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Role of Neutrophils in the Imiquimod (IMQ)-induced mouse model of Psoriasis

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Abstract

Psoriasis is a chronic skin disease associated with deregulated interplays between immune cells and keratinocytes. Neutrophil accumulation in the skin is one of the histological features that characterize psoriasis. However, the role of neutrophils in psoriasis development remains poorly understood. In this study, we utilized the imiquimod (IMQ)-induced mouse model of psoriasis to elucidate the specific contribution of neutrophils to psoriasis development. We report that neutrophils act as negative modulators of disease propagation and exacerbation by inhibiting $\gamma\delta$ T cell effector functions *via* NADPH oxidase-mediated reactive oxygen species (ROS) production, as revealed by analysing disease development/progression in neutrophil-depleted mice. We also report that Syk functions as crucial molecule mediating neutrophil and $\gamma\delta$ T cell interactions. In support of the latter findings, we demonstrate that the selective impairment of Syk-dependent signalling in neutrophils only, is sufficient to reproduce the enhancement of skin inflammation and $\gamma\delta$ T cell infiltration observed in neutrophil-depleted mice. Overall, our findings add new insights into the specific contribution of neutrophils to disease progression in the IMQ-induced mouse model of psoriasis. Considering that, similarly to mouse psoriasis, the important role of IL-17 producing $\gamma\delta$ T cells in human psoriasis has just started to emerge, it is likely that inhibitory crosstalk between neutrophils and $\gamma\delta$ T cells may exist also in human psoriasis. Neutrophils may indeed act as unexpected negative players of disease development in specific types or clinical stages of human psoriasis. Consequently, also the utilization of therapeutic interventions targeted to inhibit neutrophil functions should be carefully evaluated.

Sommario

La psoriasi è un'inflammatione cronica e recidivante della pelle, caratterizzata da un'alterata interazione tra sistema immunitario e cheratinociti. Nonostante l'infiltrazione dei granulociti neutrofili nella pelle sia una peculiarità della patologia psoriatica, il ruolo di queste cellule nella psoriasi non è ancora ben noto. In questa tesi, per studiare il ruolo dei neutrofili nella psoriasi abbiamo utilizzato un modello murino di psoriasi indotta dall'applicazione topica di imiquimod (IMQ), insieme alla somministrazione di un anticorpo specifico per la deplezione dei neutrofili. I risultati ottenuti in questo studio, hanno mostrato che i neutrofili possono interferire con la progressione della patologia inibendo in maniera specifica i linfociti T $\gamma\delta$, principali mediatori di inflammatione in questo modello sperimentale di psoriasi. I dati da noi ottenuti dimostrano che i neutrofili inibiscono la proliferazione e la produzione di IL-17 da parte dei linfociti T $\gamma\delta$ tramite la produzione di specie reattive dell'ossigeno (ROS), indotta dall'attivazione del complesso enzimatico NADPH ossidasi. Inoltre, abbiamo dimostrato che tale meccanismo inibitorio è contatto dipendente e richiede l'interazione specifica neutrofilo-linfocita T $\gamma\delta$. Infine, abbiamo identificato la chinasi Syk quale molecola cruciale implicata nella modulazione di questa capacità dei neutrofili di inibire i linfociti T $\gamma\delta$. Infatti, l'assenza specifica di Syk nei neutrofili è risultata sufficiente per riprodurre il fenotipo osservato con la deplezione completa dei neutrofili nel modello di psoriasi indotta da IMQ. L'insieme dei dati ottenuti contribuiscono quindi alla comprensione del ruolo del neutrofilo nel modello murino di psoriasi indotta dall'applicazione topica di imiquimod. Come per il modello murino, la possibile rilevanza patogenetica dei linfociti T $\gamma\delta$ sta emergendo anche nella psoriasi umana. E' quindi ipotizzabile che interazioni inibitorie tra i neutrofili e i linfociti T $\gamma\delta$ possano essere importanti anche nello sviluppo di psoriasi nell'uomo. I neutrofili potrebbero infatti esercitare un inaspettato ruolo di modulazione negativa nell'evoluzione dell'inflammatione psoriatica. Conseguentemente, l'utilizzo di terapie mirate all'inibizione delle funzioni effettrici dei neutrofili nella psoriasi dovrebbe essere valutato con cautela.

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1. Introduction

Psoriasis: general features

1.1 Epidemiology

Psoriasis is a common immune-mediated chronic skin disease that occurs in genetically predisposed individuals with an overall prevalence of 2% to 3% of the world's population (Christophers 2001). Psoriasis exhibits a bimodal distribution with a peak between 15 and 30 years and another between 50 and 60 years (Raychaudhuri & Gross 2000). Crucial traits of psoriasis, such as the high prevalence, chronicity, comorbidity and the resulting visible impact on physical appearance, make it a disorder with both a physical and psychological burden (Kimball et al. 2005). Individuals with psoriasis have indeed also an increased risk of developing other chronic and serious health diseases. These so-called comorbid diseases include psoriatic arthritis, the metabolic syndrome, cardiovascular disorders, as well as numerous other diseases such as anxiety/depression, non-alcoholic fatty liver disease, Crohn's disease, or lymphoma (Griffiths & Barker 2007; Nestle et al. 2009).

1.2 Histological hallmarks

Psoriasis is characterized by different clinical phenotypes and its classification is complex and it is therefore out of the scope of this thesis. Chronic plaque psoriasis (psoriasis vulgaris) is the most common form of the disease, accounting for about 90% of cases (Boehncke & Schön 2015). Chronic plaque psoriasis typically manifests with sharply demarcated chronic erythematous plaques covered by silvery white scales, which most commonly appear on the elbows, knees, scalp, umbilicus, and lumbar area. Histopathological features of psoriatic skin include: epidermal acanthosis (thickening of the viable epidermis layers due to an abnormal keratinocytes turnover), hyperkeratosis (thickened cornified layer), parakeratosis

(retention of nuclei in the stratum corneum) and exaggerated angiogenesis. There is also an inflammatory infiltrate within the skin layers consisting of T-lymphocytes, dendritic cells (DCs), macrophages, mast cells and neutrophils. The latter cells are responsible for the formation of subcorneal micro-abscesses, also referred to as Munro's microabscesses.

1.3 Triggering factors

Psoriasis is a chronic autoimmune skin disease, whose multifactorial pathogenesis is thought to result from a combination of genetic, environmental and immunologic factors (Deng et al. 2016).

1.3.1 Genetic triggers

The high concordance rates found in twins and families affected by psoriasis support the evidence for a strong genetic component in psoriatic disease (Bowcock 2005). Initial linkage analysis of several affected families allowed the identification of at least 12 **psoriasis-susceptibility** (PSORS) loci (Pasić et al. 2009). More recently, genome-wide association studies (GWAS) focused on these large genome regions unveiled susceptibility genes carrying single nucleotide polymorphisms (SNPs). A significant percentage of susceptibility genes is related to the immune system, spanning an array of functions that involve antigen presentation (HLA-Cw6, ERAP1, ERAP2, MICA), the IL-23 axis (IL12Bp40, IL23Ap19, IL23R, JAK2, TYK2), T-cell development and T-cells polarization (RUNX1, RUNX3, STAT3, TAGAP, IL4, IL13), innate immunity (CARD14, c-REL, TRAF3IP2, DDX58, IFIH1) and negative regulators of immune responses (TNIP1, TNFAIP3, NFKBIA, ZC3H12C, IL36RN, SOCS1) (Tsoi et al. 2012).

1.3.2 Environmental triggers

Genetics is only a part of the pathogenesis. Indeed, without certain environmental triggers, epigenetic modification and inflammatory responses, people with high genetic susceptibility may still fail to develop psoriasis, even though they are at significantly higher risk. Psoriasis is indeed known to be either triggered or

exacerbated also by non-specific exogenous factors, such as mild physical trauma (tattoos, sunburns, scratching) (Sagi & Trau 2011). Strong evidence also exists for psoriasis onset after tonsillar *Streptococcus pyogenes* infection (Gudjonsson et al. 2003). The use of various drugs, such as lithium, β -blockers, antimalarial agents, non-steroidal anti-inflammatory drugs, and angiotensin-converting enzyme inhibitors, has also been associated with onset or worsening of disease in psoriatic patients (Basavaraj et al. 2010). In addition, a possible association between vaccination and the new onset and/or exacerbation of psoriasis has been reported recently (Gunes et al. 2015).

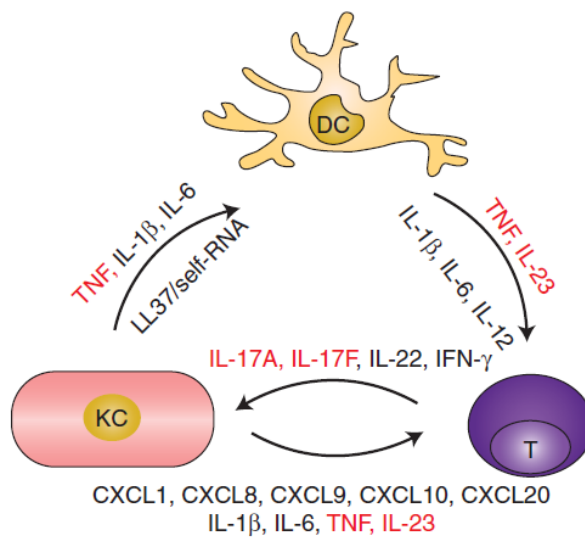
1.3.3 Epigenetics in psoriasis

Increasing data suggest an emerging role of epigenetics in psoriatic disease as a plausible link between environmental exposure and psoriasis development. In this context, methylation of genes involved in the modulation of cell proliferation and apoptosis, such as SHP-1 and p16 respectively, was found abnormal in patients with psoriasis compared to healthy controls (Chen et al. 2008), (Ruchusatsawat et al. 2006). Abnormal expression of histone acetyltransferases (HATs) and histone deacetylases (HDACs) has also been observed in psoriatic skin samples (Tovar-Castillo et al. 2007). Zhang P and colleagues demonstrated that hypoacetylation of histone H4 is observed in peripheral blood of psoriatic patients, and that the degree of hypoacetylation of histone H4 inversely correlates to disease severity (Zhang et al. 2011). Importantly, emerging evidence has indicated that HDAC inhibitors (HDAC-Is) can be used in the treatment of psoriasis (McLaughlin & La Thangue 2004). Another epigenetic mechanism found to play a role in the pathogenesis of psoriasis is RNA-associated silencing (Sonkoly et al. 2007).

1.4 Pathogenesis

In the past psoriasis was believed to be a disease primarily caused by intrinsic alterations of epidermal keratinocyte proliferation. Around 1980, however, several observations supported the central role of immune cells in the pathogenesis of psoriasis (Bos et al. 2005). Genetic data on human leukocyte (HLA) associations with psoriasis development, as well as data on the presence of oligoclonal T cells

in human psoriatic skin, further underline the importance of the immune system in psoriasis pathogenesis (Eberle et al. 2016). Loss of tolerance against putative auto-antigens, including keratins, the antimicrobial peptide LL37, heat shock proteins and the melanocytic antigen ADAMTSL5-like protein 5 (ADAMTSL5), have also been recognized as an important factor contributing to disease pathogenesis (Besgen et al. 2010; Arakawa et al. 2015). For many years, psoriasis was considered a Th1-type disease in which IFN- γ and TNF were the predominant pathogenic driver cytokines. More recently, deregulated axis involving the overproduction of IL-23, and the consequent activation of IL-17 producing T cell subsets (T17), has instead emerged as the central immune pathway driving psoriasis development (Zaba et al. 2009). IL-23 expressed by inflammatory DCs drives Th17 development and expansion. In turn, Th17-driven IL-17A and IL-22 act on keratinocytes to induce CC chemokine 20 (CCL20) and anti-microbial peptides production (Wilson et al. 2007) which sustains skin inflammation. This inflammatory loop is then amplified by the capability of keratinocyte-derived CCL20 to further recruit CCR6-bearing Th17 cells, which, in turn, further activate keratinocytes to produce CCL20 in a positive feedback manner (Chiricozzi et al. 2011). The overproduction of other inflammatory cytokines, such as IL-1, IL-36, TNF α and IL-22, is also known to trigger pivotal pathogenic pathways in human psoriasis (Johnston et al. 2017; Johnston & Gudjonsson 2014) (**Figure 1**).



From Di Meglio et al, 2014

Figure 1. Psoriasis immunopathogenesis. A pathogenic crosstalk between innate and adaptive immune cells sustained by pro-inflammatory mediators, underlies the deregulated immune response seen in psoriasis. The three main cellular players and their products are depicted in this diagram. Keratinocytes (KCs) produce key cytokines, while activated DCs present yet undefined antigens and secrete mediators leading to the differentiation and activation of IL-17-producing T cells (T, here representing both $\alpha\beta$ and $\gamma\delta$ T cells). T cells, in turn, secrete cytokines that activate KC aberrant differentiation program and induce the production of further pro-inflammatory mediators, especially chemokines (CXCL1, CXCL8) recruiting neutrophils (not shown) or other immune cells (CXCL9, CXCL10, CCL20), as well as other antimicrobial peptides (AMPs) (not shown). Critical pro-inflammatory molecules, effectively targeted by biologic drugs, are shown in red.

Among the cellular mediators, today there is a widely accepted consensus that psoriatic lesions originate as a result of a deregulated crosstalk between keratinocytes and resident cutaneous immune cell types belonging to both the adaptive and the innate branch of the immune system. However, despite the large experimental knowledge already existing on psoriasis pathogenesis, there is still an on-going debate on which is/are the immune cell type/s primarily involved in disease initiation and progression. What is now emerging is that psoriasis is a dynamic disease in which specific cell types can dominate over the others and sustain the mounting inflammation during the different phases of disease, including disease development, progression and maintenance. As anticipated above, the crucial role of DCs (Jariwala 2007) and T17 [including T helper 17 (Th17) and, to a lower extent, $\gamma\delta$ T cells] (Diani et al. 2015; Papotto et al. 2017) has been widely studied in psoriatic patients as well as in preclinical models of psoriasis (Cai et al. 2011; C. Wohn et al. 2013). By contrast, the role of myeloid cells in diseases pathogenesis (such as neutrophils, monocytes and macrophages), which are also known to infiltrate the psoriatic plaques and to display abnormal functions in psoriatic patients, is less well characterized (Deng et al. 2016; Coimbra et al. 2012; Schön et al. 2017). Focus of this thesis has been to better characterize the role of neutrophils in psoriasis pathogenesis. Particular attention has been dedicated to better understand the ability of these cells to modulate the IL-23/T17 axis and $\gamma\delta$ T cells (an innate-like T cell type of IL-17 producing T cells) during psoriasis development.

1.4.1 IL-23/IL-17A axis

IL-23 is a heterodimer of a unique IL-23p19 and shared IL-12/23p40 chains produced by both resident dermal DCs and inflammatory mDCs, as well as macrophages in psoriasis (Zaba et al. 2009). A broad bunch of evidence suggests a pathogenic role for IL-23 in psoriasis. Indeed, expression of IL-23p19, IL-23p40 and IL-23R is increased in psoriasis lesional skin (Tonel et al. 2010), along with an increased number of Th17 cells in the dermis (Lowe et al. 2008) and in the blood. Moreover intradermal injection of IL-23 in mouse skin resulted in erythema with psoriasis-like histological features (Chan et al. 2006). The clinical success of biologic drugs targeting IL-12/IL-23p40 confirms the importance of this cytokine and its associated pathways, in psoriasis. In fact, IL-23 represents a crucial mediator in psoriatic inflammation, driving the development of IL-17 and IL-22 producing Th17 cells. Importantly, IL-23 along with its receptor and downstream transcription factor STAT3, are all genes that are associated with increased susceptibility to psoriasis development. Of note, activation of STAT3 and ROR $\gamma\delta$, another Th17 characterizing transcription factor, is required for IL-17A and IL-17F expression. Th17, which largely infiltrate psoriatic skin dermis in humans, represent an important source of IL-17A, IL-17F, and IL-22 (Ortega et al. 2009; Cai et al. 2011). IL-17A and IL-17F share high structural and functional homology and primarily activate KCs to produce leukocyte recruiting chemokines, AMPs and many inflammatory mediators. IL-17A/F can also mobilize neutrophils, working as a link between adaptive and innate immunity (Pelletier et al. 2010), and further create a self-sustaining loop through IL-17A induced production of CCL20 by KCs. Besides Th17 cells, IL-17A and/or IL-22 are produced by other cell types of immune cells identified in psoriatic skin, including innate lymphoid cells (ILCs)3 and $\gamma\delta$ T cells (Chien et al. 2014; Artis & Spits 2015).

1.4.2 $\gamma\delta$ T Cells

$\gamma\delta$ T cells account for approximately 3-5% of all lymphoid cells found in the secondary lymphoid tissue and the blood whereas they are highly enriched in different epithelial tissues, such as the intestinal mucosa, the skin and the reproductive tract. These cells represent, therefore, the first line of defence against

pathogens by providing a rapid cytokine response. $\gamma\delta$ T cells are the first immune cells found in the fetus and provide immunity to new-borns prior to activation of the adaptive immune system (Sinkora et al. 2005). While $\alpha\beta$ CD4⁺T cells mostly leave the thymus as 'naive' cells that differentiate upon activation in the periphery, $\gamma\delta$ T lymphocytes are generated in the thymus from CD27⁺ $\gamma\delta$ -cell-committed progenitor, and acquire their effector function during thymic development (Muñoz-Ruiz et al. 2017). After functional rearrangement and expression of the γ and δ -TCR chains, these cells become reprogrammed depending on the TCR signalling strength: strong agonists lead differentiation towards an IFN- γ -producing lineage, which can be identified on the basis of the expression of the co-stimulatory receptor CD27 (Haas et al. 2009). In contrast low TCR signalling leads to the development of IL-17-producing $\gamma\delta$ T cells expressing ROR $\gamma\delta$ (Th17-lineage transcription factor) and other surface receptors including CD127 (IL7R) and CCR6 (Kisielow et al. 2008).

Several chemokine receptors, cytokine receptors and pattern recognition receptors (PRRs) are expressed by $\gamma\delta$ T cells and have been showed to be involved in their activation. Production of IL-23, IL-1 and IL-18 by activated DCs and macrophages promotes IL-17 production by $\gamma\delta$ T cells (Sutton et al. 2009).

Despite the fact that the activation of $\gamma\delta$ T cells relies mainly on innate cytokines, it appears to be also regulated and 'fine-tuned' by various accessory receptors such as programmed cell death protein 1 (PD-1), the B and T lymphocyte attenuator (BTLA) and the co-stimulatory receptor CD28 (Ribot et al. 2012).

Mouse skin harbours distinct subsets of $\gamma\delta$ T cells which differ according to the skin layer. In the epidermis there is a large number of $\gamma\delta$ T cells which, do to their marked dendritic morphology, have been named dendritic epidermal T cells (DECT) (Havran & Jameson 2010). DECT express the canonical V γ 5V δ 1 TCR and participate in tissue surveillance and wound healing producing keratinocyte growth factors and IFN- γ (Jameson et al. 2002). In the dermis, the V γ 4⁺ and V γ 6⁺ T cells are the most represented subsets and are responsible for pathogenic inflammatory responses mediated by the production of IL-17 (O'Brien & Born 2015). Moreover dermal $\gamma\delta$ T cells, but not epidermal $\gamma\delta$ T cells, constitutively express CCR6 and ROR $\gamma\delta$ (Cai et al. 2011). Among dermal $\gamma\delta$ T cells, resident

V γ 6⁺ cells are long-lived, self-renewing, mostly radio-resistant cells that require a source of fetal thymocytes for reconstitution. On the contrary, infiltrating V γ 4⁺ cells migrate from the lymph nodes, are radiosensitive and can be reconstituted from bone marrow progenitors (Haas et al. 2012; Roark et al. 2007). Dermal $\gamma\delta$ T cells have been shown to proliferate and secrete IL-17A, IL-17F and IL-22 when cultured in the presence of IL-23 and IL-1 β (Cai et al. 2011; Gray et al. 2011). This pathogenic signature is critical for triggering the formation of psoriasiform plaques in mice, as genetic deletion of these cytokines substantially diminishes the disease severity in the imiquimod (IMQ)-induced mouse model of psoriasis (see IMQ mouse model below) (Pantelyushin et al. 2012). Furthermore, Cai Y. and colleagues showed that skin pathology was diminished in *Tcrd*^{-/-} (T-cell receptor delta chain knock-out) mice treated with IMQ as opposed to *Tcra*^{-/-} (T-cell receptor $\alpha\beta$ knock-out) and wild-type mice, suggesting that dermal $\gamma\delta$ T cells play a major role in driving the IMQ-induced skin inflammation (Cai et al. 2011). Using the same animal model, Ramirez-Valle F. and colleagues found an expansion of a population of V γ 4⁺ V δ 4⁺ T cells that was mostly responsible for IL-17 production. IL-17 producing V γ 4⁺ V δ 4⁺ T cells were able to establish long-lived memory in the skin, persisting in the dermis for long periods of time after the initial stimulation with IMQ. Moreover, experienced V γ 4⁺ V δ 4⁺ T cells showed enhanced effector functions and mediated and exacerbated secondary inflammatory response (Ramírez-Valle et al. 2015; Hartwig et al. 2015). The observation that mice bearing a mutation in the gene encoding Sox13, which fail to develop dermal V γ 4⁺ cells but can generate dermal V γ 6⁺ cells, had lower psoriasis-like dermatitis scores than those of Sox13^{+/+} mice upon IMQ treatment, further support the pathologic role of V γ 4⁺ V δ 4⁺ T cell population in psoriasis (Gray et al. 2013). As discussed above, IL-17 producing dermal $\gamma\delta$ T cells have been extensively studied in murine models of psoriasis. In contrast to mouse skin, $\gamma\delta$ T cells are rare in healthy human skin. In fact, approximately 2-9% of all dermal cells are $\gamma\delta$ T cells (Ebert et al. 2006). Furthermore, the role of these cells in human skin, and especially their role in IL-17 signalling, remains poorly characterized. There are however few evidence for a novel V γ 9⁺V δ 2⁺ T cell subset, expressing cutaneous lymphocytes antigen CCR6, which was found to

accumulate in psoriatic skin and to be reduced into the circulation of patients. This mobilization of $CCR6^+V\gamma 9^+V\delta 2^+$ T cells observed in psoriatic patients associates with disease severity and is normalized after successful treatment of psoriasis (Laggner et al. 2011).

1.4.3 Neutrophils

Neutrophils are innate immune cells belonging to the family of polymorphonuclear leukocytes, that are well recognized as one of the major players during acute inflammation (Mantovani et al. 2011). They are typically the first leukocytes to be recruited to an inflammatory site and are capable of eliminating pathogens by multiple mechanisms. In the circulation, mature neutrophils have an average diameter of 7–10 μ m, their nucleus is segmented and their cytoplasm is enriched with granules and secretory vesicles (Mestas & Hughes 2004). Mature neutrophils develop in the bone marrow from pluripotent hematopoietic stem cells (HSC) in a process called “granulopoiesis”. Granulopoiesis is strictly dependent on the effects of several colony stimulating factors (CSFs), among which granulocyte CSF (G-CSF) is the most important, and cytokines, such as IL-6, IL-23 and IL-17 (Bugl et al. 2012). The release of neutrophils from the bone marrow to the circulation and peripheral tissue contributes to the maintenance of neutrophil homeostasis and, as such, is a tightly regulated process. Indeed, in physiological conditions the peripheral neutrophil population is maintained within a constant number by balancing the production, release and clearance of neutrophils from the circulation. The process is controlled by granulocyte colony stimulating factor (G-CSF) (Lieschke et al. 1994), which is produced in response to IL-17A synthesized by T cell populations ($\gamma\delta$ and natural killer T cells) involved in regulating neutrophil homeostasis (Ley et al. 2006). Release of IL-17A is, in turn, induced by IL-23 originating from tissue-resident macrophages and dendritic cells. During inflammation the number of neutrophils in the blood and tissues can increase up to 10-fold. With time the cells die by apoptosis and are removed by macrophages and dendritic cells. This process results in down-regulation of IL-23 synthesis by those cells and thus reduces G-CSF release (Stark et al. 2005).

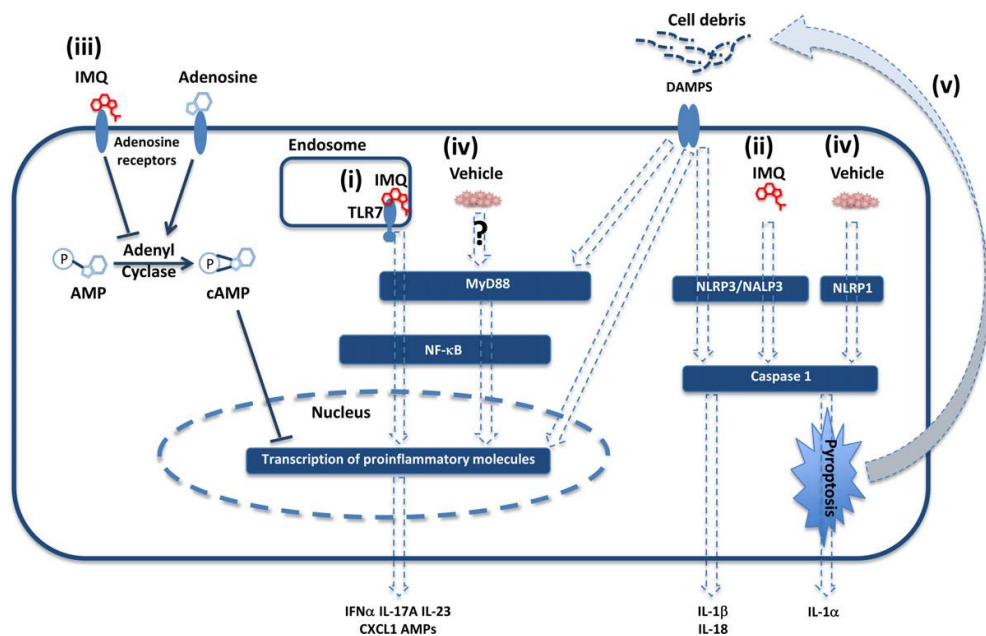
Neutrophils were always considered short-lived cells with a half-life in the circulation of approximately 1.5 and 8 hours in mice and humans, respectively. Recent studies challenged this concept, proposing that under basal conditions the average circulatory lifespan of neutrophils is up to 12.5 hours for mouse cells and 5.4 days for human neutrophils (Pillay et al. 2010). In this context, the notion that neutrophil longevity can increase several fold, both *in vitro* and *in vivo*, under inflammatory conditions has changed the view under which these cells have long been considered. Indeed, a longer lifespan may allow neutrophils to carry out more complex activities at the inflammatory sites, such as contributing to the resolution of inflammation or shaping adaptive immune responses. The classical view of neutrophils as “suicide” phagocytic cells capable only to release lytic enzymes and to produce reactive oxygen intermediates (ROI) with antimicrobial potential (Nathan 2006) has indeed been challenged by the demonstration that these cells can actually be induced to express several genes encoding key inflammatory mediators, including complement components, Fc receptors, chemokines and cytokines (Mantovani et al. 2011; Tecchio & Cassatella 2016). Thus, in response to different signals neutrophils express a vast and diverse repertoire of cytokines that are crucial to the role of neutrophils in innate and adaptive immune responses and to their role in defence and pathology. Accordingly, also the observation that neutrophils can infiltrate lymphoid organs, including spleen and lymph nodes, as well as the demonstration that neutrophils exhibit complex crosstalk with components of the innate and adaptive immune system (including DCs, T and B cells) (Scapini & Cassatella 2014) has renewed the interest in these cells within the immunology community. Importantly, locally activated neutrophils not only amplify the inflammatory process, but, surprisingly, can also actively participate in its resolution phase (Jones et al. 2016). In this context, relevant to this thesis, is the notion that the inflammatory environment can profoundly shape the functional status of neutrophils. Indeed, among the novel discoveries on neutrophil biology, concepts such as “neutrophil heterogeneity” and “neutrophil plasticity” have started to emerge, implying that, under pathological conditions, neutrophils may differentiate into discrete subsets defined by distinct phenotypic and functional profiles (Scapini et al. 2016; Silvestre-Roig et al. 2016).

Despite the fact that the origin and phenotype of the majority of these neutrophil subsets remain unclear, accumulating evidence supports the idea that these distinct neutrophil populations may play diverse, and even opposing roles in infection, inflammation and cancer immunology (Scapini et al. 2016; Silvestre-Roig et al. 2016). For instance, the controversial role of neutrophils in cancer pathogenesis is a subject of extensive investigation in the field. Indeed, evidence that neutrophils can display both immunosuppressive/pro-tumor and immunostimulatory/anti-tumor functions has been reported in both tumour-bearing mice and in cancer patients (Shaul & Fridlender 2017; Liang & Ferrara 2016). In this context, it has been suggested that neutrophils can polarize from an N1 pro-inflammatory and anti-tumoral phenotype, to an N2 anti-inflammatory and pro-tumoral phenotype (Granot & Jablonska 2015). Furthermore, it is now well established that myelopoiesis can be profoundly modified during inflammation and cancer, releasing altered mature myelocytes and myeloid-derived suppressor cells (MDSCs) that exert immunosuppressive and protumoral activity, mainly by inhibiting T cell functions (Moses & Brandau 2016; Scapini et al. 2016). Altogether, these novel aspects of neutrophil biology have shed a new light not only on the potential complex roles that neutrophils play during inflammation and immune responses, but also on the pathogenesis of several inflammatory disorders including infection, autoimmunity and cancer. In the context of psoriasis, as it will be described more in details in the aim of the study and in the discussion of this thesis, neutrophil accumulation in the skin is one of the histological features of this disease (Perera et al. 2012). In psoriatic skin, neutrophils initially infiltrate into the dermis at the early phase and later into the epidermis at the chronic phase. However, to date little is known about the pathogenic role of neutrophils *in vivo*, despite the fact that the role of IL-17 in neutrophil-mediated inflammation strongly suggests that neutrophils may also participate to psoriasis pathogenesis.

1.5 The imiquimod (IMQ) mouse model of psoriasis

Research on the pathogenesis of psoriasis has been severely hampered by the lack of a naturally occurring disorder in laboratory animals that mimics the complex phenotype and pathogenesis of the human disease (Bocheńska et al. 2017). However, a mouse model of psoriasiform dermatitis caused by the repeated topical application of Aldara™ cream [containing the Toll-like receptor 7/8 (TLR7/8) ligand IMQ (5%)] was described in 2009 (van der Fits et al. 2009). This model is currently broadly utilized to elucidate pathogenic mechanisms involved in psoriasis development as well as to evaluate possible new therapies for this disease. IMQ is a potent immune activator used for topical treatment of infections caused by *Papilloma* virus and other skin-associated abnormalities, including pre-cancerous skin lesions (Beutner & Ferenczy 1997). IMQ treatment is known to exacerbate the disease in patient already affected by psoriasis, also at distant sites, suggesting a possible systemic effect of IMQ (Wu et al. 2004). The primary mode of action of IMQ in mice is through ligation of TLR7 that activates NF-κB signalling in a MyD88-dependent fashion (Hemmi et al. 2002) (**Figure 2**). However, IMQ has been shown to have pro-inflammatory actions independently from TLR7 ligation. In fact, IMQ is a ligand for adenosine receptor for which it may act as an antagonist leading to inflammation development (Schön et al. 2006). Independently of TLR7 and MyD88 signalling, IMQ may also activate the inflammasome in keratinocytes *via* the NALP3 pathway, leading to activation of caspase 1 and production of IL-1β and IL-18 (Kanneganti et al. 2006). Repeated applications of IMQ-containing cream rapidly induces skin inflammation in mice with remarkable pathological and histological resemblance to human psoriasis, including the development of skin erythema and scaling, epidermal thickening (acanthosis), altered keratinocyte differentiation, neoangiogenesis and skin infiltration of immune cells (van der Fits et al. 2009). Interestingly, the involvement of a deregulated IL-23/IL-17 axis and the overproduction of other inflammatory cytokines, such as IL-1, IL-36 and IL-22, that are known to trigger pivotal pathogenic pathways involved in human psoriasis, also appears to be mirrored in the IMQ-induced psoriasis (Flutter & Nestle 2013a; Tortola et al. 2012; Rabeony et al. 2015). The murine cell populations that express high levels of TLR7 include

macrophages and DCs. IMQ-induced activation of Myd88 in these cells triggers the production of IL-23, which is required for the maximal T cell responses and inflammation (Costa et al. 2017; C. Wohn et al. 2013). Dermal $\gamma\delta$ T cells are the major IL-17-producing cells in the skin in response to IL-23 stimulation, while conventional $\alpha\beta$ T cells do not contribute to the development of psoriasis lesions in this model (Kalyan et al. 2014). Overall these observations indicate that IMQ-induced psoriasis in mice closely resembles human psoriasis in terms of phenotypic skin changes and that lesion development is strictly dependent upon the IL-23/IL-17 axis (Cai et al. 2011; van der Fits et al. 2009).



From Flutter B and Nestle FO 2013

Figure 2. Mechanisms of action of Aldara™. Aldara™ can activate immune responses *via* a number of different pathways including: (i) TLR7-dependent MyD88 pathway activation in immune cells, (ii) NALP3 activation of the inflammasome, by IMQ, (iii) antagonism of adenosine receptor signalling, (iv) direct activation of the inflammasome or MyD88 pathways through unknown receptors by the vehicle (v), imiquimod- or vehicle-driven cell death leading to the release of preformed IL-1 α and cell debris.

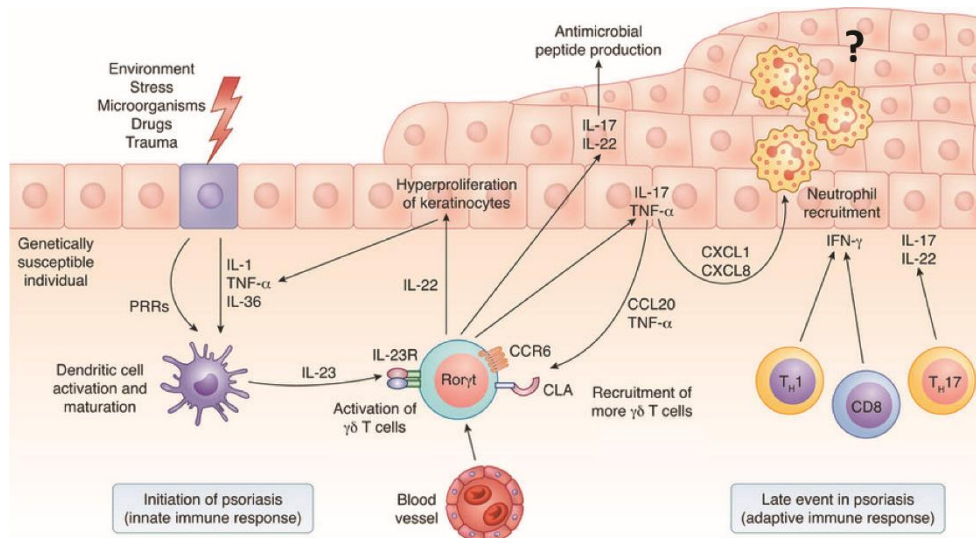
2. Aim of the study

Neutrophils are the most abundant leukocytes in humans and play a major role in driving immune responses against most type of infections. Recently, it has become clear that the functions of neutrophils go far beyond the elimination of microorganisms and that these cells may contribute to the pathogenesis of numerous chronic inflammatory disorders (Kolaczowska & Kubes 2013; Mócsai 2013). In this context, despite the fact that the presence and infiltration of neutrophils into the epidermis is one of the hallmark histologic features of psoriasis (Perera et al. 2012), the role of these cells in disease pathogenesis remains poorly understood. The most credited hypothesis views neutrophils as the principal cell mediators in the IL-17-dependent pathophysiology of psoriasis, suggesting a pro-inflammatory role of neutrophils in this disease. However, data emerging from clinical evidence do not allow drawing definitive conclusions.

Indeed, while some clinical evidence report that agranulocytosis can improve the clinical outcome in patients with different subtype of psoriasis (Pai et al. 1999; Toichi et al. 2000), other clinical trials for therapeutic interventions aimed at interfering with neutrophil recruitment or functions into the inflammatory skin (e.g. anti-human CXCL8 Abs) were not successful (Bhushan et al. 2002; Schön et al. 2017). Similar controversial results on the pathogenic role of neutrophils in psoriasis also emerge from studies in which preclinical models of this disease have been utilized (Schön et al. 2000; Singh et al. 2016; Sumida et al. 2014).

This being said, to better elucidate the role of neutrophils in psoriasis development, I utilized the imiquimod (IMQ)-induced mouse model of psoriasis, which consists of the topical administration of Aldara™ cream [containing the (TLR7/8) ligand IMQ (5%)] (Flutter & Nestle 2013b; Hawkes et al. 2016). While the crucial role of DCs and T cells (mostly $\gamma\delta$ T cells) in the development of IMQ-induced psoriasis has been elegantly demonstrated (Cai et al. 2011; Pantelyushin et al. 2012; Singh et al. 2016; Tortola et al. 2012; C. Wohn et al. 2013; Yoshiki et al. 2014), the role of neutrophils in this model remains unclear (**Figure 3**).

Indeed, neutrophil depletion resulted in a reduction of IMQ-induced psoriasis in one study (Sumida et al. 2014) or to not affect disease development in another study (Singh et al. 2016). Herein, by performing neutrophil depletion or utilizing mice carrying impairment in neutrophil functions, including *p47phox*^{-/-} mice [lacking a cytosolic subunit of the phagocyte nicotinamide dinucleotide phosphate (NADPH) oxidase (Jackson et al. 1995)] and *Syk*^{fl/fl}*Mrp8-cre*⁺ mice [carrying the specific deletion of the Syk kinase in neutrophils only (Elliott et al. 2011; Van Ziffle & Lowell 2009)], I uncovered a novel potential regulatory role of neutrophils in the IMQ-induced psoriasis.



From Becher B & Pantelyushin, 2014

Figure 3. Initiation of psoriasis by $\gamma\delta$ T cells. Several insults can directly activate DCs through pattern recognition receptors (PRRs) or indirectly through keratinocyte stress (release of IL-1, TNF- α and IL-36). Stress-sensing DCs can produce IL-23, which activates $\gamma\delta$ T cells to make TNF- α , IL-17 and IL-22, representing an early event in psoriasis that is mediated by the innate immune response. IL-22 induces keratinocyte proliferation, and TNF- α and IL-17 activate DCs and keratinocytes, leading to upregulation of adhesion molecules by the skin epithelium, angiogenesis and chemokine production. IL-17–induced chemokines include CXCL1 and CXCL8 and are responsible for the recruitment of neutrophils. IL-17R engagement by keratinocytes also leads to local production of CCL20, which attracts more circulating CCR6⁺ $\gamma\delta$ T cells. This escalates into a self-amplifying inflammatory loop that can also be mediated by the adaptive immune system.

3. Materials and Methods

3.1 Mice

Syk^{fl/fl} and *Syk^{fl/fl}Mrp8-cre⁺* mice, were previously described (Van Ziffle & Lowell 2009), *p47phox^{-/-}* mice were a gift from Prof. Romani (University of Perugia) and were previously described (Jackson et al. 1995). *Tcra^{-/-}* mice were a gift from Prof. Constantin (University of Verona). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice used in this study were on a C57BL/6 background and kept in a specific pathogen-free facility. All mouse experiments were carried out in accordance with guidelines prescribed by the Ethics Committee for the usage of laboratory animals for research purposes at the University of Verona and by the Italian Ministry of Health.

3.2 IMQ-induced psoriasis model

For induction of psoriasis-like skin inflammation, mice at 8–12 wk of age were shaved on the back with an electric clipper 1 d prior to treatment and received a daily topical dose of 62,5 mg of commercially available IMQ cream (5%) (Aldara Cream™, Meda AB) or control cream (vaseline) on their shaved backs for 3 or 6 consecutive days as previously described (van der Fits et al. 2009; Costa et al. 2017). On the fourth or seventh day, the animals were euthanized (**Figure 4**). Back skin was isolated and half was fixed in 10% formaldehyde for histopathology analysis while the other half was finely chopped and stored in RNAlater (Ambion) for quantitative real-time PCR (qRT-PCR) or digested, as described below, to achieve single-cell suspensions for flow cytometry analysis.

3.3 Neutrophil depletion

Mice were injected intra-peritoneally (i.p.) with 300 µg of rat anti-mouse Ly6G Ab (clone 1A8; BioXcell) or isotype control Rat IgG2a (clone 2A3; BioXCell), dissolved in 300 µl phosphate-buffered saline (PBS) every other day from day 0 to day 6 (**Figure 4**).

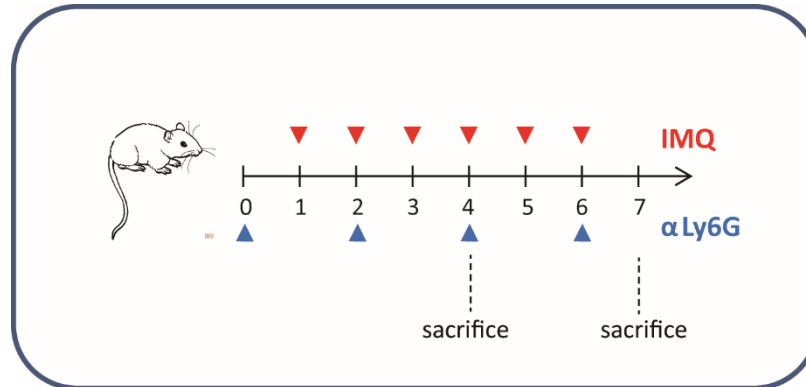


Figure 4. Neutrophil depletion in the IMQ-induced mouse model of psoriasis.

3.4 Cell preparation and flow cytometry

Skin tissue (2 cm X 2 cm) was cut from dorsal skin of the mouse. After removing subcutaneous tissue and collagen intensively with forceps, the skin was cut into small pieces and digested with 0,4 mg/ml Liberase TM (Roche Ltd.) and 0,5 mg/ml DNase I (Sigma) in RPMI 1640 medium (Sigma) for 1 hour. Single cell suspension was obtained by shredding with gentle Macs Dissociator (Miltenyi Biotec) and filtering with 70 µm and 40 µm cell strainer in series. Lymph nodes were mechanically dissociated by two frosted microscope slides and passage through a 70 µm cell strainer to yield a single-cell solution. Cells were resuspended in phosphate buffered saline containing 2 % (vol/vol) fetal calf serum, 2 mM EDTA and maintained at 4°C. For flow cytometry, 1–2×10⁶ cells were stained. Non-specific binding was blocked by pre-incubation with 0.5 µg anti-CD16/32 (2.4G2, Biolegend) and 100 µg mouse IgG (Sigma). Surface staining was performed with the following anti-mouse Abs: Ly6G(1A8), TCRαβ

(H57-597), CD62L (MEL-14), CD11b (M1/70), CD45 (30-F11), I-Ab (MHCII)(AF6-120.1), CD44 (IM7), TCR γ/δ (GL3) from Biolegends; Ly6C (AL-21), CD11c (HL3), CD3 (145-2C11) and GR-1 (RB6-8C5), from BD Biosciences. After final wash, cells were resuspended in staining/wash buffer containing 1 mg/ml propidium iodide (PI; Sigma-Aldrich) for viability staining according to the manufacturer's instructions. For intracellular cytokine staining, the cells were activated for 4 hours in phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (750 ng/ml) in the presence of brefeldin A (1 mg/ml). Thereafter, cells were surface-stained, washed, and then fixed and permeabilized using the eBioscience kit as previously described (Scapini et al. 2011). Intracellular staining was performed with anti-mouse IL-17A (TC11-18H10.1; eBioscience) or its relevant isotype control mAbs. Sample fluorescence was measured by a seven-color MACSQuant Analyzer (Miltenyi Biotec), while data analysis was performed by using FlowJo software Version 8.8.6 (Tree Star, Ashland, OR, USA).

3.5 Quantitative real-time PCR

Real-time reverse transcription-PCR was performed, as previously described (Tamassia et al. 2014), using total RNA isolated from 30 mg of the skin by RNeasy Fibrous Tissue Mini Kit (QIAGEN) and utilizing the following gene-specific primer pairs (all purchased from Invitrogen) (**Table 1**). Data were calculated by Q-Gene software (<http://www.genequantification.de/download.html>) and expressed as mean normalized expression (MNE) units after RPL32 normalization.

3.6 Skin histology

Dorsal skin samples (3 mm) were obtained by a transversal cut of the central skin area, fixed in 10% neutral buffered formalin and embedded in paraffin blocks by using a Tissue-Tek® Tissue Embedding Console System from Diapath (Bergamo, Italy). The paraffin blocks were cut into 3 μ m thick cross-section and stained with hematoxylin and eosin following the standard procedure (immersion in Mayer's hematoxylin: 2 minutes; immersion in eosin: 3 min) by using a Leica Microsystem Autostainer XL ST5010 (Milano, Italy). Epidermal thickness was

determined by measuring the average interfollicular distance under the microscope in a blinded manner. Pictures were taken using Leica DFC 300FX Digital Color Camera on a Leica DM 6000 B microscope at a 100x magnification.

3.7 Isolation of peritoneal neutrophils

Peritoneal exudates were recovered 16 hours after i.p. injection of Bio-Gel® P Polyacrylamide Beads (Bio Rad). After a passage through a 70 µM cell strainer, cell suspensions were incubated with different mAbs as described in the flow cytometry section. CD11b⁺Ly6G⁺ neutrophils were sorted using a FACS Aria™ II flow cytometer (Becton Dickinson) (> 99.0 % purity). Alternatively, neutrophils were purified using EasySep™ Mouse Neutrophil Enrichment Kit according to the manufacturer's instructions (>95% purity). Purified cells were suspended at proper concentration depending on the assay in RPMI 1640 medium supplemented with 10 % FBS, 1 % ultraglutamine and 1 % penicillin/streptomycin (BioWhittaker-Lonza), added to 96-well plates and cultured at 37°C, 5 % CO₂.

3.8 Proliferation and IL-17A production by γδ T cells

γδ T cells were isolated from single-cell suspensions of mouse spleen and lymph nodes from wild-type or *Tcra*^{-/-} mice, using the TCRγ/δ⁺ T Cell Isolation Kit (Miltenyi Biotec) (> 85.5 % purity). Proliferation assay was performed in 96-well plates pre-coated (for 1 hour at 37°C) with 10 µg ml⁻¹ anti-CD3 mAbs [(G23-8, eBioscience). 1 x 10⁵ γδ T cells /well and 2 µg/ml anti-CD28 mAbs (B122, eBioscience) were then added to the plates (at 37°C, 5 % CO₂), in the presence of 10 ng/mL IL-1β (eBioscience) *plus* 100 ng/mL IL-23 (eBioscience). After 24 hours purified peritoneal neutrophils were added to the culture at the appropriate ratio. Following a 72 hour-incubation BrdU was added to the co-cultures for additional 4 hours; supernatants were then harvested for measurement of IL- 17A by using a specific ELISA kit (R&D Systems) while proliferation was determined by BrDU incorporation by ELISA (Cell proliferation ELISA, Roche), following the manufacturer's protocol. γδ T cell inhibition of proliferation is expressed as percentage of decrease of the absorbance value (at 450 nm: revealing the amount

of BrdU incorporated in proliferating $\gamma\delta$ T cells) of each experimental conditions over the absorbance value of anti-CD3/CD28-stimulated $\gamma\delta$ T cells.

3.9 Quantification of reactive oxygen species

Oxidative stress detection was performed using the cellROX® Deep Red Flow Cytometry Assay Kit (Life Technology). 96-well plates were pre-coated (for 1 hour at 37°C) with 10 $\mu\text{g ml}^{-1}$ anti-CD3 mAbs [(G23-8, eBioscience). 1×10^5 $\gamma\delta$ T cells/well and 2 $\mu\text{g/ml}$ anti-CD28 mAbs (B122, eBioscience) were added into the plates (at 37°C, 5 % CO₂), in the presence of 10 ng/mL IL-1 β (eBioscience) *plus* 100 ng/mL IL-23 (eBioscience). The day after purified peritoneal neutrophils were added to the culture at 1:1 $\gamma\delta$ T cells-to-neutrophils ratio for 1,5 hours. Then cells were stained with the CellROX® Deep Red reagent for additional 1.5 hours. Sample fluorescence was measured by a seven-colour MACSQuant Analyzer (Miltenyi Biotec), while data analysis was performed by using FlowJo software Version 8.8.6 (Tree Star, Ashland, OR, USA).

3.10 Statistical analysis

Data were expressed as the mean \pm SD and analysed using GraphPad Prism Version 5 software (GraphPad Software, Inc.). The comparison of variables was performed using two-tailed Student *t*- test (for comparison between two groups) or a 1-way ANOVA with Bonferroni's post test (used for multiple comparisons), Dunnett's post-test (when multiple comparisons to control group were made). P-values of less than 0.05 were considered significant and symbols indicate significant increases: */#, $P < 0.05$; **/###, $P \leq 0.01$; ***/####, $P \leq 0.001$; ****/#####, $P \leq 0.0001$. Graphs were elaborated using GraphPad Prism Version 5 software (GraphPad Software, Inc.).

Table 1. List of primer sequences used for RT-PCR

Gene Name	Forward Primers	Reverse Primers
IL-36a	TGCCCACTCATTCTGACCCA	GTGCCACAGAGCAATGTGTC
IL-1α	CAGTGAAATTTGACATGGGTG	CAGGCATCTCCTTCAGCAG
S100A7	GCCTCGCTTCATGGACAC	CGGAACAGCTCTGTGATGTAGT
Lcn2	GGACCAGGGCTGTCGCTACT	GGTGGCCACTTGACATTGT
IL-17 A	ATCAGGACGCGCAAACATGA	TTGGACACGCTGAGCTTTGA
IL-22	TGACGACCAGAACATCCAGA	CGCCTTGATCTCTCCACTCT
IL-23p19	CACCTCCCTACTAGGACTCAGC	CTGCCACTGCTGACTAGAAC
IL-1β	AGATGAAGTGCTCCTTCCAG	TTGTCCATGGCCACAACAAC
Rpl32	GAAGTTCATCAGGCACCAGTC	TCTTGTGGCTCCCATAACCGA

4. Results

Neutrophil depletion reduces the progression, but not the initiation, of skin inflammation and epidermal thickening in the IMQ-induced psoriasis.

To investigate the specific contribution of neutrophils to the development of IMQ-induced psoriasis, we performed neutrophil depletion by injecting anti-Ly6G (clone 1A8) Ab, or isotype control Ab, in mice treated with IMQ (or vaseline control cream), for 3 or 6 consecutive days as originally described by Van der Fits et al. (van der Fits et al. 2009). First, we confirmed that the anti-Ly6G-treatment successfully depleted neutrophils in lymph nodes and the skin of either vaseline or IMQ-treated mice after both 3 and 6 days of treatment (**Figure 5**).

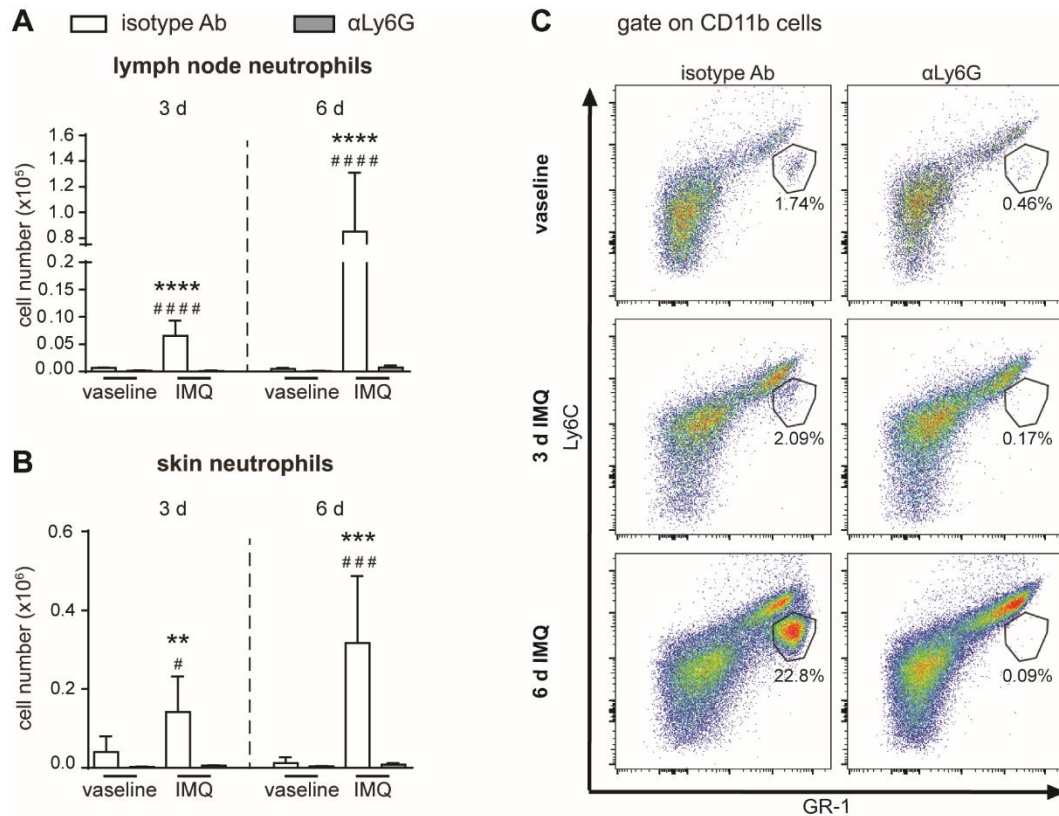


Figure 5. anti-Ly6G antibody (α Ly6G) efficiently depletes neutrophil infiltration in the lymph nodes and the skin of IMQ-treated mice. Dorsal skin of mice was topically treated with IMQ containing-cream (Aldara[®]) or vaseline, utilized as control cream, for 3 or 6 consecutive days. To deplete neutrophils, mice were injected i.p. with anti-Ly6G antibody (α Ly6G) or the isotype control (isotype Ab). **(A)** Draining lymph nodes were collected and analysed by flow cytometry. The total number of neutrophils ($CD11b^+Ly6C^{int}GR-1^{high}Ly6G^{high}$) is reported. **(B)** Total skin (2×2 cm) was digested and analysed by flow cytometry. The total number of neutrophils ($CD11b^+Ly6C^{int}GR-1^{high}Ly6G^{high}$) is reported. **(C)** Representative FACS plots showing the frequencies of $CD11b^+Ly6C^{int}GR-1^{high}Ly6G^{high}$ neutrophils in the lymph nodes of α Ly6G- or isotype Ab- treated mice after treatment with IMQ, or vaseline, for 3 or 6 consecutive days. Data are pooled from 2 separate time course experiments and are expressed as means \pm SD ($n = 5$ mice per time point). Statistical differences of IMQ-treated vs. vaseline-treated mice (#) and IMQ-treated vs. IMQ-treated mice following neutrophil depletion (*) are reported. # $P \leq 0.05$; ** $P \leq 0.01$; ####/*** $P \leq 0.001$; #####/**** $P \leq 0.0001$ by 1-way ANOVA with Bonferroni's post-test.

Interestingly, neutrophil depletion did not significantly affect epidermal thickening up to 3 days of IMQ treatment (**Figure 6A**). However, differently from what previously published (Singh et al. 2016; Sumida et al. 2014), we observed an unexpected significant increase of epidermal thickening in neutrophil-depleted mice, as compared to control mice, upon 6 days of IMQ treatment (**Figure 6A, B**). Consistently, the expression of skin-associated psoriatic genes by qRT-PCR, such as Lipocalin-2 (Lcn2) and S100 calcium binding protein A7/psoriasin (S100A7) was significantly higher in IMQ-treated mice receiving anti-Ly6G (clone 1A8) Ab, as compared to control isotype-treated mice (**Figure 7**). Strikingly, we also observed that, upon IMQ treatment, mice receiving anti-Ly6G Ab manifested a significantly increased expression of cytokines implicated in the IL-23/T17 axis, including IL-23, IL-22, IL-17, as compared to control isotype-treated mice (**Figure 7**). Neutrophil depletion, instead, did not significantly affect the expression of other inflammatory cytokines induced by IMQ treatment, such as IL-1 β , IL-36 α and IL-1 α (**Figure 7**).

Overall, these data suggest a novel potential role for neutrophils as negative modulators of disease progression and of the IL-23/T17 axis in the IMQ-induced psoriasis.

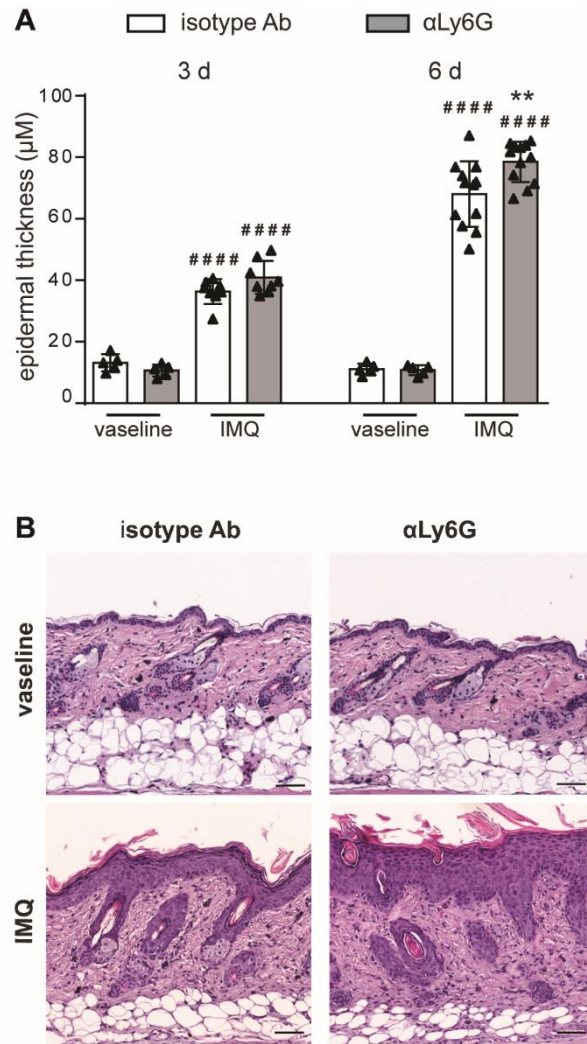


Figure 6. Increased epidermal thickening in neutrophil-depleted mice in response to IMQ treatment. Dorsal skin of mice was topically treated with vaseline or IMQ-containing cream (Aldara[®]) for 3 or 6 consecutive days. Mice were injected with the depleting antibody α Ly6G or isotype control antibody (isotype Ab). **(A)** The height of epidermal hyperplasia was measured in interfollicular epidermis on H&E-stained slides by light microscopic evaluation. Data are pooled from 3 separate time course experiments and are expressed as means \pm SD. Statistical differences of IMQ-treated vs. vaseline-treated mice (#) and IMQ-treated vs. IMQ-treated mice following neutrophil depletion (*) are reported. ** $P \leq 0.01$; ##### $P \leq 0.0001$ by 1-way ANOVA with Bonferroni's post-test. **(B)** Representative H&E-staining of dorsal skin from mice injected with isotype Ab or α Ly6G treated with vaseline or IMQ for 6 days. Original magnification, X100; original scale bars 40 μ m.

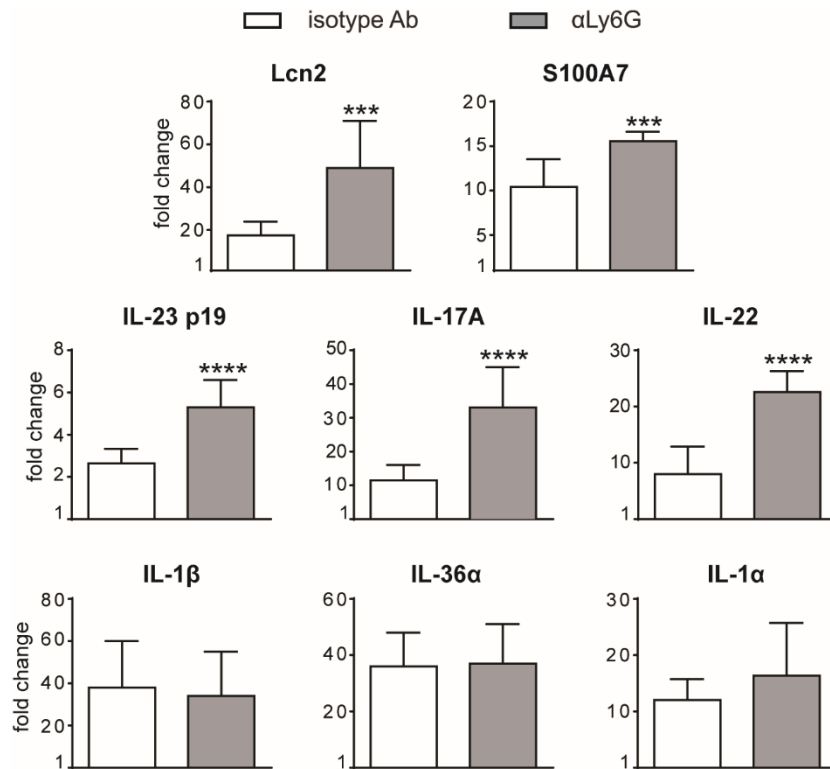


Figure 7. Gene-expression analysis of inflammatory molecules in the skin of IMQ-treated control or neutrophil-depleted mice. The dorsal skin of mice was topically treated with IMQ-containing cream (Aldara®) or vaseline for 6 consecutive days. Mice were injected with the depleting antibody α Ly6G or isotype control antibody (isotype Ab). Total skin RNA was extracted and reverse transcribed. mRNA expression of the indicated genes for IMQ-treated control or neutrophil-depleted mice is displayed as fold change of MNE units (after RPL32 normalisation) over vaseline-treated control. Data are pooled from 2 separate experiments and are expressed as means \pm SD (n = 8-12 mice). Statistical differences of IMQ-treated vs. IMQ-treated mice following neutrophil depletion (*) are reported. *** $P \leq 0.001$; **** $P \leq 0.0001$ by *t* test.

Neutrophil depletion increases the expansion and infiltration of $\gamma\delta$ T cells in lymph nodes and skin of IMQ-treated mice.

We then performed a careful characterization of the CD45⁺ cells infiltrating the draining lymph nodes and the skin of IMQ-treated mice receiving anti-Ly6G Ab or control isotype Ab by flow cytometry, utilizing the gating strategies previously described (Costa et al. 2017). Interestingly, after 6 days of IMQ treatment, we found strongly increased infiltration of $\gamma\delta$ T cells in the draining lymph nodes of neutrophil-depleted mice, $\gamma\delta$ T cells being the main pathological T cells in this mouse model of psoriasis (Cai et al. 2011; Pantelyushin et al. 2012) (**Figure 8A, B**-left panel). Besides the total number, also the number of CD44^{high}CD62L^{low} effector $\gamma\delta$ T cells and of IL-17-producing $\gamma\delta$ T cells were significantly increased, indicating that not only the infiltration but also the activation of these cells was profoundly affected by neutrophil depletion (**Figure 8B**-right panel, **C, D**). No significant differences in the infiltration of $\alpha\beta$ T cells (**Figure 8E**), monocytes/macrophages (**Figure 8F**) and DCs (**Figure 8G**) were instead found in the draining lymph nodes of anti-Ly6G-treated, as compared to control isotype-treated mice. Notably, a strong expansion of dermal $\gamma\delta$ TCR^{low} T cells was evident also in the skin of anti-Ly6G-treated, as compared to control isotype-treated mice after 6 days of IMQ treatment (**Figure 9A, B**). No significant differences in the infiltration of monocytes/macrophages (**Figure 9C**), DCs (**Figure 9D**) and TCR⁺ $\alpha\beta$ T cells (**E**) were instead found in the skin of anti-Ly6G-treated, as compared to control isotype-treated mice. It is worth to point out that, under our experimental conditions, $\gamma\delta$ T cells and neutrophils infiltrated the lymph nodes and the skin of IMQ-treated mice with similar kinetics, being the infiltration of both cell types much more consistent after 6 rather than 3 days of IMQ-treatment (**Figure 5A, B** and **figure 10A, B**).

Overall, these data point out at neutrophils as potential negative regulators of the infiltration and expansion of $\gamma\delta$ T cells in the lymph nodes and skin of IMQ-treated mice.

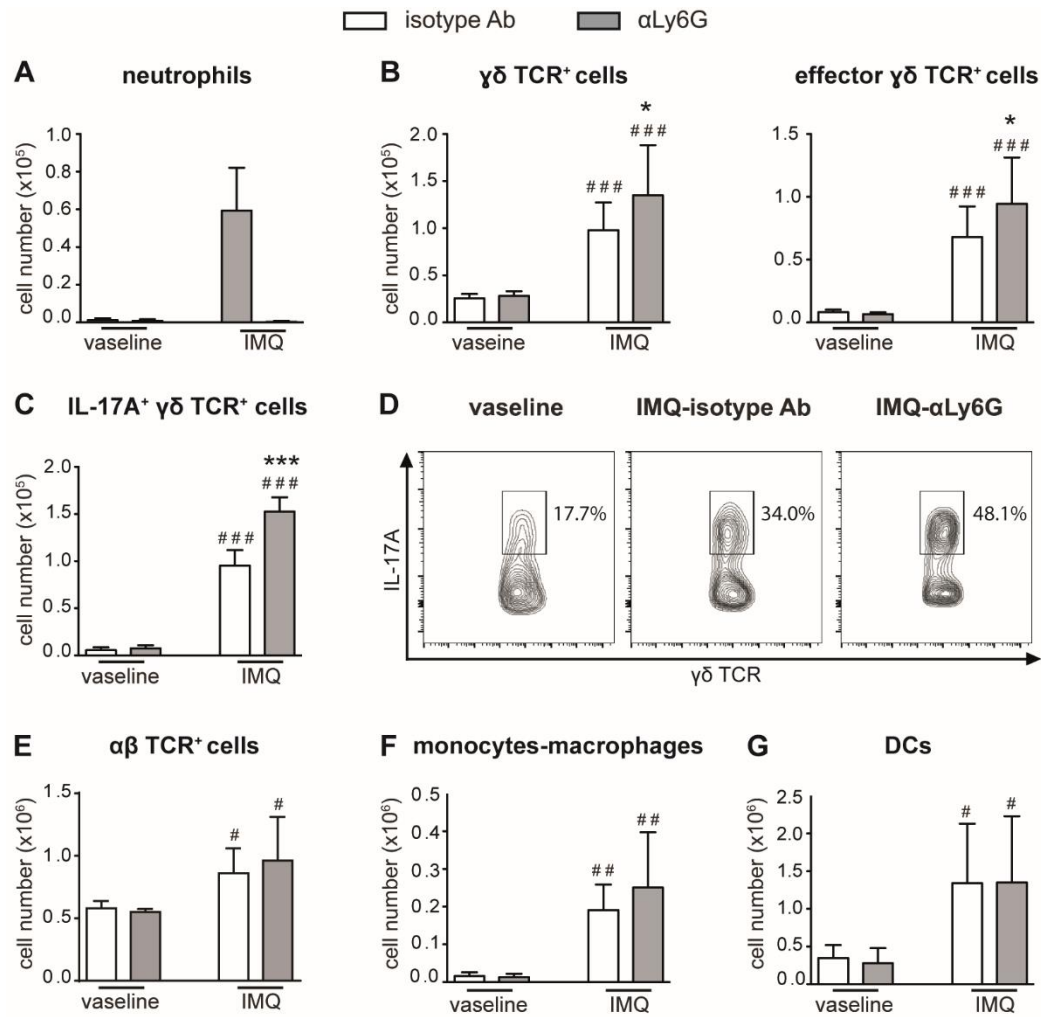


Figure 8. Infiltration of inflammatory cells in the draining lymph nodes of IMQ-treated control or neutrophil-depleted mice. The dorsal skin of mice was topically treated with IMQ-containing cream (Aldara®) or vaseline, utilized as control cream, for 6 consecutive days. Mice were injected with the depleting antibody α Ly6G or isotype control antibody (isotype Ab). Draining lymph nodes were collected and analysed by flow cytometry. Panels report: the total number of neutrophils (Ly6C^{int} Ly6G^{high} GR-1^{high}) (A), the number of total (left panel) and effector (CD44^{high}CD62L^{low}, right panel) $\gamma\delta$ TCR⁺ cells (B), the number of IL-17A-producing $\gamma\delta$ TCR⁺ cells (C), the frequencies of IL-17A-producing $\gamma\delta$ TCR⁺ cells (representative FACS plots) (D), the number of $\alpha\beta$ TCR⁺ T cells (E), the number of monocytes/M ϕ (CD11b^{high}Ly6G^{low}CD11c^{low/-}MHCII^{low/-} cells plus CD11b^{high}Ly6G^{low}CD11c^{low/-}MHCII^{high} cells) (F), and the number of DCs (CD11c^{+/high}MHCII^{high}) (G). Data are pooled from 3 separate experiments and are expressed as means \pm SD (n = 14-15 mice). Statistical differences of IMQ-treated vs. vaseline-treated mice (#) and IMQ-treated vs. IMQ-treated mice following neutrophil depletion (*) are reported. #/*P \leq 0.05; ##P \leq 0.01; ###/***P \leq 0.001 by 1-way ANOVA with Bonferroni's post-test.

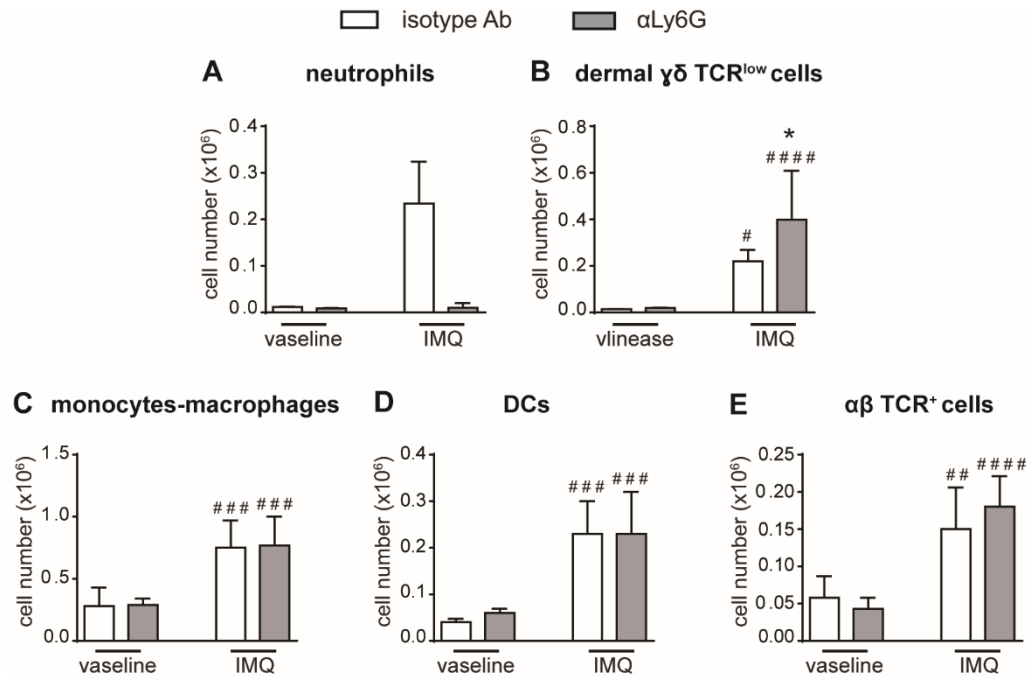


Figure 9. The infiltration of $\gamma\delta$ T cells is increased in the skin of neutrophil-depleted mice treated with IMQ. The dorsal skin of mice was topically treated with IMQ-containing cream (Aldara[®]) or vaseline for 6 consecutive days. Mice were injected with the depleting antibody α Ly6G or the isotype control antibody (isotype Ab). Total skin (2x2 cm) was digested and analysed by flow cytometry. Panels report: the total number of neutrophils (Ly6C^{int} GR-1^{high} Ly6G^{high}) (A), dermal $\gamma\delta$ TCR^{low} T cells (B), monocytes/M ϕ (CD11b^{high} Ly6G^{low/-} MHCII^{low/-} cells *plus* CD11b^{high} Ly6G^{CD11c^{low/-} MHCII^{high} cells) (C), DCs (CD11c^{+/high} MHCII^{high}) (D) and $\alpha\beta$ TCR⁺ T cells (E). Data are pooled from 2 separate experiments and are expressed as means \pm SD (n = 8-10 mice). Statistical differences of IMQ-treated vs. vaseline-treated mice (#) and IMQ-treated vs. IMQ-treated mice following neutrophil depletion (*) are reported. #/* $P \leq 0.05$; ## $P \leq 0.01$; ### $P \leq 0.001$; #### $P \leq 0.0001$ by 1-way ANOVA with Bonferroni's post-test.}

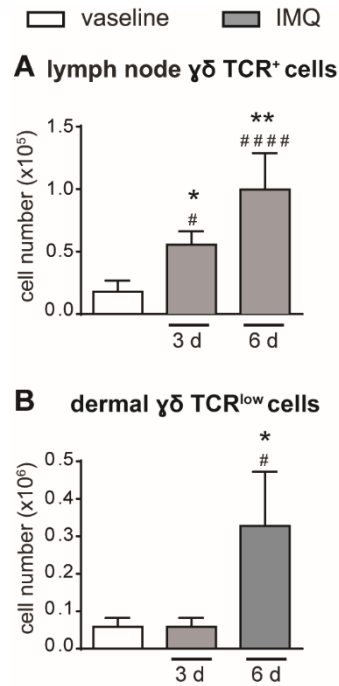


Figure 10. Skin infiltration of $\gamma\delta$ T cells in the lymph nodes and skin of mice treated with IMQ for 3 and 6 days. Dorsal skin of mice was topically treated with vaseline or IMQ-containing cream (Aldara[®]) for 3 or 6 consecutive days. **(A)** Draining lymph nodes were collected and analysed by flow cytometry. The total number of $\gamma\delta$ TCR⁺ cells is reported. **(B)** Total skin (2x2 cm) was digested and analysed by flow cytometry. The total number of dermal $\gamma\delta$ TCR^{low} T cells is reported. Data are pooled from 2 separate time course experiments and are expressed as means \pm SD (n = 5 mice). Statistical differences of IMQ-treated vs. vaseline-treated mice (#) and IMQ-treated mice after 3 days vs. IMQ-treated mice after 6 days (*) are reported. #/* $P \leq 0.05$; ** $P \leq 0.01$; #### $P \leq 0.0001$ by 1-way ANOVA with Bonferroni's post-test.

Neutrophils inhibit the proliferation and the production of IL-17 by $\gamma\delta$ T cells *via* cell contact-dependent reactive oxygen species (ROS) production.

Previous findings have highlighted the capacity of neutrophils to both positively and negatively modulate the effector functions of $\gamma\delta$ T cells (Davey et al. 2014; Kalyan et al. 2014a; Sabbione, María L. Gabelloni, et al. 2014; Hassane et al. 2017). Therefore we investigated the effect of neutrophils on the proliferation and the production of IL-17 by $\gamma\delta$ T cells stimulated with anti-CD3 Abs *plus* anti-CD28 Abs in the presence of 100 ng/mL IL-23 and 10 ng/mL IL-1 β , as previously described (Cai et al. 2011; Costa et al. 2017). As shown in **Figure 11A, B**, neutrophils inhibited both the proliferation and the production of IL-17 by activated $\gamma\delta$ T cells. Given that the degree of this inhibitory effect was dependent on the ratio with T cells (**Figure 11A, B**), in all subsequent experiments we used the 5/1 neutrophil/T cell ratio, condition in which we obtained a strong and reproducible inhibition of $\gamma\delta$ T cell functions by neutrophils. Similarly, to what published by Sabbione et al. (Sabbione, María L. Gabelloni, et al. 2014) with human neutrophils, we found that the addition of either catalase (a H₂O₂ scavenger) or of diphenyleneiodonium (DPI, a NADPH oxidase inhibitor) strongly reverted the immunosuppressive functions of mouse neutrophils on $\gamma\delta$ T cells (**Figure 11C, D**), while other inhibitors, including pentoxifylline (PTX, a degranulation inhibitor) or L-arginine [an arginase-1 (ARG1) inhibitor] were not effective (**Figure 11C, D**). Taken together, these data suggested that the inhibitory effects of neutrophils on $\gamma\delta$ T cell functions in the IMQ-induced mouse model of psoriasis involves the production of ROS. In line with these observations, neutrophils isolated from *p47^{phox}*^{-/-} mice were unable to effectively inhibit $\gamma\delta$ T cells (**Figure 12A**). Furthermore, by performing a flow cytometric measurement of ROS production, we also observed that the production of ROS by wild-type (WT) neutrophils, but not by *p47^{phox}*^{-/-} neutrophils, was strongly enhanced by the presence of $\gamma\delta$ T cells in the culture (**Figure 12B**). Finally, in line with the fact that the inhibitory functions of different immunosuppressive neutrophil populations have been shown to occur through direct cell contact-dependent mechanisms (Choi et al. 2012; Marini et al. 2017; Pillay et al. 2012; Schmielau & Finn 2001), we found that the capacity of neutrophils to inhibit $\gamma\delta$ T cell proliferation

was significantly lower if neutrophils were separated from T cells by the use of transwells (**Figure 12C**).

Overall, the results obtained so far show that neutrophils inhibit $\gamma\delta$ T cell functions *via* a cell contact-dependent triggered ROS production.

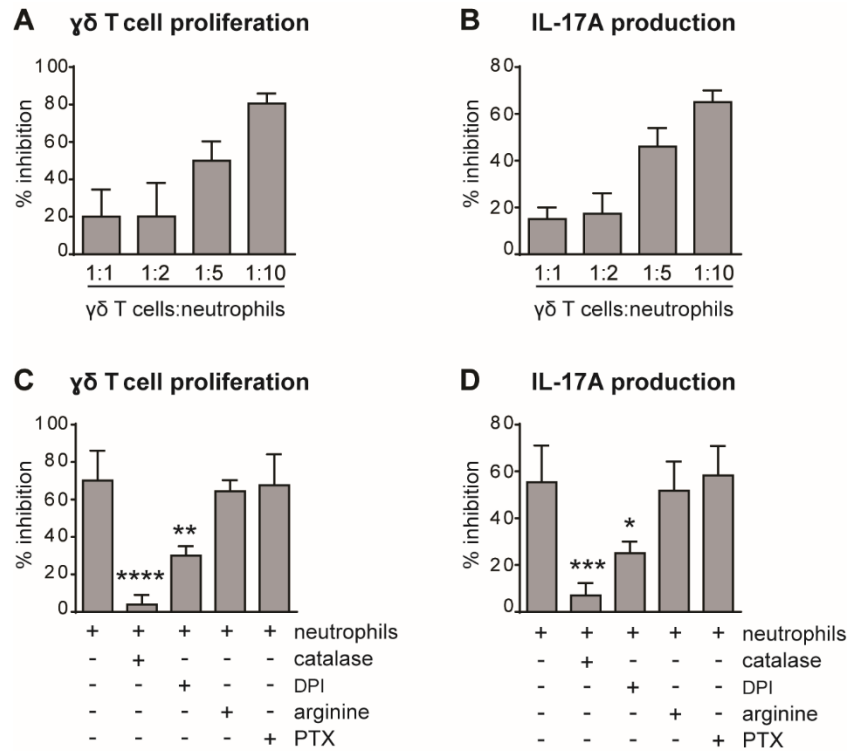


Figure 11. Neutrophils inhibit the proliferation and IL-17 production by $\gamma\delta$ T cells *via* reactive oxygen species (ROS) production. (A, B) $\gamma\delta$ T cells were stimulated with CD3/CD28, 100 ng/ml IL-23 *plus* 10 ng/ml IL-1 β and cultured for 72h in the presence or absence of neutrophils at different ratios. (C, D) $\gamma\delta$ T cells were stimulated with CD3/CD28, 100 ng/ml IL-23 *plus* 10 ng/ml IL-1 β and cultured for 72h with neutrophils added at a 1 to 5 $\gamma\delta$ T to neutrophil cell ratio, with or without inhibitors: catalase (1000 U/ml), diphenyleiodonium (DPI) (0,1 μ M), L-arginine (200 μ g/ml-1), pentoxifillin (PTX) (0,5 μ M). The percentages of inhibition of proliferation, as measured by BrdU incorporation (A, C), or IL-17A production (B, D) by $\gamma\delta$ T cells, are reported. Graph values indicate means \pm SD from 2 to 3 independent experiments. Statistical differences of the effect of neutrophils in the presence or absence of inhibitors are reported. *P \leq 0.05; **P \leq 0.01; ***P \leq 0.001; ****P \leq 0.0001 by 1-way ANOVA with Dunnett's post-test.

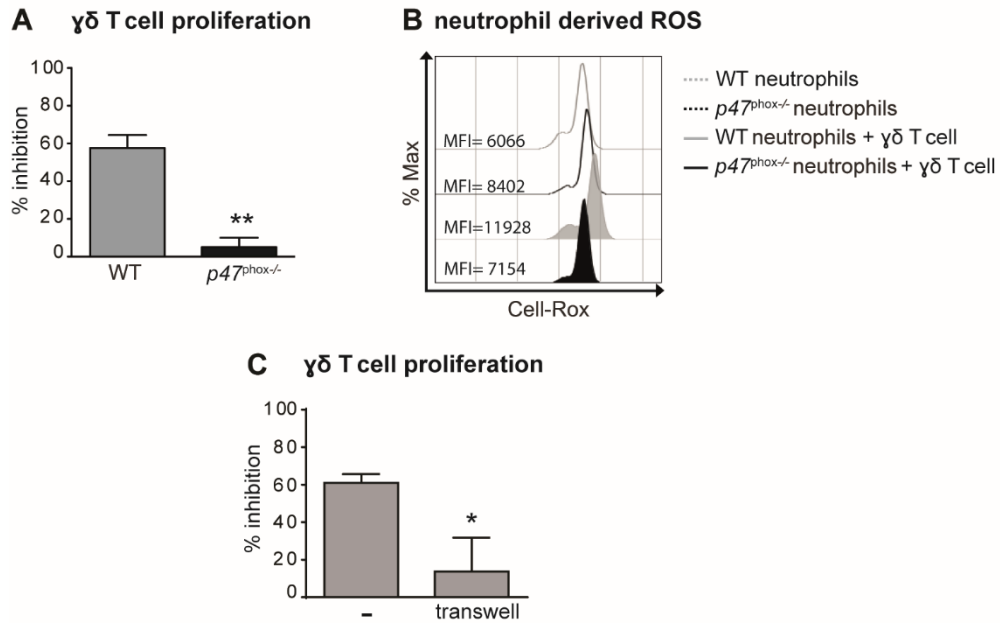


Figure 12. Neutrophil-mediated inhibition of $\gamma\delta$ T cell proliferation requires (NADPH) oxidase-dependent ROS production and direct cell-to-cell contacts. $\gamma\delta$ T cells were stimulated with CD3/CD28, 100 ng/ml IL-23 plus 10 ng/ml IL-1 β and cultured with neutrophils, from wild-type (WT) or $p47^{phox-/-}$ mice. Neutrophils were added at a 1 to 5 $\gamma\delta$ T to neutrophil cell ratio for 72 hours (A, C) or at a 1 to 1 $\gamma\delta$ T to neutrophil cell ratio for 3 hours (B). (A) Percentages of inhibition of $\gamma\delta$ T cell proliferation by neutrophils from WT or $p47^{phox-/-}$ mice as measured by BrdU incorporation. (B) Representative FACS histogram plots depicting the Cell-RoX MFI of CD11b⁺Ly6G⁺ neutrophils from WT or $p47^{phox-/-}$ mice in the presence or absence of $\gamma\delta$ T cells, as evaluated by FACS analysis. (C) Stimulated $\gamma\delta$ T cells were cultured with neutrophils under direct contact or transwell conditions. The graph shows the percentages of inhibition of $\gamma\delta$ T cell proliferation, as measured by BrdU incorporation. Graph values indicate means \pm SD from 2 independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$, by t test.

Syk signalling modulates the capacity of neutrophils to inhibit $\gamma\delta$ T cell functions and disease progression in the IMQ-mouse model of psoriasis.

Spleen tyrosine kinase (Syk), a member of non-receptor tyrosine kinases, transmits signals in neutrophils from a variety of immunoreceptors, including Fc γ receptors (Fc γ Rs) (Futosi et al. 2013), adhesion molecules, such as β 2 integrins (Mócsai et al. 2002) and P-Selectin glycoprotein ligand 1 (PSGL-1) (Stadtman et al. 2013). As a consequence, *Syk*^{-/-} neutrophils display impaired effector functions, including the production of ROS and the release of granule contents, in response to several inflammatory stimuli (Futosi et al. 2013).

Syk-based signalling in neutrophils alone was previously shown to be critical for appropriate host defence to *Staphylococcus aureus* (Van Ziffle & Lowell 2009) or the development of inflammatory arthritis (Elliott et al. 2011), suggesting the relevance of this signalling pathway in neutrophils during immune responses. Therefore, we decided to utilize mice carrying the specific deletion of Syk in neutrophils [*Syk*^{fl/fl}*Mrp8-cre*⁺ mice (Elliott et al. 2011; Van Ziffle & Lowell 2009)], available in our laboratory, as an experimental model to test whether the specific impairment of this signalling pathway in neutrophils was sufficient to affect the interactions of these cells with $\gamma\delta$ T cells and IMQ-induced psoriasis. Consistently, *Syk*^{-/-} neutrophils failed to produce ROS and to inhibit $\gamma\delta$ T cells proliferation under our experimental conditions *in vitro* (**Figure 13A, B**). These data validated therefore Syk as crucial signalling molecule involved in the modulation of neutrophil capability to inhibit $\gamma\delta$ T cell functions *via* a contact-dependent ROS production.

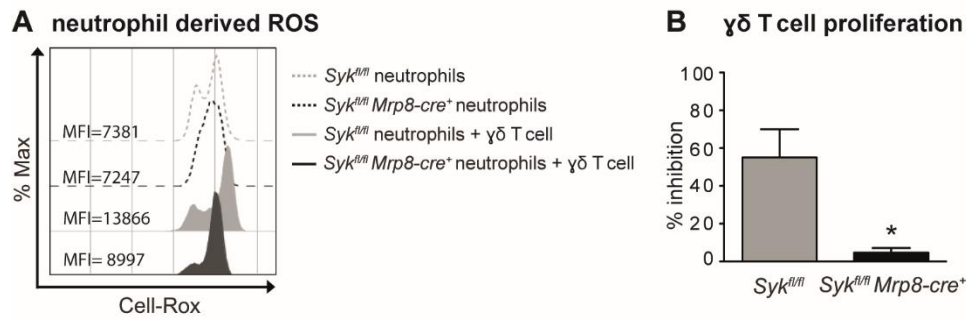


Figure 13. ROS-mediated inhibition of $\gamma\delta$ T cell proliferation by neutrophils requires the activation of Syk-dependent signalling pathways. $\gamma\delta$ T cells were stimulated with CD3/CD28, 100 ng/ml IL-23 plus 10 ng/ml IL-1 β and cultured with neutrophils from $Syk^{fl/fl}$ or $Syk^{fl/fl} Mrp8-cre^+$ mice at a 1 to 1 $\gamma\delta$ T to neutrophil cell ratio for 3 hours (A) or at a 1 to 5 $\gamma\delta$ T to neutrophil cell ratio for 72h (B). (A) Representative FACS histogram plots depicting the CellROX MFI of CD11b⁺Ly6G⁺ neutrophils from $Syk^{fl/fl}$ or $Syk^{fl/fl} Mrp8-cre^+$ mice in the presence or absence of $\gamma\delta$ T cells, as evaluated by FACS analysis. (B) Percentages of inhibition of $\gamma\delta$ T cell proliferation by neutrophils from $Syk^{fl/fl}$ or $Syk^{fl/fl} Mrp8-cre^+$ mice, as measured by BrdU incorporation. Graph values indicate means \pm SD from 2 independent experiments. * $P \leq 0.05$ by t test.

As far as IMQ-induced psoriasis experiments, we initially found that $Syk^{fl/fl} Mrp8-cre^+$ mice did not manifest, as compared to control $Syk^{fl/fl}$ mice, a significant increase of epidermal thickness, after 6 days of IMQ-treatment (Figure 14A). Interestingly, however, similarly to neutrophil-depleted mice, $Syk^{fl/fl} Mrp8-cre^+$ mice manifested, as compared to control $Syk^{fl/fl}$ mice, an enhanced expression of skin-associated psoriatic genes, such as S100A7 and Lcn2, as well as a specific increase in the expression of cytokines implicated in the IL-23/T17 axis, including IL-23, IL-22, IL-17, after 6 days of IMQ treatment (Figure 14B). Furthermore, also the number of total and activated $\gamma\delta$ T cells producing IL-17, but not of other cell types, was increased in the draining lymph nodes of $Syk^{fl/fl} Mrp8-cre^+$ mice, as compared to control mice, after 6 days of IMQ treatment (Figure 15). On a similar fashion, the number of dermal $\gamma\delta$ T cells infiltrating into the skin of IMQ-treated $Syk^{fl/fl} Mrp8-cre^+$ mice was significantly increased as compared to IMQ-treated control mice (Figure 16A, B). It is noteworthy to remark that, in line with the fact that Syk is not directly involved in controlling neutrophil migration to the

inflammatory sites (Mócsai et al. 2002), we did not notice any significant difference in the capacity of *Syk*^{-/-} neutrophils to infiltrate the lymph nodes and the skin in response to IMQ treatment (**Figure 15A** and **Figure 16A**). This observation, together with the fact that the deletion efficiency of the *Mrp8-cre* is only about 80-90 % (Elliott et al. 2011), meaning that about 10 to 20% of circulating neutrophils are *Syk*^{+/+}, can potentially explain why the specific neutrophil impairment present in *Syk*^{fl/fl}*Mrp8-cre*⁺ mice was sufficient to reproduce only the increase in skin inflammation and $\gamma\delta$ T cell infiltration, but not the overall increase in epidermal thickening observed in mice with a complete neutrophil depletion. Anyhow, data clearly suggests that Syk-dependent signalling pathways controlling neutrophil effector functions, but not neutrophil migration, are required for the neutrophil-mediated inhibition of $\gamma\delta$ T functions *in vitro* and *in vivo*.

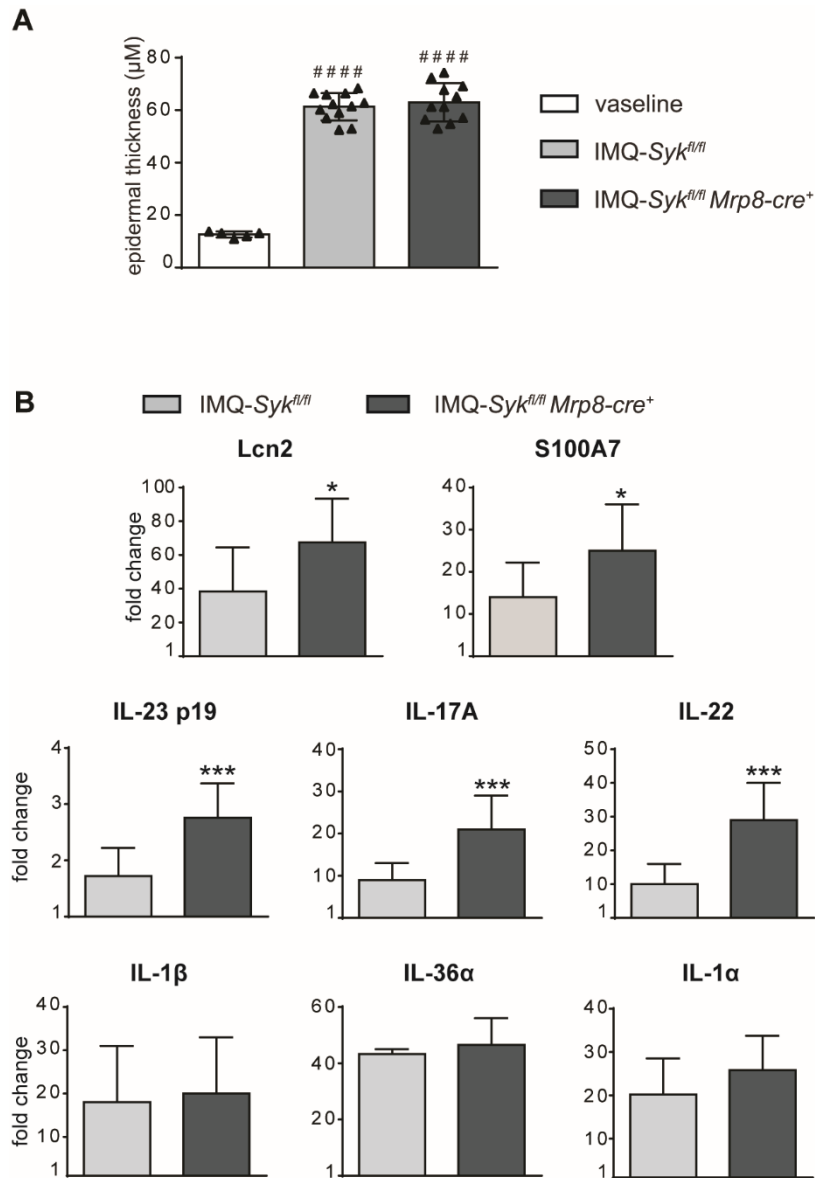


Figure 14. Epidermal thickening and gene-expression analysis of inflammatory molecules in the skin of IMQ-treated $\text{Syk}^{\text{fl/fl}}$ and $\text{Syk}^{\text{fl/fl}} \text{Mrp8-cre}^+$ mice. The dorsal skin of control $\text{Syk}^{\text{fl/fl}}$ and $\text{Syk}^{\text{fl/fl}} \text{Mrp8-cre}^+$ mice was topically treated with IMQ-containing cream (Aldara[®]) or vaseline for 6 consecutive days. (A) The height of epidermal hyperplasia (epidermal thickening) was measured in interfollicular epidermis on H&E-stained slides by light microscopic evaluation. (B) Total skin RNA was extracted and reverse transcribed. mRNA expression of the indicated genes for IMQ-treated $\text{Syk}^{\text{fl/fl}}$ and $\text{Syk}^{\text{fl/fl}} \text{Mrp8-cre}^+$ mice is displayed as fold change of MNE units (after RPL32 normalisation) over vaseline-treated controls. Data are pooled from 2 separate experiments and are expressed as means \pm SD (n = 11 mice). Statistical differences of IMQ-treated $\text{Syk}^{\text{fl/fl}}$ or $\text{Syk}^{\text{fl/fl}} \text{Mrp8-cre}^+$ mice vs. vaseline-treated mice (#) and IMQ-treated $\text{Syk}^{\text{fl/fl}}$ vs. IMQ-treated $\text{Syk}^{\text{fl/fl}} \text{Mrp8-cre}^+$ mice (*) are reported. #### $P \leq 0.0001$ by 1-way ANOVA with Bonferroni's post-test. * $P \leq 0.05$; *** $P \leq 0.001$ by *t* test.

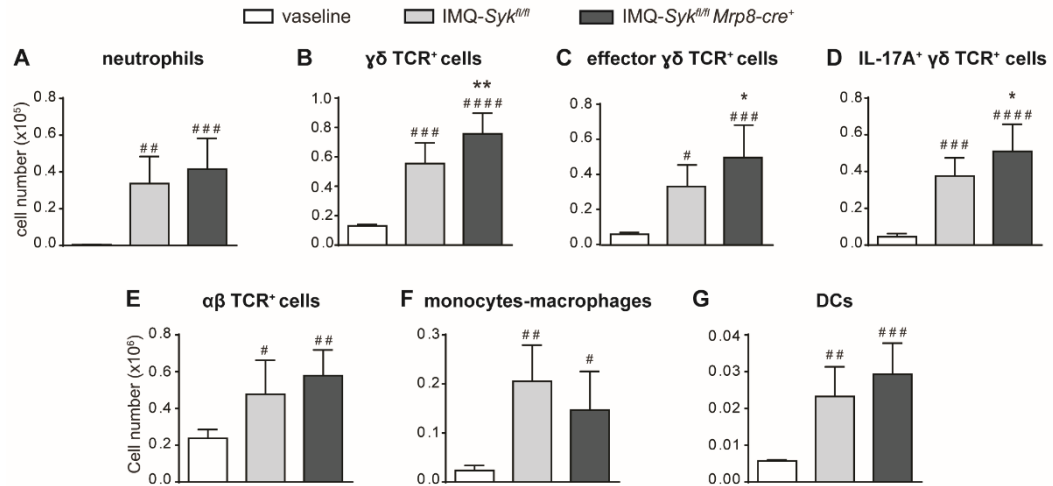


Figure 15. Infiltration of inflammatory cells in the draining lymph nodes of IMQ-treated *Syk^{fl/fl}* and *Syk^{fl/fl}Mrp8-cre⁺* mice. The dorsal skin of control *Syk^{fl/fl}* and *Syk^{fl/fl}Mrp8-cre⁺* mice was topically treated with IMQ-containing cream (Aldara[®]) or vaseline for 6 consecutive days. Draining lymph nodes were collected and analysed by flow cytometry. Panels report: the number of neutrophils (Ly6C^{int} GR-1^{high} Ly6G^{high}) (A), the number of total (B) and effector (CD44^{high} CD62L^{low}) $\gamma\delta$ TCR⁺ cells (C), the number of IL-17A-producing $\gamma\delta$ TCR⁺ cells (D), the number of $\alpha\beta$ TCR⁺ T cells (E), the number of monocytes/M ϕ (CD11b^{high}Ly6G⁺CD11c^{low/-}MHCII^{low/-} cells plus CD11b^{high}Ly6G⁺CD11c^{low/-}MHCII^{high} cells (F), and the number of DCs (CD11c^{+high}MHCII^{high}) (G). Data are pooled from 2 separate experiments and are expressed as means \pm SD (n = 11 mice). Statistical differences of IMQ-treated *Syk^{fl/fl}* or *Syk^{fl/fl}Mrp8-cre⁺* vs. vaseline-treated mice (#) and IMQ-treated *Syk^{fl/fl}* vs. IMQ-treated *Syk^{fl/fl}Mrp8-cre⁺* mice (*) are reported. #/**P* \leq 0.05; ##/***P* \leq 0.01; ###*P* \leq 0.001; ####*P* \leq 0.0001 by 1-way ANOVA with Bonferroni's post-test.

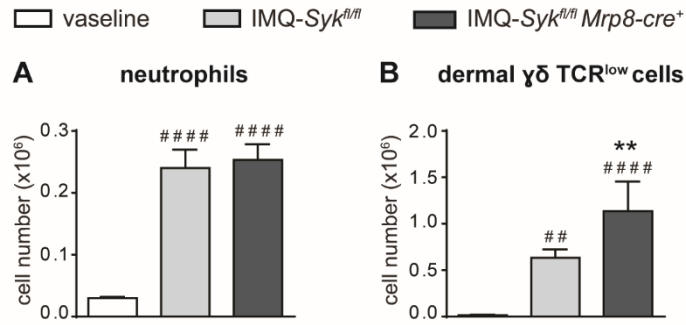


Figure 16. The infiltration of $\gamma\delta$ T cells is increased in the skin of IMQ-treated *Syk^{fl/fl}Mrp8-cre⁺* mice. The dorsal skin of control *Syk^{fl/fl}* and *Syk^{fl/fl}Mrp8-cre⁺* mice was topically treated with IMQ-containing cream (Aldara[®]) or vaseline for 6 consecutive days. Total skin (2x2 cm) was digested and analysed by flow cytometry. Panels report: the total number of neutrophils (Ly6C^{int} GR-1^{high} Ly6G^{high}) (A), dermal $\gamma\delta$ TCR^{low} T cells (B). Data are pooled from 2 separated experiments and are expressed as means \pm SD (n = 6 mice). Statistical differences of IMQ-treated *Syk^{fl/fl}* or *Syk^{fl/fl}Mrp8-cre⁺* vs. vaseline-treated mice (#) and IMQ-treated *Syk^{fl/fl}* vs. IMQ-treated *Syk^{fl/fl}Mrp8-cre⁺* mice (*) are reported. ##/** $P \leq 0.01$; #### $P \leq 0.0001$ by 1-way ANOVA with Bonferroni's post-test.

5. Discussion

Neutrophil accumulation in the skin is one of the histological features that characterize psoriasis (Boehncke & Schön 2015; Deng et al. 2016). However, the role of neutrophils in psoriasis development remains poorly understood. In this study, by utilizing the mouse model of IMQ-induced psoriasis, I uncover a novel role of neutrophils as negative regulators of disease propagation and exacerbation. In fact, neutrophil depletion resulted in an increased epidermal thickening accompanied by an increased inflammatory cell infiltration and cytokine/psoriatic gene expression. In particular, neutrophil depletion resulted in a profound exacerbation of the inflammation associated to the IL-23/T17 pathway. Interestingly, such an effect seemed to be mediated by the ability of neutrophils to inhibit, *via* NADPH oxidase-dependent ROS production, the proliferation and the production of IL-17 by $\gamma\delta$ T cells, key effector components of psoriatic inflammation in this model. Finally, we demonstrated, for the first time, that Syk function as crucial signalling molecule mediating this inhibitory crosstalk between neutrophils and $\gamma\delta$ T cells (**Figure 17**). Aim of future research is to identify the specific molecule/s involved in these cell-to-cell contact interactions between neutrophils and $\gamma\delta$ T cells that is/are responsible of triggering Syk-dependent inhibitory functions in neutrophils.

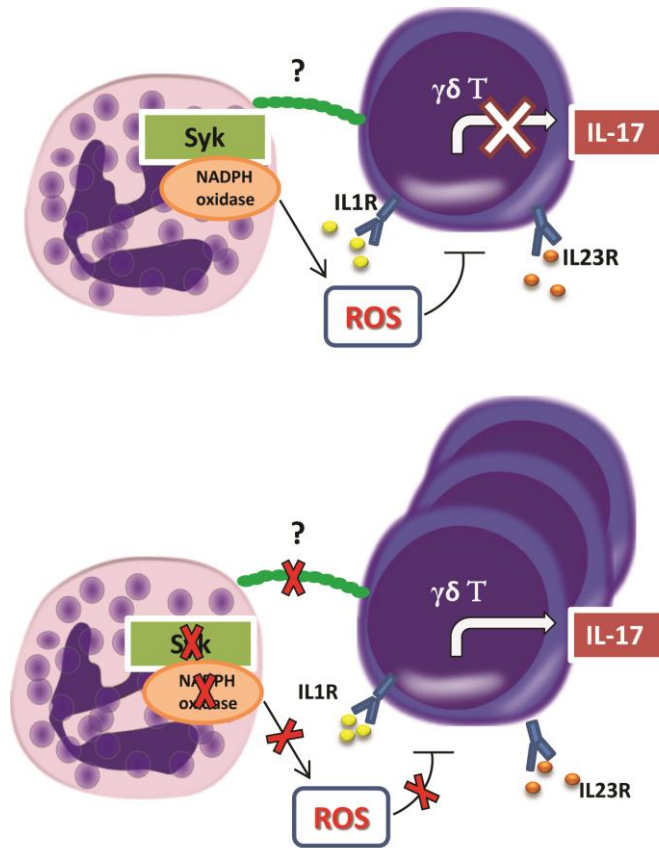


Figure 17. The ability of neutrophils to inhibit the proliferation and IL-17 production by $\gamma\delta$ T cells occurs *via* cell-contact and NADPH oxidase-dependent ROS production. We also propose Syk as important molecules involved in the modulation of this inhibitory pathway.

Neutrophils, due to their ability to both promote and inhibit inflammatory and immune responses, seem to play a rather complex role in several inflammatory diseases (Scapini & Cassatella 2014; Scapini et al. 2016; Silvestre-Roig et al. 2016; Soehnlein et al. 2017). As far as psoriasis, the current hypothesis is that neutrophils play a pro-inflammatory role in disease pathogenesis (Schön et al. 2017). This assumption is mostly based on the fact that these cells are generally linked to the IL-23/T17-related inflammatory axis and that they have been proposed to sustain skin inflammation for example by producing NETs (Lin et al. 2011; Zabieglo et al. 2015) and other inflammatory cytokines [including IL-17 and IL-22 (Dyring-Andersen et al. 2017)] or by activating IL-36 family cytokines *via* the release of proteases (Henry et al. 2016). However, to date, compelling evidence of this

pathogenic role of neutrophils in psoriasis does not exist. For instance, neutrophils have been also proposed to play a regulatory role in psoriatic inflammation *via* the release of elastase and the consequent activation of the anti-inflammatory cytokine IL-36 receptor antagonist (Macleod et al. 2016), a negative modulator of psoriasis development (Macleod et al. 2016). Only few studies have actually attempted to clarify the pathogenic role of neutrophils in disease pathogenesis by utilizing different types of preclinical models (Schön et al. 2000; Singh et al. 2016; Sumida et al. 2014). In one study performed on flaky skin mice (fsn/fsn), a spontaneously developing psoriasis-like mouse model, neutrophils were proposed to be pro-inflammatory and to promote psoriasis development. However, these conclusions were drowned based on the effect of neutrophil depletion or blockage of infiltration into the skin by injections of anti-GR-1 Ab (clone RB6-8C5) or anti- α M β 2 (CD11b/CD18; clone M1/70) Ab, respectively (Schon et al., 2000). As these types of interventions affect also several other subsets of myeloid cells, including monocytes/macrophages and DCs which also play a pivotal role in psoriasis development (Costa et al. 2017; Singh et al. 2016; Tortola et al. 2012; Christian Wohn et al. 2013), it is difficult to draw definitive conclusions on the specific role of neutrophils in this model. Similar to our work, two other studies have instead attempted to perform neutrophil depletion by utilizing the more specific anti-Ly6G Ab (clone 1A8) in the IMQ-induced mouse model of psoriasis (Singh et al. 2016; Sumida et al. 2014). However, while we found a protective role of neutrophils in this experimental model, in the study by Sumida H et al. (Sumida et al. 2014) neutrophils were shown to contribute to psoriasis development and in the study by Singh, T. et al (Singh et al. 2016) neutrophils were instead shown to not affect disease development. The reasons for these controversial results can be likely attributed to the fact that several variations to the original protocol for IMQ-induced psoriasis (e.g. Aldara dosage, treatment of back skin *versus* ears, total days of treatment) have been utilized across different laboratories. We choose to perform the mostly utilized protocol originally published by van der Fits, L. et al (application of 60 mg of Aldara cream on the shaved back for 6 days (van der Fits et al. 2009). On the contrary, in the study performed by Sumida, H. et al. (Sumida et al. 2014), psoriasis was induced by applying a lower dose (30 mg) of Aldara cream

on the shaved back for 6 days, and in the study by Singh, T. et al. (Singh et al. 2016) by applying 25 mg of Aldara cream on the ears for 4 days. In this context, it is noteworthy to remark that in our experimental conditions we did not observe any effect of neutrophil depletion on epidermal thickening after 3 days of IMQ treatment, likely because at this time point the number of neutrophils infiltrating the lymph nodes and skin was much lower than the one observed after 6 days of IMQ treatment. It is therefore possible that the lower dose and/or shorter protocol of Aldara cream utilized in the study by Sumida, H. et al. and by Singh, T. et al. may have induced a suboptimal neutrophil activation and infiltration into the skin, as compared to the one induced by our experimental protocol, leading therefore to different outcome on disease development after neutrophil depletion. In support of this hypothesis is the fact that, differently from our study, in the paper by Sumida, H. et al. the authors report a pick of neutrophil infiltration at 3 days after Aldara treatment with almost no neutrophil infiltration after 6 days (Sumida et al. 2014). The different housing conditions of the animal facilities may have also influenced the controversial results among the three studies. However, we tend to exclude this possibility as neutrophil-depleted mice treated with our experimental protocol of IMQ-induced psoriasis and housed in a different animal facility (University of California, San Francisco, USA) displayed a similar enhancement of epidermal thickening after 6 days of IMQ treatment (C.A. and C.A.L. unpublished observation).

Despite these limitations, likely intrinsic to the peculiar experimental model and conditions utilized, we believe that the important message emerging from our study is that neutrophils may acquire a regulatory role during psoriasis development throughout their preferential interactions with $\gamma\delta$ T cells. In this context, controversial observations are emerging also on the crosstalk occurring between neutrophils and $\gamma\delta$ T cells (Kalyan & Kabelitz 2014; Scapini & Cassatella 2014). For example, human neutrophils were initially shown to stimulate $\gamma\delta$ T cells (Davey et al. 2011), while more recent evidence suggests that neutrophils negatively modulate spontaneous and phosphoantigen-induced $\gamma\delta$ T-cell activation (Sabbione, María L Gabelloni, et al. 2014) or contribute to the loss of peripheral blood V γ 9V δ 2 T cells observed after long-term or high-dose

administration of zoledronate (Kalyan et al. 2014b). In mice, neutrophils were initially shown to inhibit $\gamma\delta$ T cell functions in a mouse model of *C. neoformans* infection (Wozniak et al. 2012), while a more recent paper revealed that neutrophils can actually sustain IL-17 production by $\gamma\delta$ T cells in the lung *via* IL-1 β secretion in a NLRP3-inflammasome dependent manner during the initial phase of pneumococcal infection (Hassane et al. 2017). Different mechanisms have also been reported to mediate the interactions between neutrophils and $\gamma\delta$ T cells. For instance human neutrophils have been reported to both promote (Towstyka et al. 2017) and inhibit (Fazio et al. 2014) $\gamma\delta$ T functions via the release of serine proteases. Similarly to what reported by Sabbione et al. (Sabbione, María L Gabelloni, et al. 2014) using human neutrophils, we found that the inhibitory effect of murine neutrophils on $\gamma\delta$ T cells is mediated via cell-contact depended NADPH oxidase activation and ROS release.

$\gamma\delta$ T cells are known to be particularly susceptible to oxidative stress (Marlin et al. 2017). Interestingly, several evidence support a contribution of IL-17 in Chronic Granulomatous Disease (CGD) mediated hyper-inflammation (Rieber et al. 2012) and susceptibility to autoimmune diseases (De Ravin et al. 2008). Despite the fact that these phenomena have been so far mostly linked to an expansion of Th17 lymphocytes (Horváth et al. 2011), the possible contribution of $\gamma\delta$ T cells is also starting to emerge. In this context, a strong response of IL-17 producing $\gamma\delta$ T cells was reported in *p47phox*^{-/-} mice infected with *A. fumigatus* (Romani et al. 2008). Relevant to our study is also the observation that *p47phox*^{-/-} mice were reported to develop enhanced IMQ-induced psoriasis (Kim et al. 2014). Future studies should further characterize the specific role of neutrophil-mediated inhibition of $\gamma\delta$ T cell functions in this phenomenon.

Overall this study proposes that neutrophils, instead of being promoters of inflammation as generally believed, can actually act as important negative regulators of the IMQ-mediated model of psoriasis. Considering that psoriasis consists of several different clinical phenotypes, each of them characterized by distinct balance between autoimmune and auto-inflammatory immune processes, it may be worth to further verify the effective role of neutrophils also in human psoriasis. Neutrophils may indeed act as unexpected negative players of disease

development in specific types or clinical stages of human psoriasis. Consequently, also the utilization of therapeutic interventions targeted to inhibit neutrophil functions should be carefully evaluated. In this context, similarly to mouse psoriasis, the important role IL-17 producing $\gamma\delta$ T cells in human psoriasis has just started to emerge (Cai et al. 2011; Laggner et al. 2011), indicating that possible inhibitory crosstalk between neutrophils and $\gamma\delta$ T cells may exist also in human psoriasis and play a potential role in the modulation of disease development.

6. Additional studies

During my PhD I also participated to the following studies:

Role of MyD88 signaling in the imiquimod-induced mouse model of psoriasis: focus on innate myeloid cells.

Costa S, Marini O, **Bevilacqua D**, DeFranco AL, Hou B, Lonardi S, Vermi W, Rodegher P, Panato A, Tagliaro F, Lowell CA, Cassatella MA, Girolomoni G, Scapini P.
J Leukoc Biol. 2017 Sep;102(3):791-803. doi: 10.1189/jlb.3MA0217-054RR. Epub 2017 Jun 22.

Mature CD10⁺ and immature CD10⁻ neutrophils present in G-CSF-treated donors display opposite effects on T cells.

Marini O, Costa S, **Bevilacqua D**, Calzetti F, Tamassia N, Spina C, De Sabata D, Tinazzi E, Lunardi C, Scupoli MT, Cavallini C, Zoratti E, Tinazzi I, Marchetta A, Vassanelli A, Cantini M, Gandini G, Ruzzenente A, Guglielmi A, Missale F, Vermi W, Tecchio C, Cassatella MA, Scapini P.
Blood. 2017 Mar 9;129(10):1343-1356. doi: 10.1182/blood-2016-04-713206. Epub 2017 Jan 4.

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