were taken 72 hpf and GFP fluorescence was measured in top and bottom skin and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. **p≤0.01, ****p≤0.001.

Curiously, we observed that melanocytes infiltrated the inflamed skin of Spint1a-deficient larvae, as it has been shown during wounding (Levesque et al., 2013), and PLP significantly reduced this infiltration (Figure 21). Collectively, all these results show an anti-inflammatory role for vitamin B6 in two different zebrafish psoriasis models.

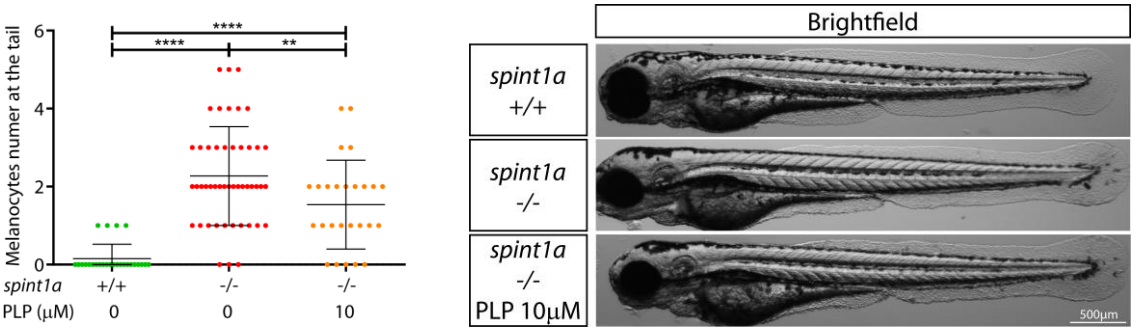


Figure 21. PLP reduces melanocytes infiltration in skin in Spint1a-deficient larvae. Zebrafish Spint1a-deficient were treated by bath immersion at 24 hpf with PLP that was refreshed at 48 hpf. Images were taken 72 hpf. The melanocytes that migrated to the final part of the tail were counted in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. **p≤0.01, ****p≤0.001.

The effect of PLP was also checked in the Clint1a-deficient model of psoriasis, which also shows skin neutrophil infiltration and keratinocyte hyperproliferation (Dodd *et al.*, 2009). The results show that PLP was also able to diminish skin Nfkb activation (Figure 22).

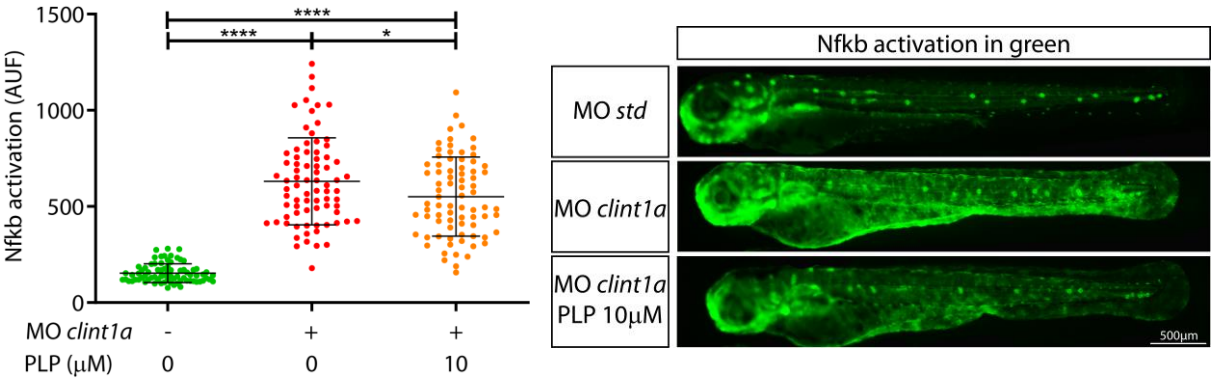


Figure 22. PLP reduces Nfkb activation in Clint1a-deficient larvae. Zebrafish one-cell *NFKB:eGFP* embryos were injected with *std* or *clint1a* MO. At 24 hpf larvae were treated by bath immersion with PLP that was refreshed at 48 hpf. Images were taken at 72 hpf and GFP fluorescence was measured in top and bottom skin and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. *p≤0.05, ****p≤0.001.

Expression of genes encoding vitamin B6-dependent enzymes are dramatically altered in psoriasis patients

As the previous results point out to the relevance of vitamin B6 in psoriasis, we analyzed the expression of the genes encoding PLP-dependent enzymes (Percudani and Peracchi, 2009) in publicly available transcriptomic data from lesional and non-lesional skin. The results show that about 60% of them presented altered mRNA levels in skin of psoriasis patients and, more interestingly, the transcript levels of 21% of them correlated with those of *IL1B* in lesional skin (Figure 23, Table 2, Figure 24). We focused our attention into *PYGL* and *G6PD* , which encode glycogen phosphorylase L and glucose-6-phosphate 1-dehydrogenase, respectively, and are involved in the glycogen catabolism and their mRNA levels were strongly induced in the lesional skin of psoriasis patients and correlated with those of *IL1B* (Figure 23B).

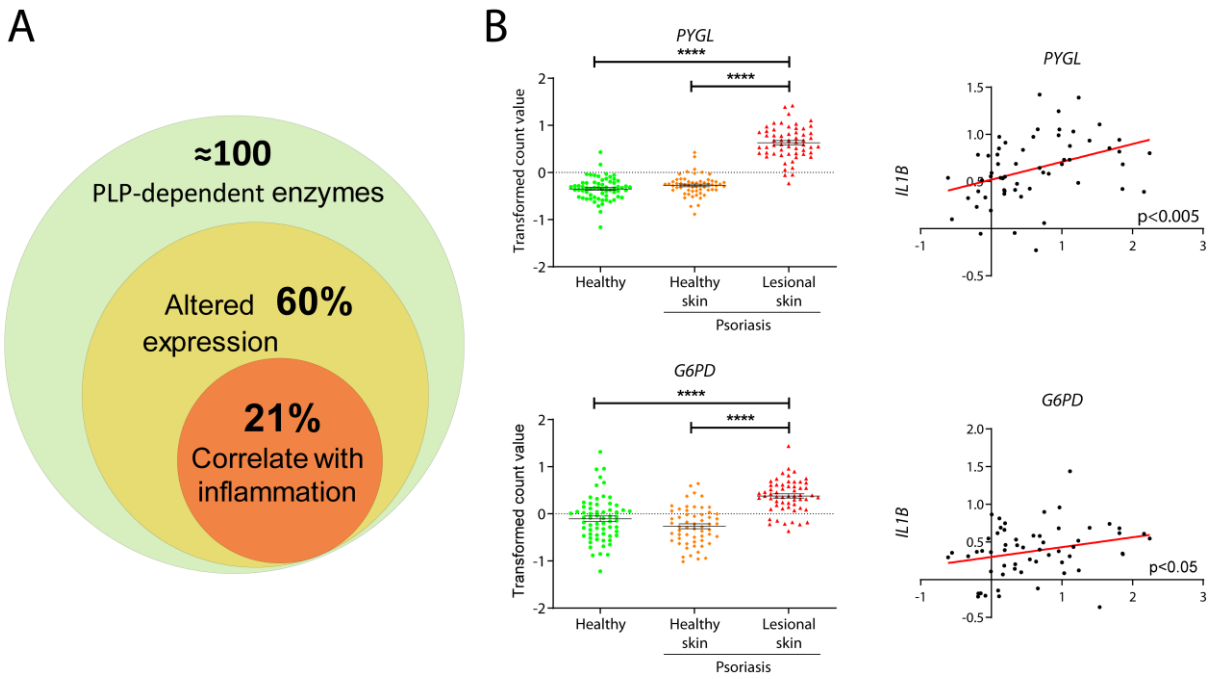
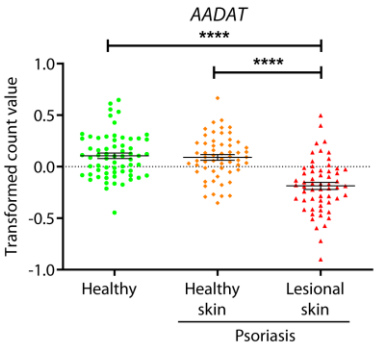
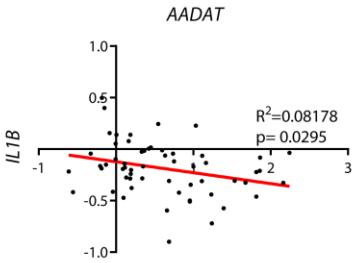


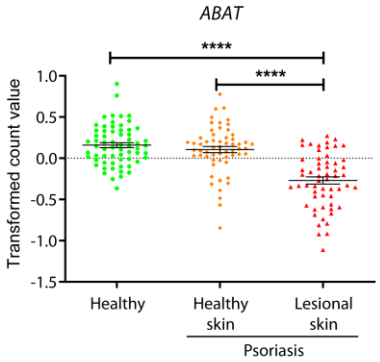
Figure 23. Altered expression of genes encoding PLP-dependent enzymes in psoriasis patients. A) Diagram of genes encoding PLP-dependent enzymes showing that among them 60% have an altered transcript levels in psoriasis patients and 21% correlate with those of the inflammation marker *IL1B*. B) Two examples of PLP-dependent enzymes that show an increased expression in psoriasis lesional skin and whose mRNA levels correlate with those of *IL1B*. List of PLP-dependent enzymes was obtained from "http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/home.pl" and transcriptomic data were obtained from the dataset GDS4602 hosted in GEO database. Each dot represents a patient and the mean ± S.E.M. for each group is also shown. ****p≤0.0001.

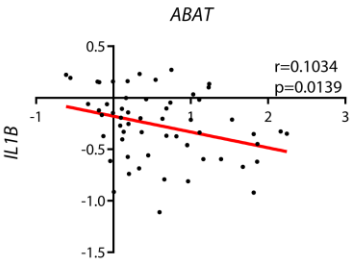
Name	UniProt ID	Gene Name
4-aminobutyrate aminotransferase, mitochondrial	P80404	*ABAT*
Aspartate aminotransferase	Q2TU84	*GIG18*
Histidine decarboxylase	P19113	*HDC*
Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Q8N5Z0	*AADAT*
Proline synthase co-transcribed bacterial homolog protein	O94903	*PROSC*
Antizyme inhibitor 1	Q96A70	*AZIN2*
5-aminolevulinate synthase, nonspecific, mitochondrial	P13196	*ALAS1*
Alanine aminotransferase 2	Q8TD30	*GPT2*
Alpha-aminoadipic semialdehyde dehydrogenase	P49419	*ALDH7A1*
Aspartate aminotransferase, cytoplasmic	P17174	*GOT1*
Aspartate aminotransferase, mitochondrial	P00505	*GOT2*
Glucose-6-phosphate 1-dehydrogenase	P11413	*G6PD*
Glycogen phosphorylase, liver form	P06737	*PYGL*
Kynureninase	Q16719	*KYNU*
O-phosphoseryl-tRNA(Sec) selenium transferase	Q9HD40	*SEPSECS*
Serine hydroxymethyltransferase	Q5BJF5	*SHMT2*
Serine palmitoyltransferase 2	O15270	*SPTLC2*

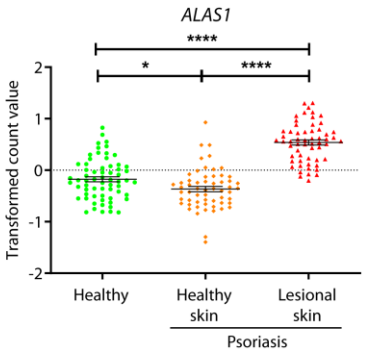
Table 2. PLP-dependent enzymes whose genes show altered and correlated transcript levels with those of the inflammation marker *IL1B* in psoriasis patient lesional skin.

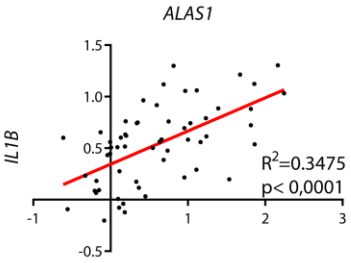

AADAT
****
****
Transformed count value
1.0
0.5
0.0
-0.5
-1.0
Healthy
Healthy skin
Lesional skin
Psoriasis


AADAT
IL1B
R2=0.08178
p= 0.0295
1.0
0.5
-0.5
-1.0
-1
2
3


ABAT
****
****
Transformed count value
1.0
0.5
0.0
-0.5
-1.0
-1.5
Healthy
Healthy skin
Lesional skin
Psoriasis


ABAT
IL1B
r=0.1034
p=0.0139
0.5
-0.5
-1.0
-1.5
-1
1
2
3


ALAS1
****
*
****
Transformed count value
2
1
0
-1
-2
Healthy
Healthy skin
Lesional skin
Psoriasis


ALAS1
IL1B
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p< 0,0001
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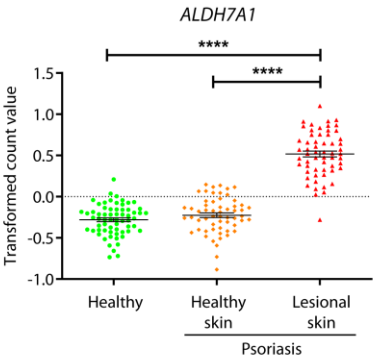
1.0
0.5
-0.5
-1
1
2
3


ALDH7A1
****
****
Transformed count value
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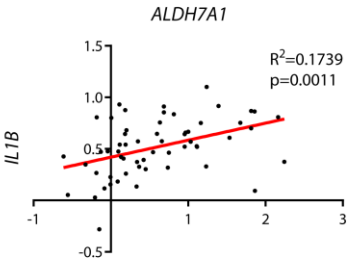
1.0
0.5
0.0
-0.5
-1.0
Healthy
Healthy skin
Lesional skin
Psoriasis


ALDH7A1
IL1B
R2=0.1739
p=0.0011
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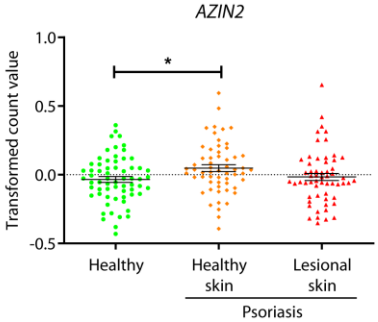
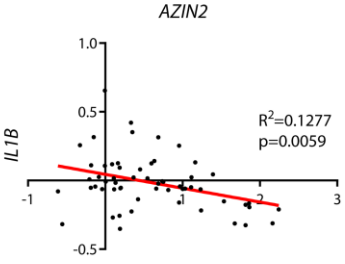
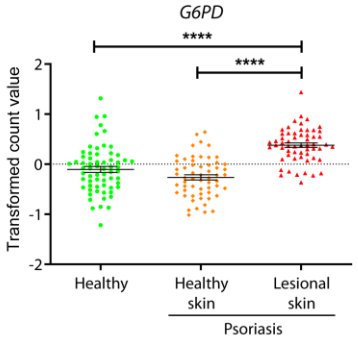
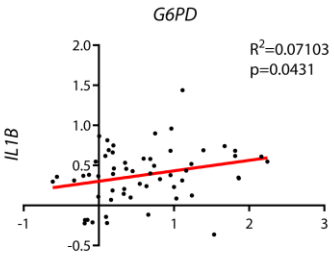
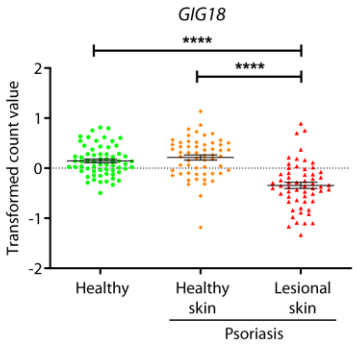
1.0
0.5
-0.5
-1
1
2
3

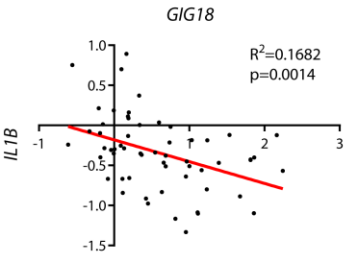

AZIN2
*
Transformed count value
1.0
0.5
0.0
-0.5
Healthy
Healthy skin
Lesional skin
Psoriasis

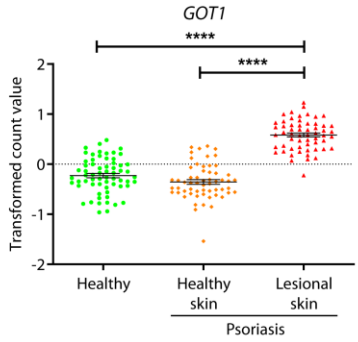

AZIN2
IL1B
R2=0.1277
p=0.0059
1.0
0.5
-0.5
-1
3


G6PD
****
****
Transformed count value
2
1
0
-1
-2
Healthy
Healthy skin
Lesional skin
Psoriasis


G6PD
R2=0.07103
p=0.0431
IL1B
2.0
1.5
1.0
0.5
-0.5
-1
1
2
3


GIG18
****
****
Transformed count value
2
1
0
-1
-2
Healthy
Healthy skin
Lesional skin
Psoriasis


GIG18
R2=0.1682
p=0.0014
IL1B
1.0
0.5
-0.5
-1.0
-1.5
-1
1
2
3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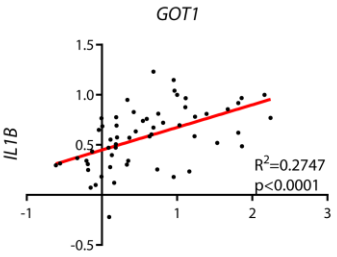
Healthy skin
Lesional skin
Psoriasis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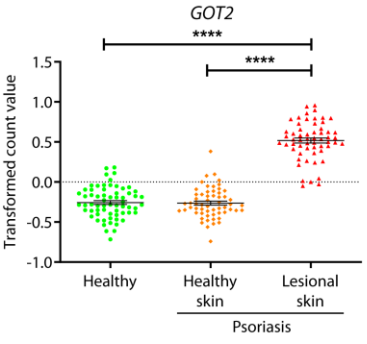
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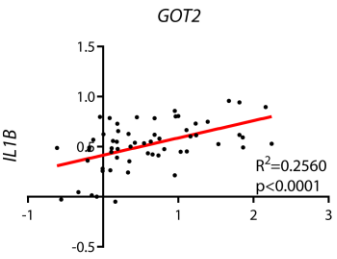
Lesional skin
Psoriasis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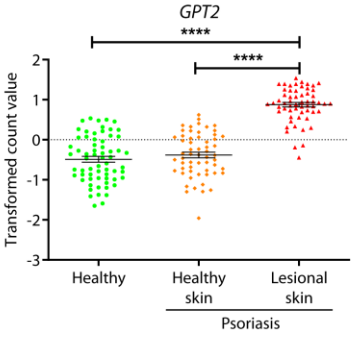
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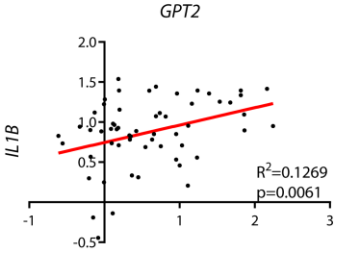
Lesional skin
Psoriasis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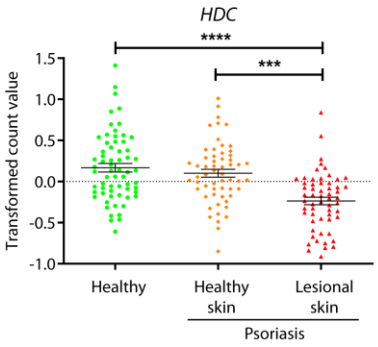
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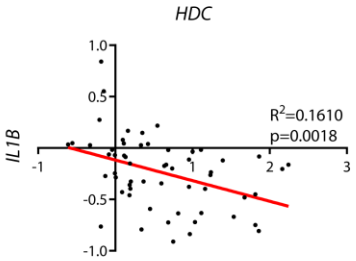
Healthy skin
Lesional skin
Psoriasis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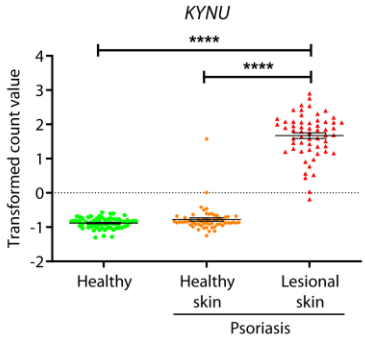
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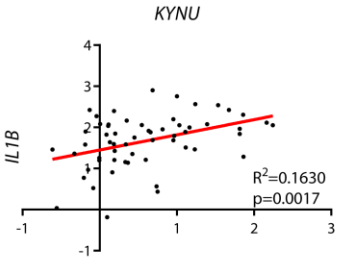
Lesional skin
Psoriasis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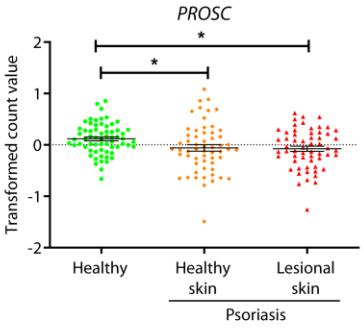
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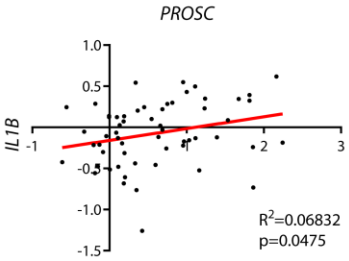
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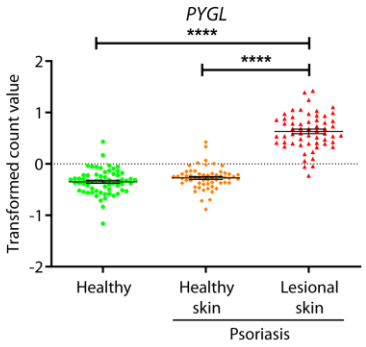
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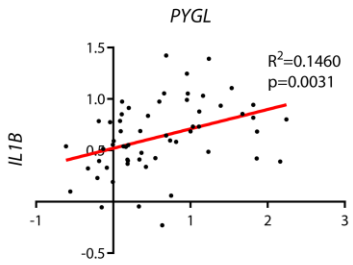
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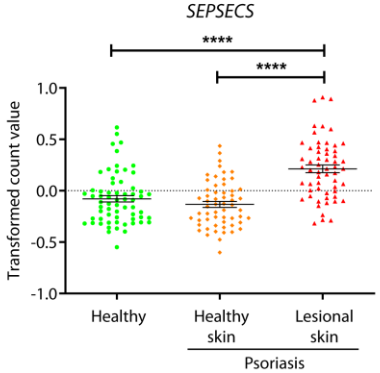
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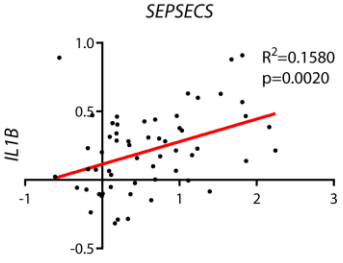
Lesional skin
Psoriasis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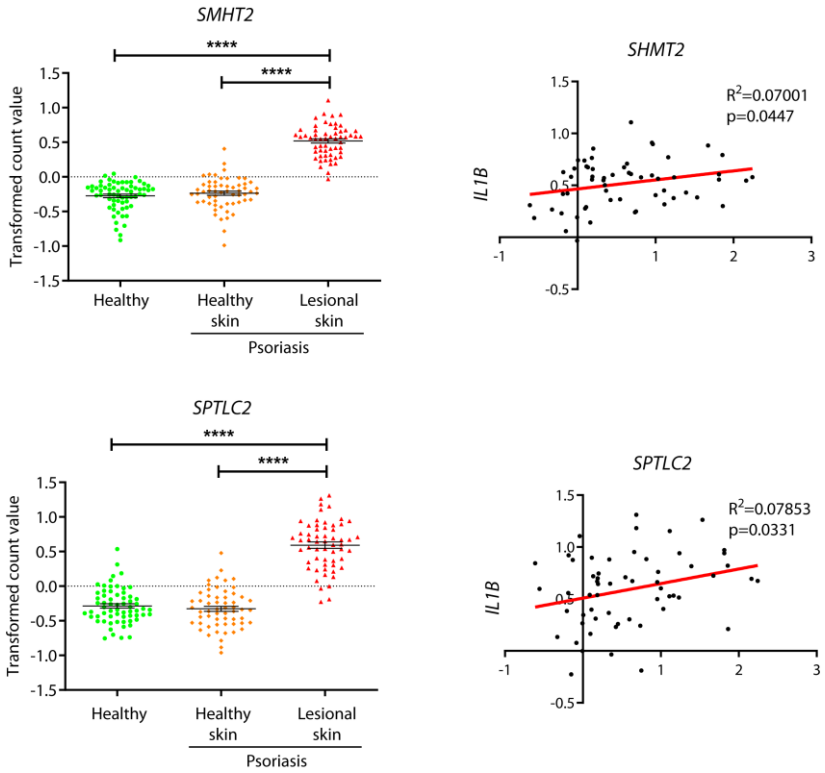

Figure 24. Genes encoding PLP-dependent enzymes that have altered transcript levels and their correlation with *IL1B* in psoriasis patient lesional skin. The list of PLP-dependent enzymes was obtained from "http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/home.pl" and transcriptomic data from the dataset GDS4602 hosted in GEO database. Each dot represents a patient and the mean ± S.E.M. for each group is also shown. *p≤0.05, ***p≤0.001, ****p≤0.0001.

To confirm the gene expression study, we analyzed PYGL and G6PD by immunohistochemistry in skin from healthy donors and psoriasis lesional skin. Controversially, PYGL was found to be expressed by keratinocytes of the basal, spinous and granular layers in both healthy and lesional skin but at reduced levels in the latter (Figure 25A). As regards G6PD, it was found to be expressed in keratinocytes of basal, spinous and granular layers at similar levels in both healthy and lesional skin (Figure 25B).

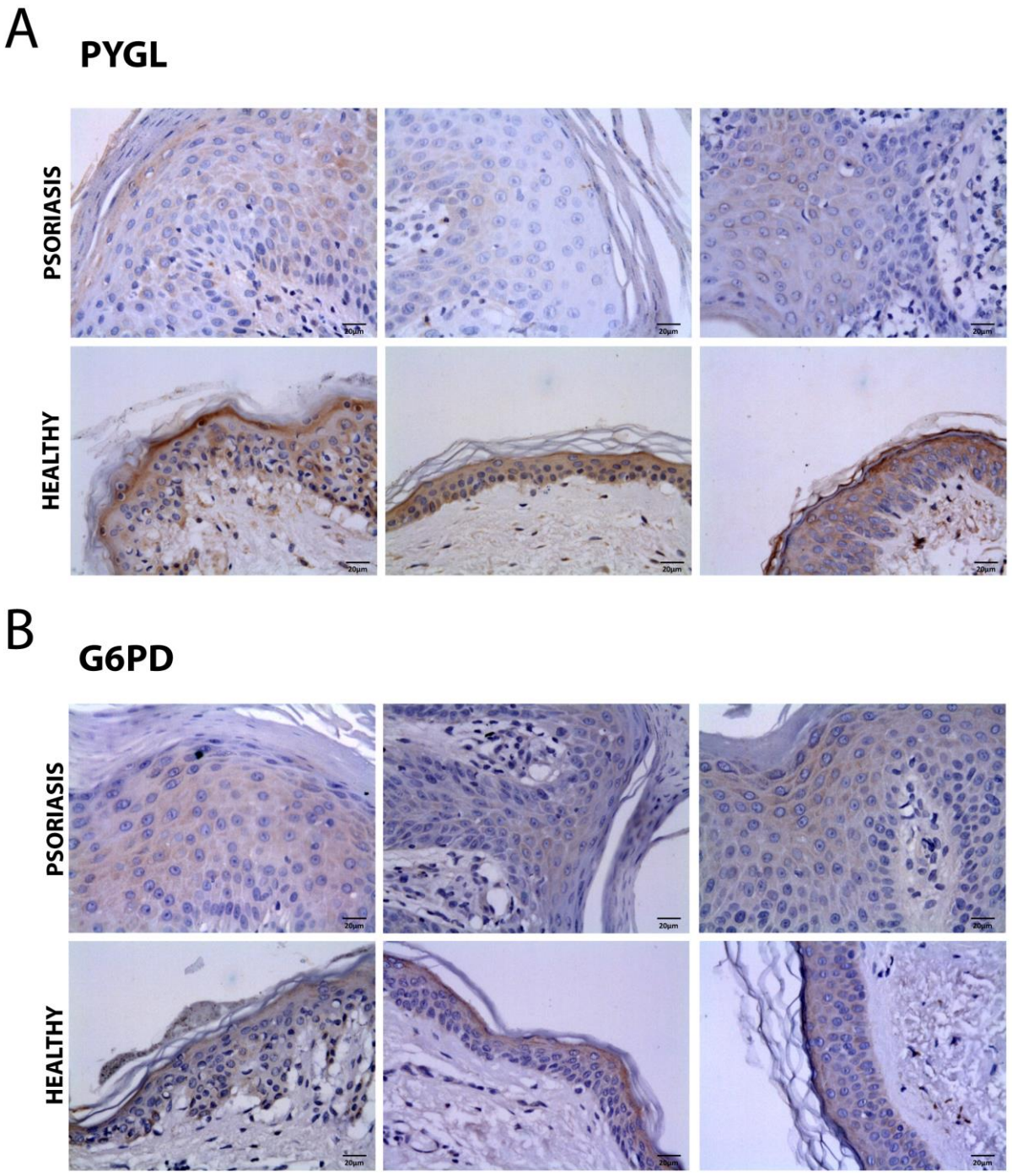

Figure 25. PYGL and G6PD immunohistochemistry in skin from healthy donors and psoriasis lesional skin. Representative images of sections from healthy and psoriatic lesional skin biopsies that have been immunostained with anti-PYGL (A) or anti-G6PD (B) antibodies and then counterstained with hematoxylin.

Pharmacological inhibition of PYGL alleviates inflammation in Spint1a-deficient zebrafish larvae and human keratinocytes

The relevance of glucose metabolism in psoriasis have been demonstrated in a mouse model deficient in the glucose transporter 1, which show normal skin homeostasis but are largely resistant to psoriasis-like disease (Zhang *et al.*, 2018). In addition, early clinical studies reported increased glycogen accumulation and glycogen synthase activity in psoriasis lesional skin (Harmon and Phizackerley, 1984; Sasai, 1970). We, therefore, analyzed the impact of pharmacological inhibition of Pygl with CP-91149 in Spint1a-deficient larvae. Inhibition of Pygl resulted in reduced neutrophil skin infiltration (Figure 26).

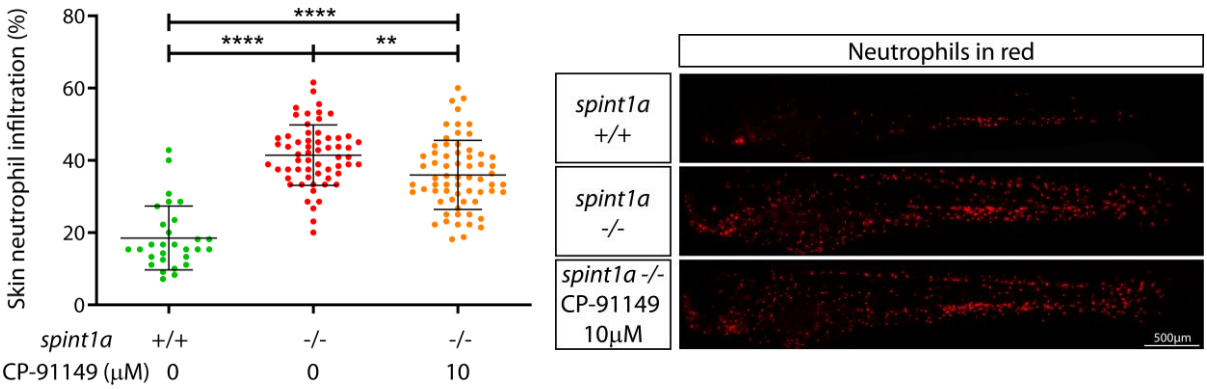

Figure 26. Pharmacological inhibition of Pygl reduces skin neutrophil infiltration in Spint1a-deficient larvae. Zebrafish Spint1a-deficient embryos with labeled neutrophils (*lyz:DsRed*) were treated by bath immersion at 24 hpf with CP-91149 that was refreshed at 48 hpf. Images were taken 72 hpf and the neutrophil mobilization from the CHT was quantified as indicated in M&M section in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. **p≤0.01, ****p≤0.0001.

Moreover, CP91149 was able to slightly diminish skin H_2O_2 production (Figure 27).

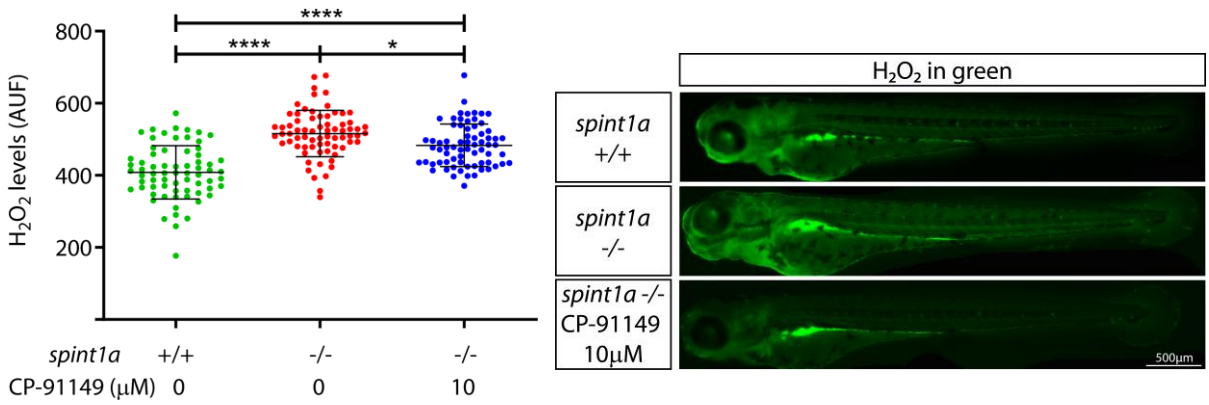

Figure 27. Pharmacological inhibition of Pygl diminishes skin hydrogen peroxide release in Spint1a-deficient larvae. Zebrafish Spint1a-deficient embryos were treated by bath immersion at 24 hpf with CP-91149 that was refreshed at 48 hpf. One hour immediately before taking images at 72 hps, larvae were incubated with 50 μM of the H_2O_2 probe acetyl-pentafluorobenzene sulphonyl fluorescein solution. GFP fluorescence was measured and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. *p≤0.05, ****p≤0.0001.

We also check the effect of Pygl inhibition in the Nfkb activation, but there were no significant differences with untreated Spint1a-deficient larvae (Figure 28).

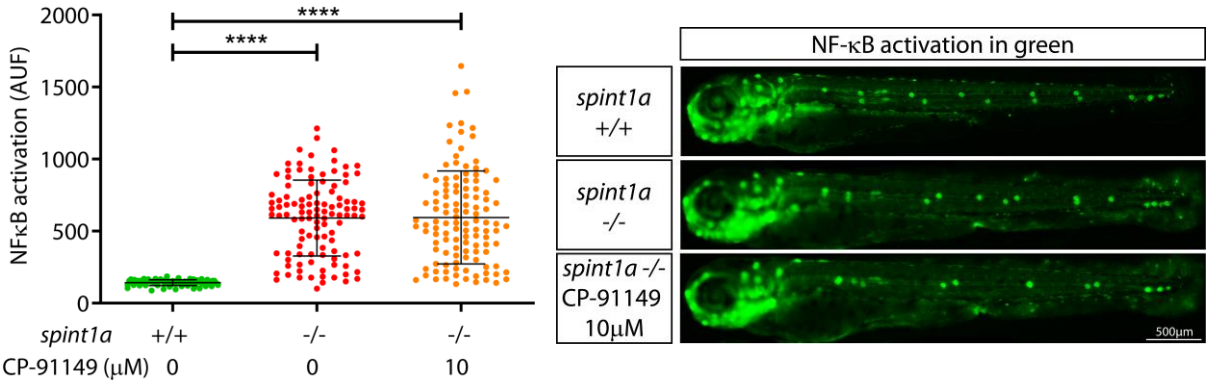

Figure 28. Pharmacological inhibition of Pygl fails to inhibit Nfkb activation in Spint1a-deficient larvae. Zebrafish Spint1a-deficient embryos with labeled Nfkb activation (*NF-κB:eGFP*) were treated by bath immersion at 24 hpf with CP-91149 that was refreshed at 48 hpf. Images were taken 72 hpf and GFP fluorescence was measured in top and bottom skin and the mean value of intensity was calculated as indicated in M&M in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. ****p≤0.001.

As observed with the PLP treatment, Pygl inhibition was also able to reduce the skin melanocyte infiltration in the tail (Figure 29).

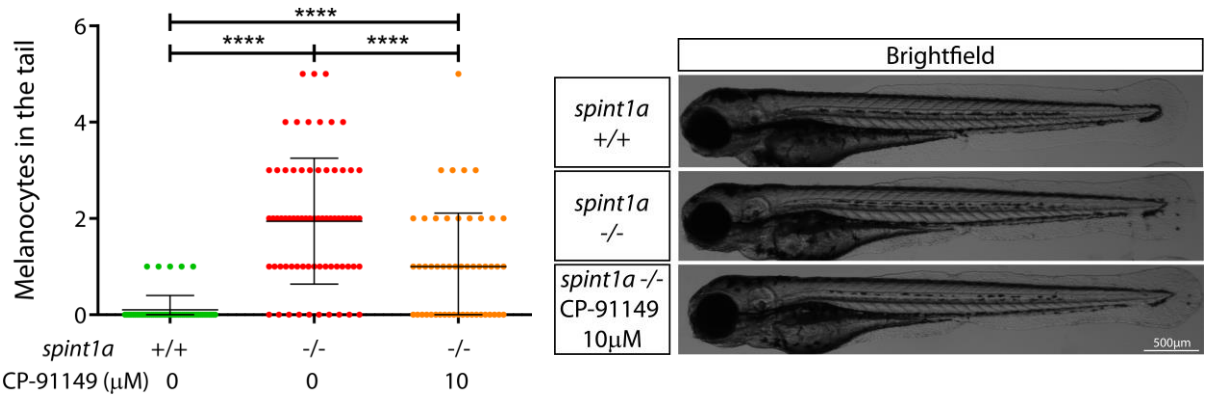

Figure 29. Pharmacological inhibition of Pygl reduces the number of melanocytes in the tail of Spint1a-deficient larvae. Zebrafish Spint1a-deficient larvae were treated by bath immersion at 24 hpf with CP-91149 that was refreshed at 48 hpf. Images were taken 72 hpf and the melanocytes that migrated to the final part of the tail were counted in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. ****p≤0.001.

Lastly, we also evaluate keratinocyte hyperplasia present in the tail region, as the number of keratinocyte accumulations, and we found that Pygl inhibition was able to reduce the number of keratinocyte accumulations (Figure 30).

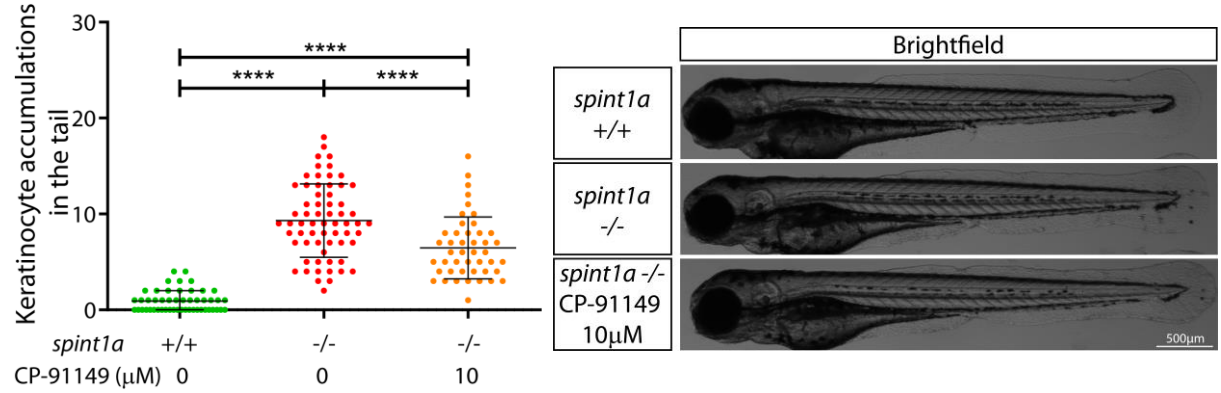

Figure 30. Pharmacological inhibition of Pygl reduces keratinocyte hyperplasia of Spint1a-deficient larvae. Zebrafish Spint1a-deficient were treated by bath immersion at 24 hpf with CP-91149 that was refreshed at 48 hpf. Images were taken 72 hp and the number keratinocyte accumulations in the tail were scored in at least 20 larvae per group in 3 different experiments. Each dot represents a larva and the mean ± S.E.M. for each group is also shown. ****p≤0.001.

To check if the PYGL inhibition was able to reduce inflammation in human keratinocytes, HaCaT keratinocytes pre-stimulated with recombinant TNFA and IL17A, two relevant cytokines driven psoriasis, were used. CP-91149 was able to fully abrogate the induction of *TNFA* and *IL1B* genes in human pre-stimulated HaCaT cells (Figure 31). Collectively, these results show that pharmacological inhibition of PYGL inhibits inflammation in zebrafish and human keratinocytes.

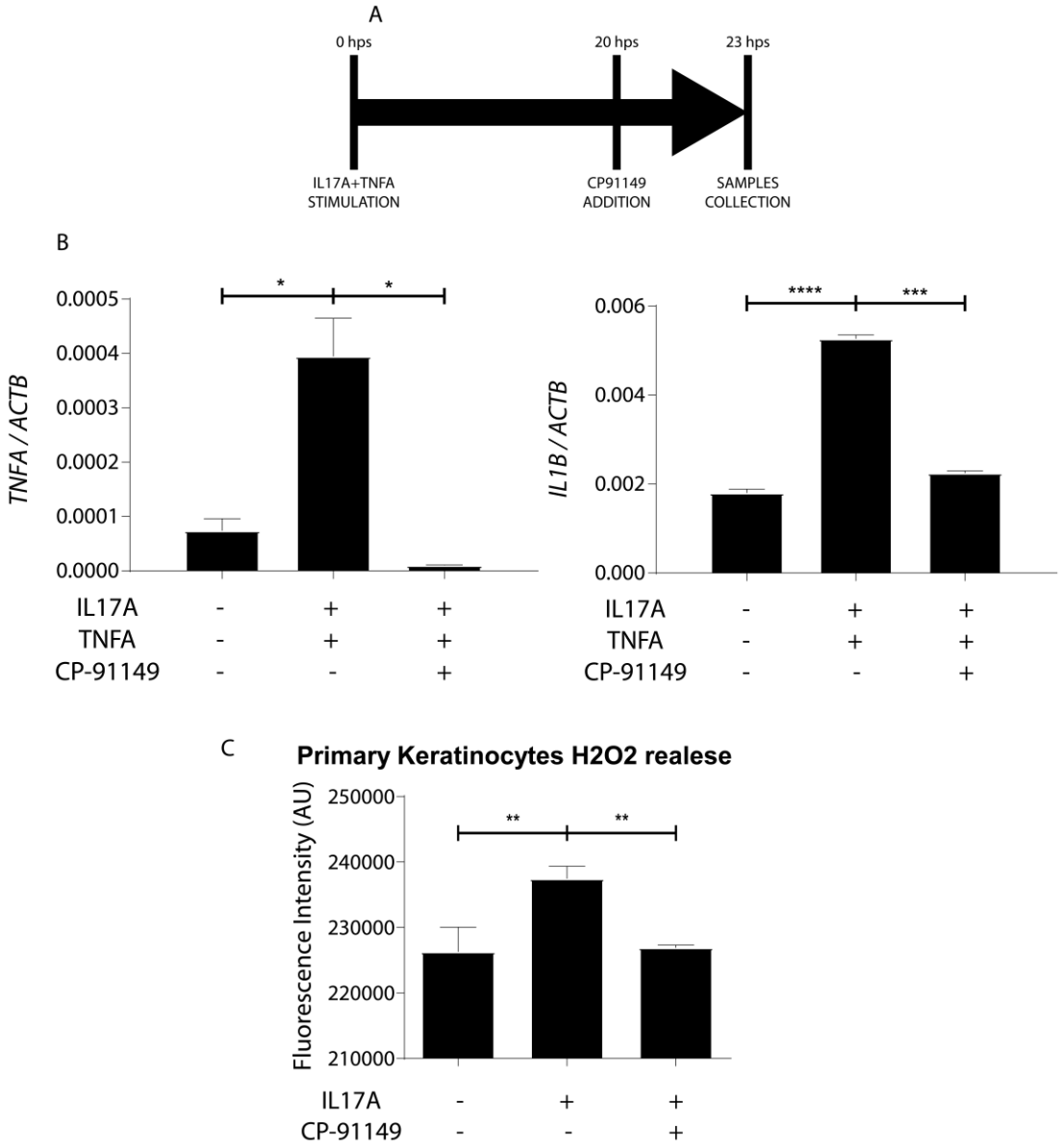

Figure 31. Pharmacological inhibition of PYGL fully abrogates the induction of *TNFA* and *IL1B* in human HaCaT keratinocytes. A) Scheme showing the stimulation procedure. Human HaCaT keratinocytes were stimulated using 100 ng/ml IL17A and 40 ng/ml TNFA for 20 hours and then 10 μM CP-91149 was added for 3 hours before samples collection. B) *TNFA* and *IL1B* transcript levels were analyzed using RT-qPCR. The PCR was performed in technical triplicate and the results are representative of two independent experiments. *p≤0.05, ***p≤0.001, ****p≤0.0001.

DISCUSSION

Vitamin B6 deficiency has been described to be involved in chronic inflammation, being its supplementation able to restore homeostasis (Casciato *et al.*, 1984; Dobbelstein *et al.*, 1974; Sorice *et al.*, 1980; Talbott *et al.*, 1987). Plasma PLP levels are usually inversely associated with different inflammation markers, like C-reactive protein (CRP), among others (Friso *et al.*, 2001; Sakakeeny *et al.*, 2012). It has also been reported that acute inflammation could cause a rise in vitamin B6 vitamers in the tissues affected and an increased catabolism to PA (Ulvik *et al.*, 2012). Psoriasis, like others inflammatory diseases, has different comorbidities among witch metabolic syndrome stands out (Boehncke *et al.*, 2011). A study focused in metabolic syndrome associated to psoriasis has pointed out to vitamin B6 as a protector component (Romani *et al.*, 2013). Collectively, all this information indicates that vitamin B6 could be involved in the development different inflammatory diseases including psoriasis. However, the mechanisms involved in the effects of vitamin B6 in chronic inflammatory disorders in general, and in particular psoriasis, are largely unknown. We anticipate that these effects will be very complex, since more than 100 enzymes used PLP as cofactor (Percudani and Peracchi, 2009)

Using transcriptomic data available from previous studies about psoriasis lesional and non lesional skin, we have found an impaired expression in all the transcripts encoding the enzymes involved in vitamin B6 metabolism in psoriasis lesional skin and some of them correlated with inflammation. Moreover, analyzing blood serum levels of the different vitamin B6 vitamers and their catabolic product PA, we have demonstrated that psoriasis patients have increased mobilization of PL, probably to reach the affected areas, and reduced PLP and vitamin B6 catabolism to PA in serum. In addition, we found a negative correlation between PAr and PASI, probably reflecting increased plama PL that compensate the slightly reduced PLP. These observations are one of the most important findings of this study because it strongly suggests the involvement of vitamin B6 in psoriasis and point out to PAr as an additional biomarker of disease severity. Similarly, reduced plasma PLP and increased PA levels were reported to be associated to oxidative stress and inflammation (Christensen *et al.*, 2012; Paul *et al.*, 2013; Sakakeeny *et al.*, 2012; Shen *et al.*, 2010). Intriguingly, although it has been shown that AOX1 actively catabolizes vitamin B6 vitamers to PA under oxidative stress because *AOX1* gene is induced by the master antioxidant response transcription factor NF-E2 related factor 2 (NRF2) (Maeda *et al.*, 2012), *AOX1* is downregulated in psoriasis lesional skin. Further studies are required to ascertain the relevance of this paradoxical observation.

To study the possible beneficious effects of vitamin B6 supplementation in psoriasis and to reveal the mechanisms involved, different zebrafish psoriasis models were used. Taking advantage of the unique characteristics for *in* vivo imaging of the zebrafish and the availability of different preclinical zebrafish models of psoriasis, we found that exogenous addition of vitamin B6 alleviates skin oxidative stress and inflammation, reducing the infiltration of neutrophils in the skin and NF-κB activation, the later playing an important role in the initiation of psoriasis (Wang *et al.*, 2018b), as well as melanocyte infiltration of inflamed areas, as it has been reported to inflamed wounds (Levesque *et al.*, 2013). Although it is important to highlight that these results show the beneficial impact of vitamin B6 supplementation in 3 models of psoriasis in which oxidative stress and skin inflammation are triggered by different genetic alterations, they do not demonstrate the mechanism involved. However, it is tempting to speculate that vitamin B6 might directly inhibit oxidative stress, since H_2O_2 has been shown to initiate and perpetuate an inflammatory loop in Tnfr2-deficient zebrafish larvae (Candel *et al.*, 2014).

One of the most important contribution of this study is the alteration of the transcript levels of 60% of the genes encoding the around 100 PLP-dependent enzymes in psoriasis lesional skin and, more importantly, that the levels of 21% of them show correlation with those of the inflammatory marker *IL1B*. As we have discussed above, the effects of exogenous vitamin B6 supplementation must be extremely complex, since it participates in many signaling pathways. Our results reveal that some of these pathways could have a huge impact in psoriasis. For example, vitamin B6 may regulate the kynurenine pathway, which is the main tryptophan catabolic pathway and has immunomodulatory effects (Wang *et al.*, 2015; Yeh and Brown, 1977). Another interesting pathway is the one involved in hydrogen sulfide (H_2S) production, which is a gaseous messenger with proinflammatory effects at high concentrations and anti-inflammatory effects at low concentrations (Bhatia, 2012; Gemici and Wallace, 2015; Whiteman and Winyard, 2011).

We focused our attention into the glycogen degradation pathway, since the genes encoding two PLP-dependent enzymes, PYGL and G6PD, show increased transcript levels in psoriasis lesional skin and positive correlation with those of *IL1B*. In addition, early clinical studies reported that psoriasis lesional skin accumulated glycogen (Halprin *et al.*, 1973; Harmon and Phizackerley, 1984; Sasai, 1970; Stankler and Walker, 1976). However, we did not observe increased PYGL and G6PD protein amount in psoriasis lesional skin but rather slightly reduced levels of PYGL. Although these results are not completely coherent, it is well-

known that PYGL enzymatic activity is regulated at post-translational levels through phosphorylation. Therefore, we decided to inhibit Pygl and G6pd using genetic and pharmacological strategies in the Spint1a-deficient zebrafish psoriasis model and, strikingly, it was found that most of the inflammation markers evaluated were alleviated, including neutrophil and melanocyte infiltration, and keratinocyte hyperplasia. However, Nfkb levels were unaltered at the time analyzed. In addition, pharmacological inhibition of PYGL in human keratinocytes pre-stimulated with TNFA and IL17A robustly declines *TNFA* and *IL1B* transcript levels. Glycogen degradation is one of the most important sources of glucose and the deprivation of glucose by genetic and pharmacological inhibition of the glucose transporter 1 (GLUT1) decreases hyperplasia in mouse model of psoriasis (Zhang *et al.*, 2018). More importantly, GLUT1 protein amount in lesional skin positively correlated with disease severity in psoriasis patients (Hodeib *et al.*, 2018). Although future experiments are required to understand the contribution of PYGL and G6PD in skin inflammation, it is also tempting to speculate about an alternative mechanism where glucose-1-phospate released from glycogen by the action of PYGL may be used by G6PD to generate NADPH that, in turn, may fuel NADPH oxidases, such as DUOX1, to produce H_2O_2, which mediates the skin inflammatory loop in the Tnfr2-deficient zebrafish psoriasis model and is strongly expressed in psoriasis patient lesional skin (Candel *et al.*, 2014). Blocking this pathway, we could be affecting both reactions, but future experiments are required to discern which one is causing more impact.

In conclusions, the results from this thesis point out to the relevance of vitamin B6 in the development of psoriasis and PAr as an attractive marker for this disease. The zebrafish preclinical models used have revealed the anti-inflammatory role of vitamin B6 supplementation in skin inflammation induced by different genetic alterations and have uncovered PYGL and G6PD as novel, putative therapeutic target for psoriasis. Preclinical human 3D psoriasis skin models should be the following step to confirm the usefulness vitamin B6 supplementation and PYGL and G6PD inhibition for the treatment of this disease.

CONCLUSIONS

1. The expression of genes encoding the enzymes involved in vitamin B6 metabolism is altered in psoriasis lesional skin.

2. The PAr index negatively correlates with PASI and, therefore, can be used as a prognostic marker of disease severity.

3. The expression of 60% of genes encoding PLP-dependent enzymes are altered in psoriasis lesional skin and the expression of 21% of them correlates with inflammation.

4. The PLP-dependent enzymes PYGL and G6PD show decreased and similar protein levels, respectively, in psoriasis lesional skin.

5. Vitamin B6 vitamers alleviate H_2O_2 production, Nfkb activation, neutrophil and melanocyte skin infiltration, and keratinocyte hyperplasia in three different zebrafish preclinical models of psoriasis.

6. Pharmacological inhibition of the PLP-dependent enzyme PYGL reduces H_2O_2 production, neutrophil and melanocyte skin infiltration, and keratinocyte hyperplasia, but fails to inhibit Nfkb activation, in the Spint1a-deficient zebrafish model of psoriasis. In addition, pharmacological inhibition of PYGL in human keratinocytes pre-stimulated with TNFA and IL17A robustly reduces the induction of *IL1B* and *TNFA*.

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RESUMEN EN CASTELLANO

La inmunidad es el balance entre la protección frente a agentes externos y la tolerancia para evitar alergias o enfermedades autoinmunitarias (Waller and Sampson, 2018). Diferentes moléculas, células y tejidos participan en una respuesta coordinada formando el sistema inmunitario. Dependiendo del tiempo de reacción y la especificidad de la respuesta se clasifica en dos tipos principales que están coordinadas y reguladas entre sí (Dempsey *et al.*, 2003; Parkin and Cohen, 2001). Por un lado, el sistema inmunitario innato es la respuesta más rápida frente a infecciones, compuesta principalmente por moléculas solubles y diferentes tipos celulares como mastocitos, células asesinas naturales, dendríticas y mieloides, llamadas neutrófilos y macrófagos, que detectan señales de daño y liberan quimioquinas o citoquinas para atraer más células inmunitarias (Abbas *et al.*, 2019; McComb *et al.*, 2013; Medzhitov, 2007). Por otro lado, el sistema inmunitario adaptativo es específico y desarrolla memoria para futuros encuentros, dividiéndose en respuesta humoral llevada a cabo por linfocitos B que reconocen patógenos libres y producen anticuerpos (Abbas *et al.*, 2019), y en respuesta mediada por células que está basada en los linfocitos T que reconocen antígenos presentados por el complejo mayor de histocompatibilidad (Neefjes *et al.*, 2011). Los linfocitos que expresan receptores que reconocen componentes del propio organismo son eliminados, pero en ocasiones esto no ocurre y se pueden desarrollar enfermedades autoinmunitarias (Gregersen and Behrens, 2006).

La respuesta del sistema inmunitario a estas señales provoca una respuesta fisiopatológica llamada inflamación que tiene el propósito de eliminar el agente que provoca el daño (Chovatiya and Medzhitov, 2014). Macrófagos y células epiteliales comienzan la respuesta inflamatoria tras la detección de un estímulo, y neutrófilos y monocitos que se diferenciarán a macrófagos migran al lugar de inflamación (Schmid-Schonbein, 2006) gracias a la producción de citoquinas y quimiocinas inflamatorias como TNFA, IL1B o CXCL8 que inducen expresión endotelial de moléculas de adhesión, vasodilatación y permeabilidad de los vasos causando hinchazón (Silva, 2010). Si después de la eliminación del daño la inflamación restaura las condiciones fisiológicas normales, esa respuesta se denomina inflamación aguda (Kumar, 2018). En caso de que esta respuesta se mantenga a largo plazo se denomina inflamación crónica y los neutrófilos se desgranulan, los linfocitos se activan y los fibroblastos liberan mediadores que inducen más infiltración (Huether and McCance, 2017). El aumento de los casos de enfermedades inflamatorias crónicas como la artritis reumatoide, la diabetes o la psoriasis han convertido a estas enfermedades en una de las mayores causas de morbilidad y mortalidad en los países desarrollados.

Entre estas enfermedades destaca la psoriasis que acepta al 3% de la población mundial y que tiene una etiología compleja y no totalmente conocida (Lebwohl, 2003). La psoriasis se puede manifestar de diferentes formas (Lebwohl, 2018; Raposo and Torres, 2016; Renton, 2014; Syed and Khachemoune, 2011), pero su fisiopatología está caracterizada por un incremento anormal en la proliferación de los queratinocitos causando descamación y eritema y una gran infiltración de células inmunitarias en las diferentes capas de la piel causando picor, quemazón y dolor (Ippagunta *et al.*, 2016). Se ha propuesto que los queratinocitos son activados y liberan mediadores inflamatorios y moléculas señalizadoras, como las interleuquinas IL17 e IL23, que reclutan neutrófilos, macrófagos y mastocitos que amplifican la inflamación y desencadenan la enfermedad (Albanesi *et al.*, 2018; Candel *et al.*, 2014; Schubert and Christophers, 1985). A pesar de que se han desarrollado diferentes tratamientos para la psoriasis, como los anticuerpos frente a sus principales interleuquinas (Malakouti *et al.*, 2015; Paek *et al.*, 2018; Reich *et al.*, 2015) o mediante fototerapia (Zhang and Wu, 2018), solo son efectivos de manera temporal y ninguno de ellos se ha demostrado eficaz a largo plazo.

Por esta razón es necesaria la investigación de nuevas dianas terapéuticas y posibles tratamientos. Para ello se han utilizado diferentes modelos *in vitro*, *ex vivo* e *in vivo* (Bochenska *et al*., 2017; Danilenko, 2008; Hawkes *et al.*, 2018; Wcislo-Dziadecka *et al.*, 2018). La mayor parte de los estudios *in vivo* en modelos animales se han hecho en alguno de los más de cuarenta modelos de psoriasis que se han descrito en ratón (Hawkes *et al.*, 2018), pero no todos ellos han sido correctamente validados y algunos manifiestan diferentes enfermedades de la piel (Swindell *et al.*, 2017a). En este estudio se han utilizado como modelos cultivos de queratinocitos humanos y el pez cebra.

El pez cebra es un pez de agua dulce de pequeño tamaño, tiene un gran número de descendencia cada semana, lo que permite tener mayor número de individuos por experimento y aumenta la reproducibilidad (Meshalkina *et al.*, 2017), se desarrolla rápidamente de forma externa, pudiendo modificar genéticamente de manera sencilla el embrión desde el estado de una sola célula (Ablain *et al.*, 2018), y tras la fertilización y durante las primeras etapas del desarrollo sus larvas son transparentes, lo que permite el seguimiento *in vivo* de diferentes moléculas o tipos celulares. El genoma del pez cebra está completamente secuenciado y comparte un 71.4% con el genoma humano (Howe *et al.*, 2013; Shim *et al.*, 2016). La estructura de la piel es similar a la de humanos (Le Guellec *et al.*, 2004) y estudios previos han mostrado que el pez cebra puede ser un buen modelo para estudiar inflamación (de Oliveira *et al.*, 2015; de Oliveira *et al.*, 2014) y existen diferentes líneas de pez cebra como modelos de psoriasis

(Candel *et al.*, 2014; Carney *et al.*, 2007; Dodd *et al.*, 2009; Mathias *et al.*, 2007; Sonawane *et al.*, 2005; Webb *et al.*, 2008; Zimmermann *et al.*, 2008).

Este trabajo se centra en el estudio del posible papel de la vitamina B6 en el desarrollo de la psoriasis. La vitamina B6 es una vitamina soluble que incluye diferentes vitámeros (Coburn, 1996), cuya forma más activa es PLP, que los humanos obtienen de la dieta y de la microbiota (Said, 2015). Debido a su característica estructura química, la vitamina B6 es capaz de disminuir las especies reactivas de oxígeno *in vitro* (Kannan and Jain, 2004), puede ayudar en el plegamiento de determinadas enzimas (Cellini *et al.*, 2014) y es un quelante de metales (Wondrak and Jacobson, 2012). Aunque la deficiencia de vitamina B6 no es común en los países desarrollados, bajos niveles de esta se asocian con gran variedad de enfermedades (Cheng *et al.*, 2008; Chiang *et al.*, 2005; Dalery *et al.*, 1995; Eussen *et al.*, 2010; Friedman *et al.*, 2004; Hron *et al.*, 2007; Johansson *et al.*, 2014; Kelly *et al.*, 2003; Larsson *et al.*, 2010; Le Marchand *et al.*, 2011; Lurie *et al.*, 2012; Roubenoff *et al.*, 1995; Selhub *et al.*, 2013; Verhoef *et al.*, 1996; Wu *et al.*, 2013).

La deficiencia de vitamina B6 se ha descrito en diferentes estudios como un factor que altera la respuesta inmunitaria y su suplementación restaura la condición homeostática (Casciato *et al.*, 1984; Dobbelstein *et al.*, 1974; Sorice *et al.*, 1980; Talbott *et al.*, 1987). PLP normalmente está inversamente asociado con diferentes marcadores de inflamación, como la proteína reactiva C entre otros, en clínica (Friso *et al.*, 2001; Sakakeeny *et al.*, 2012). Se ha descrito que la inflamación aguda puede causar un incremento de la vitamina B6 en los tejidos afectados y un incremento de su catabolismo para formar PA (Ulvik *et al.*, 2012). La psoriasis, como otras enfermedades inflamatorias, tiene diferentes comorbilidades ente las que destaca el síndrome metabólico (Boehncke *et al.*, 2011). Un estudio enfocado en el síndrome metabólico asociado a psoriasis señala a la vitamina B6 como un componente protector (Romani *et al.*, 2013). Toda esta información indica que la vitamina B6 podría estar involucrada en el desarrollo de diferentes enfermedades inflamatorias incluida la psoriasis.

Utilizando datos transcriptómicos disponibles de estudios previos sobre psoriasis en muestras de piel lesionada y no lesionada, encontramos una expresión alterada en todos los transcritos de las enzimas involucradas en el metabolismo de la vitamina B6 en piel de psoriasis lesionada y algunos de ellos correlacionaban con un marcador de inflamación. Además, analizando los niveles en suero sanguíneo de los diferentes vitámeros de la vitamina B6 y sus productos de catabolismo demostramos que los pacientes de psoriasis tienen una movilización

de PL mayor, probablemente para llegar al área afectada, mientras que el catabolismo de vitamina B6 a PA está reducido en suero de pacientes de psoriasis. Además, el índice PAr, PA(PL/PLP), correlaciona con el PASI. Este es uno de los descubrimientos más importantes en este estudio porque demuestra que PAr podría ser utilizado clínicamente como medida de la severidad de la enfermedad.

Estos resultados muestran que el metabolismo vitamina B6 está alterado en los pacientes de psoriasis tanto sistémica como localmente, señalando a la vitamina B6 como marcador de la severidad de la psoriasis y posible tratamiento para la misma. Para discernir el posible efecto beneficioso de la vitamina B6 en situación de inflamación, se utilizaron distintos modelos de psoriasis generados en pez cebra. Debido a las características especiales para toma de imágenes *in vivo* del pez cebra, utilizando diferentes modelos preclínicos de psoriasis vimos que la adición exógena de vitamina B6 mejora el estrés oxidativo y la inflamación, reduciendo la infiltración de neutrófilos en la piel que es una de las características de las lesiones de psoriasis, disminuyendo los niveles de H_2O_2 en la piel y la activación de NF-κB. Estos resultados muestran el impacto beneficioso de la suplementación con vitamina B6 cuando hay condiciones de inflamación, pero no demuestra si este efecto se debe a la acción directa la vitamina B6 o si alguna de las rutas dependientes de vitamina B6 es la responsable.

Paradójicamente, en inflamación el catabolismo de la vitamina B6 está normalmente incrementado mostrando mayor PAr, pero encontramos que en psoriasis este está disminuido en suero, aunque puede ser debido a una compensación por los mayores niveles de PL. Normalmente AOX1 degrada vitamina B6 a PA cuando hay estrés oxidativo porque el gen *AOX1* está regulado por el factor transcripcional NRF2 (Maeda *et al.*, 2012), pero en la piel lesionada de psoriasis *AOX1* se está expresando por debajo de lo normal. Esta situación puede ser clave, mostrando que el efecto de la vitamina B6 en psoriasis es debido a rutas dependientes de vitamina B6 en lugar de a sus características físicas o químicas.

Analizando los niveles transcriptómicos de alrededor de 100 genes que cifran enzimas dependientes de PLP en piel de controles sanos y de pacientes de psoriasis lesionada y no lesionada, encontramos que el 60% de ellos presentan una expresión alterada en pacientes de psoriasis y, aún más relevante, el 21% correlaciona con la expresión de *IL1B* en la piel lesionada de psoriasis. Algunas de estas enzimas están involucradas en rutas que pueden tener un gran impacto en el organismo. Por ejemplo, la ruta de la quinurenina que es la principal responsable del catabolismo del triptófano y puede tener efectos inmunomoduladores (Wang

et al., 2015; Yeh and Brown, 1977). Otra de las rutas es la involucrada en la producción de H_2S, que es un mediador gaseoso con propiedades proinflamatorias a altas concentraciones y efectos antiinflamatorios a bajas concentraciones (Bhatia, 2012; Gemici and Wallace, 2015; Wallace and Wang, 2015; Whiteman and Winyard, 2011).

Pero centramos nuestra atención en la ruta de degradación del glucógeno que tiene dos enzimas dependientes de PLP, PYGL y G6PD, que tienen aumentados sus niveles de transcritos en la piel lesionada de psoriasis y correlacionan con la expresión de *IL1B*. Estudios clínicos previos han descrito que las lesiones de psoriasis acumulan glucógeno (Halprin *et al.*, 1973; Harmon and Phizackerley, 1984; Sasai, 1970; Stankler and Walker, 1976), por lo que esta ruta podría estar relacionada con el desarrollo de la enfermedad. Cuando se analizaron las muestras de piel se encontraron niveles reducidos de proteína PYGL en pacientes de psoriasis y similares niveles de G6PD. Estos resultados no son completamente coherentes con los anteriores, pero podrían estar mostrando una expresión alterada de alguno de los componentes de la ruta. Además, la actividad enzimática de PYGL se regula mediante fosforilación. Para clarificar si esta ruta puede estar implicada en el proceso de inflamación, decidimos bloquear PYGL con un inhibidor específico en un modelo de psoriasis en pez cebra y encontramos que la mayoría de los marcadores de inflamación evaluados mejoraban cuando se aplicaba el inhibidor de PYGL. Se obtuvieron resultados similares cuando se inhibió PYGL en cultivos celulares de queratinocitos humanos.

La degradación del glucógeno es una de las fuentes más importantes de glucosa y la privación de esta puede llevar a la disminución de la proliferación de los queratinocitos. Pero además esta ruta termina con la acción de G6PD liberando NADPH, que es uno de los sustratos necesarios para las NADPH oxidasas, como DUOX1, para producir H_2O_2. Bloqueando esta ruta, podríamos estar afectando a ambas reacciones. Para discernir cuál de ellas tiene un mayor impacto hace falta realizar más experimentos.

Los resultados de esta tesis señalan a la vitamina B6 como un factor importante en el desarrollo de psoriasis en humanos. Confirmando el papel antiinflamatorio de la vitamina B6 en diferentes modelos de psoriasis y mostrando que la vitamina B6 podría ser considerada como un tratamiento clínico frente a enfermedades inflamatorias de la piel como, por ejemplo, la psoriasis. Además, el PAr podría ser utilizado como un marcador de la gravedad de la psoriasis.

ANNEXE I

Participation in publications during the PhD

1. **Martinez-Navarro, F.J.**, Martinez-Menchon, T., Mulero, V., Galindo-Villegas, J., 2019. Models of human psoriasis: Zebrafish the newly appointed player. Dev Comp Immunol 97, 76-87.

2. Tyrkalska, S. D., Pérez-Oliva, A. B., Rodríguez-Ruiz, L., Martinez-Morcillo, F.J., Alcaraz-Pérez, F., **Martinez-Navarro, F.J**, Lachaud, C., Ahmed N., Schroeder T., Pardo-Sánchez, I., Candel, S., Lopez-Muñoz, A., Choudhuri, A., Rossman, M.P., Zon, L., Cayuela, M.L., Garcia-Moreno, D., Mulero, V., 2019. Inflammasome Regulates Hematopoiesis through Cleavage of the Master Erythroid Transcription Factor GATA1. Immunity, 51(1), 50-63.e5.

3. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., de Oliveira, S., Candel, S., Cabas I., García-Ayala, A., Martínez-Menchón, T., Corbalán-Vélez, R., Mesa del Castillo, P., Cayuela, M.L., Perez-Oliva, A.B., Garcia-Moreno, D., Mulero, V., 2019. Hydrogen peroxide in neutrophil inflammation: Lesson from the zebrafish. Antioxidant & Redox Signaling (under review)

ANNEXE II

Contributions to scientific during the PhD

1. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Vitamin B6 reduces skin inflammation in a zebrafish model of psoriasis. 39 Congreso de la Sociedad Española de Inmunología, Alicante (Spain). 5-7 May 2016. Oral communication.

2. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Impact of oxidative stress in psoriasis: A role for XHD and NRF2. 39 Congreso de la Sociedad Española de Inmunología, Alicante (Spain). 5-7 May 2016. Poster.

3. García-Moreno, D., Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Martínez-Menchón, T., Corbalán-Vélez, R., Peñalver-Meseguer, J., Mulero, V. A link between NAD^+ biosynthesis and psoriasis. 39 Congreso de la Sociedad Española de Inmunología, Alicante (Spain). 5-7 May 2016. Poster.

4. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Martínez-Menchón, T., Corbalán-Vélez, R., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. A crucial role of NAD^+ metabolites in the regulation of skin inflammation. 40 Congreso de la Sociedad Española de Inmunología, Zaragoza (Spain). 25-27 May 2016. Poster.

5. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Vitamin B6 decreases skin inflammation in a zebrafish model of psoriasis. II Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 31 May-2 June 2016. Poster.

6. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Impact of oxidative stress in psoriasis: A role for XHD and NRF2. II Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 31 May-2 June 2016. Poster.

7. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Vitamin B6 decreases skin inflammation in a zebrafish model of psoriasis. IV Jornadas de Inicio a la Investigación en Biología, Murcia (Spain). 8-23 June 2016. Oral communication.

8. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Vitamin B6 decreases skin inflammation in a zebrafish model of psoriasis. 10th Annual Congress of the Spanish Federation of Biotechnologists, Asturias (Spain). 13-15 July 2016. Oral communication.

9. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. A link between vitamin B6 and H_2S in oxidative stress-induced skin inflammation. I Jornada de Investigación del IMIB-Arrixaca, Murcia (Spain). 21 November 2016. Oral communication.

10. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Martínez-Menchón, T., Corbalán-Vélez, R., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. A preclinical zebrafish psoriasis model reveals that vitamin B6 and H2S are involved in skin inflammation. 40 Congreso de la Sociedad Española de Inmunología, Zaragoza (Spain). 25-27 May 2017. Poster.

11. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. A crucial role of NAD+ metabolites in the regulation of skin inflammation. 40 Congreso de la Sociedad Española de Inmunología, Zaragoza (Spain). 25-27 May 2017. Poster.

12. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Vitamin B6 reduces skin inflammation in a zebrafish model of psoriasis. III Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 30 May-1 June 2017. Oral communication.

13. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. NAD+ metabolites regulate skin inflammation. III Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 30 May-1 June 2017. Oral communication.

14. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Valera-Pérez, A., Corbalán-Vélez, R., Martínez-Menchón, T., Peñalver-Meseguer, J., … Mulero, V. Modelling chronic inflammatory and infectious diseases using the zebrafish. Congreso Nacional de Biotecnología (Biotec 2017), Murcia (Spain). 18- 21 June 2017. Oral communication.

15. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Martínez-Menchón, T., Corbalán-Vélez, R., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. Preclinical zebrafish inflammation models reveal that vitamin B6 and H2S are involved in psoriasis skin inflammation. 11th Annual Biotechnology Congress (BAC), León (Murcia). 12-14 July 2017. Poster.

16. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Martínez-Menchón, T., Corbalán-Vélez, R., Peñalver-Meseguer, J., García-Moreno, D., Mulero, V. A preclinical zebrafish inflammation models show that vitamin B6 and H2S are involved in psoriasis. II Jornadas Científicas del IMIB-Arrixaca, Murcia (Spain). 27 November 2017. Comunicación oral.

17. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Vitamin B6 plays an essential role in psoriasis. IV Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 29-31 May 2018. Oral communication.

18. Fatas-Lalana, B., **Martínez-Navarro, F.J.**, Martínez-Menchón, T., Corbalán-Vélez, R., Garcia-Moreno, Pérez-Oliva, A. B., D., Mulero, V. Modeling the role of Treg and IL-17D in psoriasis. IV Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 29-31 May 2018. Oral communication.

19. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Pharmacological inhibition of Nampt for skin inflammatory disorder treatment. IV Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 29-31 May 2018. Oral communication.

20. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Vitamin B6 plays a crucial role in psoriasis. 11th Zebrafish Disease Models (ZDM11), Leiden (Holland). 10-13 July 2018. Poster, Flash talk and oral communication in RIG Workshop.

21. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Pharmacological inhibition of Nampt ameliorates skin inflammation in a preclinical zebrafish model. 11th Zebrafish Disease Models (ZDM11), Leiden (Holland). 10-13 July 2018. Oral communication.

22. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Vitamin B6 and H_2Splay a crucial role in psoriasis. III Jornadas Científicas del IMIB-Arrixaca, Murcia (Spain). 19-20 November 2018. Poster.

23. Martínez-Morcillo, F.J., **Martínez-Navarro, F.J.**, Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Nampt enzymatic activity inhibition restores epithelial integrity and skin inflammation in a preclinical zebrafish model. III Jornadas Científicas del IMIB-Arrixaca, Murcia (Spain). 19-20 November 2018. Oral communication.

24. Pérez-Oliva, A. B., Rodriguez-Ruiz, L., Tyrkalska, S., **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Pardo-Sanchez, I., Alcaraz-Perez, F., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. The inflammasome regulates hematopoiesis through cleavage of the erythroid transcription factor GATA1. III Jornadas Científicas del IMIB-Arrixaca, Murcia (Spain). 19-20 November 2018. Oral communication.

25. **Martínez-Navarro, F.J.**, Martínez-Morcillo, F.J., Corbalán-Vélez, R., Martínez-Menchón, T., Pérez-Oliva, A. B., Zon, L.I., Cayuela, M.L., Garcia-Moreno, D., Mulero, V. Vitamin B6 and H_2S play an essential role in psoriasis. V Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 29-31 May 2019. Oral communication.

ANNEXE III

Research stays in other laboratories during the PhD

Host institution:

Australian Regenerative Medicine Institute. Monash University. Clayton, Melbourne, Australia.

Responsable person in the Host:

Graham Lieschke.

Stay period:

From: January 14th, 2019.

To: May 1st, 2019.

Duration: 15 weeks and 3 days.