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PRECISION MEDICINE IN PSORIASIS: GENETIC INFLUENCE ON RESPONSIVENESS TO ANTI-ILs THERAPIES AND CLINICAL IMPLICATION OF PHARMACOLOGICAL TREATMENT WITH ANTI-TNF-α

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Precision medicine in psoriasis: Genetic influence on responsiveness to anti-ILs therapies and clinical implication of pharmacological treatment with anti-TNF-α

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SYNOPSIS OF THE STUDY

Background:

Psoriasis is a multifactorial disorder caused by inherited susceptibility alleles and environmental factors. In the current pathogenic mechanisms model, the cross-talk between autoreactive T-cells and resident keratinocytes plays a central role for the initiation and progression of disease. Early upstream events occurring in psoriasis include induction of innate immunity responses triggered by keratinocyte-derived autoantigens, which can activate dendritic cells (DC). DC, in turn, drive expansion of T lymphocytes, typically T helper 17 in the initial phase and IFN-γ-producing T cells during the chronic phase of the disease, by releasing IL-23 and IL-12, respectively. T cells present in active psoriatic skin establish a cytokine milieu, responsible for the local aberrant inflammatory responses and the impaired differentiation and cornification processes in the epidermis.

Genetic epidemiologic studies put in evidence that the disease inheritability is up to 60-90%, which is one of the major reported for multifactorial diseases. In support of this, studies carried out on genome-wide genotyping platforms have now identified 63 psoriasis susceptibility loci. Specific single-nucleotide polymorphisms (SNPs) were, thus, identified in genes involved in inflammatory pathways, epidermal differentiation functions, as well as in innate and adaptive immune responses. However, the major genetic determinant of psoriasis resides in PSORS1 locus, mapping to the MHC region on chromosome 6p21. This locus spans the MHC class I region and encompasses nine genes, including *HLA-C, CDSN* and *CCHCR1*, that are highly polymorphic. In particular, *HLA-Cw6* allele is known as the strongest psoriasis susceptibility genetic factor and supposed to be involved in antigen presentation to $CD8^+$ T cells.

Immunomodulation with biologics targeting pathogenic molecules are highly effective in the treatment of psoriasis, as well as of various immune-mediated inflammatory diseases. Nowadays, a variety of biological therapies are available for psoriatic patients. These agents are potentially highly effective, and include the anti-TNF biologics, the anti-IL-12/23 inhibitor, the newer class of biologicals targeting IL-17 and its receptor, and the lastly identified anti-IL-23p19 drugs. These agents are potentially highly effective, even though they may differ in time until a clinically satisfactory response is reached. In addition, a variable percentage of patients does not or only partially respond to biological therapies or develops cutaneous reactions, namely paradoxical psoriasis, as side effects. These often requires the interruption of the imputable drug and switching to other therapies.

Several evidences have related the mechanisms underlying drug response variability to the presence of specific genetic variants. To date, SNPs located in *HLA-C*, *TNFAIP3*, *TNFA*,

TNFRSF1B, *IL-12B* and *IL-23A* genes have been associated to different response to anti-TNF and anti-IL-12/IL-23 drugs. Genetic factors have also been postulated to play a pivotal role in the development of paradoxical psoriasiform reactions to anti-TNFs.

Research hypothesis and aims:

Considering the variable response to biological drugs, the possible undesirable side-effects and, not least, the high cost of biological therapies, the identification of genetic biomarkers to predict treatment response of psoriatic patients to biological drugs, either in terms of efficacy/inefficacy or safety improvement of the drugs, could greatly impact clinical decisions. We hypothesize that the variability of response of psoriatic patients to biologics, in particular to anti-IL-17A or to anti-IL-12/IL-23 drugs, as well as of patients treated with TNF blockers and developing psoriasis-like paradoxical reactions, can be attributed to their genetic background.

Therefore, the present research aimed at identifying: i) the genetic variants of psoriasis-related risk loci associating with clinical responsiveness to anti-IL17A or anti-IL12/IL-23 drugs in two large cohorts of patients affected by mild-to-severe plaque psoriasis; ii) the genetic variants or SNPs and the immunological profiles, associating with the development of paradoxical psoriasis in patients undergone anti-TNF therapy for hidradenitis suppurativa (HS) condition.

Materials and Methods:

A panel of SNPs associated with psoriasis-related risk *loci* were analyzed in two cohorts of patients diagnosed with moderate-to-severe chronic plaque-type psoriasis, treated with secukinumab (n = 63) or ustekinumab (n = 150). The severity of psoriasis and response to treatment were evaluated using the Psoriasis Area and Severity Index (PASI) score and then at follow-up visits on weeks 8, 16, 24, 40, 56, 64, 72, 88, 100 (secukinumab) or on weeks 4, 12, 28, 40, 52, 64, 76, 88, 100 (ustekinumab). The selected SNPoma, composed of n = 44 SNPs, highly represented in the psoriatic populations (minor allele frequency > 0.3) and potentially implicated in immune responses (T-cell signaling, antigen presentation), as well as inflammatory pathways and skin barrier function, were evaluated by a Next-Generation Sequencing (NGS) technology. Differences between the groups based on the clinical response to anti-ILs (≥ 75% reduction of PASI score, PASI75; ≥ 90% reduction of PASI, PASI90; 100% reduction of PASI, PASI100) were evaluated by statistical test and univariate logistic regression analysis.

SNP analysis was also performed on three HS patients, who developed paradoxical psoriasis following adalimumab therapy for HS condition. Immunological profiles were examined by

immunohistochemistry and real-time PCR in skin biopsies kept from paradoxical skin lesions, as well as by flow cytometry on blood and skin-derived T cells. The immunological patterns of paradoxical psoriasis were compared with those present in canonical psoriasis.

Results:

A panel of SNPs in HLA-C region were found to associate to a better response to secukinumab treatment. In particular, a significant association between four SNPs in HLA-C region, namely *HLA-Cw6* v1 (classical *HLA-Cw6*), *HLA-Cw6* v2, *HLA-Cw6* v3, *HLA-Cw6* LD, and response to the drug was found. Psoriatic patients carrying *HLA-Cw6* v1 reached PASI100 faster than HLA-Cw6-neg patients, and maintained this result up to week 24. *HLA-Cw6*-pos patients also showed a tendency to greater respond to secukinumab, in terms of achievement of PASI90 and PASI100. However, the most significant associations were observed for *HLA-Cw6* v2 and HLA-Cw6 LD variants, whose presence in psoriatic patients was associated to the achievements of PASI75 or PASI 90 starting from week 16 or week 4, respectively, up to week 56. Interestingly, the absence of *HLA-Cw6* v3 allele in psoriatic population guaranteed a better response to secukinumab, in terms of achievement of PASI75 at different time-points of evaluation (weeks 24, 40, 56, 64, 72, 88, 100).

Differently from secukinumab-treated patients, ustekinumab-treated cohort was strongly influenced by *HLA-Cw6* v1 allele status, but not by *HLA-Cw6* LD, *HLA-Cw6* v2, or *HLA-Cw6* v3 variants. The association between *HLA-Cw6* v1 allele presence and response to ustekinumab was significant for patients reaching PASI90 or PASI100, starting from week 12 up to week 100. In addition, two SNPs in *TNFA* gene determined a greater and long-lasting response to ustekinumab. Similarly, the presence or absence of two SNPs in *CDSN* gene, respectively, strongly associated with a good response to ustekinumab (PASI90), which was maintained up to 100 weeks. PASI90 was also reached by the majority of ustekinumab-treated patients carrying SNP in *CCHCR1* gene.

The SNP analysis of the three HS patients with paradoxical reactions showed that they carried out allelic variants in genes predisposing to psoriasis. Among them, SNPs in *ERAP1*, *NFKBIZ* and *TNFAIP* genes and in the *HLA-C* genomic region were found. Moreover, paradoxical psoriasiform skin reactions showed immunological features common to acute psoriasis, characterized by cellular players of innate immunity. In addition, type I IFNs typical of acute psoriasis were highly expressed in paradoxical skin reactions, concomitantly to other innate immunity molecules, such as the catheledicin LL37 and lymphotoxin (LT)-α and LT-β. Differently from classical psoriasis, psoriasiform lesions of HS patients showed a reduced

number of IFN-γ and TNF-α-releasing T lymphocytes. On the contrary, IL-22 immunoreactivity significantly augmented together with the IL-36γ staining.

Implication of the study /Conclusions:

SNPs in HLA-C region, including *HLA-Cw6* psoriasis allele, and in *CDSN*, *CCHCR1* and *TNFA* genes, all mapping in PSORS1 locus, were found to associate to a better response to secukinumab or to ustekinumab treatments in two large cohorts of psoriatic patients. Thus, determination of these SNP status could be useful to predict the clinical response to secukinumab or ustekinumab therapies.

The present research also identified a panel of allelic variants present in HLA-C region, as well as in *ERAP1*, *NFKBIZ* and *TNFAIP3* genes in three patients showing paradoxical psoriasis reactions after anti-TNF-α therapy. Investigations on the immunological profiles of patients with paradoxical psoriasis permitted to unveil new pathogenic mechanisms involving innate immunity pathways, and in common with acute psoriasis.

As a whole, these data are potentially of great interest since very few studies investigated the association between polymorphisms and paradoxical psoriasis. In the future, it will be necessary to extend the analysis of SNPs predisposing to psoriasis in larger cohorts of patients manifesting paradoxical skin reactions to anti-TNF drugs, but also in a population successfully responding to anti-TNF treatment to identify possible difference in the genetic background of the patients. Considering the increased incidence of paradoxical psoriasiform reactions, it becomes increasingly necessary to investigate the immunological and genetic profiles of patients developing these reactions, in order to understand the pathogenic mechanisms and to predict the risk of developing paradoxical effects.

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Clinical features of psoriasis

Psoriasis is a chronic, relapsing immune-mediated disease involving the skin of genetically predisposed individuals. It affects approximately 2-3% of the general population and can develop nearly equally in women and men, across all socioeconomic groups and at any age, following a bimodal age of onset. In > 50% of patients it presents in the first three decades of life, in particular, the mean age for the first presentation can range from 15 to 22 years of age, with a second peak occurring at 57-60 years [1].

Individual lesions vary from pinpoint to large plaque, or even generalized erythroderma. More specifically, the clinical spectrum of psoriasis includes several variants, such as plaque, guttate, inverse, erythrodermic, pustular, palmo-plantar, and drug-associated psoriasis. Plaque-type psoriasis, occurring in 85–90% of affected patients, is the most common and well-recognized morphologic presentation of psoriasis and can be fairly easily diagnosed as characteristic red colored plaques with well-defined borders and silvery-white dry scale, located on the scalp, extensor surfaces of the elbows and knees (Fig. 1a), trunk, limbs and lumbosacral area (Fig. 1b), although it can be more extensive. In approximately one-third of patients, more than 10% of the body is covered, and this is termed moderate-to-severe psoriasis. Clinical disease can also be assessed by a trained health-care practitioner, using the Psoriasis Activity and Severity Index (PASI) score. This tool ranks severity and area of erythema (redness), induration (thickness), and desquamation (scale) of the plaques in different body sections, with 72 as the maximal score. A baseline PASI score is assigned, the score is then re-evaluated at various time points in subsequent visits to estimate the efficacy of the assigned treatment. In this way, the progress of psoriasis is calculated, and most clinical studies consider that a 75% improvement from baseline is required for the treatment to be considered successful (reported as PASI75).

Evidence shows an association of psoriasis with arthritis, depression, inflammatory bowel disease and cardiovascular diseases. Several other co-morbidity conditions have been proposed as related to the chronic inflammatory status of psoriasis, such as metabolic syndrome, atherosclerosis, nonalcoholic fatty liver disease, chronic obstructive pulmonary disease, osteoporosis and celiac disease. Co-morbidities related to lifestyle (i.e. smoking habit, alcohol consumption, anxiety) or treatments (i.e. dyslipidemia, nephrotoxicity, hypertension, hepatotoxicity, skin cancer) are also notable [2].

The classic histological features of psoriasis can help explain the clinical appearance (Fig. 1c). The epidermis is greatly thickened (acanthosis), as the keratinocytes move through the

epidermis over 4–5 days, a ten-folds acceleration, but the rate of desquamation remains the same, thus leading to epidermal hyperplasia. There is a loss of the normal granular layer, thickened stratum corneum (hyperkeratosis), and retention of nuclei in the upper layers and stratum corneum (parakeratosis). The epidermis is infiltrated by neutrophils which are collect in Kogoj pustules and Munro's microabscesses. In the dermis, an inflammatory infiltrate composed mainly by $CD3^+$ T cells, dendritic cells, macrophages, mast cells and neutrophils are observed. The erythema of psoriasis lesions is due to a greater number of dilated dermal blood vessels in the dermal papillae.

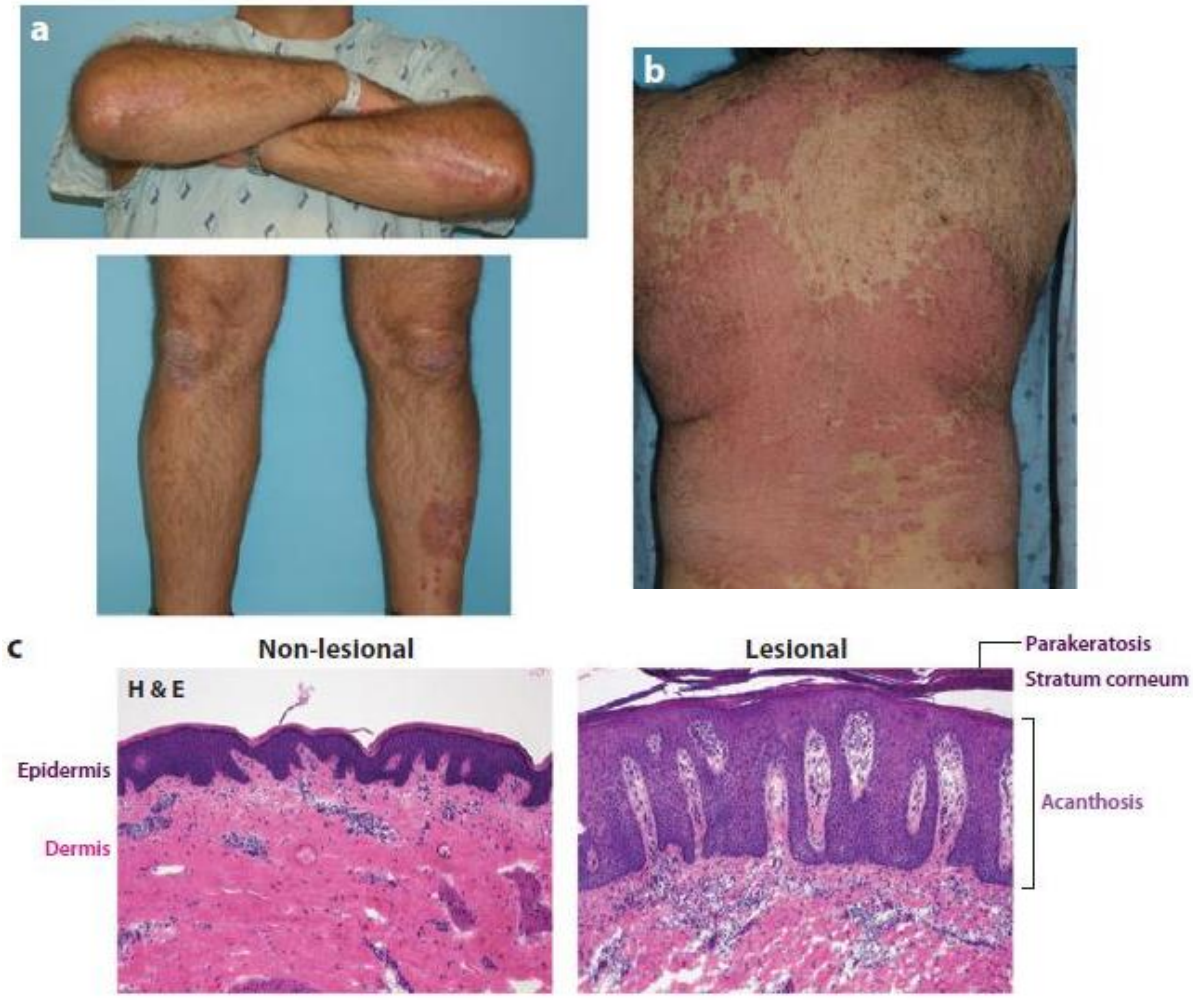


Figure 1. **Clinical and histological features of psoriasis**. (*a*) Clinical appearance of chronic psoriasis vulgaris, showing well-defined erythematous scaly plaques of psoriasis on elbows and knees. (*b*) Back showing more extensive psoriasis lesions. (*c*) Histology of nonlesional and lesional skin biopsy at the same magnification, with hematoxylin and eosin stain (H&E). The epidermis is seen as a dark layer due to keratinocyte nuclei and forms an undulating border with the pink dermis below. Nuclei of resident structural and immune cells are seen in the dermis. Lesional psoriasis skin shows a greatly thickened epidermis (acanthosis) with elongations into the dermis (rete ridges). Retention of nuclei (parakeratosis) can be seen in the thickened stratum corneum. There is a dramatic increase in the number of cells in the dermis, composed predominantly of DCs and T cells. All images 10x magnification. From figure 3 of ref [3].

Lesions can spontaneously resolve, although rarely. Resolving lesions after therapy can be encased by a distinctive rim of blanching (Woronoff's ring), predictive of clearing and histologically characterized by orthokeratosis, that is thickening of the stratum corneum without parakeratosis and restoration of the stratum granulosum.

1.2 Pathogenic mechanism and genetic predisposition to psoriasis

Initiation of complex diseases, such as psoriasis, takes place in genetically predisposed individuals in which a dysregulated immune response occurs following exposure to certain environmental triggers. In particular, psoriasis can be triggered by many environmental factors that can induce psoriasis *de novo* or exacerbate skin lesions. Trigger factors range from nonspecific stimuli including injury and trauma (termed the Koebner effect) to more specific triggers such as infection by pathogens (i. e. streptococci) or drugs (i. e. lithium, interferon (IFN)-α). Altough the exact mechanisms of the induction of psoriasis are not fully elucidated, all of these factor generate a pathogenetic cascade involving dynamic interactions between multiple cell types and numerous cytokines, that culminates in the expansion of lesional and/or circulating T cells in the psoriatic skin [3].

The epidermis and derma of the psoriatic skin have a central role in the reaction of the stimuli previously listed, as they are being able to participate in both innate or adaptative immune responses. Specifically, different cell types can participate such as Langerhans cells (LCs), myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs), macrophages, T lymphocytes and neutrophils [4,5]. Among all, a key role is played by keratinocytes which can: 1) proliferate in response to cytokines such as IL-22, to accelerate loss of surface and eliminate pathogens; 2) increase synthesis of innate effector molecules such as antimicrobial peptides (AMPs), and 3) direct migration of new T-cell subsets and other immune effector cells into the skin through production of chemokines [6,7]. In particular, injury to the skin causes cell death and the production of the endogenous AMPs, such as LL37, by keratinocytes. DNA fragment/LL37 complexes bind to intracellular Toll-like receptor 9 (TLR9) in pDCs, which causes induction of type I interferons (IFN-α and -β). In parallel, RNA fragments/LL37 complexes activate pDCs through TLR7, and mDCs through TLR8. Specifically, DCs increase the production of IL-12 and IL-23, essential for T-cell differentiation towards Th1/Th17 cells, respectively, and consequent T lymphocytes accumulation into the skin. Concomitantly, epidermal injury can trigger high-level production of CC chemokine ligands20 (CCL20) in keratinocytes, which in turn has the ability to attract $CD11c^{+}$ mDCs into the dermis, as well as CC chemokine receptor6 ($CCR6^{+}$) IL-17-producing T cells. Alternatively, infection may

activate innate immune pathways leading to production of tumor necrosis factor (TNF)- α or IFN-α in the skin. These cytokines, then, have the ability to induce chemokines, which would control recruitment of specific leukocyte effector population. In particular TNF-α serves to induce CCL20, which leads to mDC, Th17 and neutrophil recruitment, and IFN-α leads to maturation of mDCs with the ability to drive Th1 responses [8].

At this point, the switch from innate immune response of early disease to adaptative immune response of chronic disease takes place. Hence, activated mDCs migrates into regional lymph nodes and drive T-cell activation with consequence production of the principal cytokines found in psoriasis (Fig. 2a) [9].

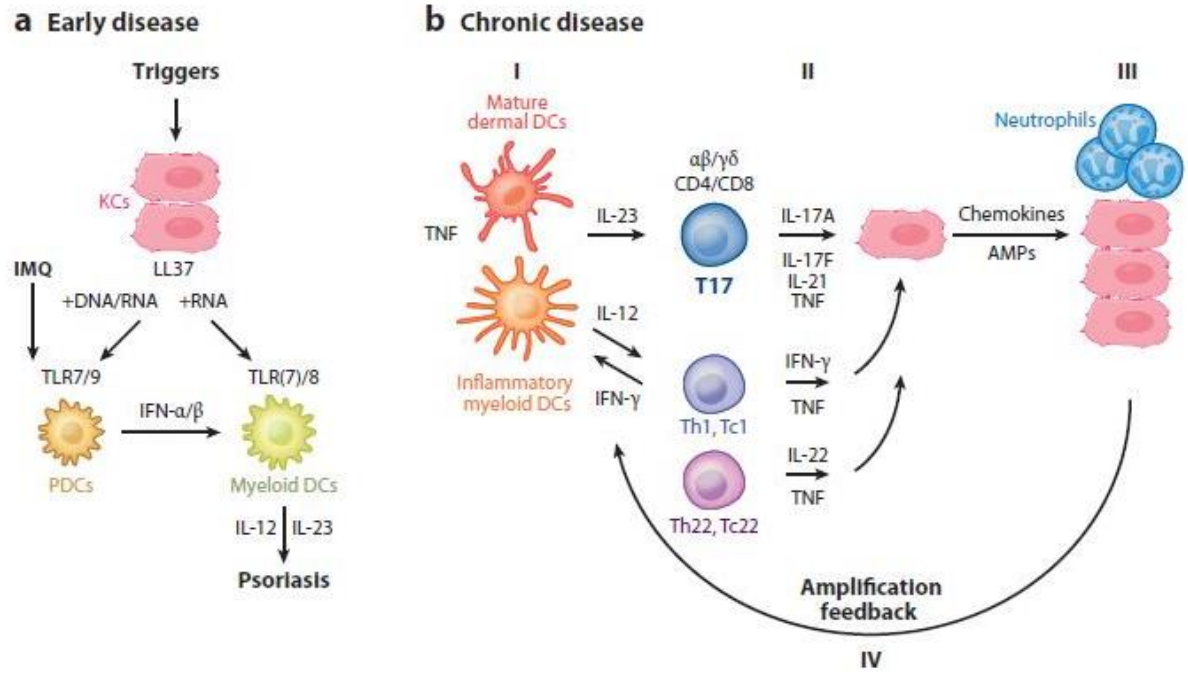


Figure 2. **Pathways for initiation and maintenance of psoriasis**. (*a*) Early disease: Imiquimod (IMQ), a TLR7 agonist, can activate plasmacytoid dendritic cells (pDCs) to produce interferons (IFN). LL37, a peptide derived from cathelicidin, may have an important role in the initiation of psoriasis lesions via this pathway. LL37 released from keratinocytes (KCs) can bind to nucleic acids to activate pDCs to release IFN-α/β. LL37/RNA complexes can also activate resident myeloid DCs to produce IL-12 and IL-23, key psoriatic cytokines. (*b*) Chronic disease: The major pathogenic pathway in psoriasis occurs when (I) mature dermal DCs and inflammatory myeloid DCs produce cytokines such as IL-23 and IL-12. (II) These cytokines activate T17 (Th17 and Tc17), Th1, and Th22 cells to contribute to the cytokine milieu and further act on keratinocytes. (III) From figure 4 of ref [3].

In this manner, Th1 activation, leads to an increased production of IFN-γ that induces synthesis of chemokines (CXCL9, CXCL10 and CXCL11) and can recruit more Th1 cells. Likewise, Th17 activation, leads to IL-17 release that activates CCL20, CXCL1, CXCL2 and CXCL8 synthesis, as well as the recruitment of more IL-17 and neutrophils into the skin. Activation of Th22 cells results in increased production of IL-22, which induces keratinocyte hyperplasia, with increased synthesis of S100 proteins and other AMPs, with consequent accelerated loss of surface keratinocytes and elimination of pathogens. In parallel, $CD8^+$ T-cell populations make the same range of cytokines, so these have been termed Tc1, Tc17, and Tc22, respectively [10–12].

These different subsets of T cells have a different hierarchical role in the T cell-mediated inflammatory cascade of psoriatic skin, and it is possible to distinguish the subsets that initiate the disease, those involved in the establishment of the self-sustaining amplification loop that leads to the cutaneous clinical manifestation [13]. Of note, the development of chronic disease activity may also be supported by mature DCs (DC-$LAMP^+$) that form cellular clusters with T cells in the dermis, and this structure can be considered as a form of induced skin-association lymphoid tissues (iSALT) or tertiary lymphoid tissue. Psoriasis could also results from failure to turn-off inflammation, which is perpetuated by this cutaneous tertiary lymphoid tissue (Fig. 2b) [3].

Finally, the inflammatory cytochine milieu also influences the immune functions of fibroblasts and endothelium, with the latter being critical for leukocyte trafficking and extravasation [14].

While the associations linking environmental triggers with dysregulated immune processes is well documented in psoriasis, epidemiological studies have repeatedly demonstrated that this condition has an important genetic component (Fig. 3).

The genetic basis of psoriasis has long been recognized, since family members of patients with psoriasis are at greater risk of developing the disease. That a genetic component may account for this finding is supported by studies among twins where the concordance rate of psoriasis is approximately 70% in monozygotic twins and 20% in dizygotic twins, depending on the study and population [15].

It is known that three billion base pairs exist in the human genome, and only 3-5% of these sequences code for proteins. A disease-causing mutation is usually quite rare (< 1%) and is commonly found in the coding or regulatory region. Although the psoriasis in mainly linked to polymorphisms within the human leukocyte antigen (HLA) locus, particularly HLA-Cw6 [16], the genetic factors of psoriasis is complex. It is assumed that there is no single disease gene, but, rather, a complex set of gene variants, resulting in an aberrant response to environmental

factors, and also in an intricate mode of inheritance, not explained by simple Mendelian genetics.

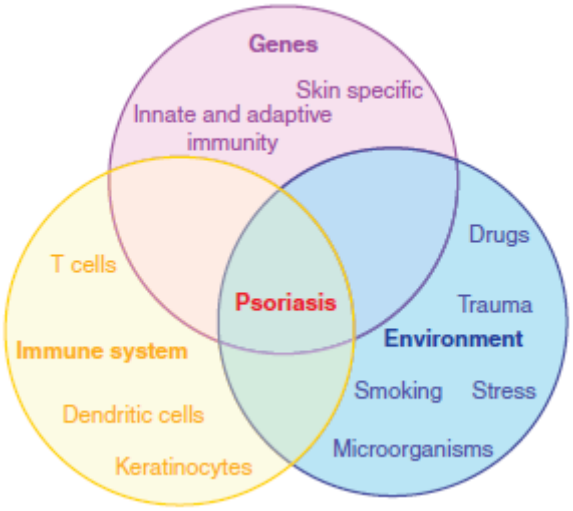


Figure 3**. Psoriasis etiopathogenesis**. Disease takes place in genetically predisposed individuals, carrying one or more psoriasis susceptibility genes (either skin specific or of immunological function) in which a dysregulated immune response (involving DC, T cells, and KCs) occurs, following exposure to certain environmental triggers. From figure 2 of ref [15].

Several psoriasis single nucleotide polymorphisms (SNPs) have been identified and thanks to classic genome-wide association study (GWAS) and linkage analysis, many other loci and polymorphism have emerged as imparting some susceptibility to psoriasis as well, and has identified at least 34 chromosomal loci with statistically significant linkage to psoriasis; these loci are called psoriasis susceptibility 1 through 34 (PSORS1 through PSORS34) [17,18].

The genes identified from GWAS can be grouped into four pathways (Fig. 4) [19]. Firstly, skin barrier function pathways have been strongly associated with psoriasis, with several studies that identified the LCE (late cornified envelope) gene cluster (*LCE3A*, *LCE3C* and *LCE3D*) higly expressed in psoriatic skin. Candidate gene studies have also implicated β-defensin gene cluster (*DEFB*) on chromosome 8 and interferon induced with helicase C domain 1 (*IFIH1*), as involved, the first one in protection against microbial invasion and the latest in induction of interferon response to viral RNA. A significant association was found between rs2740091 and rs2737532 in *DEFB4* and predisposition to psoriasis in Caucasian patients [20]. A similar association was reported for rs17716942 in *IFIH1* and rs4085613, rs4845454, rs1886734, rs4112788, rs6701216, rs4112788 in *LCE* gene.

The second and potentially the largest pathway, in terms of numbers of genetic loci implicated in psoriasis, consist on T-cell signaling, i. e. *ZNF313* (zinc finger protein 313), SOCS1 (suppressor of cytokine signaling1), STAT3/5A/5B (signal transducer and activator of

transcription3/5A/5B), *IL12B*, *IL23A* and *IL23R*, gene involved in the regulation of driving of Th17 responses. Variants within the genes *IL12B*, *IL23R* and *IL23A*, involved in the IL-23 signalling pathway, are all strongly associated with psoriasis susceptibility along with *ZNF313/RNF114*, which is also involved in T-cell activation. In particular, GWAS showed the combination of rs3212227 and rs6887695 in *IL12B* as a risk haplotype in psoriasis, and an association between rs11209026 and rs7530511 in *IL23R* gene and predisposition to psoriasis [21,22]. Other IL12B and IL23R susceptibility loci, identified in caucasian patients, include rs2201841-rs2066808 and rs2082412-rs2546890, respectively [23,24]. Also in *CTLA4* (cytotoxic T-lymphocyte antigen), encoding a protein that downregulates activation of T lymphocytes, an association with the GG haplotype of rs3087243-rs231775 was observed [25]. On the other side, T-cell activation and the ZNF313/RNF114 SNPs rs2235617 and rs495337) were found associated with psoriasis in a Caucasian cohort of patients.

Thirdly, the candidate genes identified from GWAS were found within the nuclear factor κB (NF-κB) pathway, and in a genetic region that is potentially involved in the modulation of Th2 immune responses.

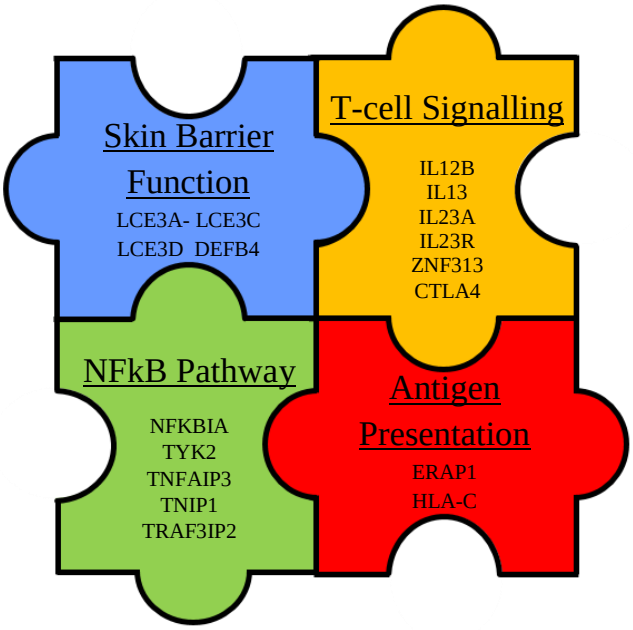


Figure 4. **The psoriasis genetic pathway**. A schematic representation of genetic variants within psoriasis-related risk loci, highly represented in the psoriatic population (allele frequency ≥ 0.3), involved in four broad immunological intersected processes, such as those involved in skin barrier function, inflammatory pathways (NFkB Pathway) and immune responses (T-cell signaling and antigen presentation). Notes: LCE3A/3C/3D, late cornified envelope 3A/3C/3D; DEFB4, defensin beta 4; NFKBIA, NFkB inhibitor alpha; TYK2, tyrosine kinase 2; TNFAIP3, TNF alpha-induced protein3; TNIP1, TNFAIP3 interacting protein 1; TRAF3IP2, TNF receptor-associated factor 3 interacting protein 2; IL12B/13/23A/23R, interleukin-12B/13/23A/23R; ZNF313, zinc finger protein 313; CTLA4, cytotoxic T-lymphocyte associated protein 4; ERAP1, endoplasmic reticulum aminopeptidase 1; HLA-C, human leukocyte antigen-C. Modified from figure 1 of ref [19].

In several studies, a number of evidences of susceptibility genes on TNF-α–induced protein 3 (*TNFAIP3*) (rs610604, rs6920220, rs10499194, rs5029939) and TNFAIP3-interacting protein 1 (*TNIP1*) (rs17728338) were found. *TNFAIP3* encodes a protein interacting with the products of *TNIP1*, that regulates the activity of NF-κB and also induces keratinocyte hyperproliferation [26]. In another GWAS, TNF receptor-associated factor 3- interacting protein 2 (*TRAF3IP2*) (rs13210247, rs339805500, rs240993), a gene encodes a protein that interact with *v-rel* reticuloendothelialiosis viral oncogene (*NF-κB/REL*) complexes, was associated with psoriasis in Caucasian cohort of patients [24]. In the same study, an association with the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (*NF-κBIA*, rs2145623) was also identified.

Nevertheless, the pathway implicated in antigen presentation to the adaptive immune system, and involving *HLA-C* and *ERAP1* genes, has been associated with psoriasis pathogenesis. This pathway will be extensively described in the next section.

1.2.1 PSORS1 locus

The first genetic association studies in psoriasis were carried out in 1972 and were focused on the major histocompatibility complex (MHC) region, on chromosome 6p21.3 [27]. But it is in 1997, when the first linkage studies to map psoriasis susceptibility to the HLA region were conducted [28].

The first region spanning 250 kb within the MHC, designated just PSORS1, was identified by genome-wide linkage scans, and has the highest odds ratio (OR) of any PSORS loci (approximately 3.0) [29]. Four genes within the region have been the major focus of investigation because of the strong association of polymorphic coding-sequence variants with psoriasis vulgaris. HLA-C (associated variant, HLA-Cw6) encodes a class I MHC protein, CDSN (associated variant, allele 5) encodes corneodesmosin, CCHCR1 (associated variant, *WWCC) encodes the coiled-coil, x-helical rod protein 1 and TNF-α. However, the absolute identification of the causative gene at this locus has proven to be challenging because of the extensive linkage disequilibrium (i.e. the tendency for particular alleles at two or more loci to be inherited together more often than would be predicted by chance) present within the MHC.

HLA-C plays a central role in the immune system by presenting pathogenic proteins derived from cytosol and its gene contains 8 exons. Exon one encodes the leader peptide, exons 2 and 3 encode the alpha1 and alpha2 domain, which both bind the peptide, exon 4 encodes the alpha3 domain, exon 5 encodes the transmembrane region, and exons 6 and 7 encode the cytoplasmic

tail. Polymorphisms within exon 2 and exon 3 are responsible for the peptide binding specificity of each class I molecule. Over one hundred HLA-C alleles have been described. Nevertheless, the analysis of high-density polymorphism, have indicated several SNPs within the minimal promoter region of HLA-C result in differential expression of various alleles and also contribute by affecting HLA-C expression [30,31]. In particular, a significantly higher frequency of HLA-Cw6 is associated with early-onset (type I), compared with late-onset (type II) psoriasis. Recently, exciting evidence has emerged for an interaction between the HLA-C and ERAP1 (involved in trimming peptides to enable effective loading and processing onto MHC class I) loci in psoriasis [32]. Even so, despite the strong genetic evidence and the obvious immunological function of HLA-C to regulate both innate and adaptative response, functional studies addressing the precise mechanism by which HLA-Cw6 alleles predispose to psoriasis are still missing and no HLA-Cw6-specific antigen or interacting protein has been identified to date.

The other proposals for the causative gene of PSORS1, the CDSN (a protein that is uniquely expressed in the granular and cornified layers of the epidermis and up-regulated specifically in psoriasis) with non-parametric study yielded significant linkage and association of allele 5 of the gene (CD*5) with psoriasis and found that 38% of haplotypes containing this allele did not contain the HLA-Cw6 allele.

Furthermore, CCHCR1 (ubiquitously protein overexpressed in psoriatic epidermis that has a role in keratinocyte proliferation) was first proposed as a potential candidate-gene when 12 coding variants were discovered within its [33]. These variants were tested for association along with CD*5 and HLA-Cw6 and the results showed significant association for an Arg-Arg synonymous SNP in the CCHCR1 gene. Four SNP haplotype of CCHCR1 has been found to be significantly associated with psoriasis at a level similar to those obtained for HLA-Cw6 [34], and supposed to be transmitted in LD.

Finally, among the genes enconded within PSORS1 region, the TNF-α showed a strong association with early-onset psoriasis in Caucasian patients, in particular the rs361525 *A allele was more frequent and the rs1800629 *A allele was less frequent in patient with type I psoriasis than in controls, although no differences were found between these polymorphism and type II psoriasis [35,36].

1.3 Pharmacological treatments and possible outcomes

Management of psoriasis in daily clinical practice is highly variable, although there is no cure, there are multiple effective treatment options, mainly targets different aspects of the inflammatory pathway. Depending on the severity of psoriasis, location of disease, relevant comorbidities, and patient preference, the appropriate treatment can be chosen. According to the guidelines, for mild disease (defined by PASI levels equal or less than 10), topical agents are commonly used [37]. Among these, emollients keep the skin moist and soft, mid- to high-potency topical corticosteroids reduce inflammation, vitamin D analogues calcipotriol reduce the proliferation of keratinocytes and modulate T cell and DCs function. These topical treatments are efficacious and can be safely initiated and prescribed. Instead, for moderate-to-severe psoriasis (defined by PASI levels exceeding 10), along with these topical treatment, phototherapy and different systemic therapy can be used. Usually, narrow band (311nm wavelength) UVB phototherapy and PUVA photochemotherapy are a good treatment options. For patients with more severe skin involvement and refractory symptoms, conventional oral systemic therapies are usually initiated, wich include retinoids (derivative of vitamin A with anti-proliferative and anti-inflammatory effect), methotrexate (folic acid antagonist, which inhibits purine synthesis), or cyclosporine (calcineurin inhibitor, which inhibits T cell activation and IL-2 cytockine production) [37–39].

When systemic agents are unable to control the lesions, then biologic agents are considered, which generally demonstrate greater efficacy than oral systemic agents.

Biological drugs are a relatively new type of treatment that can be designed to target specific immune pathways. Specific cytokines pertinent to the development of disease have been selected as drug targets in the hope of effective suppression of pathogenic immune responses, whilst reducing the risk of global suppression of protective immunity. As the pathogenic mechanisms have become better defined, there has been a shift towards the design of more targeted treatments in psoriasis [9].

Biological agents are recombinant molecules that are designed on the basis of genetic sequences from various organisms and that are often similar or identical to proteins produced by human beings. They include fusion proteins, recombinant proteins (i.e, cytokines, selective receptors) and monoclonal antibodies [39].

The first biologic agent developed specifically for a dermatologic disease was alefacept (which has been withdrawn from the market), a T-cell-targeted biologic agent. Dermatologists have subsequently moved from serendipitous choices among the available therapeutic options to targeted intervention based on increased insights into the pathogenesis of psoriasis. There was

therefore a development of antibody or fusion protein selective targeting of key cytokines mediators of inflammation. These latest biologic therapies in psoriasis, with cytokines-targeted agents, are highly effective and well tolerated overall [40].
Right now, the three most frequently used classes of biologics for the treatment of psoriasis include anti-TNF-α agents, anti-IL-12/IL-23 agent, anti-IL-17, and anti-IL-23 inhibitors [41].

1.3.1 Biologic treatments: anti-TNFs, anti-ILs

More than 1-2 million patients have been treated with TNF-α inhibitors over the past 10 years for inflammatory diseases, such as Crohn's disease, inflammatory arthritis, ankylosing spondylitis, and ulcerative colitis. The experience with psoriasis and psoriatic arthritis (PsA) is less extensive, even if they can be prescribed as monotherapies, or in combination with traditional systemic agents. TNF-α inhibitors comprehend adalimumab, etanercept, infliximab, certolizumab pegol and golimumab drugs [42,43].

- Adalimumab is a recombinant, fully human, immunoglobulin G1 (IgG1) monoclonal antibody, self-administered subcutaneously in a dose of 80 mg at week 0, 40 mg at week 1 and then 40 mg on alternate weeks. In a 24-week study, adalimumab substantially improved joint and skin manifestations of psoriasis, with 54% of patients achieving PASI75. Real-life study showed that adalimumab, in the aforementioned dose, significantly improved psoriasis, with 58% of patients reaching PASI 75 at 60 weeks [44]. Patients had substantial improvements in their quality of life [39]. It is currently the only biologic labelled for the specific indication of pediatric and severe fingernail psoriasis, as well as for other immune-mediated inflammatory conditons, such as hidradenitis suppurativa (HS). A predictive response biomarker was recently identified: HLA-Cw6 negative patients were more likely to respond to adalimumab than to ustekinumab [45].
- Etanercept is a recombinant fusion protein formed by the extracellular domain of TNF-α receptor 2 and the Fc portion of human IgG1. It is self-administered subcutaneously at a dose of 50 mg twice weekly for 3 months and then weekly. Pivotal trials with this dose, showed an achievement of PASI75 in 49% of patients by 12 weeks. Etanercept is highly effective in PsA, with a reduction in the signs and symptoms of joint disease in 73–87% of patients at 12 weeks of treatment [39]. Recent meta-analysis of real-life evidence highlights etanercept as the anti-TNF-α with the lowest survival rate (66% at

year 1, 41% at year 4) compared to adalimumab, infliximab and ustekinumab. In addition, it was the most discontinued due to the loss of efficacy [46].

- Infliximab is a monoclonal chimeric antibody comprising a murine variable region and human IgG1 (heavy and kappa light) chain constant domain regions. Infliximab is given as an intravenous infusion, optimally in a dose of 5 mg/kg over 2–3 h at weeks 0, 2, and 6, and at regular 8-week intervals thereafter. It is the only TNF-α inhibitor with weight base dosing. This regimen gives rapid and marked improvement as an induction therapy, with 82% of patients achieving PASI75 improvement at week 10 [39]. Real-life data indicate a drug survival dropping from 61% at year 1 to 42% at year 4 (it is most frequently discontinued for adverse effects) [46].

Recently, there has been increasing comprehension of the value of the Th17 lineage of T cells and related cytokines, such as IL-17 and IL-23, particularly in the pathogenesis of inflammatory skin diseases. New drugs that are designed to inhibit steps in this pathway, have shown meaningful effectiveness in treatment of PsA and moderate-to-severe plaque psoriasis.

Among them, IL-12/IL-23 inhibitor directs towards the homonymous pathway by binding and inhibiting p40 subunit shared by both IL-12 and IL-23 [11,47]. The latter are produced by antigen-presenting cells, such as macrophages and dendritic cells, and the p40 subunit binds to the IL-12 receptor β1 (IL-12Rβ1), located on the surface of Th1 and NK immune cells. The second subunits of IL-12 and IL-23 are p35 and p19, respectively, through which the ILs can bind to the specific IL-12Rβ2 and IL-23R receptors. Recently, three biological drugs targeting specifically p19 subunit of IL-23 have been developed and resulted to be particularly effective in the treatment of exacerbations of psoriatic skin and joint symptoms. This novel class of IL-23 inhibitors includes guselkumab, risankizumab and tildrakizumab [42].

- Ustekinumab is a fully human monoclonal IgG1 antibody that binds to the common p40 subunit of IL-12 and IL-23, thus inhibiting their activity and presumably, the T-cell pathways that they influence, Th1 and Th17, respectively. Ustekinumab is approved for the treatment of psoriasis, PsA and paediatric psoriasis, in a weight-based regimen: 45 mg for patients less than 100 kg and 90 mg for those who are greater, administered subcutaneously at an interval of 4 weeks after the first injection, and then every 12 weeks. Real-world highlights that ustekinumab was associated the highest drug survival compared with anti-TNF-α and was also the least likely to be discontinued due to adverse effects [46].

  Efficacy and long-term safety of ustekinumab in the treatment of moderate-to-severe real-life chronic plaque psoriasis was demonstrated in a cohort of adult patients,

observed retrospectively over a time period of 8 years. Efficacy was observed in 378 patients, among which >80% achieved a PASI score of <3 and PASI 75, 90 and 100 response in 76.2%, 61.9% and 57.1% of patients, respectively. Of note, predictor variables for improved PASI response (after 2 years) were HLA-Cw6-positive patients, female gender and BMI <30 Kg/m^2. Ustekinumab was generally well-tolerated without evidence of cumulative toxicity or organ toxicity [44,48].

- Guselkumab is a fully human IgG1 monoclonal antibody and was the first directed against the p19 subunit of IL-23. In phase-III studies, VOYAGE 1 and 2, 73.3% and 70% of patients achieved PASI90 at 16 weeks. Notably, in both studies, clinical response to guselkumab was maintained after 100 weeks of treatment. Moreover, in NAVIGATE phase-III study, guselkumab proved to be effective in patients who did not respond to ustekinumab [49,50].

On the other side, two IL-17A inhibitors and one IL-17 receptor (IL-17R) blockers have been developed for the treatment of psoriasis, PsA and axial spondyloarthritis, as well as other immune-mediated inflammatory conditions. The efficacy of the IL-17A inhibitors has elevated the standard care for patients with severe psoriasis to the extent that PASI90, instead of PASI75, should now be considered as the criterion for assessment of treatment response. IL-17 class of inhibitors encompasses secukinumab, ixekizumab, and brodalumab [11,42,47]. Other two anti-IL-17A/F inhibitors, namely bimekizumab and netakimab, have been also recently developed.

- Secukinumab is a fully human monoclonal IgG1 antibody that targets IL-17A, blocking its binding with IL-17R. Secukinumab is administered subcutaneously at doses of 300 or 150 mg once weekly for 5 weeks, and then every 4 weeks. Secukinumab introduced a new era in the management of psoriasis, shifting possible psoriasis treatment outcomes from PASI75 to PASI90 or PASI100, which are now recognized goals for the treatment of psoriasis in Italian guidelines [51]. A multicenter retrospective real-life study on 324 secukinumab treatment patients have been conducted. This study revealed the achievement of PASI90 from week 24 to week 84, confirming the results of clinical trials, in terms of effectiveness, in a more complicated set of psoriatic patients (with comorbidities, polypharmacy, multi-drug failure) [52]. These results are in agreement with the extension phase of the SUPREME study, which demonstrated efficacy in psoriatic patients achieving PASI100 from week 16 to week 72. In parallel, although HLA-Cw6-positive and -negative patients have distinct clinical features, the SUPREME study showed that secukinumab achieved similar clinical responses in both cohorts after

72 weeks of treatment, underlined also the unnecessary determination of HLA-Cw6 status for this therapy [53,54].

Secukinumab had the highest number of PASI 100 responders, but also the lowest drug survival among all the biologics [55].

- Ixekizumab is a high-affinity, humanized monoclonal IgG4 antibody that binds IL-17A, used for psoriasis and PsA, 160 mg subcutaneously at week 0 and then 80 mg at week 2, 4, 8, 10, 12 and every 4 weeks thereafter. The efficacy of ixekizumab in a cohort of real-life psoriasis patients, has been evaluated by two multicenter retrospective studies [56,57]. The percentage of patients achieving PASI 75/90/100 at 12-16 weeks of treatment was 87.5%, 50.0% and 39.6%, respectively. These rates were generally maintained at weeks 24 and 52, whit PASI75, PASI90 and PASI100 responses between 83-88%, 58-59% and 42-27%, respectively. The percentage of patients who achieved PASI75 and PASI90 at week 52 was higher in patients who were naïve to biologic agents. Overall, this agent appears to have a similar good effect of secukinumab in psoriasis [58].
- Brodalumab is a fully human monoclonal IgG2 antibody that blocks the IL-17A receptor activity, that has been approved (210 mg s.c. at weeks 0, 1, 2 and every 2 weeks thereafter) for the treatment of psoriasis vulgaris, Psa, and pustular psoriasis. The drug inhibits the biological activity of IL-17A, IL-17F and other IL-17 isoforms, by impeding their binding to the IL-17RA subunit. Brodalumab is highly efficacious in the treatment of psoriasis, as demonstrated in three phase III study, patients showing PASI75/90/100 response at week 12 in 83.3%, 70.3% and 37%, respectively [59].

1.3.2 Undesirable and paradoxical effects of biologics

Targeted biological agents have dramatically changed the treatment landscape of immune-mediated inflammatory diseases showing to be very effective in treating various dermatological, rheumatological, and systemic diseases [60,61].

Although the overall safety and tolerability profile is acceptable, some patients develop adverse reactions that are not expected according to the mechanism of action. Such reactions have been denoted as paradoxical reactions and comprise a *de novo* or worsening immune-mediated condition that would normally respond to the biologic agent that causes them.

The hypotheses proposed to explain the pathogenesis of such reactions include one or more of the following mechanisms as *primum movens*: (a) an imbalance in cytokine production, with an overproduction of IFN-α (by inhibiting pDC maturation), (b) a shift in cutaneous immune

response pattern, with altered lymphocyte recruitment and migration, in part mediated by CXCL10, (c) a spatial shift of activated innate immune cells to the skin, and (d) an imbalance or dysfunction of regulatory T-cells [62]. Importantly, chronic immune responses are absent in paradoxical psoriasis induced by TNF-α blockers, with innate inflammatory processes predominant and not followed by expansion of autoreactive T cells. These processes are concomitant to dermal accumulation of immature pDC and type I IFN overexpression [63] (Fig. 5).

Some biologic therapies also favor granulomatous reactions, traditionally, because the role of TNF-α in granuloma formation and infections, such as tuberculosis. The incidence of infections is slightly greater with the two antibodies infliximab and adalimumab than with the fusion protein etanercept [39].

Most paradoxical reactions have been reported in association with anti-TNF-α therapy, but this is not solely involved and other cytochine and T-cell pathways are potential key players, in the pathogenesis of this paradoxical effect, explaining the appearance of psoriasiform lesions, although rarely, in patients treated with different biological therapy such as rituximab, anakinra and tocilizumab, as well as cases associated with biologic therapies, such as ustekinumab, secukinumab and ixekizumab, are increasingly reported in the literature (Fig. 5).

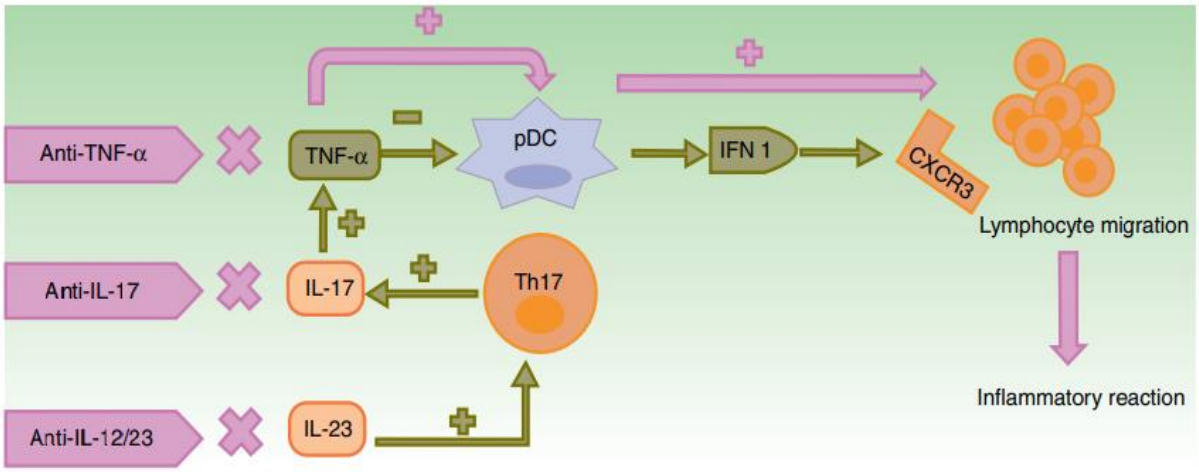


Figure 5. **Pathogenic mechanisms proposed to explain paradoxical reactions**. In normal conditions, TNF-α inhibits pDCs, which produce IFN-α. Use of anti-TNF- α molecules leads to an excess of IFN-α which, in turn, promotes expression of CXCR3 in T cells, thereby allowing migration to the inflamed tissue. The use of other biologic agents with different mechanisms of action such as ustekinumab (anti-IL-12/23) and secukinumab and ixekizumab (anti-IL-17A) indirectly leads to a decrease in TNF-α concentrations, with the aforementioned consequences. Notes: CXCR3, CXC3 chemokine receptor; IFN, interferon; IL, interleukin; pDC, plasmacytoid dendritic cell; Th cell, T helper cell; TNF, tumor necrosis factor. From figure 1 of ref [68].

The skin is frequently involved and, even if considered rare to uncommon, these cutaneous manifestations are an important cause of biologic agent discontinuation. In fact, management of these reactions consists of topical or systemic skin-directed therapies, depending on the severity and extension of the cutaneous picture, and it is generally associated with switching over to other disease-modifying regimens for treating the underlying rheumatologic condition. But in most cases, these reactions may require the interruption of the imputable drug, and no other biologics are approved for diseases like in HS. Therefore, it is important to understand the pathogenesis of these reactions, and the genetic susceptibility in these patients.

The 2–5% of patients treated with TNF-α antagonists can develop paradoxical psoriasiform skin lesions [64–66], whereas other reactions, such as eczematous and lichenoid eruptions, HS, pyoderma gangrenosum, Sweet's syndrome and granulomatous skin diseases, occur much more rarely. As well as from a clinical point of view, these cutaneous paradoxical reaction represent an intriguing immunological dilemma, whose unraveling may improve our knowledge of the pathogenesis of chronic inflammatory diseases [62,67–69].

Of note, preliminary studies support the role of specific polymorphisms in paradoxical psoriasis induced by anti-TNF-α therapy [70]. It is thus likely that paradoxical reactions occur in patients with an underlying genetic predisposition and that advances in this field will enable identification of those individuals at risk of developing such reactions.

1.4 Pharmacogenetics of psoriasis

Pharmacogenetics is a term used to describe the study of the association between genetic polymorphism and response to a drug. Pharmacogenetic findings have potential to greatly impact clinical decisions, to minimize costs of treatment, as well as to improve treatment safety by reducing adverse events [17].

The most common pharmacogenetic approach utilized to assess the variability in the efficacy and toxicity of psoriasis treatments has been the evaluation of SNPs present in genes encoding drug-metabolizing enzymes, drug transporters, and receptors. Then, genetic variants have been associated with response to traditional systemic agents and response to biologic drugs used for the therapy of psoriasis.

1.4.1 Genetic influence on responsiveness to anti-TNFs

A number of studies have been performed to study the genetic influence on responsiveness to anti-TNFs. TNF-α-related genes were the most frequently investigated in the pharmacogenetic studies, especially in associations with responses to etanercept. It was found that patients with the G/G, but not with the A/A genotype on rs1800629 may predict a better response to etanercept. This polymorphism gained attention because it was also associated with TNF-α secretion and circulating levels [71]. Futhermore, patients with G/G or G/A genotype on rs1800610 had a better response to etanercept ($p = 0.001$) [72]. Gallo *et al*. reported that the rs361525 *GG, rs1799724 *CT/ TT, and rs1799964 *TT genotypes in TNF-α were all correlated with an increased therapeutic response to anti-TNFs ($p = 0.049$; $p = 0.004$; $p = 0.041$, respectively) [73]. SNPs in TNF-α were also evaluated by studies by De Simone *et al*., confirming the association of good drug response with rs361525 *G allele and rs1800629 *GG genotype ($p = 0.03$; $p = 0.001$, respectively) [74]. Among polymorphisms in TNF-α promoter, the G/G genotype on rs361525 had a better response to TNF-α inhibitors ($p = 0.049$) [73], and the carriage of TNF-857C was associated with positive response to drug treatment in patients treated with etanercept ($p = 0.002$ and $p = 0.001$, respectively). None of these SNPs were associated with responsiveness to treatment with infliximab or adalimumab [68].

Researchers expanded their interests also to polymorphisms in genes codifying TNF-α receptors and involved in the TNF-α intracellular signaling. Individuals with polymorphisms -676 and -196 were reported to favor response to etanercept [72]. In a study on psoriatic arthritis (PsA), it was found that the TNFR1A, a member of the TNF-receptor superfamily, contributed to response to anti-TNF-α treatment, specifically, SNP rs767455 [75]. TNFRSF10A, also

known as TRAIL1, is a receptor that mediates the signaling cascade for cellular apoptosis. The CC genotype of SNP rs20575 was associated with response to infliximab at 6 months in patients with PsA. Murdaca *et al*. reported that the A/A genotype of this SNP was also associated with a decreased response to etanercept [72]. The polymorphism 676 T/G in TNFRSF1B, that encodes for the TNF receptor II, is associated with higher whole blood TNF-α production and was associated with positive response to etanercept (p = 0.001) [76]. The TNFRSF1B rs1061622 G allele was increased in frequency among HLA-Cw6 positive psoriasis patients [77]. This finding seems to conflict the results in another study, which stated that the TNFRSF1B SNP rs1061622 can predict good response to etanercept, but not to infliximab or adalimumab [78].

Individuals with the G allele of TNFAIP3 SNP rs610604 were associated with good response to therapy with all TNF-α inhibitors (p = 0.05) and etanercept (p = 0.016) [78,79]. The T allele of TNFAIP3 SNP rs2230926 was associated with good response to therapy of all TNF-α inhibitors [78,79].

Collectively, these studies demonstrate a prominent role of genetic variants involved in TNF-α signaling with response to TNF-α inhibitors.

Treatment	Gene	Variation	Ref/Alt	Locus	Efficacy	Adverse events	Ref.
TNFα inhibitors	TNFα promoter	SNP	G/A	rs361525	↑ Efficacy	none	[73,76]
	TNFα promoter	SNP	T/C	rs1799964	↑ Efficacy	none	[73]
	TNFα promoter	SNP	G/A	rs1800629	↑ Efficacy (etanercept)	none	[72]
	TNFα promoter	SNP	G/A	rs1800610	↑ Efficacy (etanercept)	none	[72]
	TNFα promoter	SNP	C/T	rs1799724	↑ Efficacy (etanercept)	none	[76]
	TNFα promoter	SNP	C/T T/T	rs1799724	↓ Efficacy	none	[76]
	TNFRSF1B	SNP	T/G	rs1061622	↑ Efficacy (etanercept)	none	[77]
	TNFRSF1B	SNP	T/G	rs1061622	↓ Efficacy	none	[78]
	TNFAIP3	SNP	G/T	rs610604	↑ Efficacy	none	[78,79]
	TNFAIP3	SNP	T/C	rs2230926	↑ Efficacy	none	[78,79]

Table 1. **Pharmacogenetic findings related to TNF-α antagonists in psoriasis treatment**. Summary of variations in TNF-α signaling associated with efficacy and adverse events of TNFα inhibitors in psoriasis patients. Note: SNP: single-nucleotide polymorphism; Ref/Alt: Reference base/Alteration base; Locus: SNP identification number at NCBI; rs, reference *SNP* ID number; TNF: tumor necrosis factor; TNFRSF1B: Tumor Necrosis Factor Receptor Subfamily, Member 1B; TNFAIP3: Tumor Necrosis Factor-Alpha-Induced Protein 3.

In addition to genetic variants involved in TNF-α signaling, genes involved in Th17 signaling also appear to affect response to TNF-α inhibitors. The IL-23R (rs11209026) GG genotype was reported as being correlated with an increased therapeutic response at 6 months to TNF-α inhibitors ($p = 0.006$) [73]. Variants in IL- 17F were examined for an association with response to anti-TNF-α agents. A positive association was found between rs763780 in IL-17F and a positive response to infliximab at 3 and 6 months ($p = 0.023$; $p = 0.020$, respectively), and at the same locus, a negative response to adalimumab at 6 months was reported ($p = 0.004$) [80].

Treatment	Gene	Variation	Ref/Alt	Locus	Efficacy	Adverse events	Ref.
TNFα inhibitors	IL-23R	SNP	G/A	rs11209026	↑ Efficacy	none	[73]
	IL-23R	SNP	G/A	rs11209026	none	↑ Adverse events	[81]
	IL-17F	SNP	T/C	rs763780	↑ Efficacy (infliximab)	none	[80]
	IL-17F	SNP	T/C	rs763780	↓ Efficacy (adalimumab)	none	[80]
	PDE3A	SNP	G/A	rs3794271	↑ Efficacy	none	[82]
	MyD88	SNP	A/G	rs7744	↑ Efficacy (etanercept)	none	[72]
	FBXL19	SNP	G/A	rs10782001	none	↑ Adverse events	[81]
	CTLA4	SNP	G/A	rs3087243	none	↑ Adverse events	[81]
	SLC12A8	SNP	G/A	rs651630	none	↑ Adverse events	[81]
	TAP1	SNP	T/C	rs1800453	none	↑ Adverse events	[81]
	HLA	$Cw6^+$			↓ Efficacy	none	[73]
	HLA	$Cw6^-$			↑ Efficacy	none	[83]
	HLA	$Cw6^+$			↑ Efficacy	none	[84]
	HLA-C	SNP	C/T	rs10484554	↑ Efficacy	none	[85]
	HLA-DRB1 encoding SE	Allele *04:04			↑ Efficacy (etanercept)	none	[72]
	HLA-DRB1 encoding SE	Allele *01:01			↑ Efficacy (etanercept)	none	[72]

Table 2. **Pharmacogenetic findings related to TNF-α antagonists in psoriasis treatment**. Summary of variations associated with efficacy and adverse events of TNFα inhibitors in psoriasis patients. Notes: SNP, single-nucleotide polymorphism; Ref/Alt, Reference base/Alteration base; Locus, SNP identification number at NCBI; rs, reference *SNP* ID number; IL23R, interleukin-23 receptor; IL17F, interleukin-17F; PDE3A, Phosphodiesterase 3A; MyD88, Myeloid differentiation primary response 88; FBXL19, F-Box And Leucine Rich Repeat Protein 19; CTLA4, Cytotoxic T-Lymphocyte Antigen 4; SLC12A8, Solute carrier family 12 member 8; TAP1, Transporter 1 ATP Binding Cassette Subfamily B Member; HLA-C, human leukocyte antigen-C; HLA-DRB1, human leukocytes antigen-DRB1.

Furthermore, phosphodiesterase 3A (PDE3A) and related proteins are associated with active transport of different organic molecule, toxins and drugs and also shown to be constitutively expressed in human keratinocytes. Concerning PDE3A, the SNP rs3794271 AA genotype was significantly associated with higher TNF-α inhibitor efficacy in patients with psoriasis (p = 0.0031) [82]. Another gene that have a central role in the immune response, both innate and adaptive, is myeloid differentiation primary response gene 88 (MyD88), and patients with rs7744 were associated with good response to etanercept [72].

Regarding HLA-Cw6, positive patients were found to have a poor response to TNF-α inhibitor and negative patients were reported as having a higher response to anti-TNF-α [73,83]. However, a more recent study conflicts these findings and showed a trend toward better response amongst HLA-Cw6 positive patients and TNF-α inhibitor [84]. In particular, none of the HLA haplotypes were associated with adalimumab response, but Masouri *et al*. found an HLA-C SNP rs10484554, that was related to the efficacy of all anti-TNF agents, especially adalimumab (p = 0.007) [85]. And lastly, HLA-DRB1 encoding SE alleles *01:01 and *04:04 were both associated with good response to etanercept [72].

1.4.2 Genetic influence on responsiveness to anti-IL-12/IL-23 and anti-IL-17 drugs

Fewer pharmacogenetic studies on anti-IL-12/IL-23 and anti-IL-17 drugs have been performed, as compared to TNF-α blockers.

Ustekinumab has demonstrated high efficacy and acceptable safety profile in psoriasis treatment by inhibiting IL-12/IL-23 inflammatory pathways [86]. However, it has been reported that genes involved in the IL-12/23 signaling pathway doesn't play a head role in patient response to anti-IL-12/IL-23 treatment. None of the SNPs studied in IL-12 or IL-23 were associated with ustekinumab response [85,87]. Only in a study, IL-12B polymorphism (rs3213094) has been associated with response to ustekinumab, and CT genotype was recognized as a predictor of better response to the drug (p = 0.017) [88]. In the same study, GG genotype of rs610604 (TNFAIP3) has been associated with poor response to ustekinumab (p = 0.031). Two TNF-related SNPs were investigated for an association with ustekinumab response, but no associations were found [83,85,87].

Regarding IL-17 gene, Prieto-Perez *et al*. demonstrated that in a cohort of 70 psoriasis patients the rs763780 SNP in *IL-17F* gene associated a no response to ustekinumab at both 3 and 6 months of treatment (p = 0.022 and p = 0.016, respectively) [80].

Moreover, few studies focused on associations with different HLA-C genes [17,41]. Three of five studies found higher response rates to ustekinumab among patients who were positive for HLA-Cw6 [83,87,89]. The favourable response among HLA-Cw6-positive patients persisted after 1 year of treatment in two studies [87,89]. Differences between HLA-Cw6 positive and negative patients decreased after 2 and 3 years of treatment [89]. These data partly contrast with results of Masouri *et al*. revealing an association between HLA-Cw6 (rs10484554) and a good response to anti-TNF therapy but not to ustekinumab treatment, even though the ustekinumab-treated group was smaller (n = 22) than the anti-TNF-*a*-treated one (n = 250) [85].

Treatment	Gene	Variation	Ref/Alt	Locus	Efficacy	Adverse events	Ref.
IL-12/23 inhibitor	IL-12B	SNP	G/C	rs6887695	↑ Efficacy	none	[87]
	IL-12B	SNP	C/G,T	rs3213094	↑ Efficacy (ustekinumab)	none	[88]
	IL-12B	SNP	T/G	rs3212227	↓ Efficacy	none	[87]
	TNFAIP3	SNP	G/T	rs610604	↓ Efficacy (ustekinumab)	none	[88]
	LCE3B/3C	DEL				none	[83]
	IL-6	SNP	C/G	rs1800795	↓ Efficacy	none	[87]
	HLA	Cw6		6p21.33	↑ Efficacy	none	[83]
	HLA-C	SNP	C/T	rs10484554	↓ Efficacy	none	[85]
	ERAP1	SNP	A/C	rs151823	↑ Efficacy	none	[85]
	ERAP1	SNP	C/A,G,T	rs26653	↑ Efficacy	none	[85]
	IL-17F	SNP	T/C	rs763780	↓ Efficacy	none	[80]
	TNFRSF1B	SNP	T/G	rs1061622	↓ Efficacy	none	[77]
IL-17 inhibitor	HLA	Cw6		6p21.33	no difference	none	[53]

Table 3. **Pharmacogenetic findings related to ILs antagonists in psoriasis treatment**. Summary of variations associated with efficacy and adverse events of IL-12/23 and IL-17 inhibitors in psoriasis patients. Notes: SNP, single-nucleotide polymorphism; Ref/Alt, Reference base/Alteration base; Locus, SNP identification number at NCBI; rs, reference *SNP* ID number; IL12B, interleukin-12B; TNFAIP3, Tumor Necrosis Factor-Alpha-Induced Protein 3; LCE3B/3C, Late Cornified Envelope 3B/3C; IL-6, interleukin-6; HLA-C, human leukocyte antigen-C; ERAP1, Endoplasmic Reticulum Aminopeptidase 1; IL-17F, interleukin-17F; TNFRSF1B, Tumor Necrosis Factor Receptor Subfamily Member 1B.

In addition, the SNPs rs151823 and rs26653 in the *ERAP1* gene were found to be associated with ustekinumab efficacy [85], and the carriage of SNPs in both HLA-C (HLA-Cw6) and TNFRSF1B (rs1061622) genes increased the risk for negative response to the anti-IL-12/IL-23 drug (p = 0.05) [77]. Finally, a cohort of 51 patients with psoriasis treated with ustekinumab has been evaluated in association studies with HLA-Cw6 and TNFAIP3 rs610604 polymorphisms, as well as *LCE3B/3C* gene deletion. Better and faster response to ustekinumab was observed in HLA-Cw6 positive patients (p = 0.008), whereas no significant association

with response was observed for the other two investigated genes [83]. On the other hand, Galluzzo *et al*. suggested that a combination of genetic factors predicts response to ustekinumab better than a single factor. Multiparameter logistic regression analysis revealed that HLA-Cw6 was a better predictor of positive response to ustekinumab in the absence of the IL-12B AA genotype (i.e. rs3212227) or the IL-6 GG genotype (i.e. rs1800795), and in the presence of the IL-12B GG (i.e. rs6887695) genotype [87].

So far, very few pharmacogenetic studies on anti-IL-17 drugs has been performed. No SNPs has been associated with a positive response to the treatment, and the SUPREME group study concluded that secukinumab was equally effective in both Cw6-positive and Cw6-negative patients reaching PASI90 up to week 72 [53].

1.5 Pharmacogenomics of psoriasis

The evolution of biotechnology and the sequencing of human DNA have allowed the creation of pharmacogenomics, a branch of genetics that analyzes human genes, the RNAs and proteins encoded by them, and the inter-and intra-individual variations in expression and function in relation to drug response. Differently from pharmacogenetic studies, few pharmacogenomic associations have been carried out in psoriatic patients. This could be mainly attributed to the high costs of high-throughput genomic investigations, such as Next-Generation Sequencing (NGS), even though in recent years, they have consistently declined, making pharmacogenomic investigations more feasible.

Thus, studies analyzing psoriasis transcriptome after 4-month treatment with methotrexate found that responders to methotrexate showed a decrease of mRNA expression of Th-related genes (i.e., Th1,Th17, and Th22) compared with non-responders [41]. Similarly, a recent study showed that most biomarkers in lesional skin returned to near nonlesional levels after 3-month therapy with etanercept. However, a subset of 248 genes did not reach 75% improvement, including IL-12, IL-17, IL-22, IFN-γ, and CXCL8 [90]. Other studies have uncovered that mRNA expression levels of TLR2 and 9, as well as key notch signaling pathway genes (i.e. NOTCH1, NOTCH2, and JAGGED1) were significantly reduced in etanercept-treated patients [91,92]. TLR2 and 9 expression was found to decrease in psoriasis lesional skin also after treatment with infliximab [91,92]. Microarray analysis demonstrated different patterns of gene expression in blood and lesional skin of patients after treatment with infliximab, in particular of genes related to cell differentiation, proliferation, and apoptosis [93].

Finally, Ovejero-Benito MC *et al*. conducted the first pharmacoepigenetic study in patients with moderate-to-severe psoriasis treated with anti-TNF drugs but failed to find differences in DNA

methylation between excellent responders and partial responders to anti-TNF drugs [94]. Nevertheless, three CpGs (cytosines bound to guanines by phosphates) were differentially methylated between excellent responders and partial responders to adalimumab [94].

2. MATERIALS AND METHODS

2.1 Patients

2.1.1 Psoriatic patients

In this retrospective study we reviewed data of 213 subjects affected by moderate-to-severe plaque-type psoriasis, who started biologic treatments at the University of Rome "Tor Vergata", Department of Dermatology/Fondazione Policlinico Tor Vergata (in collaboration with Dr. M. Talamonti, Prof. L. Bianchi and Dr. M. Galluzzo) and at IDI-IRCCS of Rome, Centro di Ricerche Integrate per la PSOriasi/ Laboratory of Experimental Immunology (in collaboration with Dr. C. Albanesi and Dr. S. Pallotta). N = 63 patients undergone secukinumab treatment (150 mg) and n = 150 received ustekinumab (45 mg for patients < 100kg and 90 mg for patients >100kg), all administered following AIFA criteria. The inclusion criteria were the following: PASI score >10, Body Surface Area (BSA) >10%, Dermatology Life Quality Index (DLQI) >10. Of note, both biologic drugs were used in monotherapy and was not combined with conventional systemics or topical therapies to improve or maintain efficacy.

For each patient enrolled (aged 18- to 65- year-olds), personal data, as well as anthropometric and clinical data were collected in accordance with the guidelines of the Declaration of Helsinki and were annotated in an electronic database specifically programmed and created *ad hoc* for the study.

Concerning clinical data, for each patient was reported: age of onset of the disease, localization, familiarity, co-morbidities of the endocrine-metabolic, cardiocirculatory, genitourinary, gastoinstestinal, respiratory and osteomuscular system, as well as lifestyles (consumption of alcohol and/or tobacco). The presence of an articular involvement was also reported. The severity of psoriasis and response to treatment were evaluated using the PASI score at baseline and, then, at follow-up visits on weeks 8, 16, 24, 40, 56, 64, 72, 88, 100 (secukinumab) or on weeks 4, 12, 28, 40, 52, 64, 76, 88, 100 (ustekinumab). Clinical efficacy of anti-ILs was assessed in terms of the 75%, 90% and 100% improvement of PASI score compared to baseline (PASI75, PASI90 and PASI100).

2.1.2 Patients with paradoxical psoriasis

Three subjects affected by HS and developing paradoxical psoriasis after treatment with adalimumab were included in the study. The HS patients developed psoriasiform skin lesions after 3-6 months treatment with adalimumab (40 mg, weekly). Patients were recruited at IDI-IRCCS of Rome (in collaboration with Dr. L. Fania and Dr. C. Mazzanti). For each patient,

clinical data were collected. In particular, HURLEY and Sartorius score, and response to adalimumab therapy have been annotated together with clinical data concerning paradoxical psoriasis, including PASI, clinical subtype, localization of illness, familiarity for psoriasis, other concomitant manifestations, infections and lifestyles.

2.1.3 Blood and skin samples

Two-ml blood samples were collected from each psoriatic patient or from patients developing paradoxical psoriasis, to isolate DNA. 20-ml blood samples were also collected from patients developing paradoxical psoriasis to isolate peripheral blood monuclear cells (PBMC). Eight-mm skin biopsies were taken from psoriasiform lesions and divided into two parts for immunohistochemistry and isolation of skin-infiltrating T lymphocytes. Clinical data, as well as skin biopsies and blood were collected from patients after written informed consent. The study was conducted in accordance with the guidelines of the Declaration of Helsinki.
Blood and skin samples were also obtained from three patients affected by classical plaque-type psoriasis (PASI8, 11,5 and 10).

2.2 SNP analysis

DNA was extracted from blood of patients by QIAcube system with QIAmp DNA kit (Qiagen, Hilden, Germany) and sequenced by NGS technology. SNPs were selected based on an extensive review of articles on the association between psoriasis and SNPs or response to biologics [24,95–99]. The selected SNPoma is composed of n = 44 SNPs, highly represented in the psoriatic populations (minor allele frequency > 0.3), and potentially implicated in immune responses (T-cell signalling, antigen presentation), as well as inflammatory pathways (cytokine-dependent signalling) and skin barrier function (Table 4-6).
SNP array was analysed by targeted sequencing, using NGS TruSeq Custom Amplicon (TSCA) Low-Input kit and was performed on MiSeq platform using a V2 Nano kit (Illumina, San Diego, USA), according to manufacturer' instructions. Amplified libraries were quantify using Qubit Fluorometer and Agilent 2100 Bioanalyzer with dsDNA HS assay and High Sensitivity DNA kit, respectively. Sequencing data were collected, aggregated and filtered by using a set of *ad hoc* bioinformatics script. Basically, a top-down approach was applied to select all positive calls with a read depth > 50x and allelic frequency of 0.3. Moreover, variant's functional annotations were verified with the latest version of ANNOVAR and IGV was used to check peculiar variants of interest.

Antigen presentation			
Genome position	**Gene**	**REF/ALT**	**SNP name**
Chr5:96101944	ERAP1	A/G	ERAP1_v1
Chr5:96101959	ERAP1	C/T	ERAP1_v2
Chr5:96124330	ERAP1	T/C	ERAP1_v3
Chr5:96124447	ERAP1	G/C	ERAP1_v4
Chr5:96124453	ERAP1	A/G	ERAP1_v5
Chr5:96139250	ERAP1	C/G	ERAP1_v6
Chr6:31252925	HLA-C region	C/T	HLA-Cw6_LD
Chr6:31252951	HLA-C region	G/T	HLA-Cw6_v5
Chr6:31253034	HLA-C region	T/G	HLA-Cw6_v6
Chr6:31266085	HLA-C region	C/G	HLA-Cw6_v7
Chr6:31266090	HLA-C region	G/A	HLA-Cw6_v1
Chr6:31266117	HLA-C region	A/C	HLA-Cw6_v8
Chr6:31266151	HLA-C region	G/T	HLA-Cw6_v9
Chr6:31266189	HLA-C region	AA/TG,AG	HLA-Cw6_v10
Chr6:31266190	HLA-C region	A/G	HLA-Cw6_v11
Chr6:31266207	HLA-C region	CA/TG	HLA-Cw6_v3
Chr6:31274380	HLA-C region	T/C	HLA-Cw6_v4
Chr6:31274449	HLA-C region	C/A	HLA-Cw6_v12
Chr6:31274513	HLA-C region	A/G	HLA-Cw6_v13
Chr6:31274518	HLA-C region	T/TCGGGGAGTCCAGCAGGTCC, TCCGGGAGTCCAGCAGGTCC	HLA-Cw6_v14
Chr6:31274555	HLA-C region	C/T	HLA-Cw6_v2
Chr6:31274580	HLA-C region	CAGCCAA/GAGCCAA,CAACCAG	HLA-Cw6_v15
Chr6:31274582	HLA-C region	GCCAA/ACCAG,GCCAG	HLA-Cw6_v16
Chr6:31274584	HLA-C region	CA/C	HLA-Cw6_v17
Chr6:31274586	HLA-C region	A/G	HLA-Cw6_v18
Chr6:31274619	HLA-C region	A/G	HLA-Cw6_v19
Chr6:31274634	HLA-C region	T/C	HLA-Cw6_v20
Chr6:31431780	HLA-B region	T/G	HLA-Cw6_v21
Chr6:31431820	HLA-B region	C/T	HLA-Cw6_v22
Chr6:31431874	HLA-B region	G/T	HLA-Cw6_v23

Table 4. **List of the analyzed SNPs involved in antigen presentation**. Notes: Genome position, UCSC Genome Browser; REF/ALT, reference base/alteration base; Chr, chromosome; ERAP1, endoplasmic reticulum aminopeptidase 1; HLA-C, human leucocyte antigen-C.

Skin barrier function			
Genome position	**Gene**	**REF/ALT**	**SNP name**
Chr6:31084163	CDSN	A/G	CDSN_v1
Chr6:31084170	CDSN	A/C	CDSN_v2
Chr6:31084191	CDSN	T/C	CDSN_v3
Chr6:31084288	CDSN	T/C	CDSN_v4
Chr6:31084435	CDSN	G/A	CDSN_v5
Chr6:31084787	CDSN	A/G	CDSN_v6
Chr6:31084792	CDSN	C/T	CDSN_v7
Chr6:31110391	CCHCR1	G/C	CCHCR1_v1
Chr6:31112737	CCHCR1	C/A	CCHCR1_v2
Chr6:31114182	CCHCR1	A/G	CCHCR1_v3
Chr6:31122482	CCHCR1	G/A	CCHCR1_v4
Chr6:31122500	CCHCR1	GCC/ACC,GCT	CCHCR1_v5
Chr6:31122502	CCHCR1	C/T	CCHCR1_v6
Chr6:31122564	CCHCR1	C/G	CCHCR1_v7

Table 5. **List of the analyzed SNPs involved in skin barrier**. Notes: Genome position, UCSC Genome Browser; REF/ALT, reference base/alteration base; Chr, chromosome; CDSN, corneodesmosin; CCHCR1, coiled-coil alpha-helical rod protein 1.

NF-kB pathway and T-cell activation			
Genome position	**Gene**	**REF/ALT**	**SNP name**
Chr1:67658803	IL23R	G/A	IL23R_v1
Chr1:67670213	IL23R	G/A	IL23R_v2
Chr1:67705900	IL23R	G/A	IL23R_v3
Chr1:67705958	IL23R	G/A	IL23R_v4
Chr2:113820124	IL1F5	C/T	IL1F5
Chr3:101576029	NFKBIZ	T/TACTTTTAGAAAGCTTTAATAACC	NFKBIZ_v1
Chr3:101663555	NFKBIZ	A/G	NFKBIZ_v2
Chr5:150467189	TNIP1	G/C	TNIP1
Chr5:158742950	IL12B	T/G	IL12B_v1
Chr5:158759900	IL12B	A/G	IL12B_v2
Chr6:31543031	TNFα	G/A	TNFα_v3
Chr6:31543101	TNFα	G/A	TNFα_v4
Chr6:31543827	TNFα	G/A	TNFα_v1
Chr6:31543943	TNFα	G/GTGAA	TNFα_v2
Chr6:52101739	IL17F	T/C	IL17F_v1
Chr6:52101758	IL17F	C/T	IL17F_v2
Chr6:52101844	IL17F	T/C	IL17F_v3
Chr6:111577761	Act1	A/G	Act1_v1
Chr6:111913262	Act1	C/T	Act1_v2
Chr6:111922720	Act1	A/G	Act1_v3
Chr6:138196066	TNFAIP3	T/G	TNFAIP3_v1
Chr6:138199417	TNFAIP3	G/T	TNFAIP3_v2
Chr17:78157811	CARD14	T/G	CARD14
Chr19:10469975	TYK2	A/C	TYK2_v1
Chr19:10472933	TYK2	A/G	TYK2_v2
Chr22:17565035	IL17RA	G/A	IL17RA

Table 6. **List of the analyzed SNPs potentially implicated in immune responses activation of T-cell signalling, as well as cytokine-dependent signalling**. Notes: Genome position, UCSC Genome Browser; REF/ALT, reference base/alteration base; Chr, chromosome; NFKBIZ, NF-κB inhibitor zeta; TRAF3IP2, TRAF3 interacting protein 2; TNFAIP3, TNF alpha induced protein 3; TYK2, tyrosine kinase 2; IL17RA, IL-17 receptor A.

2.3 Immunohistochemistry

Five-μm paraffin-embedded skin sections were stained with H&E or processed for immunohistochemistry. The primary Abs used were as follows: anti-BDCA2 (DDX0043-TDS, Dendritics, Lyon, France), anti-CD15 (#347420, BD Biosciences, Milan, Italy), anti-IL-17A (#AF-317-NA, R&D Systems, Abingdon, UK), anti-lymphotoxin (LT)-α (#SC8302, Santa Cruz Biotechnology, Dallas, TX), anti-IL-22 (#NB100-733, Novus Biologicals, Centennial, CO), anti-IFN-κ (#H00056832-M01, Abnova, Taiwan), anti-CD117 and anti-CD11C (#MONX10234 and #MON3371, Monosan, Uden, Netherlands), anti-CD68 and anti-CD3 (#P02246IT and #A0452, Dako, Glostruk, Denmark). The following Abs came from Abcam (Cambridge, UK): anti-IFN-γ (#AB218426), anti-IL-36γ (#AB156783), anti-IFN-β1

(#AB180616), and anti-LT-β (Cat#AB64835). Secondary biotinylated mAbs and staining kits (Vector Laboratories, Burlingame, CA, USA) were used to develop immunoreactivity. Sections were counterstained with Mayer's hematoxylin.

2.4 RNA analysis

In vivo expression analysis of immunological profile of HS patients was evaluated by real-time PCR analysis performed on RNA extracted from 80-μm paraffin-embedded skin sections by RecoverAll Total Nucleic Acid Isolation kit (Life technologies, Carlsbad, CA, USA). mRNA was reverse-transcribed into cDNA using Superscript IV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Relative gene expression was quantified using specific Sybergreen assays and analysed using QuantStudio5 Real-Time PCR System (Thermo-Fisher Scientific, Waltham, MIT, USA). Gene expression levels were determined by normalizing to GAPDH mRNA expression.

Gene	Sequence
IFN-α2A	Fw 5' TCTGCTATGACCATGACACGAT 3' Rv 5' CAGCATGGTCCTCTGTAAGGG 3'
IFN-β	Fw 5' CAGCAATTTTCAGTGTCAGAAGC 3' Rv 5' TCATCCTGTCCTTGAGGCAGT 3'
IFN-λ1	Fw 5' AGGCTTCTCCAGGTGAGGGA 3' Rv 5' TCCAGGACCTTCAGCGTCAG 3'
IFN-λ2	Fw 5' GGGCCTGTATCCAGCCTCAG 3' Rv 5' GAGCCGGTACAGCCAATGGT 3'
IFN-λ3	Fw 5' GGGCCTGTATCCAGCCTCAG 3' Rv 5' GGTGCAGCCAATGGTGGAG 3'
LL-37	Fw 5' TTTTGCGGAATCTTGTACCCA 3' Rv 5' TCTCAGAGCCCAGAAGCCTG 3'
GAPDH	Fw 5' TGGACCTGACCTGCCGTCTA 3' Rv 5' CCCTGTTGCTGTAGCCAAATTC 3'

Table 7. **List of the primer sequences used for Real-time PCR analysis on human samples**. Notes: IFN-α2A, interferon-α2A; IFN-β, interferon-β; IFN-λ1, interferon- λ1; IFN-λ2, interferon- λ2; IFN-λ3, interferon- λ3; LL-37, cathelicidn antimicrobial peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Fw, forward; Rv, reverse.

2.5 T skin isolation and flow cytometry analysis

T lymphocytes were isolated from skin biopsies as previously described [100]. In brief, skin biopsies were placed in culture in RPMI 1640 supplemented with 2 mM glutamine, 1 mM

sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-mercaptoethanol, 1% penicillin/streptomycin (all Invitrogen; RPMI complete), and 5% human serum (Sigma-Aldrich) supplemented with 60 U/ml IL-2. After 7-12 days, T cells emigrated from tissue samples were collected and phenotypically characterized.

In parallel, PBMC were isolated form 20-ml sample of peripheral blood samples by gradient centrifugation with Ficoll-Hypaque (Lymphoprep, Nycomed-Pharma, Oslo, Norway). T skin and PBMC were phenotypically characterized by staining with the following mAbs: anti-IFN-γ-FITC (#B27), -CD4-PE (#RPA-T4), -CD8-PeRcP (#SK1), -CD3-FITC (#HIT3a) (BD Biosciences); anti-TNF-α-FITC (#6n1E7, Miltenyi Biotec, Bergisch**,** Germany), -IL-17-PE (#eBio64DEC17, EBiosciences, Frankfurt, Germany); anti-IL-22-PeRcP (#142928, R&D Systems). Acquisitions were performed using an Attune Nxt (Life Technologies, Carlsbad, CA, USA). Analyses were performed using Flow logic software (Miltenyi).

2.6 Statistical analysis

Drug response data were analysed by last- observation- carried- forward (LOCF) method, where if a patient dropped out of the study the last value available was "carried forward" until the next observation point of the treatment. Differences between the groups (allele-pos- or -neg patients) based on the clinical response to anti-ILs was evaluated by χ^2- test. Univariate logistic regression analysis was also performed to combine genetic data of single SNP and clinical responses to biologic therapy, expressed as PASI75 90 or 100. The association between drug response and genetic data was estimated calculating the odds ratio (OR), its standard error and 95% confidence interval (CI), using the STATA 14.2 software (StataCorp, College Station, TX, USA). Deviation from null hypothesis was considered significant at p-value < 0.05.

The significance of differences in the numbers of immunoreactive cells in skin biopsies were calculated using the unpaired Student's *t*-test. Unpaired non-parametric Mann-Whitney U-test was used to compare differences in mRNA content in skin biopsies of HS and psoriatic patients. Statistical analysis was performed with Prism v.5.0 (GraphPad company, St Diego, CA, USA), and values are expressed as the mean + SD. All testing was two-sided, and values of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Pharmacogenomic studies on psoriatic populations

3.1.1 Clinical classification of psoriatic patients

In the subgroup of patients undergone Secukinumab's treatment, 42 males and 21 females aged between 22 and 76 years, with a baseline PASI score > 10.0 were included. Patient demographics and disease characteristics at baseline are described in Table 8.

In the subgroup of patients undergone Ustekinumab's treatment, 95 males and 55 females aged between 18 and 85 years affected by moderate-to-severe chronic plaque psoriasis, were included. Patient demographics and disease characteristics at baseline are described in Table 9.

Characteristic	N = 63
Male/female, n	42/21
	Mean ± Standard deviation (range)
Age, years	45.0 ± 13.1 (22-76)
BMI, Kg/m^2	31.1 ± 9.2 (18.9-41.8)
Male	32.7 (18.9-41.3)
Female	27.6 (20.4-41.80)
Age at disease onset, years	22.8 ± 12.1 (3-62)
PASI	18.9 ± 12.1 (8.0-56.7)
Biologics before anti-IL-17A therapy, n (%)	
0 prior biologics	47 (74.5)
1 prior biologics	6 (9.5)
2 prior biologics	4 (6.5)
≥ 3 prior biologics	6 (9.5)
Comorbidities, n	29
Hypertension	17
Type 2 diabetes mellitus	6
Hyperlipidemia	3
Psychiatric diseases	1
Bipolar disorder	0
Depressive disorder	1
Anxiety disorder	0
Obesity	19
Class I (BMI 30.0-34.9 kg/m^2)	12
Class II (BMI 35.0-39.9 kg/m^2)	4
Class III (BMI ≥40.0 kg/m^2)	3

Table 8. **Patient demographics and disease characteristics at baseline-Secukinumab's treatment.** Table indicates number of Caucasian patients undergone secukinumab treatment include on the sudy. Anthropometric and clinical data were shown as Mean ± Standard deviation (range). Notes: BMI, body mass index; PASI, psoriasis area severity index.

Characteristic	N = 150
Male/female, n	95/55
	Mean ± Standard deviation (range)
Age, years	51.2 ± 13.7 (18-85)
BMI, Kg/m^2	29.4 ± 18.0 (18.4-51.1)
Male	28.1 (19.7-51.1)
Female	27.2 (18.4-39.8)
Age at disease onset, years	29.6 ± 16.1 (1-72)
PASI	19.1 ± 10.9 (5.0-62)
Biologics before anti-IL-12/23 therapy, n (%)	
0 prior biologics	84 (56)
1 prior biologics	36 (24)
2 prior biologics	23 (15.3)
≥ 3 prior biologics	7 (4.7)
Comorbidities, n	23
Hypertension	46
Type 2 diabetes mellitus	7
Hyperlipidemia	10
Psychiatric diseases	8
Bipolar disorder	0
Depressive disorder	3
Anxiety disorder	5
Obesity	40
Class I (BMI 30.0-34.9 kg/m^2)	26
Class II (BMI 35.0-39.9 kg/m^2)	10
Class III (BMI ≥40.0 kg/m^2)	4

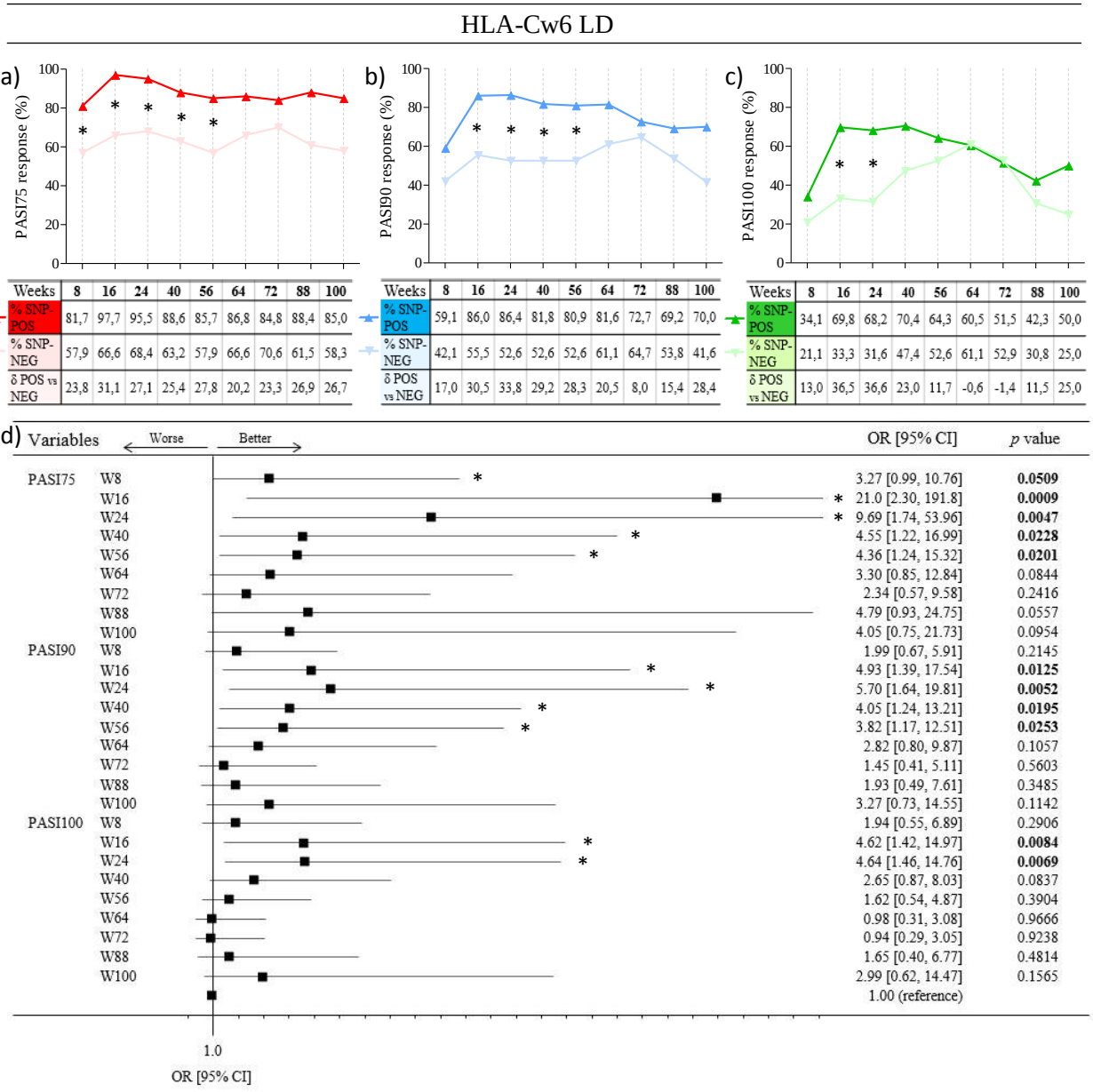
Table 9. **Patient demographics and disease characteristics at baseline-Ustekinumab's treatment.** Table indicates number of Caucasian patients undergone ustekinuamb treatment include on the sudy. Anthropometric and clinical data were shown as Mean ± Standard deviation (range). Notes as in table 8.

3.1.2 Association analysis between SNPs and response to the anti-IL-17A biologic secukinumab

In order to evaluate whether response to the anti-IL-17A depended on allele variants presence or absence, we analyzed 44 SNPs predisposing to psoriasis. We studied the efficacy of the drug by calculating the 75%, 90% and 100% improvement of the PASI score (PASI75, PASI90 and PASI100), respectively up to 2 years of treatment with secukinumab. A logistic regression analysis evaluating the relationship between single independent variables (SNP status) and dependent variables (PASI75, PASI90 and PASI100 response at weeks 8, 16, 24, 40, 56, 64, 72, 88 and 100), was performed.

We found a significant association between four SNPs present in the HLA-C region, namely HLA-Cw6 LD, HLA-Cw6 v1, HLA-Cw6 v2, HLA-Cw6 v3, and response to secukinumab (Figg. 6-9). Concerning the HLA-Cw6 LD variant, the PASI75 end-point was significantly reached by the majority of psoriatic patients carrying out the SNP at week 8 (81.7%, 36 of 44), at week 16 (97.7%, 42 of 43), at week 24 (95.5%, 42 of 44), at week 40 (88.6%, 39 of 44), at

week 56 (85.7%, 36 of 42), as compared to SNP-negative patients (Fig. 6a and d). Starting from week 64, the association between the achievement of PASI75 and presence of HLA-Cw6 LD variant was not statistically significant, even if the Δ between the curves relative to the percentage of positive and negative patients increased starting from week 88 to week 100 (Fig. 6a). Similar results were observed when the PASI90 end-point was evaluated, with the 86% (37 of 43), 86.4% (38 of 44), 81.8% (36 of 44) and 80.9% (34 of 42) of psoriatic patients carrying HLA-Cw6 LD variant being responsive to secukinumab at week 16, 24, 40 and 56, respectively (Fig. 6b and d).



Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	81,7	97,7	95,5	88,6	85,7	86,8	84,8	88,4	85,0
% SNP-NEG	57,9	66,6	68,4	63,2	57,9	66,6	70,6	61,5	58,3
δ POS vs NEG	23,8	31,1	27,1	25,4	27,8	20,2	23,3	26,9	26,7

Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	59,1	86,0	86,4	81,8	80,9	81,6	72,7	69,2	70,0
% SNP-NEG	42,1	55,5	52,6	52,6	52,6	61,1	64,7	53,8	41,6
δ POS vs NEG	17,0	30,5	33,8	29,2	28,3	20,5	8,0	15,4	28,4

Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	34,1	69,8	68,2	70,4	64,3	60,5	51,5	42,3	50,0
% SNP-NEG	21,1	33,3	31,6	47,4	52,6	61,1	52,9	30,8	25,0
δ POS vs NEG	13,0	36,5	36,6	23,0	11,7	-0,6	-1,4	11,5	25,0

		OR [95% CI]	*p* value
PASI75	W8	3.27 [0.99, 10.76]	**0.0509**
	W16	21.0 [2.30, 191.8]	**0.0009**
	W24	9.69 [1.74, 53.96]	**0.0047**
	W40	4.55 [1.22, 16.99]	**0.0228**
	W56	4.36 [1.24, 15.32]	**0.0201**
	W64	3.30 [0.85, 12.84]	0.0844
	W72	2.34 [0.57, 9.58]	0.2416
	W88	4.79 [0.93, 24.75]	0.0557
	W100	4.05 [0.75, 21.73]	0.0954
PASI90	W8	1.99 [0.67, 5.91]	0.2145
	W16	4.93 [1.39, 17.54]	**0.0125**
	W24	5.70 [1.64, 19.81]	**0.0052**
	W40	4.05 [1.24, 13.21]	**0.0195**
	W56	3.82 [1.17, 12.51]	**0.0253**
	W64	2.82 [0.80, 9.87]	0.1057
	W72	1.45 [0.41, 5.11]	0.5603
	W88	1.93 [0.49, 7.61]	0.3485
	W100	3.27 [0.73, 14.55]	0.1142
PASI100	W8	1.94 [0.55, 6.89]	0.2906
	W16	4.62 [1.42, 14.97]	**0.0084**
	W24	4.64 [1.46, 14.76]	**0.0069**
	W40	2.65 [0.87, 8.03]	0.0837
	W56	1.62 [0.54, 4.87]	0.3904
	W64	0.98 [0.31, 3.08]	0.9666
	W72	0.94 [0.29, 3.05]	0.9238
	W88	1.65 [0.40, 6.77]	0.4814
	W100	2.99 [0.62, 14.47]	0.1565
		1.00 (reference)	

Figure 6. **Clinical response to secukinumab between week 8 and week 100 on patients carrying or not HLA-Cw6 LD variant**. Proportion of HLA-Cw6 LD -positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 LD and response to secukinumab (d). Notes: PASI75, ≥ 75% reduction of PASI score; PASI90, ≥ 90% reduction of PASI; PASI100, 100% reduction of PASI; OR, odds ratio; CI, confidence interval; *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$

In contrast, PASI100 was significantly reached only by SNP-positive patients at week 16 (69.8%, 30 of 43) and 24 (68.2, 30 of 44) (Fig. 6c and d).

Univariate logistic regression analysis also revealed a significant association between HLA-Cw6 v1 and response to secukinumab. In particular, we observed the achievement of PASI75, PASI 90, or PASI 100 by patients carrying out the HLA-Cw6 v1 at weeks 16 and 56, at weeks 24 and 40, or at weeks 8, 16 and 24, respectively. At these time points and for the indicated PASI, differences between HLA-Cw6 v1 positive and negative patients were statistically significant, as evaluated by chi^2 tests (Fig. 7).

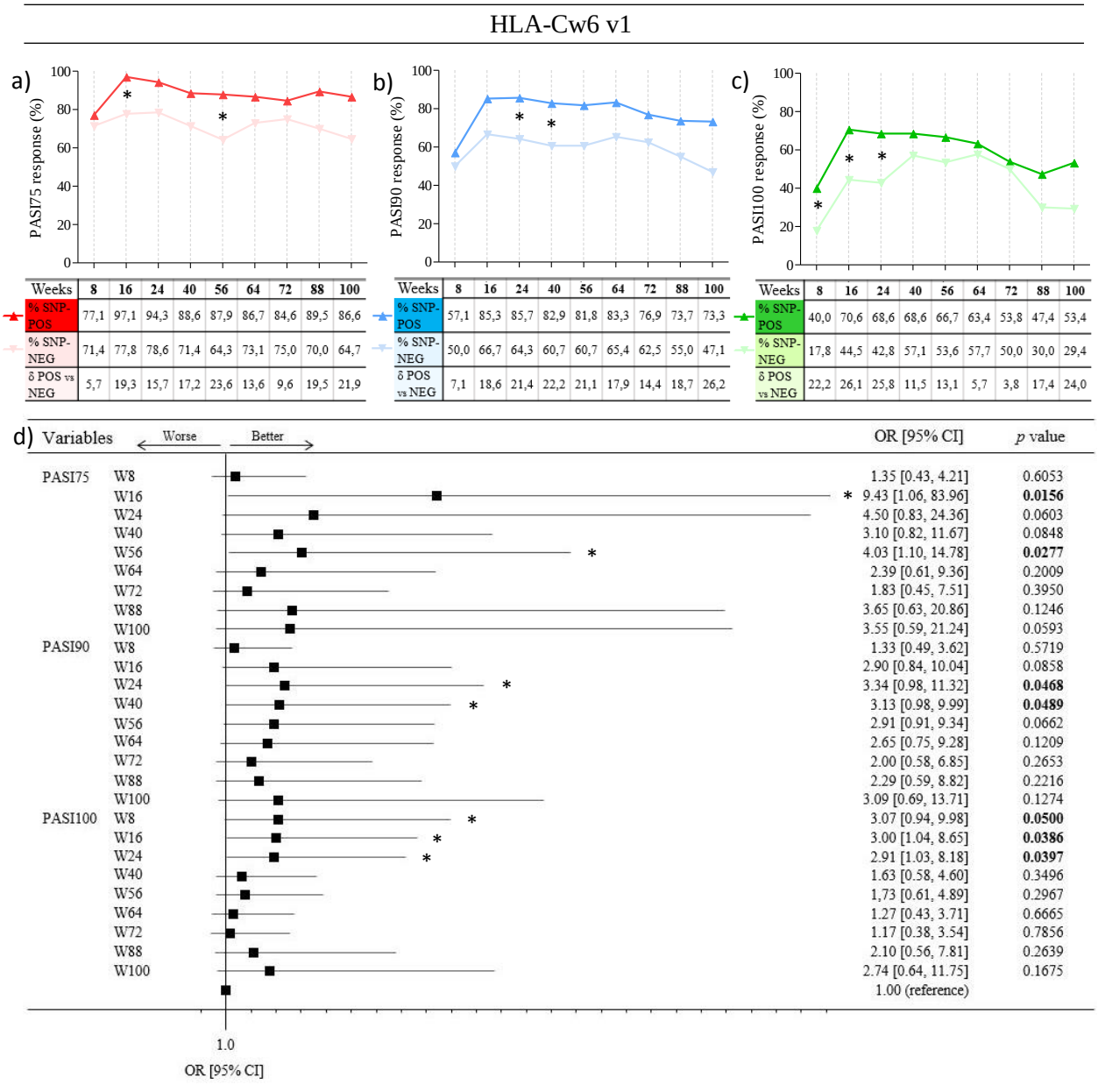
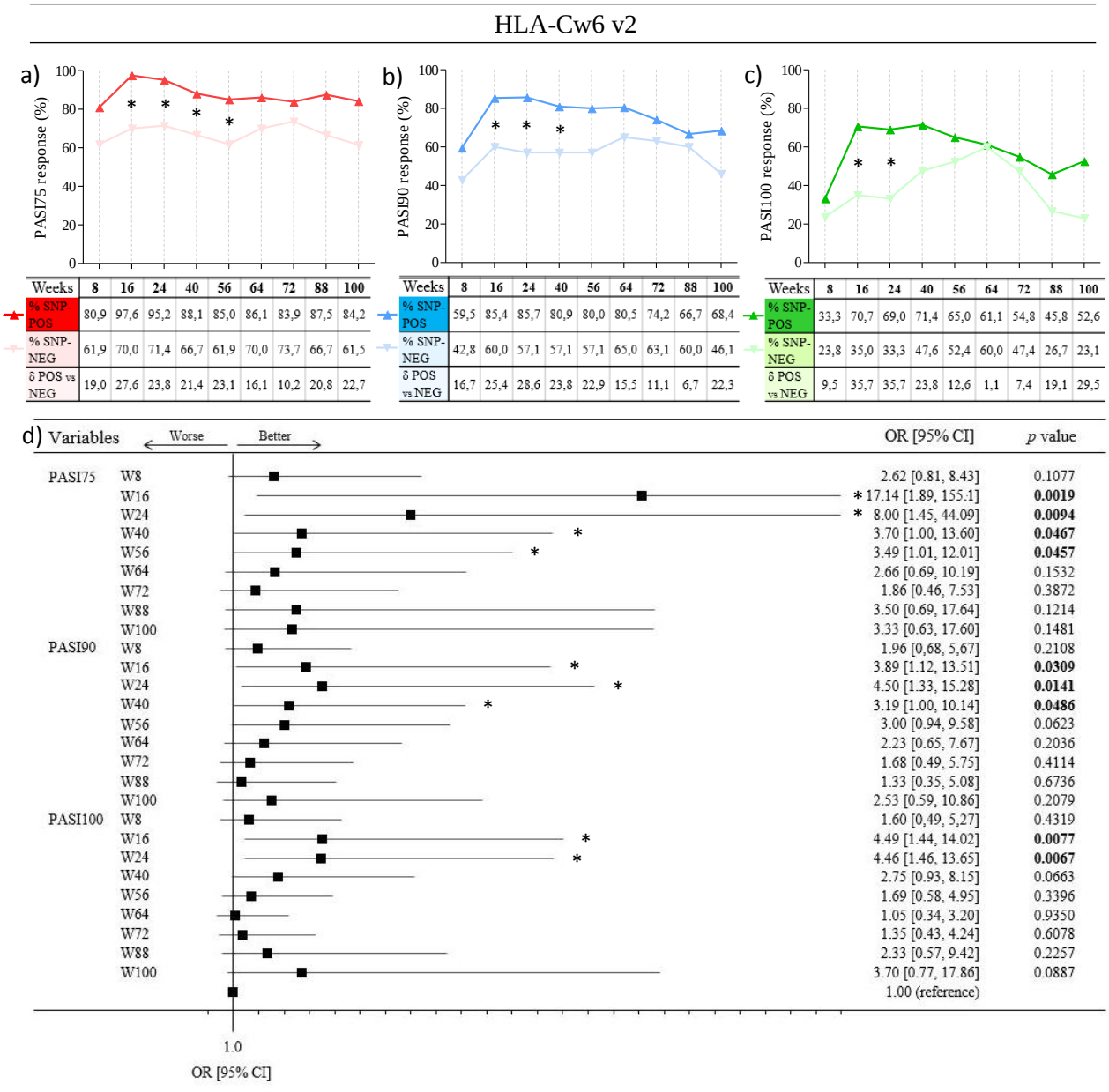


Figure 7. **Clinical response to secukinumab between week 8 and week 100 on patients carrying or not HLA-Cw6 v1 variant.** Proportion of HLA-Cw6 v1-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 v1 and response to secukinumab (d). Notes as in Figure 7. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

Moreover, a new association was found between HLA-Cw6 v2 variant in the HLA-C region and response to secukinumab. In particular, a significant proportion of HLA-Cw6 v2 positive patients experienced PASI75 at different time points: 97.6% (40 of 41), 95.2% (40 of 42), 88.1% (37 of 42) and 85% (34 of 40) at week 16, 24, 40 and 56, respectively (Fig 8a and d). Similar results were obtained when PASI90 and PASI100 achievement was considered, with the exception of weeks 40 and 56 (Fig. 8b-c-d).



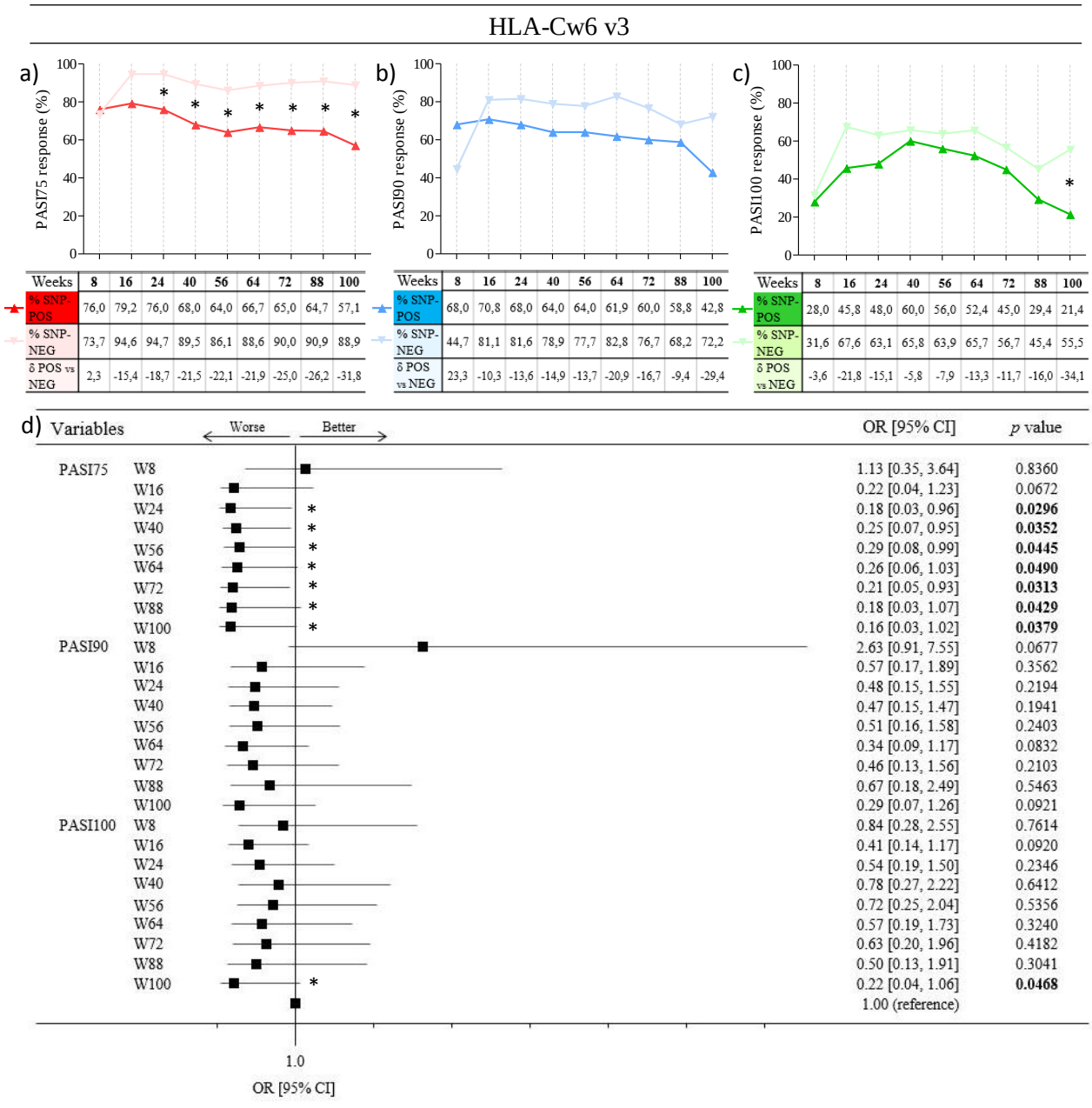
Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	80,9	97,6	95,2	88,1	85,0	86,1	83,9	87,5	84,2
% SNP-NEG	61,9	70,0	71,4	66,7	61,9	70,0	73,7	66,7	61,5
δ POS vs NEG	19,0	27,6	23,8	21,4	23,1	16,1	10,2	20,8	22,7

Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	59,5	85,4	85,7	80,9	80,0	80,5	74,2	66,7	68,4
% SNP-NEG	42,8	60,0	57,1	57,1	57,1	65,0	63,1	60,0	46,1
δ POS vs NEG	16,7	25,4	28,6	23,8	22,9	15,5	11,1	6,7	22,3

Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	33,3	70,7	69,0	71,4	65,0	61,1	54,8	45,8	52,6
% SNP-NEG	23,8	35,0	33,3	47,6	52,4	60,0	47,4	26,7	23,1
δ POS vs NEG	9,5	35,7	35,7	23,8	12,6	1,1	7,4	19,1	29,5

Figure 8. **Clinical response to secukinumab between week 8 and week 100 on patients carrying or not HLA-Cw6 v2 variant.** Proportion of HLA-Cw6 v2-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 v2 and response to secukinumab (d). Notes as in Figure 7. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

Finally, differently from the other SNPs of the HLA-C region, HLA-Cw6 v3 variant was found to be associated to a worse response of psoriatic patients to secukinumab. In fact, the majority of HLA-Cw6 v3-negative patients reached PASI75 at week 24 (94.7%, 36 of 38), at week 40 (89.5%, 34 of 38), at week 56 (86.1%, 31 of 36) at week 64 (88.6%, 31 of 35), at week 72 (90%, 27 of 30), at week 88 (90.9%, 20 of 22), at week 100 (88.9%, 16 of 18), as compared HLA-Cw6 v3-positive patients (Fig. 9).



Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	76,0	79,2	76,0	68,0	64,0	66,7	65,0	64,7	57,1
% SNP-NEG	73,7	94,6	94,7	89,5	86,1	88,6	90,0	90,9	88,9
δ POS vs NEG	2,3	-15,4	-18,7	-21,5	-22,1	-21,9	-25,0	-26,2	-31,8

Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	68,0	70,8	68,0	64,0	64,0	61,9	60,0	58,8	42,8
% SNP-NEG	44,7	81,1	81,6	78,9	77,7	82,8	76,7	68,2	72,2
δ POS vs NEG	23,3	-10,3	-13,6	-14,9	-13,7	-20,9	-16,7	-9,4	-29,4

Weeks	8	16	24	40	56	64	72	88	100
% SNP-POS	28,0	45,8	48,0	60,0	56,0	52,4	45,0	29,4	21,4
% SNP-NEG	31,6	67,6	63,1	65,8	63,9	65,7	56,7	45,4	55,5
δ POS vs NEG	-3,6	-21,8	-15,1	-5,8	-7,9	-13,3	-11,7	-16,0	-34,1

		OR [95% CI]	p value
PASI75	W8	1.13 [0.35, 3.64]	0.8360
	W16	0.22 [0.04, 1.23]	0.0672
	W24	0.18 [0.03, 0.96]	**0.0296**
	W40	0.25 [0.07, 0.95]	**0.0352**
	W56	0.29 [0.08, 0.99]	**0.0445**
	W64	0.26 [0.06, 1.03]	**0.0490**
	W72	0.21 [0.05, 0.93]	**0.0313**
	W88	0.18 [0.03, 1.07]	**0.0429**
	W100	0.16 [0.03, 1.02]	**0.0379**
PASI90	W8	2.63 [0.91, 7.55]	0.0677
	W16	0.57 [0.17, 1.89]	0.3562
	W24	0.48 [0.15, 1.55]	0.2194
	W40	0.47 [0.15, 1.47]	0.1941
	W56	0.51 [0.16, 1.58]	0.2403
	W64	0.34 [0.09, 1.17]	0.0832
	W72	0.46 [0.13, 1.56]	0.2103
	W88	0.67 [0.18, 2.49]	0.5463
	W100	0.29 [0.07, 1.26]	0.0921
PASI100	W8	0.84 [0.28, 2.55]	0.7614
	W16	0.41 [0.14, 1.17]	0.0920
	W24	0.54 [0.19, 1.50]	0.2346
	W40	0.78 [0.27, 2.22]	0.6412
	W56	0.72 [0.25, 2.04]	0.5356
	W64	0.57 [0.19, 1.73]	0.3240
	W72	0.63 [0.20, 1.96]	0.4182
	W88	0.50 [0.13, 1.91]	0.3041
	W100	0.22 [0.04, 1.06]	**0.0468**
		1.00 (reference)	

Figure 9. **Clinical response to secukinumab between week 8 and week 100 on patients carrying or not HLA-Cw6 v3 variant.** Proportion of HLA-Cw6 v3-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 v3 and response to secukinumab (d). Notes as in Figure 7. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

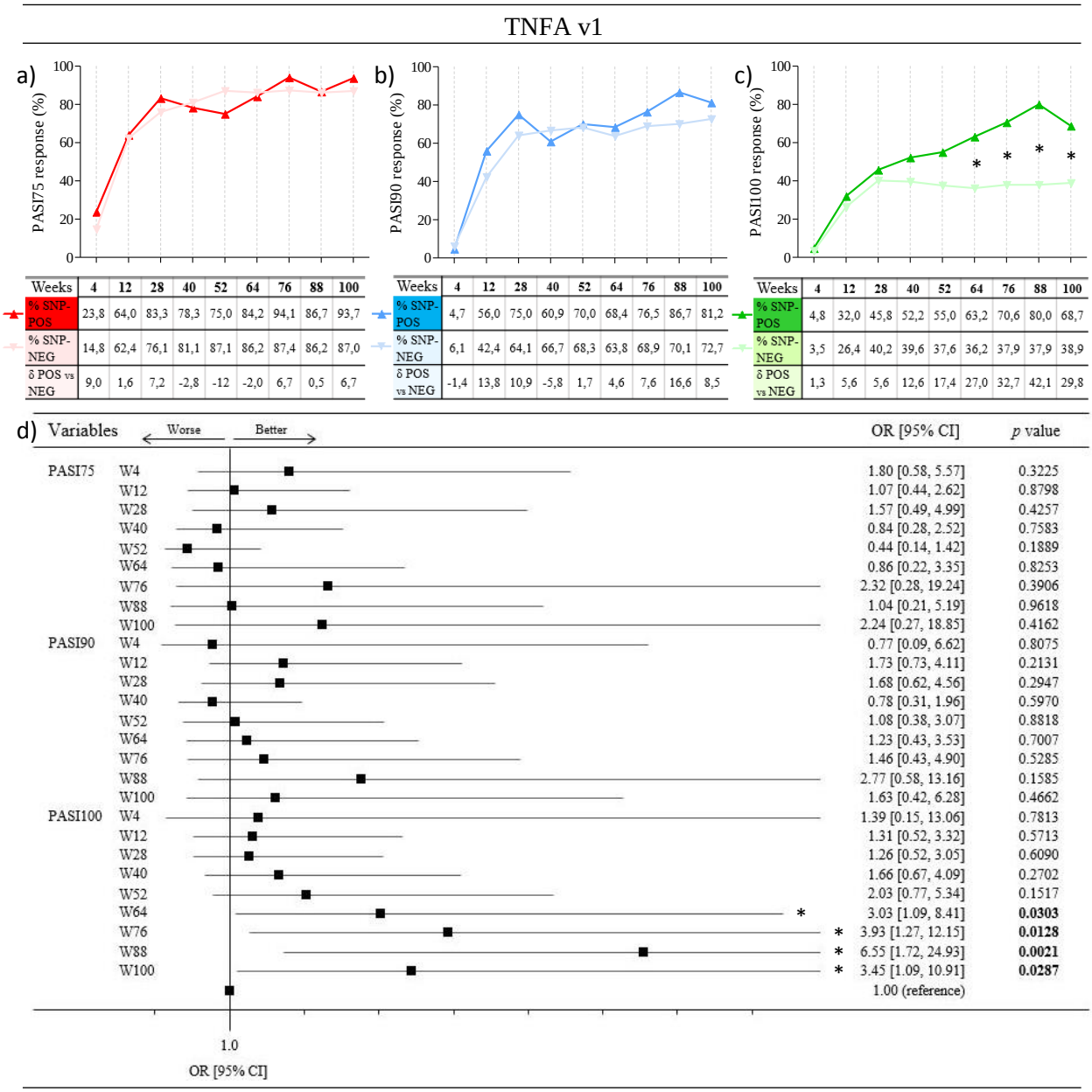
No significant associations were found for the other analyzed SNPs and response to secukinumab (data not shown), thus suggesting that only the allele variants in HLA-C region could have a predominant role in the response to biological drugs targeting IL-17.

3.1.3 Association of SNPs and response to ustekinumab, a blocker of IL-12/IL-23 p40 subunit

The analysis of 44 SNPs predisposing to psoriasis was also conducted on a subgroup of psoriatic patients undergone treatment with the anti-IL-12/IL-23 p40 ustekinumab biological, and the response to the drug was evaluated up to 2 years.

We found a significant association between SNPs in *TNFA* (TNFA v1, TNFA v2), *CDSN* (CDSN v2, CDSN v3) and *CCHCR1* (CCHCR1 v5) genes, as well as SNPs in the HLA-C region (HLA-Cw6 LD, HLA-Cw6 v1, HLA-Cw6 v2) and positive response to the drug. Concerning the allele variants in *TNFA* gene, we observed a significant association between TNFA v1 and achievement of PASI100 after ustekinumab treatment at weeks 64 (63.2%, 12 of 19), 76 (70.6%, 12 of 17), 88 (80.0%, 12 of 15) and 100 (68.7%, 11 of 16) (Fig. 10c and d). Similar results were observed for TNFA v2, present in an intron variant of *TNFA*, with the PASI90 being reached by 72.7% of TNFA v2-negative psoriatic patients at week 64 (Fig. 11b, c and d). PASI100 achievement was observed in the 50.7%, 55.1% or 52.3% of TNFA v2-negative patients at weeks 76, 88 or 100, respectively (Fig. 11b, c and d).

Moreover, we also found a significant association between the CDSN v2 variant in *CDSN* gene and response to ustekinumab. SNP absence in psoriatic patients was significantly associated with the achievement of PASI75 at week 64 (92.5% 62 of 67), and PASI90 at week 64 (76.1%, 51 of 67), week 76 (79%, 49 of 62), week 88 (80%, 48 of 60), and week 100 (82.1%, 46 of 56). Similar results were obtained when PASI100 achievement was considered. In particular, the majority of SNP-negative patients obtained an improvement of PASI score of 100% at week 52 (50%, 36 of 72), 64 (49.3%, 33 of 62), 76 (51.6%, 32 of 62), 88 (56.7%, 34 of 60), 100 (53.6%, 30 of 56) (Fig. 12). Regarding CDSN v3 variant, the PASI75 was reached by 92.3% (24 of 26) and 92% (23 of 25) of positive patients at week 12 and 28, respectively (Fig. 13 a and d). The achievement of PASI90 by patients carrying out SNP was observed at week 12 (65.4%, 17 of 26), 64 (82.6%, 19 of 23), 76 (86.9%, 20 of 23), 88 (90.9%, 20 of 22) and 100 (90.5%, 19 of 21) (Fig. 13 b and d). PASI100 was instead significantly reached only at week 28 by 60% of positive patients (15 of 25) (Fig. 13 c and d).



Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	23,8	64,0	83,3	78,3	75,0	84,2	94,1	86,7	93,7
% SNP-NEG	14,8	62,4	76,1	81,1	87,1	86,2	87,4	86,2	87,0
δ POS vs NEG	9,0	1,6	7,2	-2,8	-12	-2,0	6,7	0,5	6,7

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	4,7	56,0	75,0	60,9	70,0	68,4	76,5	86,7	81,2
% SNP-NEG	6,1	42,4	64,1	66,7	68,3	63,8	68,9	70,1	72,7
δ POS vs NEG	-1,4	13,8	10,9	-5,8	1,7	4,6	7,6	16,6	8,5

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	4,8	32,0	45,8	52,2	55,0	63,2	70,6	80,0	68,7
% SNP-NEG	3,5	26,4	40,2	39,6	37,6	36,2	37,9	37,9	38,9
δ POS vs NEG	1,3	5,6	5,6	12,6	17,4	27,0	32,7	42,1	29,8

		OR [95% CI]	p value
PASI75	W4	1.80 [0.58, 5.57]	0.3225
	W12	1.07 [0.44, 2.62]	0.8798
	W28	1.57 [0.49, 4.99]	0.4257
	W40	0.84 [0.28, 2.52]	0.7583
	W52	0.44 [0.14, 1.42]	0.1889
	W64	0.86 [0.22, 3.35]	0.8253
	W76	2.32 [0.28, 19.24]	0.3906
	W88	1.04 [0.21, 5.19]	0.9618
	W100	2.24 [0.27, 18.85]	0.4162
PASI90	W4	0.77 [0.09, 6.62]	0.8075
	W12	1.73 [0.73, 4.11]	0.2131
	W28	1.68 [0.62, 4.56]	0.2947
	W40	0.78 [0.31, 1.96]	0.5970
	W52	1.08 [0.38, 3.07]	0.8818
	W64	1.23 [0.43, 3.53]	0.7007
	W76	1.46 [0.43, 4.90]	0.5285
	W88	2.77 [0.58, 13.16]	0.1585
	W100	1.63 [0.42, 6.28]	0.4662
PASI100	W4	1.39 [0.15, 13.06]	0.7813
	W12	1.31 [0.52, 3.32]	0.5713
	W28	1.26 [0.52, 3.05]	0.6090
	W40	1.66 [0.67, 4.09]	0.2702
	W52	2.03 [0.77, 5.34]	0.1517
	W64	3.03 [1.09, 8.41] *	**0.0303**
	W76	* 3.93 [1.27, 12.15] *	**0.0128**
	W88	* 6.55 [1.72, 24.93] *	**0.0021**
	W100	* 3.45 [1.09, 10.91] *	**0.0287**
		1.00 (reference)	

Figure 10. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not TNFA v1 variant.** Proportion of TNFA v1-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between TNFA v1 and response to ustekinumab (d). Notes: PASI75, ≥ 75% reduction of PASI score; PASI90, ≥ 90% reduction of PASI; PASI100, 100% reduction of PASI; OR, odds ratio; CI, confidence interval; *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$

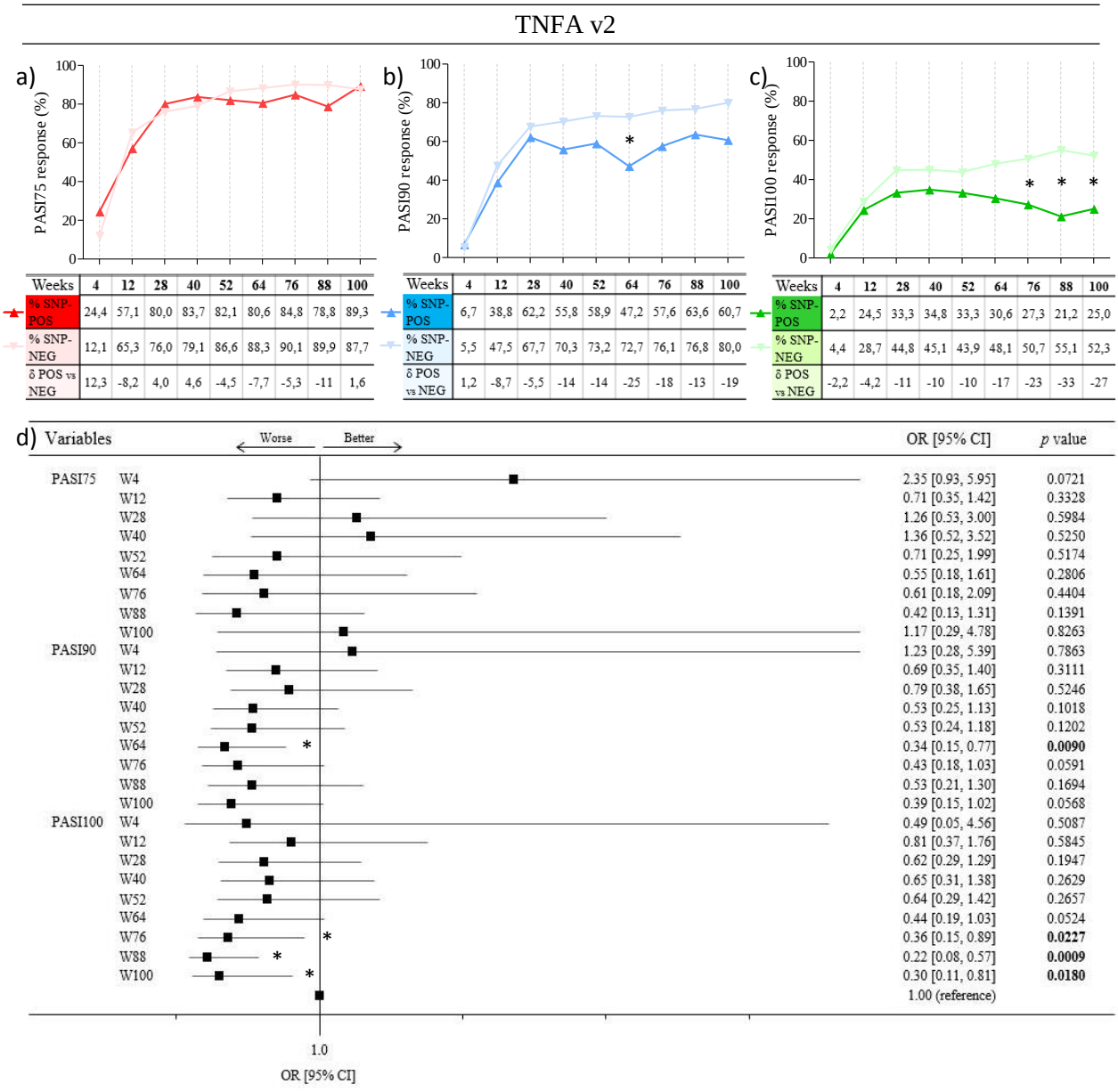
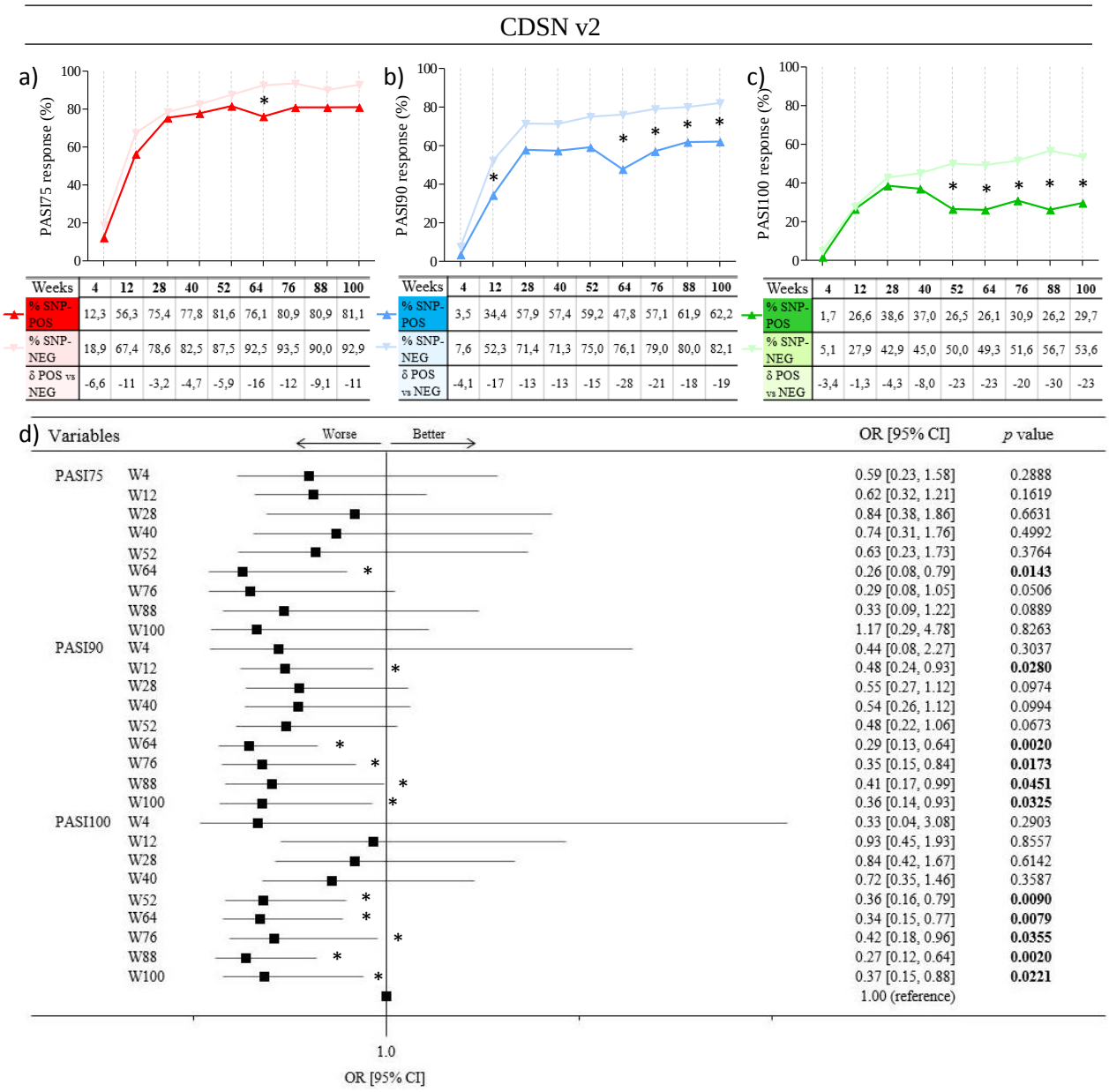


Figure 11. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not TNFA v2 variant.** Proportion of TNFA v2-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between TNFA v2 and response to ustekinumab (d). Notes as in Figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.



Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	12,3	56,3	75,4	77,8	81,6	76,1	80,9	80,9	81,1
% SNP-NEG	18,9	67,4	78,6	82,5	87,5	92,5	93,5	90,0	92,9
δ POS vs NEG	-6,6	-11	-3,2	-4,7	-5,9	-16	-12	-9,1	-11

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	3,5	34,4	57,9	57,4	59,2	47,8	57,1	61,9	62,2
% SNP-NEG	7,6	52,3	71,4	71,3	75,0	76,1	79,0	80,0	82,1
δ POS vs NEG	-4,1	-17	-13	-13	-15	-28	-21	-18	-19

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	1,7	26,6	38,6	37,0	26,5	26,1	30,9	26,2	29,7
% SNP-NEG	5,1	27,9	42,9	45,0	50,0	49,3	51,6	56,7	53,6
δ POS vs NEG	-3,4	-1,3	-4,3	-8,0	-23	-23	-20	-30	-23

		OR [95% CI]	*p* value
PASI75	W4	0.59 [0.23, 1.58]	0.2888
	W12	0.62 [0.32, 1.21]	0.1619
	W28	0.84 [0.38, 1.86]	0.6631
	W40	0.74 [0.31, 1.76]	0.4992
	W52	0.63 [0.23, 1.73]	0.3764
	W64	0.26 [0.08, 0.79]	**0.0143**
	W76	0.29 [0.08, 1.05]	0.0506
	W88	0.33 [0.09, 1.22]	0.0889
	W100	1.17 [0.29, 4.78]	0.8263
PASI90	W4	0.44 [0.08, 2.27]	0.3037
	W12	0.48 [0.24, 0.93]	**0.0280**
	W28	0.55 [0.27, 1.12]	0.0974
	W40	0.54 [0.26, 1.12]	0.0994
	W52	0.48 [0.22, 1.06]	0.0673
	W64	0.29 [0.13, 0.64]	**0.0020**
	W76	0.35 [0.15, 0.84]	**0.0173**
	W88	0.41 [0.17, 0.99]	**0.0451**
	W100	0.36 [0.14, 0.93]	**0.0325**
PASI100	W4	0.33 [0.04, 3.08]	0.2903
	W12	0.93 [0.45, 1.93]	0.8557
	W28	0.84 [0.42, 1.67]	0.6142
	W40	0.72 [0.35, 1.46]	0.3587
	W52	0.36 [0.16, 0.79]	**0.0090**
	W64	0.34 [0.15, 0.77]	**0.0079**
	W76	0.42 [0.18, 0.96]	**0.0355**
	W88	0.27 [0.12, 0.64]	**0.0020**
	W100	0.37 [0.15, 0.88]	**0.0221**
		1.00 (reference)	

Figure 12. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not CDSN v2 variant.** Proportion of CDSN v2-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between CDSN v2 variant and response to ustekinumab (d). Notes as in Figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

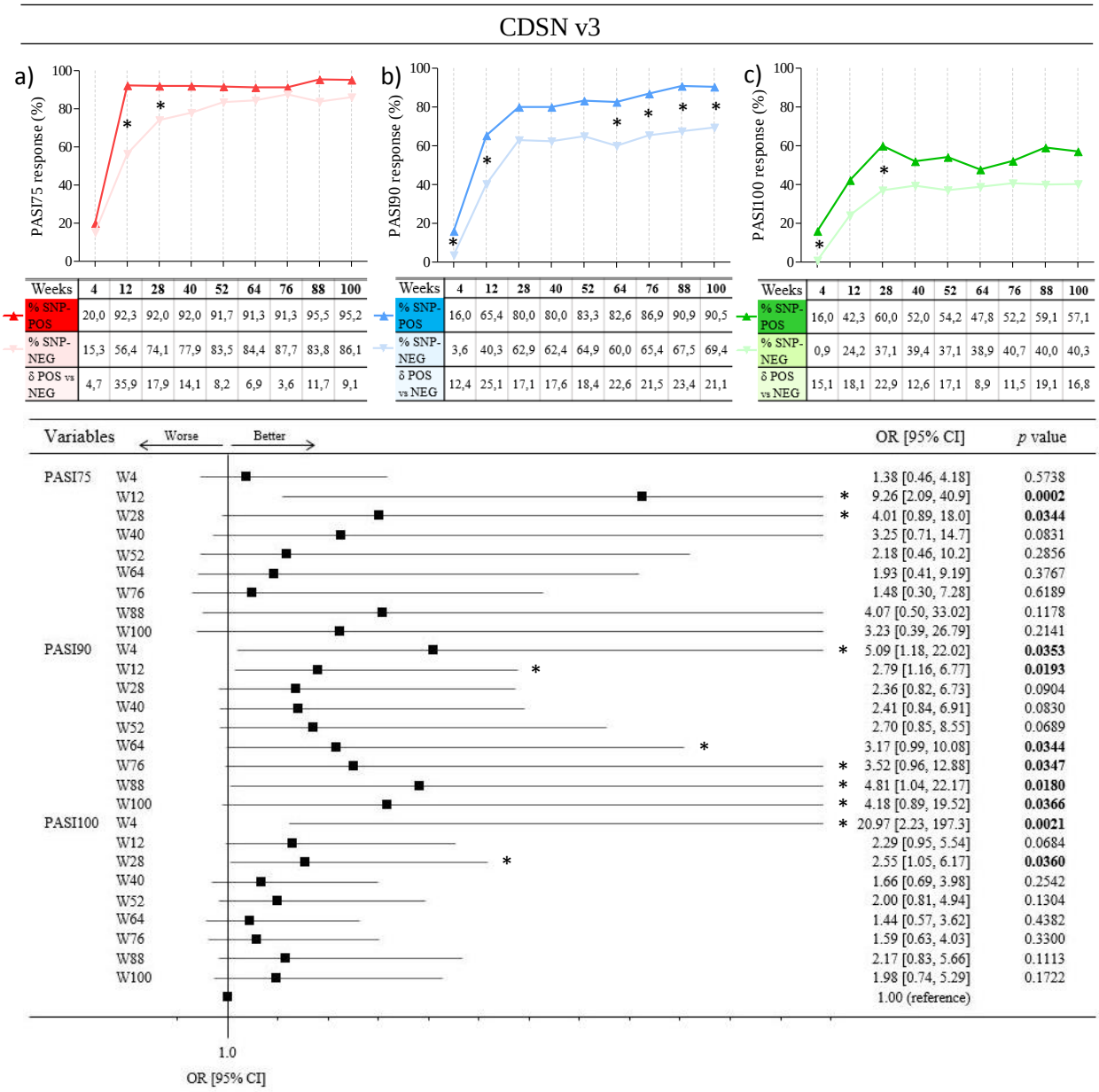
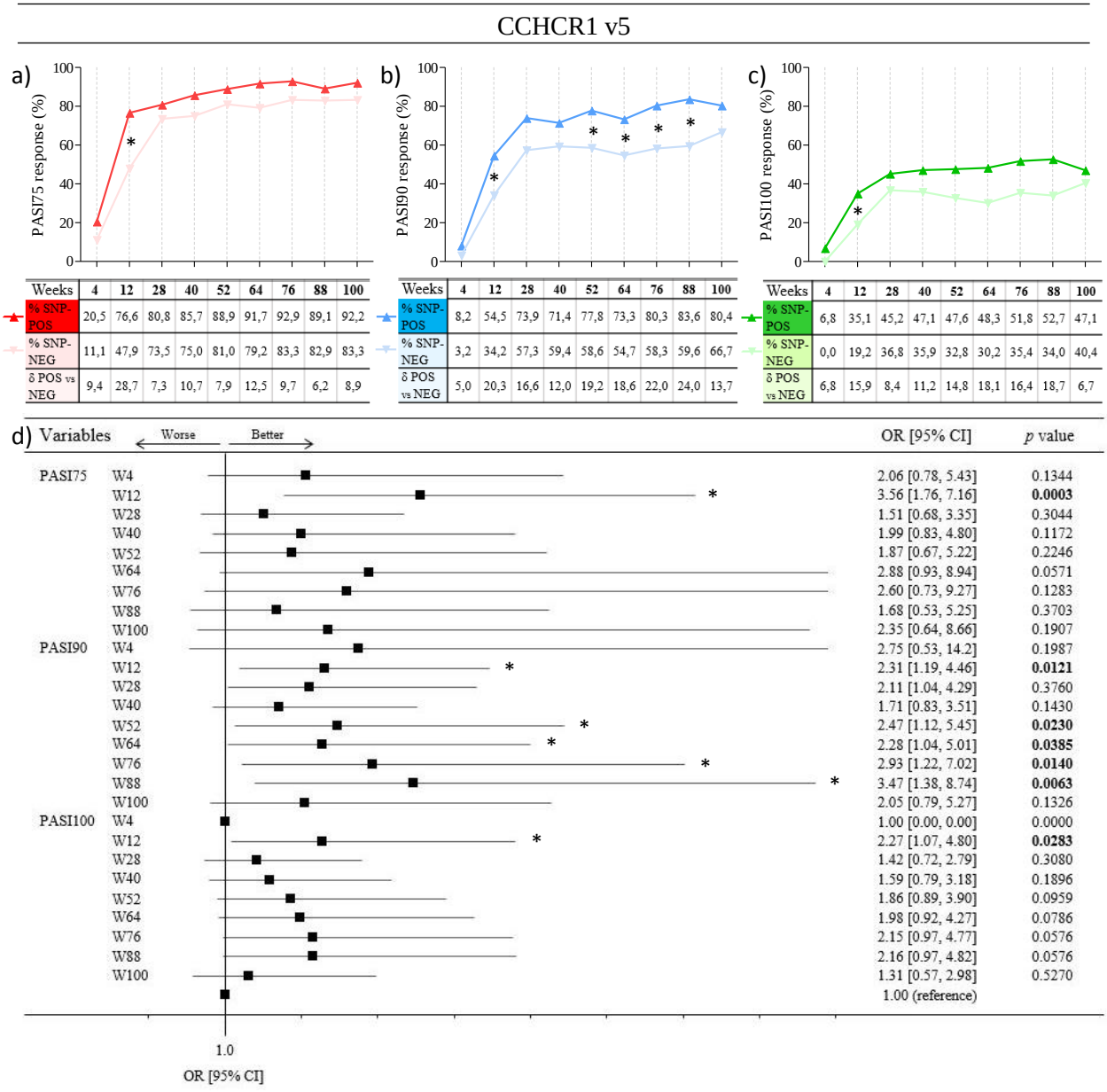


Figure 13. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not CDSN v3 variant.** Proportion of CDSN v3-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between CDSN v3 variant and response to ustekinumab (d). Notes as in Figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

Logistic regression analysis also revealed a significant association between the CCHCR1 v5 variant in the *CCHCR1* gene and response to ustekinumab. In particular, we found that the association between the achievement of PASI90 and presence of CCHCR1 v5 was statistically significant, starting from week 12 to 88 for the all time points examined, with the exception of week 28 and 40 (Fig.14 b and d). A significant association was also observed between the achievement of PASI100 at week 12 (35.1%, 27 of 77) and presence of CCHCR1 v5 variant (Fig. 14 c and d).



Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	20,5	76,6	80,8	85,7	88,9	91,7	92,9	89,1	92,2
% SNP-NEG	11,1	47,9	73,5	75,0	81,0	79,2	83,3	82,9	83,3
δ POS vs NEG	9,4	28,7	7,3	10,7	7,9	12,5	9,7	6,2	8,9

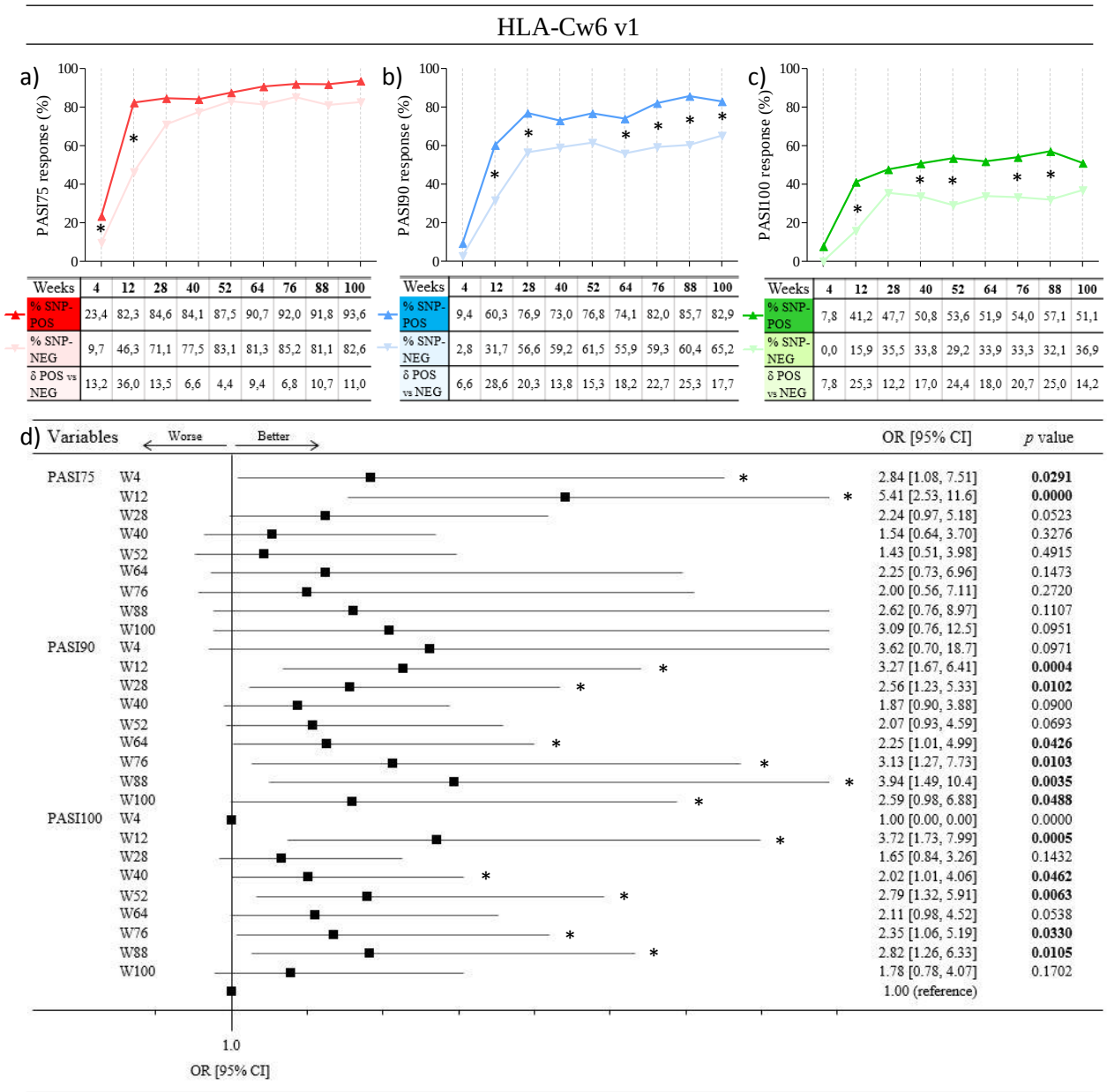
Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	8,2	54,5	73,9	71,4	77,8	73,3	80,3	83,6	80,4
% SNP-NEG	3,2	34,2	57,3	59,4	58,6	54,7	58,3	59,6	66,7
δ POS vs NEG	5,0	20,3	16,6	12,0	19,2	18,6	22,0	24,0	13,7

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	6,8	35,1	45,2	47,1	47,6	48,3	51,8	52,7	47,1
% SNP-NEG	0,0	19,2	36,8	35,9	32,8	30,2	35,4	34,0	40,4
δ POS vs NEG	6,8	15,9	8,4	11,2	14,8	18,1	16,4	18,7	6,7

Variables		OR [95% CI]	*p* value
PASI75	W4	2.06 [0.78, 5.43]	0.1344
	W12	3.56 [1.76, 7.16]	**0.0003**
	W28	1.51 [0.68, 3.35]	0.3044
	W40	1.99 [0.83, 4.80]	0.1172
	W52	1.87 [0.67, 5.22]	0.2246
	W64	2.88 [0.93, 8.94]	0.0571
	W76	2.60 [0.73, 9.27]	0.1283
	W88	1.68 [0.53, 5.25]	0.3703
	W100	2.35 [0.64, 8.66]	0.1907
PASI90	W4	2.75 [0.53, 14.2]	0.1987
	W12	2.31 [1.19, 4.46]	**0.0121**
	W28	2.11 [1.04, 4.29]	0.3760
	W40	1.71 [0.83, 3.51]	0.1430
	W52	2.47 [1.12, 5.45]	**0.0230**
	W64	2.28 [1.04, 5.01]	**0.0385**
	W76	2.93 [1.22, 7.02]	**0.0140**
	W88	3.47 [1.38, 8.74]	**0.0063**
	W100	2.05 [0.79, 5.27]	0.1326
PASI100	W4	1.00 [0.00, 0.00]	0.0000
	W12	2.27 [1.07, 4.80]	**0.0283**
	W28	1.42 [0.72, 2.79]	0.3080
	W40	1.59 [0.79, 3.18]	0.1896
	W52	1.86 [0.89, 3.90]	0.0959
	W64	1.98 [0.92, 4.27]	0.0786
	W76	2.15 [0.97, 4.77]	0.0576
	W88	2.16 [0.97, 4.82]	0.0576
	W100	1.31 [0.57, 2.98]	0.5270
		1.00 (reference)	

Figure 14. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not CCHCR1 v5 variant.** Proportion of CCHCR1 v5-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between CCHCR1 v5 variant and response to ustekinumab (d). Notes as in figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

Finally, accordingly to previously published results [89], we found a significant association between HLA-Cw6 classical variant (v1) presence in patients and response to ustekinumab treatment. In particular, HLA-Cw6 allele presence was significantly associated to the achievement of PASI90 at week 12 (60.3%, 41 of 68 patients), 28 (76.9%, 50 of 65), 64 (74.1%, 40 of 54), 76 (82%, 41 of 50), 88 (85.7%, 42 of 49) and 100 (82.9%, 39 of 47) (Fig. 15).



Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	23,4	82,3	84,6	84,1	87,5	90,7	92,0	91,8	93,6
% SNP-NEG	9,7	46,3	71,1	77,5	83,1	81,3	85,2	81,1	82,6
δ POS vs NEG	13,2	36,0	13,5	6,6	4,4	9,4	6,8	10,7	11,0

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	9,4	60,3	76,9	73,0	76,8	74,1	82,0	85,7	82,9
% SNP-NEG	2,8	31,7	56,6	59,2	61,5	55,9	59,3	60,4	65,2
δ POS vs NEG	6,6	28,6	20,3	13,8	15,3	18,2	22,7	25,3	17,7

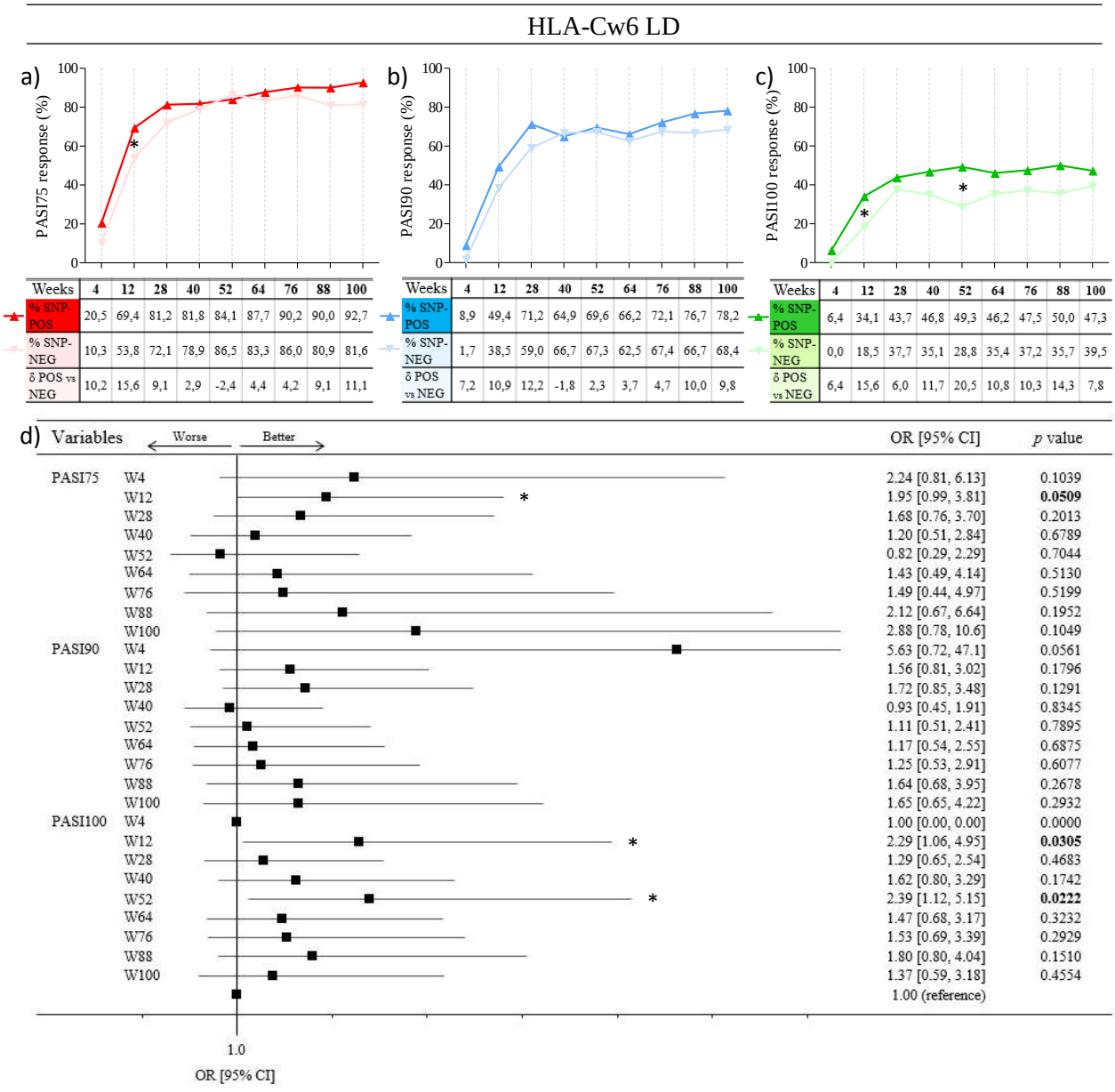
Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	7,8	41,2	47,7	50,8	53,6	51,9	54,0	57,1	51,1
% SNP-NEG	0,0	15,9	35,5	33,8	29,2	33,9	33,3	32,1	36,9
δ POS vs NEG	7,8	25,3	12,2	17,0	24,4	18,0	20,7	25,0	14,2

		OR [95% CI]	p value
PASI75	W4	2.84 [1.08, 7.51]	**0.0291**
	W12	5.41 [2.53, 11.6]	**0.0000**
	W28	2.24 [0.97, 5.18]	0.0523
	W40	1.54 [0.64, 3.70]	0.3276
	W52	1.43 [0.51, 3.98]	0.4915
	W64	2.25 [0.73, 6.96]	0.1473
	W76	2.00 [0.56, 7.11]	0.2720
	W88	2.62 [0.76, 8.97]	0.1107
	W100	3.09 [0.76, 12.5]	0.0951
PASI90	W4	3.62 [0.70, 18.7]	0.0971
	W12	3.27 [1.67, 6.41]	**0.0004**
	W28	2.56 [1.23, 5.33]	**0.0102**
	W40	1.87 [0.90, 3.88]	0.0900
	W52	2.07 [0.93, 4.59]	0.0693
	W64	2.25 [1.01, 4.99]	**0.0426**
	W76	3.13 [1.27, 7.73]	**0.0103**
	W88	3.94 [1.49, 10.4]	**0.0035**
	W100	2.59 [0.98, 6.88]	**0.0488**
PASI100	W4	1.00 [0.00, 0.00]	0.0000
	W12	3.72 [1.73, 7.99]	**0.0005**
	W28	1.65 [0.84, 3.26]	0.1432
	W40	2.02 [1.01, 4.06]	**0.0462**
	W52	2.79 [1.32, 5.91]	**0.0063**
	W64	2.11 [0.98, 4.52]	0.0538
	W76	2.35 [1.06, 5.19]	**0.0330**
	W88	2.82 [1.26, 6.33]	**0.0105**
	W100	1.78 [0.78, 4.07]	0.1702
		1.00 (reference)	

Figure 15. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not HLA-Cw6 v1 variant.** Proportion of HLA-Cw6 v1-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 v1 variant and response to ustekinumab (d). Notes as in Figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

Importantly, *HLA-Cw6* allele presence was significantly associated with a complete remission of the disease, being PASI100 reached by a significant proportion of psoriatic of patients (41.2% at week 12, 50.8% at week 40, 53.6% at week 52, 54% at week 76, and 57.1% at week 88) (Fig. 15).

Differently from secukinumab studies, the other HLA-C allele variants studied (HLA-Cw6 LD and HLA-Cw6 v2) only occasionally have been found significantly associated with responsiveness to ustekinumab (Fig. 16 and 17).



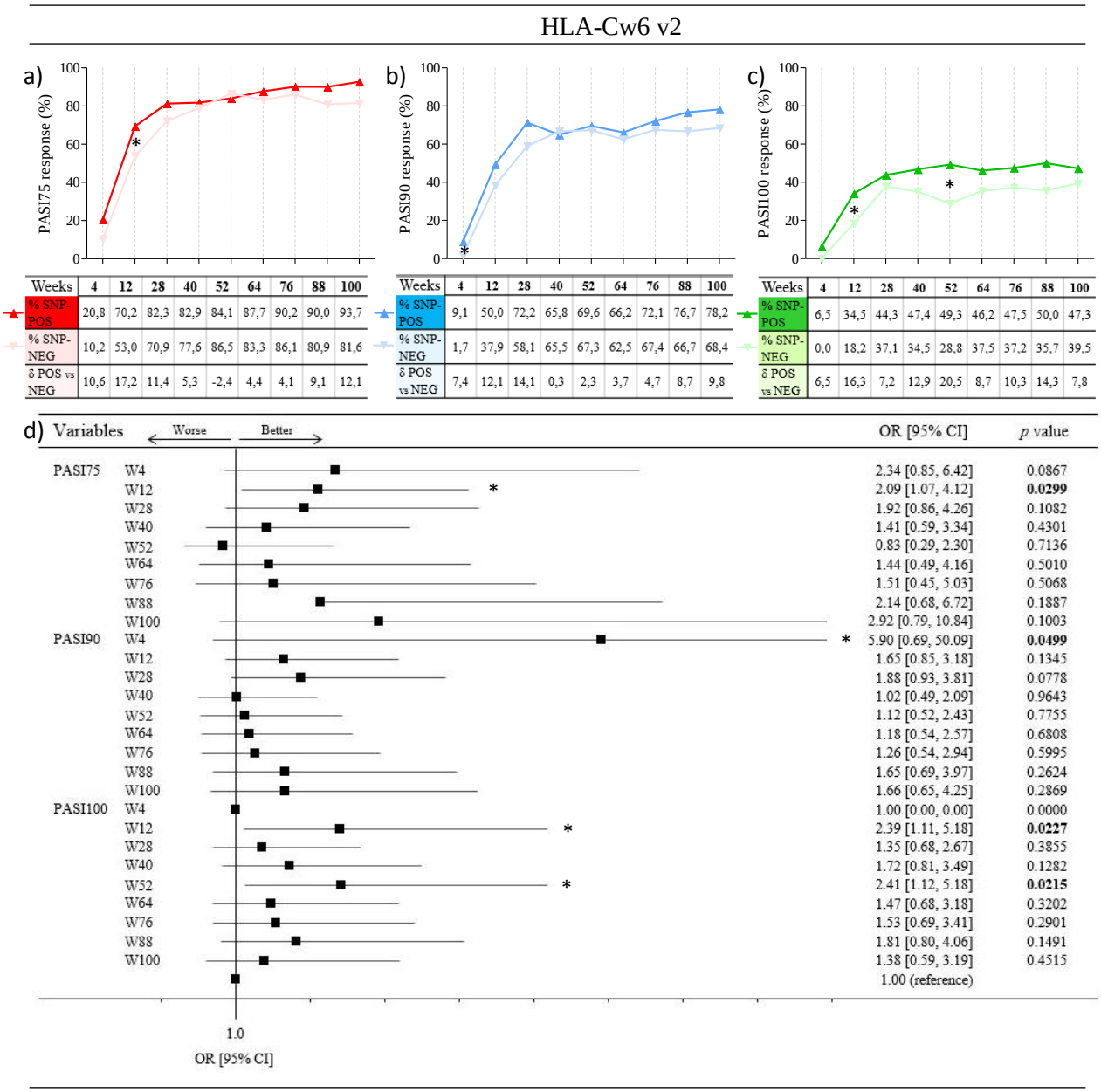
Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	20,5	69,4	81,2	81,8	84,1	87,7	90,2	90,0	92,7
% SNP-NEG	10,3	53,8	72,1	78,9	86,5	83,3	86,0	80,9	81,6
δ POS vs NEG	10,2	15,6	9,1	2,9	-2,4	4,4	4,2	9,1	11,1

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	8,9	49,4	71,2	64,9	69,6	66,2	72,1	76,7	78,2
% SNP-NEG	1,7	38,5	59,0	66,7	67,3	62,5	67,4	66,7	68,4
δ POS vs NEG	7,2	10,9	12,2	-1,8	2,3	3,7	4,7	10,0	9,8

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	6,4	34,1	43,7	46,8	49,3	46,2	47,5	50,0	47,3
% SNP-NEG	0,0	18,5	37,7	35,1	28,8	35,4	37,2	35,7	39,5
δ POS vs NEG	6,4	15,6	6,0	11,7	20,5	10,8	10,3	14,3	7,8

		OR [95% CI]	*p* value
PASI75	W4	2.24 [0.81, 6.13]	0.1039
	W12	1.95 [0.99, 3.81]	**0.0509**
	W28	1.68 [0.76, 3.70]	0.2013
	W40	1.20 [0.51, 2.84]	0.6789
	W52	0.82 [0.29, 2.29]	0.7044
	W64	1.43 [0.49, 4.14]	0.5130
	W76	1.49 [0.44, 4.97]	0.5199
	W88	2.12 [0.67, 6.64]	0.1952
	W100	2.88 [0.78, 10.6]	0.1049
PASI90	W4	5.63 [0.72, 47.1]	0.0561
	W12	1.56 [0.81, 3.02]	0.1796
	W28	1.72 [0.85, 3.48]	0.1291
	W40	0.93 [0.45, 1.91]	0.8345
	W52	1.11 [0.51, 2.41]	0.7895
	W64	1.17 [0.54, 2.55]	0.6875
	W76	1.25 [0.53, 2.91]	0.6077
	W88	1.64 [0.68, 3.95]	0.2678
	W100	1.65 [0.65, 4.22]	0.2932
PASI100	W4	1.00 [0.00, 0.00]	0.0000
	W12	2.29 [1.06, 4.95]	**0.0305**
	W28	1.29 [0.65, 2.54]	0.4683
	W40	1.62 [0.80, 3.29]	0.1742
	W52	2.39 [1.12, 5.15]	**0.0222**
	W64	1.47 [0.68, 3.17]	0.3232
	W76	1.53 [0.69, 3.39]	0.2929
	W88	1.80 [0.80, 4.04]	0.1510
	W100	1.37 [0.59, 3.18]	0.4554
		1.00 (reference)	

Figure 16. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not HLA-Cw6 LD variant.** Proportion of HLA-Cw6 LD-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 LD variant and response to ustekinumab (d). Notes as in Figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.



Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	20,8	70,2	82,3	82,9	84,1	87,7	90,2	90,0	93,7
% SNP-NEG	10,2	53,0	70,9	77,6	86,5	83,3	86,1	80,9	81,6
δ POS vs NEG	10,6	17,2	11,4	5,3	-2,4	4,4	4,1	9,1	12,1

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	9,1	50,0	72,2	65,8	69,6	66,2	72,1	76,7	78,2
% SNP-NEG	1,7	37,9	58,1	65,5	67,3	62,5	67,4	66,7	68,4
δ POS vs NEG	7,4	12,1	14,1	0,3	2,3	3,7	4,7	8,7	9,8

Weeks	4	12	28	40	52	64	76	88	100
% SNP-POS	6,5	34,5	44,3	47,4	49,3	46,2	47,5	50,0	47,3
% SNP-NEG	0,0	18,2	37,1	34,5	28,8	37,5	37,2	35,7	39,5
δ POS vs NEG	6,5	16,3	7,2	12,9	20,5	8,7	10,3	14,3	7,8

		OR [95% CI]	*p* value
PASI75	W4	2.34 [0.85, 6.42]	0.0867
	W12 *	2.09 [1.07, 4.12]	**0.0299**
	W28	1.92 [0.86, 4.26]	0.1082
	W40	1.41 [0.59, 3.34]	0.4301
	W52	0.83 [0.29, 2.30]	0.7136
	W64	1.44 [0.49, 4.16]	0.5010
	W76	1.51 [0.45, 5.03]	0.5068
	W88	2.14 [0.68, 6.72]	0.1887
	W100	2.92 [0.79, 10.84]	0.1003
PASI90	W4 *	5.90 [0.69, 50.09]	**0.0499**
	W12	1.65 [0.85, 3.18]	0.1345
	W28	1.88 [0.93, 3.81]	0.0778
	W40	1.02 [0.49, 2.09]	0.9643
	W52	1.12 [0.52, 2.43]	0.7755
	W64	1.18 [0.54, 2.57]	0.6808
	W76	1.26 [0.54, 2.94]	0.5995
	W88	1.65 [0.69, 3.97]	0.2624
	W100	1.66 [0.65, 4.25]	0.2869
PASI100	W4	1.00 [0.00, 0.00]	0.0000
	W12 *	2.39 [1.11, 5.18]	**0.0227**
	W28	1.35 [0.68, 2.67]	0.3855
	W40	1.72 [0.81, 3.49]	0.1282
	W52 *	2.41 [1.12, 5.18]	**0.0215**
	W64	1.47 [0.68, 3.18]	0.3202
	W76	1.53 [0.69, 3.41]	0.2901
	W88	1.81 [0.80, 4.06]	0.1491
	W100	1.38 [0.59, 3.19]	0.4515
		1.00 (reference)	

Figure 17. **Clinical response to ustekinumab between week 4 and week 100 on patients carrying or not HLA-Cw6 v2 variant.** Proportion of HLA-Cw6 v2-positive and -negative patients achieving PASI75 (a), PASI90 (b), PASI100 (c). Univariate logistic regression for association analysis between HLA-Cw6 v2 variant and response to ustekinumab (d). Notes as in Figure 11. *p* values for categorical variables were calculated using the logistic model, * $p < 0.05$.

3.2 Studies on the paradoxical psoriasis-like manifestations induced by the anti-TNF-α therapy

SNP analysis was in parallel conducted on three patients affected by HS, who developed psoriasiform reactions following anti-TNF-α therapy with adalimumab. In parallel to the genetic profiles, the immunological profiles of these patients have also been investigated.

3.2.1 Clinical characterization of patients manifesting paradoxical psoriasis reactions after adalimumab treatment

We analyzed three patients affected by severe HS developing paradoxical reactions following a therapy with the anti-TNF adalimumab. Adalimumab was administered following AIFA criteria for HS condition. After paradoxical psoriasis manifestation, adalimumab was discontinued.

Patient 1, a 48-year-old Caucasian woman, showed nodules, fistulas and sinus tracts in the inguinal and perianal region (Hurley III, Sartorius score: 41.5). After three months of therapy with anti-TNF-α, patient 1 developed psoriasiform eruptions (PASI 6.8), with pustular lesions and erythemato-scaly lesions on the plantar region and lower limbs, respectively (Fig. 18).

A similar pattern of HS severity was observed in patient 2 (Hurley III, Sartorius score: 41.5), a 40-year-old Caucasian man, showing erythematous-pustular lesions in the palmo-plantar region and erythemato-scaly plaques on the legs and scalp, ascribed to psoriasiform dermatitis (PASI 5.2), arisen after two months with adalimumab (Fig. 18). Patient 2 concomitantly showed alopecia areata on the scalp and some eczematous-like skin lesions. Patient 3, a 27-year-old Caucasian man, was affected by severe HS (Hurley III, Sartorius score: 61.5) characterized by comedones, nodules, and fistulas in the inguinal, gluteal and abdominal region. Patient 3 developed pustular lesions on palmo-plantar regions, and erythemato-scaly plaques on the legs, scalp, elbows and trunk (PASI 5.6) after three months of biological therapy (Fig. 18). He refused to undergo a punch biopsy, and, therefore, we could not perform the histological and immunological exams.

Histological examinations of psoriasiform lesions of patient 1 and 2, showed epidermal hyperplasia with parakeratosis, papillary vessel ectasia and perivascular infiltrate compatible with a psoriasiform dermatitis, with different amounts of intraepidermal or subcorneal neutrophilic infiltration (Fig. 18). Interestingly, some eczematiform spongiotic areas overlapping with the psoriasis-like histological pattern were present in skin lesions of patient 2.

Of note, paradoxical psoriasis regressed in all patients, when adalimumab was discontinued. These finding suggests that paradoxical psoriasis is a transient side-effect induced by TNF blockade, with clinical and histological presentations resembling psoriasis.

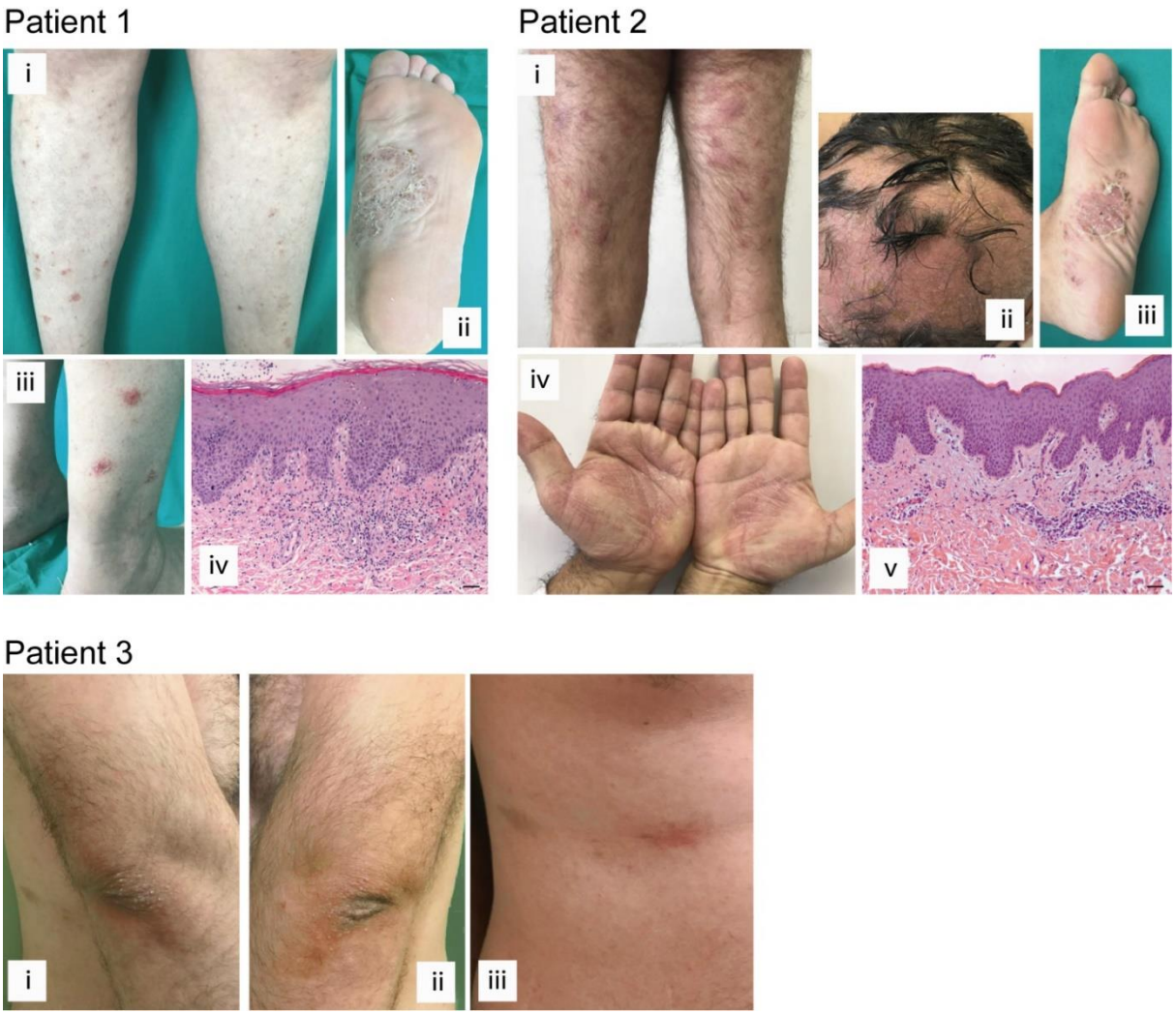


Figure 18. **Clinical and histological presentation of paradoxical psoriasis induced by anti-TNF-α therapy in HS patients**. Cutaneous lesions of patient 1, 2 and 3 affected by severe HS, presenting paradoxical psoriasiform reaction after anti-TNF-α treatment. Patient 1 panels shows paradoxical erythemato-squamous plaques localized on lower limbs (i and iii) and pustular lesions on the plantar region (ii). Patient 2 similarly shows erythemato-squamous plaques on the limbs (i), pustular lesions on the palmo-plantar region and severe form of alopecia areata involving part of the scalp (ii-iv). Patient 3 panels reveals an erythematosus patches, with mild desquamation on the elbows and trunk (i-iii). H&E staining for the corresponding histopathology of patients 1 (iv) and patient 2 (v) was also performed. Bars, 200 μM.

3.2.2 SNP analysis of patients developing paradoxical psoriasis

In order to understand whether paradoxical reactions had a genetic basis, the presence or absence of 44 SNPs predisposing to psoriasis in the DNA of the three patients have been analyzed by using NGS technology. To this end, the same SNP array used for the pharmacogenomic studies on secukinumab and ustekinumab has been employed.

Allelic variants in genes predisposing to classical psoriasis, including SNPs in *ERAP1* and *HLA-C* region, were detected in all the HS patients with paradoxical reactions, either in homozygosis or in heterozygosis condition (Table 10). Three SNPs in *ERAP1* (rs30187, rs30186, rs26653) and nine variants in *HLA-C* (HLA-Cw6 LD, rs9264942, rs10484554, rs2524095, rs28383849, rs9264944, rs2853922, rs147538049, rs9264946) were, in fact, found in all patients, with SNPs differently distributing in the three patients. None of them showed classical *HLA-Cw6* allele, even though patient 1 and 3 carried out three-point SNPs (rs2524095, rs2853922, rs386698994) mapping nearby HLA-Cw6 SNP position (rs17192540) (Table 10). On the contrary, patient 2 showed mostly genetic polymorphisms (rs9264942, rs10484554, rs28383849, rs9264944, rs147538049, rs9264946) present in the genomic region containing a second variant of *HLA-Cw6* (Table 10). All HS patients carried out SNPs in *NFKBIZ* (rs3217713) and *TNFAIP3* (rs610604) genes, codifying IKBζ and A20 protein, respectively. Interestingly, patient 3 showed the higher number of psoriasis-related SNPs, sharing a number of SNPs with patient 2 (rs7637230 /rs4819554 /rs3132554 /rs10542126 /rs3130983) and rs280519 with patient 1 (Table 2). Patient 3 also carried out other two SNPs in *CDSN* (rs1062470 /rs707913) and three SNPs in *CCHCR1* (rs1576 /rs130079 /rs746647) (Table 10).

Although rs11209026 in *IL23R* gene has been previously associated with paradoxical psoriasiform reactions to anti-TNFs [81], we could not find this SNP in none of HS patients. Other two SNPs in *IL23R*, rs72676067 and rs1004819, were instead detected in patient 2 and 1, respectively.

Antigen presentation				
dbSNP ID	**Gene**	**Patient 1**	**Patient 2**	**Patient 3**
rs30187	ERAP1	●	◑	◑
rs30186	ERAP1	●	◑	●
rs11743410	ERAP1	○	○	◑
rs26653	ERAP1	●	◑	●
rs114395371	HLA-C region	○	◑	○
rs17192540	HLA-C region	○	○	○
rs2524095	HLA-C region	●	○	●
rs2853922	HLA-C region	●	○	●
rs386698994	HLA-C region	●	○	◑
rs79709508	HLA-C region	●	●	○
rs28383849	HLA-C region	○	◑	○
rs10484554	HLA-C region	○	◑	○
rs147538049	HLA-C region	○	◑	○
rs9264944	HLA-C region	○	◑	○
rs9264946	HLA-C region	○	◑	○
NF-κB pathway and T-cell activation				
dbSNP ID	**Gene**	**Patient 1**	**Patient 2**	**Patient 3**
rs72676067	IL23R	○	◑	○
rs1004819	IL23R	◑	○	○
rs41313262	IL23R	○	○	◑
rs11209026	IL23R	○	○	○
rs3217713	NFKBIZ	●	●	●
rs7637230	NFKBIZ	○	◑	●
rs2546890	IL12B	○	◑	◑
rs1800610	TNFα	○	◑	○
rs2397084	IL17F	◑	○	○
rs71562288	TRAF3IP2	○	○	◑
rs33980500	TRAF3IP2	○	◑	○
rs610604	TNFAIP3	◑	◑	●
rs12720356	TYK2	○	○	◑
rs280519	TYK2	◑	○	●
rs4819554	IL17RA	○	◑	●
Skin barrier function				
dbSNP ID	**Gene**	**Patient 1**	**Patient 2**	**Patient 3**
rs3132554	CDSN	○	◑	◑
rs1042127	CDSN	○	◑	○
rs1042126	CDSN	○	◑	◑
rs1062470	CDSN	○	○	◑
rs707913	CDSN	○	○	◑
rs3130983	CDSN	○	◑	◑
rs1576	CCHCR1	○	○	●
rs130079	CCHCR1	○	○	◑
rs746647	CCHCR1	○	○	●
rs130075	CCHCR1	◑	○	○

Table 10: **SNPs carried out by HS patients developing paradoxical psoriasis after anti-TNF-α therapy**. SNP-carrying genes were classified accordingly to their functions (i. e. control of antigen presentation, NF-κB pathway and T-cell activation, skin barrier). Note: dbSNP ID, data base SNP identification number at NCBI; rs, reference *SNP* ID number; ERAP1, endoplasmic reticulum aminopeptidase 1; NFKBIZ, NF-κB inhibitor zeta; TRAF3IP2, TRAF3 interacting protein 2; TNFAIP3, TNF alpha induced protein 3; TYK2, tyrosine kinase 2; IL17RA, IL-17 receptor A; CDSN, corneodesmosin; CCHCR1, coiled-coil alpha-helical rod protein 1. rs17192540 and rs79709508 were relative to HLA-Cw6 and HLA-Cw6 2nd allelic variant (HLA-Cw6 2v).

● : homozygotic variant; ◑ : heterozygotic variant; ○ : wild type.

3.2.3 Immunohistochemical and molecular characterization of psoriasiform skin lesions

Next, leukocyte subpopulations were characterized in paradoxical psoriasiform lesions, and compared to those present in classical plaque-type psoriasis. The contribution of innate immunity pathways, in particular the presence of innate immunity cells subpopulations was characterized by immunohistochemistry. In line with previous studies [63,101], $BDCA2^+$ pDC in the dermis were significantly more abundant than in classical psoriasis (~ 2.7-fold increase). In parallel, the increase of $CD15^+$ neutrophils, c-kit/$CD117^+$ mast cells, $CD68^+$ macrophages and monocytes in the dermis of both paradoxical skin reactions was observed (~ 3.8-, 3.5- and 1.8-fold increase, respectively), if compared with LS and NLS plaque-type psoriasis (Fig. 19). On the contrary, $CD11c^+$ DC immunoreactivity substantially decreased in both the dermis and epidermis of paradoxical lesions, as compared to plaque-type psoriasis (~ 1.5-fold-decrease). A similar number of $CD3^+$ cells were detected in paradoxical skin lesions and classical psoriasis (Fig. 19).

In parallel, the local expression of psoriasis-related cytokines, namely IL-17A, IFN-γ, IL-22, and IL-36γ , were evaluated by immunohistochemistry [102]. A local overproduction of these cytokines has been revealed. As shown in Figure 20, IFN-γ immunoreactivity decreased in psoriasiform reactions of patients 1 and 2, as compared to classical psoriasis. In contrast, psoriasiform lesions showed an increased number of infiltrating $IL\text{-}22^+$ leukocytes, in particular in cells having a macrophage-like morphology (~ 2.1-fold increase). Due the numerous neutrophils present in the dermis of paradoxical reactions, IL-36γ positivity also enhanced, as compared to classical psoriasis. However, IL-36γ expression in the epidermal compartment was similar (Fig. 20).

Furthermore, since the inflammatory infiltrate pattern in paradoxical psoriasis strongly resembles that present in acute psoriasis, selected innate immunity molecules potentially involved in triggering of psoriasis has been also investigated. The type I IFN-β was expressed in paradoxical skin lesions, mainly in keratinocytes, at levels significantly higher than classical psoriasis (~ 1.9-fold increase). IFN-β expression was also detected in cells with a T-cell- and DC-like morphology, as well as in endothelial cells. Epidermis of psoriasiform reactions was also immunoreactive for IFN-κ, another keratinocyte-derived type I IFN [103]. IFN-κ expression was similar in the two psoriasis conditions, even if it showed different subcellular localization within keratinocytes, being it cytoplasmic in psoriasiform lesions and membrane-bound in classical psoriasis (Fig. 21). IFN-κ staining was also present in cells with a monocyte- or DC-like morphology, at comparable levels in classical and unclassical psoriasis (Fig. 21).

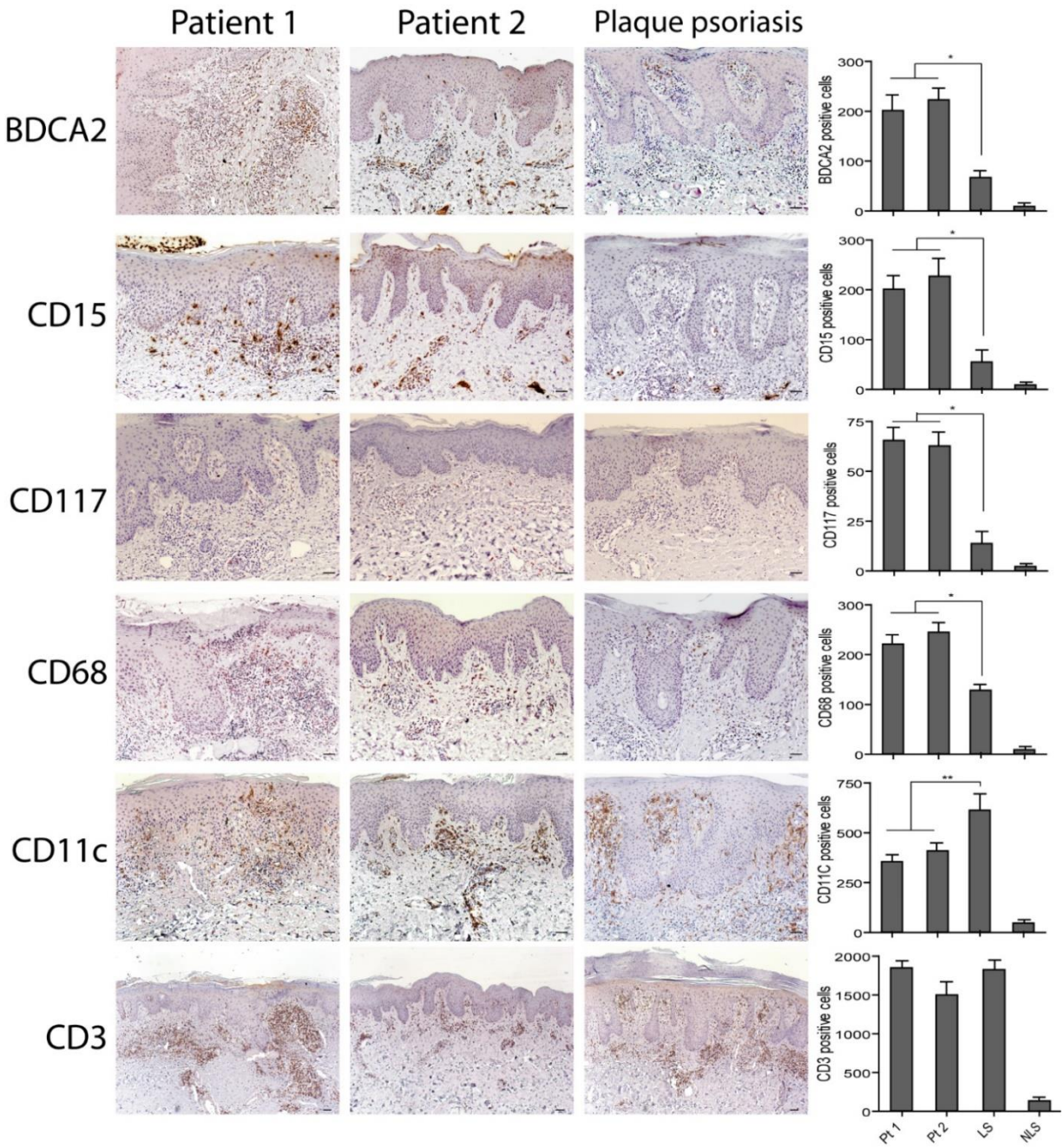


Figure 19. **Innate immunity cells highly infiltrate paradoxical psoriasis skin lesions**. Immunohistochemistry analysis of paradoxical skin reactions obtained from patient 1 (Pt1) and 2 (Pt2) shows an increase of positive BDCA2, CD15, CD117, CD68 cells, a reduction of CD11c cells and similar values of CD3 cells, when compared with psoriasis. Lesional (LS) and nonlesional (NLS) skin of the same psoriatic patient ($n = 3$) was analyzed. Slides were analyzed by two pathologists with experience in dermatology field. Positive cells were counted in five adjacent fields at a magnification of 200X. Graphs show the mean of number of positive cells ± SD *per* three sections. One out of three representative stainings is shown. *$p < 0.01$, **$p < 0.05$ *vs* classical psoriasis. Bars, 200 μM.

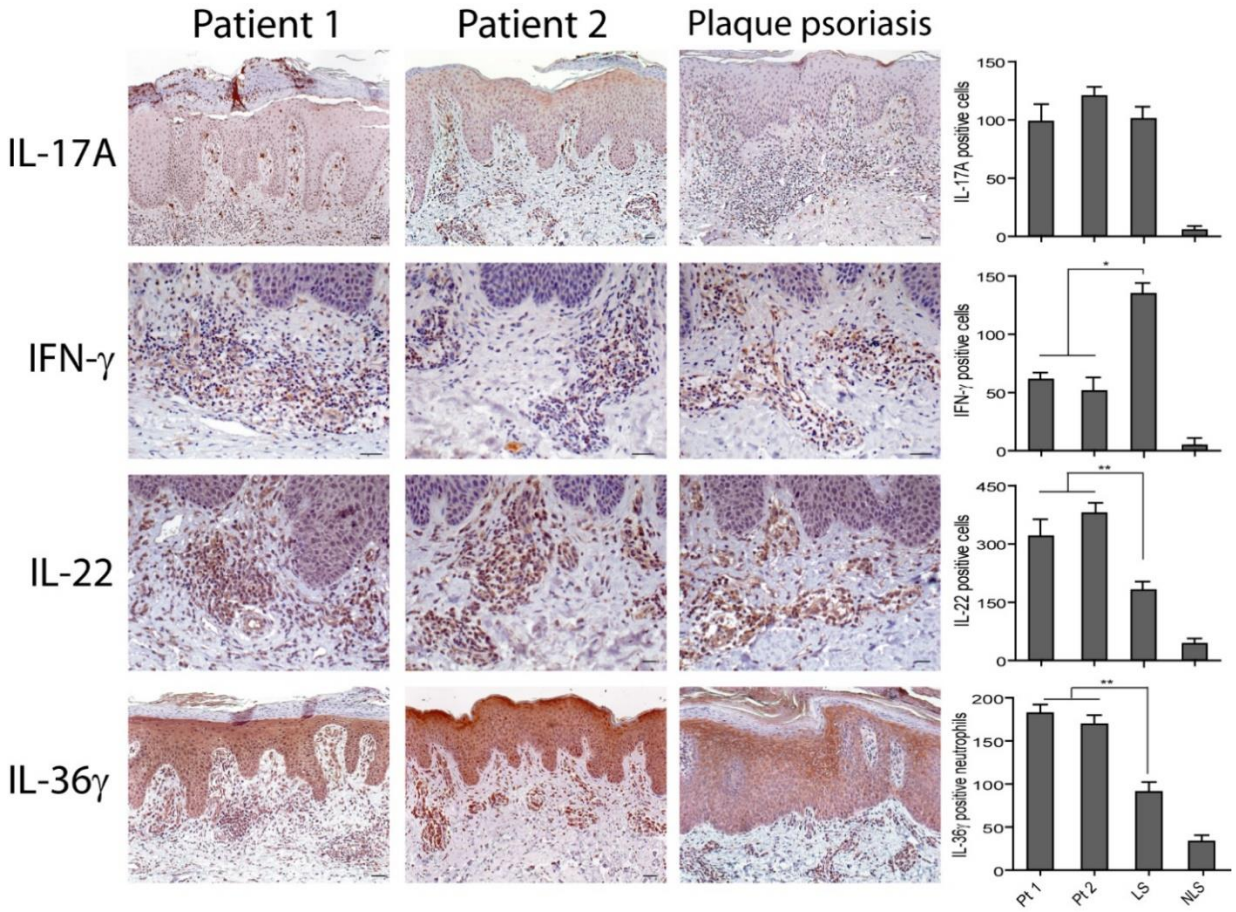


Figure 20. **Expression of psoriasis-related cytokines in paradoxical psoriasiform reactions**. Immunohistochemistry analysis performed on paradoxical skin lesions obtained from patients 1 (Pt1) and 2 (Pt2) shows similar values of IL-17A$^+$ cells, a reduction of dermal IFN-γ$^+$ cells and an increase of IL-22$^+$ or IL-36γ$^+$ cells, when compared with psoriatic skin lesions. Lesional (LS) and nonlesional (NLS) skin of the same psoriatic patient (n = 3) was analyzed. Graphs show the mean of number of positive cells ± SD *per* three sections. One out of three representative stainings is shown. *p < 0.01, **p < 0.05, *vs* classical psoriasis. Bars, 200 μM.

Finally, LT-α and LT-β, two members of the TNF family cytokine, also known as TNF-β and TNF-C, were investigated, as possibly deregulated by anti-TNF-α therapy. Conversely, both lymphotoxins were strongly overexpressed in paradoxical skin reactions, especially in keratinocytes of the basal layer epidermis (Fig. 21), suggesting an important role in the early phase of psoriasis, not investigated yet.

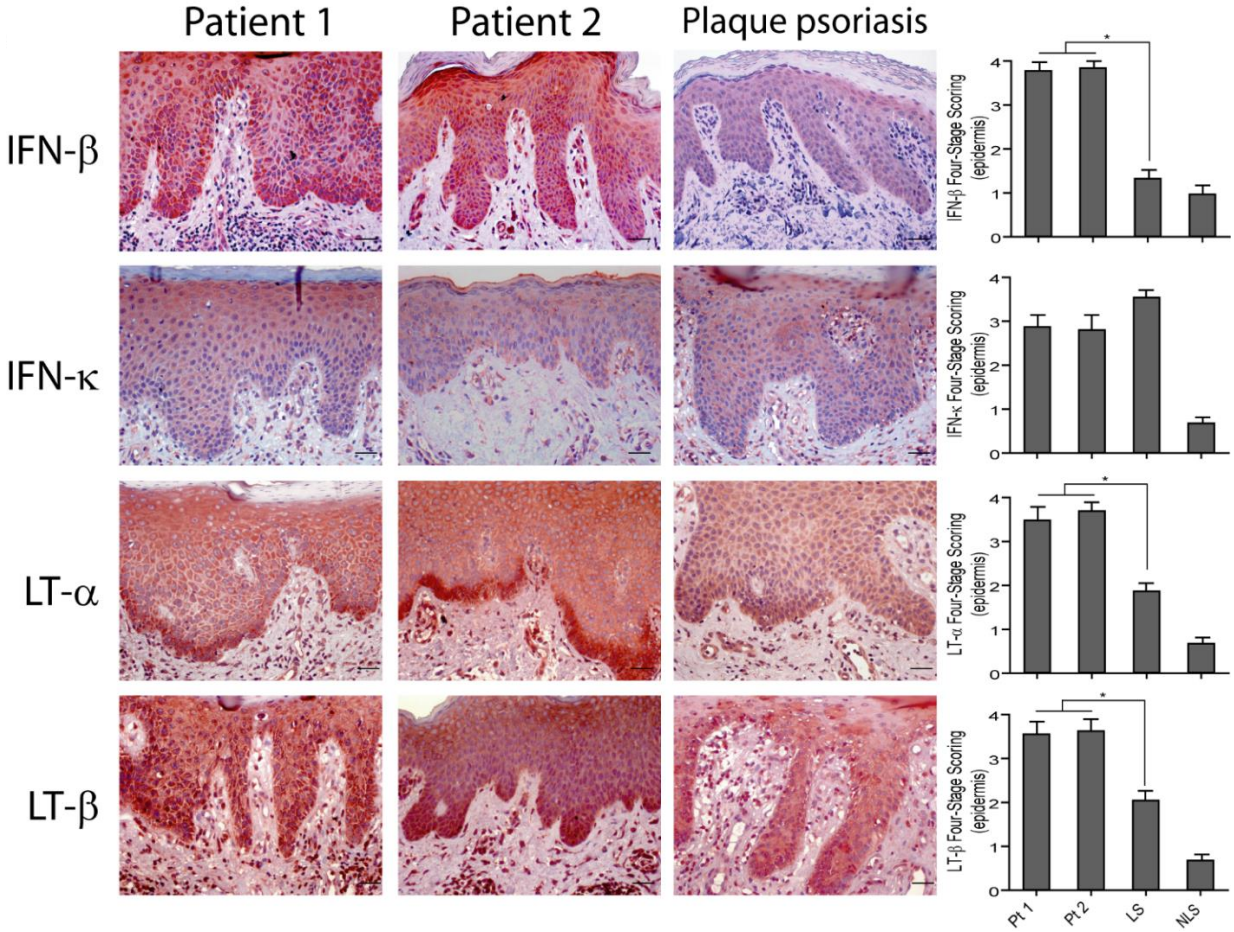


Figure 21. **Innate immunity molecules are overexpressed in the skin of HS patients after TNF-α treatment**. Immunohistochemistry analysis of paradoxical skin reactions obtained from patients 1 (Pt1) and 2 (Pt2) shows an increase of IFN-β, LT-α, LT-β, and similar IFN-κ positivity, when compared with psoriatic skin lesions. Lesional (LS) and nonlesional (NLS) skin of the same psoriatic patient (n = 3) was analyzed. Graphs show the mean ± SD of semiquantitative, four-stage scoring, ranging from negative immunoreactivity (0) to strong immunoreactivity (4+) and relative to the epidermal expression of the indicated molecules. One out of three representative stainings is shown. *p < 0.01, vs classical psoriasis. Bars, 200 μM.

Innate immunity molecules potentially involved in the pathogenesis of psoriasis were then analyzed at mRNA expression levels. Type I IFN-β mRNA was expressed in paradoxical skin lesions, at levels higher than classical psoriasis. Similarly, IFN-α2a and IFN-λ1, but not IFN-λ2 and IFN-λ3, were greatly increased in paradoxical psoriasis, as compared to plaque psoriasis (Fig. 22 and data not shown). Of note, LL-37 mRNA was also strongly expressed in psoriasiform skin lesions, at levels higher than classical psoriasis (Fig. 22).

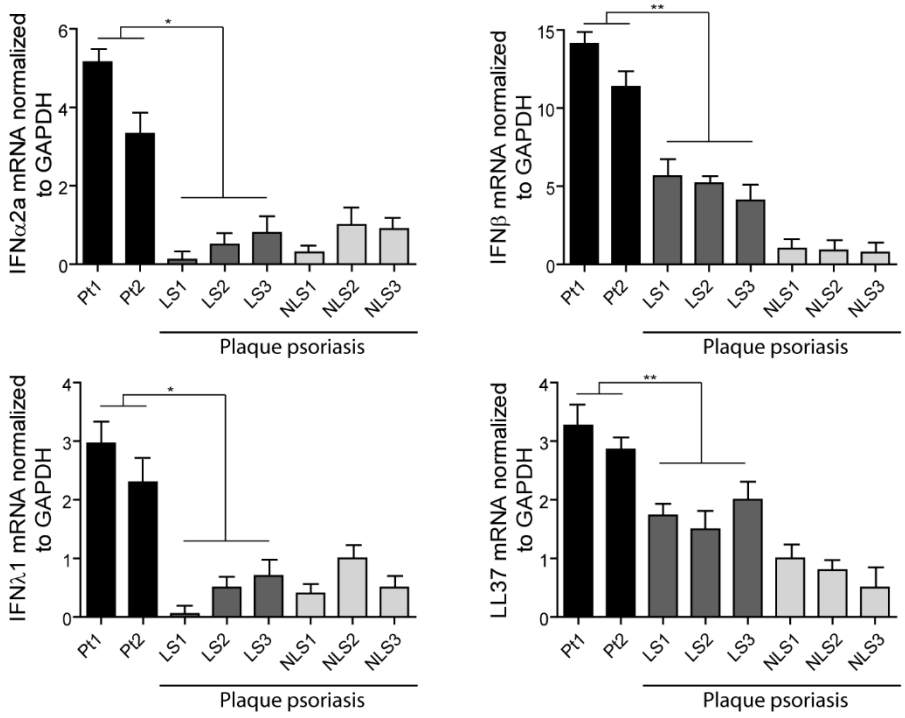


Figure 22: **Innate immunity molecules are overexpressed in the skin of HS patients after TNF-α treatment**. mRNA expression of IFNα2a, IFNβ, IFNλ1 and LL37 was analyzed by real-time PCR in skin lesions of patients 1 (Pt1) and 2 (Pt2) and in skin biopsies from LS and NLS skin of three psoriatic patients. mRNA values were normalized to GAPDH mRNA. Values obtained from triplicate experiments were averaged, and data presented as means of 2^-ΔΔCT ± SD. *$p < 0.01$, **$p < 0.05$.

3.2.4 Immunological profile analysis of patients developing paradoxical reactions

Next, immunophenotypical characterization of T-skin and PBMC isolated from HS patients developing paradoxical psoriasiform reaction, was performed. FACS analysis of the T-cells isolated from skin biopsies showed a significant reduction of IFN-γ^+ $CD3^+$ cells in patients 1 and 2, when compared to $CD3^+$ cells isolated from classical psoriasis (~ 7- and 1.7-fold decrease, respectively) (Fig. 23). The reduction of IFN-γ positivity was also observed in circulating $CD3^+$ cells of patients 1 and 2 (Fig. 23). Similarly, TNF-α positivity was lower in

T-skin lymphocytes of patients 1 and 2. TNF-α positivity of circulating $CD3^+$ cells was instead lower only in patient 1, as compared to patient 2 and patients with classical psoriasis (Fig. 23). Moreover, IL-17A positivity was comparable in patient 1 and psoriatic patients, whereas it was very high in T cells isolated from skin of patient 2, where a mixed population of T cells, either responsible for the psoriasiform or eczematous reactions, is likely present (Fig. 20). IL-22 was similar in T cells of psoriasiform lesions and classical psoriasis, whereas it was substantially reduced in PBMC (Fig. 23). $CD3^+$ cells from skin biopsies of HS patients were enriched in $CD8^+$, but not in $CD4^+$ cells, when compared to PBMC isolated from the same patients. $CD3^+$ T-skin cells of classical psoriasis showed instead an enrichment of both $CD4^+$ and $CD8^+$ subpopulations (Fig. 24).

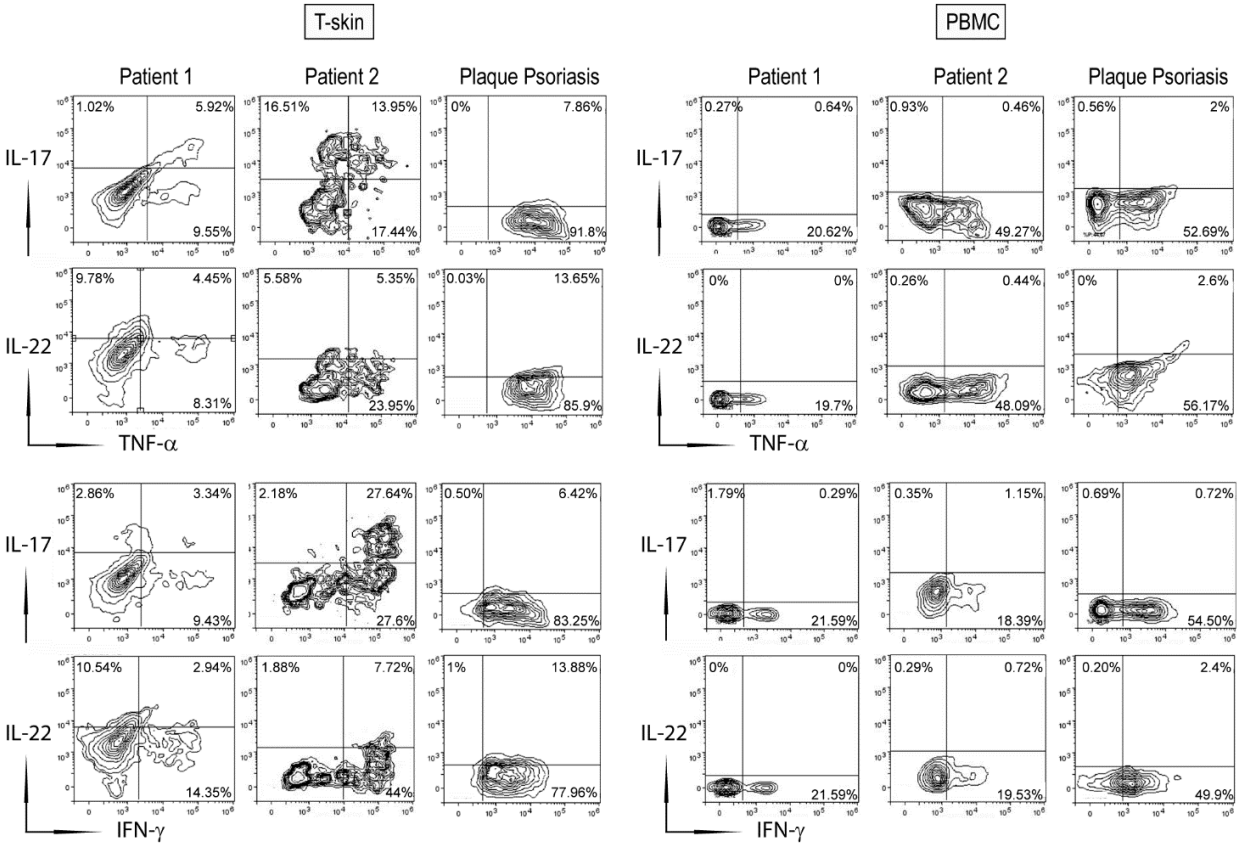


Figure 23: **Immunophenotypical characterization of T-skin and PBMC isolated from HS patients with psoriasiform lesion**s. T-skin cell lines (left panel) and PBMCs (right panel) were isolated from biopsies and blood, respectively, of patients 1 and 2 and from psoriatic patients (*n = 2*). Co-expression of IL-17, IL-22, TNF-α or IFN-γ on gated $CD3^+$ cells, were analyzed by flow cytometry. Percentage of positive fluorescent cells is shown in each quadrant. Results show the mean values of data obtained for one representative experiment out of three experiments.

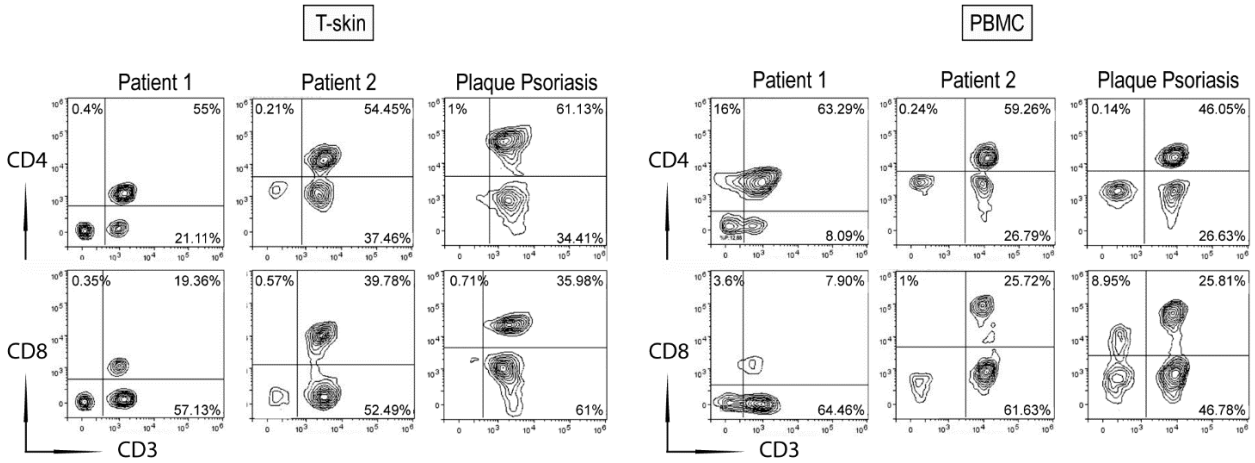


Figure 24: **Immunophenotypical characterization of T-skin and PBMC isolated from HS patients with psoriasiform lesions.** T-skin cell lines (left panel) and PBMCs (right panel) were isolated from biopsies and blood, respectively, of patients 1 and 2 and from psoriatic patients (*n = 2*). Surface CD4, CD8 and CD3, were analyzed by flow cytometry. Percentage of positive fluorescent cells is shown in each quadrant. Results show the mean values of data obtained for one representative experiment out of three experiments.

DISCUSSION

Most inflammatory skin disorders, such as psoriasis, are multifaceted diseases, as they are polygenic, clinically and pathogenically complex, as well as multifactorial, meaning that multiple genetic, epigenetic, lifestyle, and environmental factors play a role in the manifestation of the disease.

Identification of robust biomarkers that reflect the various clinical psoriasis phenotypes, and which eventually form the basis for stratification of endotypes, can help in making diagnosis (diagnostic biomarkers) and for prognosis indication (prognostic biomarkers). Also predictive biomarkers, having the potential to identify the individuals that are more or less likely to respond to a given drug, could be differentially carried out by patients based on the complexity/heterogeneity of their disease status.

Among predictive biomarkers, a number of pharmacogenetic markers, namely variations of DNA or RNA characteristics as related to drug response, have been identified for several disease conditions, including psoriasis. In particular, polymorphisms in genes encoding drug-metabolizing enzymes, transporters and drug targets, accounting for up to 95% of interpatient variability, were found to be the most frequent type of variations identified in relation to response to systemic drugs used for psoriasis treatment, in particular to methotrexate and cyclosporine [17]. On the other hand, in the last decade, some SNPs in psoriasis susceptibility genes have been related to response to the biological drugs [17,104].

However, until now, pharmacogenomic studies in psoriasis have been underpowered to produce reliable results and the majority have not recorded treatment response or toxicities prospectively in an objective and reproducible manner [105]. Many of the published studies to date have adopted a candidate gene approach, focusing on single gene polymorphisms based on existing knowledge of the metabolic or immune pathways of psoriasis treatments producing conflicting or nonsignificant results for the most part. Therefore, a validation of these results in adequately powered patient cohorts would be essential before the pharmacogenomic markers can be used to predict treatment response in the clinical setting. In addition, the discovery of new pharmacogenomic biomarkers, simultaneously present in different genes involved in intersected pathogenic pathways, and which could be predictive for the responsiveness of psoriatic patients to biological drugs, including drugs of more recent identification and employment, such as the newer class of biologicals targeting IL-17A and its receptor and the lastly identified anti-IL-23 drugs, would be important.

The advent of biological agents has significantly improved the clinical management of psoriasis, although these treatments have been so far associated with variable response degrees,

in terms of efficacy and safety. Nowadays, a variety of biological therapies are available for psoriatic patients. These agents are potentially highly effective, even though they may differ in time until a clinically satisfactory response is reached [48,87,89,106]. In addition, a variable percentage of psoriatic patients does not or only partially respond/s to biological therapies (primary failure) or even, after an initial response, loses responsiveness over time (secondary failure). This often requires switching to other biological drugs, with increased healthcare costs. Of note, biological drugs, in particular the anti-TNFs, can even worsen psoriasis condition by inducing paradoxical cutaneous reactions and other side effects [66]. These paradoxical events, in particular the psoriasis-like reactions also occur in patients with severe immune-mediated inflammatory conditions, including HS [64–66]. The reason why anti-TNFs induce paradoxical psoriasiform reactions only in a portion of subjects affected by different autoimmune conditions, and mostly with a similar phenotype is still unknown. A growing number of scientific evidence supports the influence of the genetic background in predisposing to paradoxical psoriasis or other skin inflammatory conditions, together with certain environmental factors [81]. In addition, the presence of specific haplotypes associated with a hyperactivation of innate immunity pathways, in particular with pDC activation and/or type I IFN and TNF-α signaling could lead to paradoxical manifestations.

Therefore, the discovery of genetic biomarkers to predict treatment response of psoriatic patients to biological drugs, either in terms of efficacy/inefficacy or safety improvement of the drugs, have potential to greatly impact clinical decisions and, eventually, in the development of individually tailored treatment.

On this subject, only few SNPs in *HLA-Cw6, TNFAIP3, TNFA, TNFRSF1B, IL12B* and *IL-23A* or in *IL-23R, FBXL19, CTLA4, SLC12A8* and *TAP1* genes have been found to influence the therapeutic response of psoriatic patients to anti-TNFs and ustekinumab biologics [24,65,109,76,79,96–99,107,108], or to associate with paradoxical psoriasiform reactions induced by anti-TNFs, respectively [81]. To date no genetic associations influencing the clinical response to anti-IL-17A or anti-IL-23 are known.

The present research identified a set of SNPs in genes psoriasis-related risk loci in two large cohorts of patients affected by mild-to-severe plaque psoriasis undergone therapy with IL17A (n = 63 patients), or IL-12/IL-23 (n = 150 patients) blockers, associating with clinical responsiveness to anti-ILs drugs. Concerning secukinumab-treated patients, a significant association between four single SNPs in the *HLA-C* genomic region, namely *HLA-Cw6* v1, *HLA-Cw6* LD, *HLA-Cw6* v2, or *HLA-Cw6* v3 and response to the drug was found. Importantly, psoriatic patients carrying *HLA-Cw6* classical variant (v1) reached PASI100 faster (week 8)

than HLA-Cw6-neg patients, and maintained this result up to week 24. *HLA-Cw6*-pos patients also showed a tendency to greater respond to secukinumab, in terms of achievement of PASI90 and PASI100. These data partly contrast with previous findings of the SUPREME study demonstrating that secukinumab has high efficacy irrespective of *HLA-Cw6* status in psoriatic patients [53,54]. However, these studies linked *HLA-Cw6* allele presence and responses of psoriatic patients to secukinumab in terms of achievements of PASI90.

In the present study, the most significant associations were observed for *HLA-Cw6* v2 and HLA-Cw6 LD variants, whose presence in psoriatic patients was associated to the achievements of PASI75 or PASI 90 starting from week 16 or week 4, respectively, up to week 56. Interestingly, the absence of *HLA-Cw6* v3 allele in psoriatic population guaranteed a better response to secukinumab, in terms of achievement of PASI75 at different time-points of evaluation (weeks 24, 40, 56, 64, 72, 88, 100).

Although, no functional evidence correlating the presence or absence of *HLA-Cw6* variants and response to secukinumab exist, it is plausible that these alleles allow or not the presentation of epitopes present in different putative antigens, such as the disintegrin and metalloprotease domain containing thrombospondin type I motif-like 5 (ADAMTSL5), a protein identified as autoantigen presented by melanocytes in a HLA-Cw6-restricted fashion [97], and recently localized in keratinocytes throughout the psoriatic epidermis [110,111]. Among autoantigens possibly presented by *HLA-Cw6* allele, the cathelecidin LL37 can be recognized by circulating $CD8^+$ T cells with a cytokine and skin-homing receptor profile (IFN-γ^{high}, IL-17^{high}, CLA^+, $CCR6^+$, and $CCR10^+$) typical of psoriatic skin T cells [112,113]. Most recently, phospholipase A2 group IVD (PLA2G4D) was also identified in psoriatic keratinocytes as important player in the generation of psoriasis autoantigens [113]. The latter include nonprotein neolipids that are recognized by CD1a-restricted T cells, thereby inducing the production of IL-22 and IL-17A [114]. These lipid antigens could be transferred from keratinocytes to neighboring antigen-presenting cells through released exosomes, similarly to what observed for $tryptase^+$ mast cells of psoriatic lesions [114].

Differently from secukinumab-treated patients, ustekinumab-treated cohort was strongly influenced by *HLA-Cw6* v1 allele status, but not by *HLA-Cw6* LD, *HLA-Cw6* v2, or *HLA-Cw6* v3 variants. The association between *HLA-Cw6* v1 allele presence and response to ustekinumab was significant for patients reaching PASI90 or PASI100, starting from week 12 up to week 100. These results extend previous findings by Talamonti *et al.* showing that *HLA-Cw6* status can predict the efficacy of ustekinumab treatment, in terms of achievement of PASI50 and PASI75 up to 52 weeks [89], and phase III studies reporting that a higher percentage of *HLA-*

Cw6-pos patients achieved PASI, 50, 75, 90 and 100 up to week 28 compared to of *HLA-Cw6*-neg patients [115–117]. The efficacy of ustekinumab, thus, would depend on the presence *HLA-Cw6* allele possibly as activator of antigen-specific $CD8^+$ T cell-mediated lymphocyte immune responses driven by IL-12/IL-23 signaling. However, differently from secukinumab-regulated immune responses, which are uniquely dependent on IL-17A action, the ustekinumab-regulated immunity pathways are dependent on IL-12 and IL-23, and thus on both IFN-γ and IL-17A action. Of note, the presence of TNFA v1 variant or absence of TNFA v2 in *TNFA* gene determined a greater and long-lasting response to ustekinumab. Similarly, the presence or absence of CDSN v2 or CDSN v3 variants of *CDSN* gene, respectively, strongly associated with a good response to ustekinumab (PASI90), which was maintained up to 100 weeks. PASI90 was also reached by the majority of ustekinumab-treated patients carrying CCHCR1 v5 variant in *CCHCR1* gene. It is noteworthy that *HLA-C*, *TNFA, CDSN* and *CCHCR1* genes all map in PSORS1 locus on chromosome 6p21.3, which remains the strongest susceptibility locus for psoriasis. That PSORS1 region have a predominant effect on psoriasis manifestation is also suggested by the findings of the present study showing that no statistically significant associations between allele variants of genes mapping in other locus or PSORS and response to secukinumab or ustekinumab were found. Indeed, univariate logistical regression analysis is not appropriate to highlight possible association between group of SNPs, maybe transmitted in linkage (for instance *HLA-Cw6* v1 and other *HLA-Cw6* alleles), and response to drugs. Therefore, multivariate logistical regression analysis could unveil additional significant associations, as in the case of *IL12B* rs3212227 SNP and response to ustekinumab, that was found to be more successful in *HLA-Cw6*-pos/*IL12B* rs3212227-pos than in *HLA-Cw6*-neg/*IL12B* rs3212227-neg psoriatic patients [87].

Several evidences showed that treatment with anti-TNFs is also influenced by the genetic background of psoriatic patients, which thus can show variable responses, including adverse side-effects, such as exacerbation of psoriasis or manifestation of other skin pathological conditions. In addition, anti-TNF therapy can induce psoriasiform manifestations in patients affected by immune-mediated inflammatory diseases unrelated to psoriasis. Among the genetic variants influencing the clinical response to anti-TNF therapy, those relative to *IL-23R, FBXL19, CTLA4, SLC12A8* and *TAP1* genes have been found to associate with paradoxical psoriasis [81]. For instance, three SNPs in *IL23R* gene (rs10489628, rs10789229, and rs1343151) associated with paradoxical reactions in patients affected by Crohn's disease undergone therapy with infliximab [118]. Moreover, patients with inflammatory bowel disease developing severe psoriasiform skin lesions and/or anti-TNF-induced alopecia, carried out the

rs7530511 SNP in the *IL23R* gene, as well as the rare coding *IL23R* variant rs11209026 [119], known to be associated with decreased IL-23 signal transduction and Th17 cytokine production [120,121]. The rs11209026 allele was not instead found in none of the three HS patients analyzed for this study [122]. On the contrary, HS patients carried out numerous allelic variants in the *HLA-C*. None of patients showed the major *HLA-Cw6* psoriasis allele, even though other SNPs in the proximity of HLA-Cw6 and neighboring to other HLA-C variants were found. Concomitantly, HS patients carried out allelic variants in *ERAP1* gene. However, due to the lack of antigen-specific $CD8^+$ T-cell responses in HS patients, the link between the presence of SNPs in HLA-C region/*ERAP1* gene and susceptibility to paradoxical psoriasis is apparently missing. Indeed, other than having a role in MHC class I antigen presentation, ERAP1 is involved in the activation of innate immunity pathways, by inducing inflammasome and production of cytokines and chemokines (i. e. IL-6, TNF-α and CCL2) [123]. Importantly, allelic variants of *ERAP1* leading to missense mutation increases ERAP1 capability to induce inflammation in autoimmune diseases [124]. HS patients also carried out polymorphisms in *NFKBIZ* and *TNFAIP3*, which could be responsible for an NF-κB hyperactivation, as demonstrated for other pathological conditions [125,126].

A growing number of scientific evidence supports the influence of specific haplotypes associated with a hyperactivation of innate immunity pathways, in particular with pDC activation and/or type I IFN and TNF-α signaling, leading to paradoxical manifestations [81]. Consistently, allelic variants in *NFKBIZ* and *TNFAIP3* might determine the enhanced type I IFN expression observed in paradoxical lesions, as both IKBζ and A20 can transcriptionally regulate IFNs expression, respectively, *via* activation and inhibition of NF-κB [127]. Variants in *TNFAIP3*, in particular those imparting lower A20 expression, might be responsible for an uncontrolled IFN-β expression, as demonstrated by silencing TNFAIP3 mRNA expression in vascular model of inflammation [128].

Although no functional evidence correlating the presence of SNPs and anti-TNF drug responses exist, it is plausible that the allelic variants could predispose to paradoxical psoriasis by determining an amplification of the innate skin immunity circuits that are overactive in psoriasiform lesions. In this study, we observed, in fact, a marked dermal accumulation of innate immunity cells, including pDC, neutrophils, mast cells, and macrophages, together with impressive expression levels of innate immunity molecules. Among them, IFN-α2a, IFN-β and IFN-λ1 are overexpressed in the skin of HS patients following anti-TNF-α therapy.

Also LT-α and LT-β, as well as LL37 were detected at very high levels in paradoxical psoriasis, when compared to classical psoriasis.

Transient type I IFN upregulation has already been described in classical psoriasis, during the acute phase of disease development, as well as in paradoxical psoriasis [81,129]. Concomitantly, IFN-β is known to be expressed by pDC in both conditions [129]. We found that IFN-β, together with IFN-κ and lymphotoxins were impressively expressed in the epidermal compartment of paradoxical skin reaction, other than in pDC and leukocytes infiltrating the dermis. Type I IFNs and lymphotoxins released by keratinocytes might have a fundamental pathogenic role in paradoxical psoriasis. However, the mechanisms by which these molecules promote psoriatic skin phenotype are not yet known, neither in paradoxical nor in classical psoriasis. On the contrary, the immunological function of IFN-α has been extensively studied, especially in classical psoriasis, where it is known to induce Th17 responses [130]. In paradoxical psoriasis, IFN-α could have a different role, being antigen-specific Th17 responses absent. It could induce chemokines at the epidermal level, such as CXCL10 and CXCL9, responsible for the recruitment of DC and nonspecific T cells. These inflammatory cells could in turn sustain and amplify local inflammatory responses in paradoxical reactions [131].

The induction of innate immunity processes in paradoxical psoriasis is dependent on the loss of TNF-α function in limiting the innate immune responses in the skin, as previously demonstrated [129]. In fact, TNF-α blocking determined the accumulation of pDC and inhibition of their maturation. As a consequence, pDC could release very high levels of the type I IFN-α6 and IFN-β, being, thus, responsible for paradoxical psoriasis. Together with pDC, we found other innate immunity cells present in psoriasiform lesions of HS patients. Among them, $CD15^+$ neutrophils, c-kit/$CD117^+$ mast cells, $CD68^+$ macrophages and monocytes abundantly infiltrate the dermis of paradoxical skin reactions. This pattern of leukocyte subpopulations is very similar to that found in early psoriasis, and is consistent with the overactive innate immunity processes present during the initial phase of inflammation, as previously shown [5]. Similarly to pDC, innate immune cells could be recruited by chemokines released by keratinocytes, fibroblasts and endothelial cells (i. e. CCL20, chemerin), whose expression depends on type I IFNs produced by resident skin cells themselves. In fact, other than controlling the expression of type I IFNs in pDC, TNF-α might negatively regulate these molecules in keratinocytes, which notoriously also contribute to the induction of innate immunity pathways in acute psoriasis [6]. This hypothesis is supported by the findings that type I IFNs are induced in keratinocytes of paradoxical psoriasis and *vice versa* are present at low levels in chronic plaque

psoriasis. It would be important to confirm the high expression of innate immunity mediators following TNF-α blocking *in vitro*, in primary keratinocyte cultures, as demonstrated for cultured pDC [63].

Analysis of T-cell infiltrate of paradoxical skin reactions demonstrated a significant reduction of IFN-γ- or TNF-α-producing $CD3^+$ cells in paradoxical psoriasis, when compared to chronic psoriasis. However, $CD8^+$ and IL-17^+ lymphocytes were present in paradoxical psoriasiform reactions, at levels comparable to psoriasis, even if it is conceivable that they were nonspecifically recruited. The absence of bursting of type-I T-cell response in paradoxical skin reactions was not surprising, if we consider that it is typical of the chronic phase in classical psoriasis [6,7,132]. On the contrary, IL-22-producing cells increased in psoriasiform reactions of HS patients, even though positive cells showed mostly a macrophage-like morphology. IL-22 overexpression could be responsible for hyperproliferation and de-differentiation of keratinocytes typical of the epidermis of paradoxical psoriasiform lesions. Finally, although an inflammatory cytokine milieu, inducing the local production of chemokines and cytokines by resident skin cells, can be effectively established in paradoxical psoriasis, it seems to be not sufficient to induce the chronicization of psoriasiform reactions in HS patients, possibly for the lacking of DC and T-cell activation by causative antigen(s) of psoriasis.

In conclusion, this study identified new genetic predictors for response to secukinumab and to ustekinumab, as well as associated new psoriasis susceptibility polymorphisms potentially predictive for the development of paradoxical psoriasiform reactions evoked by anti-TNF therapy. This study also identified for the first time the immunological mediators whose expression is influenced by TNF-α blocking and likely responsible for paradoxical psoriasis development, even though an extension of analysis in a larger cohort of patients will be necessary.

Considering the high drug response interpatient variability and the augmented incidence of paradoxical psoriasiform reactions among anti-TNF-treated patients, it is becoming increasingly necessary to investigate the genetic profiles, together with cutaneous immunological profiles of patients undergoing to therapies with biologics. However, the analysis of genetic polymorphisms could, in the future, be used not only to predict response to biological therapy but also to improve resource allocation and reduce exposure of patients to unnecessary toxicity and adverse effects.

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APPENDICES

I. Training activities

Courses:

- "Ion S5 and Chef System Workflow" -IDI-IRCCS, 29/05/2019, Roma, Italy (24h)
- "The use of statistics in biomedical research and R software" -Consiglio Nazionale delle Ricerche, 14-17/05/2019, Roma, Italy
- "Statistica medica con STATA" -Università di Verona, 19/09/2018-05/10/2018, Verona, Italy
- "Theory and Computational Practice for Genome Variant analysis using high throughput sequencing data" -Università di Verona, 11-13/12/2017, Verona, Italy
- "The use of statistics in biomedical research" -Istituto Santa Lucia, 21-23/06/2017, Rome, Italy

Congresses and meetings:

- 24° World Congress of Dermatology (WCD2019), 10-15/06/2019, Milan, Italy
- 2nd European Workshop on Skin Immune Mediated Inflammatory Diseases (SIMID) -11-13/10/2018, Verona, Italy
- XV FISV Congress, Università LaSapienza, 18-21/09/2018, Rome, Italy
- International Retreat of PhD students in Immunology -Università di Verona, 6-7/10/2017, Verona, Italy

II. Research activities

Abstracts:

- Galluzzo M, Albanesi C, Scaglione GL, **Morelli M**, Madonna S, D'Adamio S, Scarponi C, Pallotta S, Capoluongo E, De Paolis E, De Bonis M, Andreani M, Galluccio T, Locatelli F, Bianchi L and Talamonti M "Precision medicine in psoriasis: pharmacogenetics of ustekinumab and secukinumab response in a large cohort of psoriatic patients", 24° World Congress of Dermatology (WCD2019), 10-15/06/2019, Milan, Italy

- Fania L, **Morelli M**, Scarponi C, Scopelliti F, Cattani C, Cavani A, Capoluongo E, De Paolis E, De Bonis M, Scaglione GL, Tonanzi T, Pilla MA, Pagnanelli G, Mazzanti C, Madonna S, Albanesi C "Immunological and genetic profiles of drug-induced paradoxical psoriasis", 24° World Congress of Dermatology (WCD2019), 10-15/06/2019, Milan, Italy

- Failla CM, Albanesi C, Capriotti L, Scarponi C, Mercurio L, Facchiano F, **Morelli M**, Cordella M, Pagnanelli G, Cavani A and Madonna S "Interleukin (IL)-17/IL-36 axis in the crosstalk between endothelial cells and keratinocytes in inflammatory skin responses", XV FISV Congress, Università LaSapienza, 18-21/09/2018, Rome, Italy

- Mercurio L, **Morelli M**, Scarponi C, Eisenmesser E, Pagnanelli G, Dinarello CA, Albanesi C, Madonna S "IL-38 has an anti-inflammatory action in psoriasis and its expression

correlates with disease severity and therapeutic response to anti-IL-17 treatment", XV FISV Congress, Università LaSapienza, 18-21/09/2018, Rome, Italy

Scientific publications:

- Lauffer F, Jargosch M, Baghin V, Krause L, Kempf W, Absmaier-Kijak M, **Morelli M**, Madonna S, Marsais F, Lepescheux L, Albanesi C, Mueller NS, Theis FJ, Schmidt-Weber C, Eyerich S, Biedermann T, Vandeghinste N, Steidl S, Eyerich K. "IL-17C amplifies epithelial inflammation in human psoriasis and atopic eczema" *European Academy of Dermatology and Venereology*. 2019; 10.1111/jdv.16126

- Fania L, **Morelli M**, Scarponi C, Mercurio L, Scopelliti F, Cattani C, Scaglione GL, Tonanzi T, Pilla MA, Pagnanelli G, Mazzanti C, Girolomoni G, Cavani A, Madonna S, Albanesi C. "Paradoxical psoriasis induced by TNF-α blockade shows immunological features typical of the early phase of psoriasis development" *The Journal of Pathology: Clinical Research.* 2019; 10.1002/cjp2.147

- Mercurio L, **Morelli M**, Scarponi C, Eisenmesser EZ, Doti N, Pagnanelli G, Gubinelli E, Mazzanti C, Cavani A, Ruvo M, Dinarello CA, Albanesi C, Madonna S "IL-38 has an anti-inflammatory action in psoriasis and its expression correlates with disease severity and therapeutic response to anti-IL-17A treatment" *Cell Death & Disease*, 2018; 10.1038/s41419-018-1143-3

- **Morelli M**, Scarponi C, Mercurio L, Facchiano F, Pallotta S, Madonna S, Girolomoni G, Albanesi C "Selective immunomodulation of inflammatory pathways in keratinocytes by the Janus kinase (JAK) inhibitor tofacitinib. Implication for employment of JAK-targeting drugs in psoriasis" *Journal of Immunology Research* 2018; 10.1155/2018/7897263

- Madonna S, Scarponi C, **Morelli M**, Sestito R, Scognamiglio PL, Marasco D, Albanesi C "SOCS3 inhibits the pathological effects of IL-22 in non-melanoma skin tumor-derived keratinocytes" *Oncotarget* 2017; 10.18632/oncotarget.15629

III. Manuscripts

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ORIGINAL ARTICLE

Paradoxical psoriasis induced by TNF-α blockade shows immunological features typical of the early phase of psoriasis development

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Abstract

Immunomodulation with anti-TNF-α is highly effective in the treatment of various immune-mediated inflammatory diseases, including hidradenitis suppurativa (HS). However, this may be responsible for unexpected paradoxical psoriasiform reactions. The pathogenic mechanisms underlying the induction of these events are not clear, even though the involvement of innate immune responses driven by plasmacytoid dendritic cells (pDC) has been described. In addition, the genetic predisposition to psoriasis of patients could be determinant. In this study, we investigated the immunological and genetic profiles of three HS patients without psoriasis who developed paradoxical psoriasiform reactions following anti-TNF-α therapy with adalimumab. We found that paradoxical psoriasiform skin reactions show immunological features common to the early phases of psoriasis development, characterized by cellular players of innate immunity, such as pDC, neutrophils, mast cells, macrophages, and monocytes. In addition, IFN-β and IFN-α2a, two type I IFNs typical of early psoriasis, were highly expressed in paradoxical skin reactions. Concomitantly, other innate immunity molecules, such as the cathelicidin LL37 and lymphotoxin (LT)-α and LT-β were overproduced. Interestingly, these innate immunity molecules were abundantly expressed by keratinocytes, in addition to the inflammatory infiltrate. In contrast to classical psoriasis, psoriasiform lesions of HS patients showed a reduced number of IFN-γ and TNF-α-releasing T lymphocytes. On the contrary, IL-22 immunoreactivity was significantly augmented together with the IL-36γ staining in leukocytes infiltrating the dermis. Finally, we found that all HS patients with paradoxical reactions carried allelic variants in genes predisposing to psoriasis. Among them, SNPs in *ERAP1*, *NFKBIZ*, and *TNFAIP* genes and in the *HLA-C* genomic region were found.

Keywords: psoriasis; hidradenitis suppurativa; anti-TNF-α therapy; paradoxical psoriasis; skin inflammation; innate immunity; type I IFN; lymphotoxin

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No conflicts of interest were declared.

Introduction

TNF-α blockers are efficaciously utilized in the treatment of various immune-mediated diseases, such as psoriasis, rheumatoid arthritis and, more recently, hidradenitis suppurativa (HS) [1,2]. However, cutaneous reactions, such as eczematous and psoriasiform lesions, and other side effects have been reported [3–5]. Some of these adverse reactions are considered as paradoxical effects and, in particular, 2–5% of patients treated with TNF-α antagonists develop paradoxical psoriasiform skin lesions [6–9]. These reactions may require the interruption of the imputable drug, and no other biologics are approved for diseases like in HS. Therefore, it is important to understand the pathogenesis of these reactions, and a possible genetic susceptibility should be examined in these patients.

Psoriasis is a chronic inflammatory skin disease mediated by autoreactive T cells, which produces epidermal keratinocyte hyperproliferation with aberrant differentiation and senescence [10,11]. Early upstream events occurring in psoriasis include induction of innate immunity responses, primarily depending on keratinocytes activated by mechanical trauma, pathogens or drugs. At this initial phase, keratinocytes establish innate immunity circuits involving neutrophils, mast cells and macrophages and, importantly, enable plasmacytoid dendritic cell (pDC)- and myeloid DC (mDC)-driven responses [12–14]. Local production of type I IFN, as well as TNF-α and IL-6, by pDC and mDC unleashes adaptive immune responses, with expansion of T lymphocytes, typically Th17 and Th22 in the initial phase and Th1 cells during the chronic phase of the disease [10,11,15]. Hence, lymphokines released in skin lesions, in particular IL-17, IL-22, and IFN-γ, further amplify local immune responses [10,16–18]. Chronic immune responses are absent in paradoxical psoriasis induced by TNF-α blockers, with innate inflammatory processes predominant and not followed by expansion of autoreactive T cells [19]. These processes are concomitant with dermal accumulation of immature pDC and type I IFN overexpression [19].

Several studies have shown that intrinsic defects in genes controlling T-cell commitment and keratinocyte inflammatory activation are associated with psoriasis [20,21]. Among them, the *HLA-Cw6* allele represents the strongest genetic risk variant associated with psoriasis [22]. The *HLA-Cw6* haplotype might influence antigen presentation and immune responses, especially when associated with variants in the *ERAP1* gene, encoding an aminopeptidase involved in the formation of the peptides loaded on MHC class I molecules [23]. Interestingly, a number of allelic variants were found in genes encoding signal transducers associated with IL-17 or TNF-α, such as *NFKBIZ* and *TNFAIP3*, encoding IKBζ and A20 proteins, respectively [24]. Both IKBζ and A20 proteins regulate IL-17- and TNF-α-induced molecular signaling, being an activator and a negative regulator of NF-κB respectively [25].

Here, we report the immunological and genetic profiles of HS patients who developed psoriasiform reactions following anti-TNF-α therapy with adalimumab. We found that paradoxical psoriasiform skin predominantly shows immunological features common to early psoriasis, characterized by a massive infiltrate of innate immunity cells and local overproduction of innate immunity molecules. In contrast to classical psoriasis, psoriasiform lesions showed an increased number of infiltrating IL-22$^+$ leukocytes. Finally, we found that all the HS patients with paradoxical reactions carried allelic variants in genes predisposing to classical psoriasis, including SNPs in the *HLA-C* genomic region.

Material and methods

Patients and samples

Three patients affected by HS, who developed psoriasiform skin lesions after treatment with adalimumab (40 mg, weekly), and three patients affected by classical plaque-type psoriasis (PASI 8, 11.5, and 10) were included in the study. Clinical data, as well as skin biopsies and blood, were collected from patients with the permission of the IDI-IRCCS Local Ethics Committee (Prot. CE 475/2016).

8-mm skin biopsies were taken from psoriasiform lesions arising in HS patients or from 1.5-month old psoriatic plaques. Biopsies were divided into two parts for immunohistochemistry and isolation of skin-infiltrating T lymphocytes. A 2-ml sample of peripheral blood was used to extract DNA, whereas a 20-ml sample was used to isolate peripheral blood mononuclear cells (PBMCs).

Immunohistochemistry

5-μm paraffin-embedded skin sections were stained with H&E or processed for immunohistochemistry. The primary antibodies used were as follows: anti-BDCA2 (DDX0043-TDS, Dendritics, Lyon, France), anti-CD15 (#347420, BD Biosciences, Milan, Italy), anti-IL-17A (#AF-317-NA, R&D Systems, Abingdon, UK), anti-lymphotoxin (LT)-α (#SC8302, Santa Cruz Biotechnology, Dallas, TX, USA), anti-IL-22 (#NB100-733, Novus Biologicals, Centennial, CO, USA), anti-IFN-κ (#H00056832-M01, Abnova, Taiwan), anti-CD117 and anti-CD11C (#MONX10234 and #MON3371, Monosan, Uden, Netherlands), anti-CD68 and anti-CD3 (#P02246IT and #A0452, Dako, Glostruk, Denmark). The following antibodies came from Abcam (Cambridge, UK): anti-IFN-γ (#AB218426), anti-IL-36γ (#AB156783), anti-IFN-β1 (#AB180616), and anti-LT-β (Cat#AB64835). Immunoreactivities were developed using the 3,3′-diaminobenzidine *HRP* substrate. Sections were counterstained with Mayer's hematoxylin.

T-cell isolation from skin biopsies and FACS analysis

T lymphocytes were isolated from skin biopsies as previously described [26]. After 4–7 days, cells that

had emigrated from biopsies were collected and characterized phenotypically. Lymphocytes were stained with the following monoclonal antibodies (mAbs): anti-IFN-γ-FITC (#B27), -CD4-PE (#RPA-T4), -CD8-PeRcP (#SK1), -CD3-FITC (#HIT3a) (BD Biosciences); anti-TNF-α-FITC (#6n1E7, Miltenyi Biotec, Bergisch, Germany), -IL-17-PE (#eBio64DEC17, EBiosciences, Frankfurt, Germany); anti-IL-22-PeRcP (#142928, R&D Systems). Acquisitions were performed using an Attune Nxt (Life Technologies, Carlsbad, CA, USA). Analyses were performed using Flow logic software (Miltenyi Biotec).

Real-time PCR analysis

Total RNA was extracted from skin biopsies using RecoverAll Total Nucleic Acid Isolation (Life Technologies), and analyzed by real-time PCR [27]. The primer sets were as follows:

IFN-α2A, 5′TCTGCTATGACCATGACACGAT3′/5′CAGCATGGTCCTCTGTAAGGG3′;
IFN-β, 5′CAGCAATTTTCAGTGTCAGAAGC3′/5′TCATCCTGTCCTTGAGGCAGT3′;
IFN-λ1, 5′AGGCTTCTCCAGGTGAGGGA3′/5′TCCAGGACCTTCAGCGTCAG3′;
IFN-λ2, 5′GGGCCTGTATCCAGCCTCAG3′/5′GAGCCGGTACAGCCAATGGT3′;
IFN-λ3, 5′GGGCCTGTATCCAGCCTCAG3′/5′GGTGCAGCCAATGGTGGAG3′/;
LL-37, 5′TTTTGCGGAATCTTGTACCCA3′/5′TCTCAGAGCCCAGAAGCCTG3′;
GAPDH, 5′TGGACCTGACCTGCCGTCTA3′/5′CCCTGTTGCTGTAGCCAAATTC3′.

Samples were analyzed using the QuantStudio5 Real-Time PCR System (Thermo-Fisher Scientific, Waltham, MA, USA).

SNP analysis

DNA was extracted from blood using the QIAcube® system (Qiagen, Hilden, Germany). SNPs were selected based on an extensive review of articles on the association between psoriasis and SNPs or response to biologics [23,24,28–32]. The SNP panel was analyzed by targeted sequencing, using NGS TruSeq Custom Amplicon kit and the MiSeq platform (Illumina, San Diego, CA, USA). SNPs are listed in supplementary material, Table S1 together with additional SNPs near the investigated genomic regions. Positive calls were selected applying a read depth >50X and allelic frequency >0.3. Variants' annotations were verified with ANNOVAR on hg19.

Statistics

Wilcoxon's signed rank test (SigmaStat; San Rafael, CA, USA) was used to compare differences in mRNA content in skin biopsies of HS and psoriatic patients. The significance of differences in the numbers of immunoreactive cells in skin biopsies was calculated using the unpaired Student's *t*-test. Statistical analysis was performed with Prism v.5.0 (Graphpad, La Jolla, CA, USA), and values are expressed as the mean + SD. Values of $p < 0.05$ were considered significant.

Results

Clinical characterization of paradoxical psoriasis

We analyzed three patients affected by severe HS, and who developed paradoxical psoriasiform reactions following treatment with adalimumab. Patient 1, a 48-year-old Caucasian woman, showed nodules, fistulas and sinus tracts in the inguinal and perianal regions (Hurley III, Sartorius score: 41.5). After 3 months of therapy with anti-TNF-α, she developed psoriasiform eruptions (PASI 6.8), with pustular lesions and erythemato-scaly lesions on the plantar region and lower limbs, respectively (Figure 1). A similar pattern of HS severity was observed in patient 2 (Hurley III, Sartorius score: 41.5), a 40-year-old Caucasian man, showing erythematous-pustular lesions in the palmo-plantar regions and erythemato-scaly plaques on the legs and scalp, ascribed to psoriasiform dermatitis (PASI 5.2), arising after 2 months treatment with adalimumab (Figure 1). He concomitantly showed alopecia areata on the scalp and some eczematous-like skin lesions. Patient 3, a 27-year-old Caucasian man, was affected by severe HS (Hurley III, Sartorius score: 61.5) characterized by comedones, nodules, and fistulas in the inguinal, gluteal and abdominal regions. He developed pustular lesions in the palmo-plantar regions, and erythemato-scaly plaques on the legs, scalp, elbows, and trunk (PASI 5.6) after 3 months of biological therapy (Figure 1). He refused to undergo a punch biopsy and, therefore, we could not perform the histological and immunological analyses. Paradoxical psoriasis regressed in all patients when adalimumab was discontinued. Interestingly, all three patients examined had a positive family history for psoriasis and, additionally, patient 1 reported other cases of HS among first-degree relatives.

Histological examination of the psoriasiform lesions of patients 1 and 2 showed epidermal hyperplasia with parakeratosis, papillary vessel ectasia and perivascular infiltrate compatible with a psoriasiform dermatitis (Figure 1). A $CD15^+$ neutrophilic infiltrate was

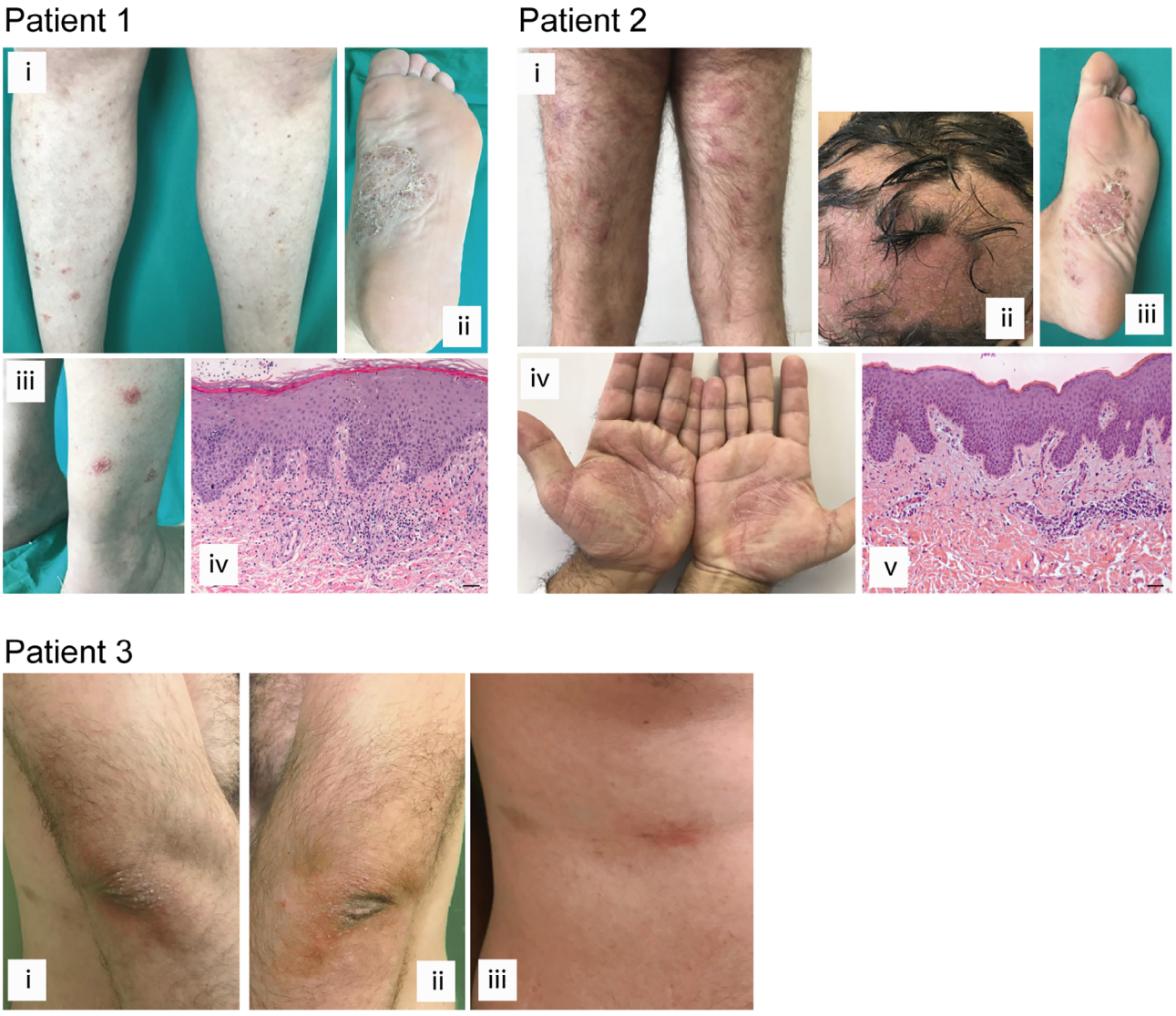

Figure 1. Clinical and histological presentation of paradoxical psoriasis induced by anti-TNF-α therapy in HS patients. Cutaneous lesions of patients 1–3 affected by severe HS, presenting paradoxical psoriasiform reaction after anti-TNF-α treatment. The patient 1 panels show paradoxical erythemato-squamous plaques localized on the lower limbs (i and iii) and pustular lesions in the plantar region (ii). Patient 2 similarly shows erythemato-squamous plaques on the limbs (i), pustular lesions in the palmo-plantar region and a severe form of alopecia areata involving part of the scalp (ii–iv).The patient 3 panels reveal erythematosus patches with mild desquamation on the elbows and trunk (i–iii). H&E staining for the corresponding histopathology of patients 1 (iv) and patient 2 (v) was also performed. Scale bars, 200 μM.

abundant in the dermal compartment and present in corneal abscesses (Figures 1 and 2). Interestingly, some eczematiform spongiotic areas overlapping with the psoriasis-like histological pattern were present in the skin lesions of patient 2.

Innate immunity cells highly infiltrate paradoxical psoriasiform lesions

Leukocyte subpopulations were characterized in paradoxical psoriasiform lesions, and compared to those present in classical plaque-type psoriasis. In line with previous studies [19,33], paradoxical psoriasis exhibited a prominent infiltrate of BDCA2$^+$ pDCs in the dermis, significantly more abundant than in classical psoriasis (~2.7-fold increase). In parallel, a significant increase of CD15$^+$ neutrophils, c-kit/CD117$^+$ mast cells, CD68$^+$ macrophages and monocytes in the dermis of paradoxical skin reactions was observed (~3.8-, 3.5-, and 1.8-fold increase, respectively) (Figure 2). In contrast, CD3$^+$ cells were similar and CD11c$^+$ DCs were less abundant (~1.5 fold-decrease) (Figure 2).

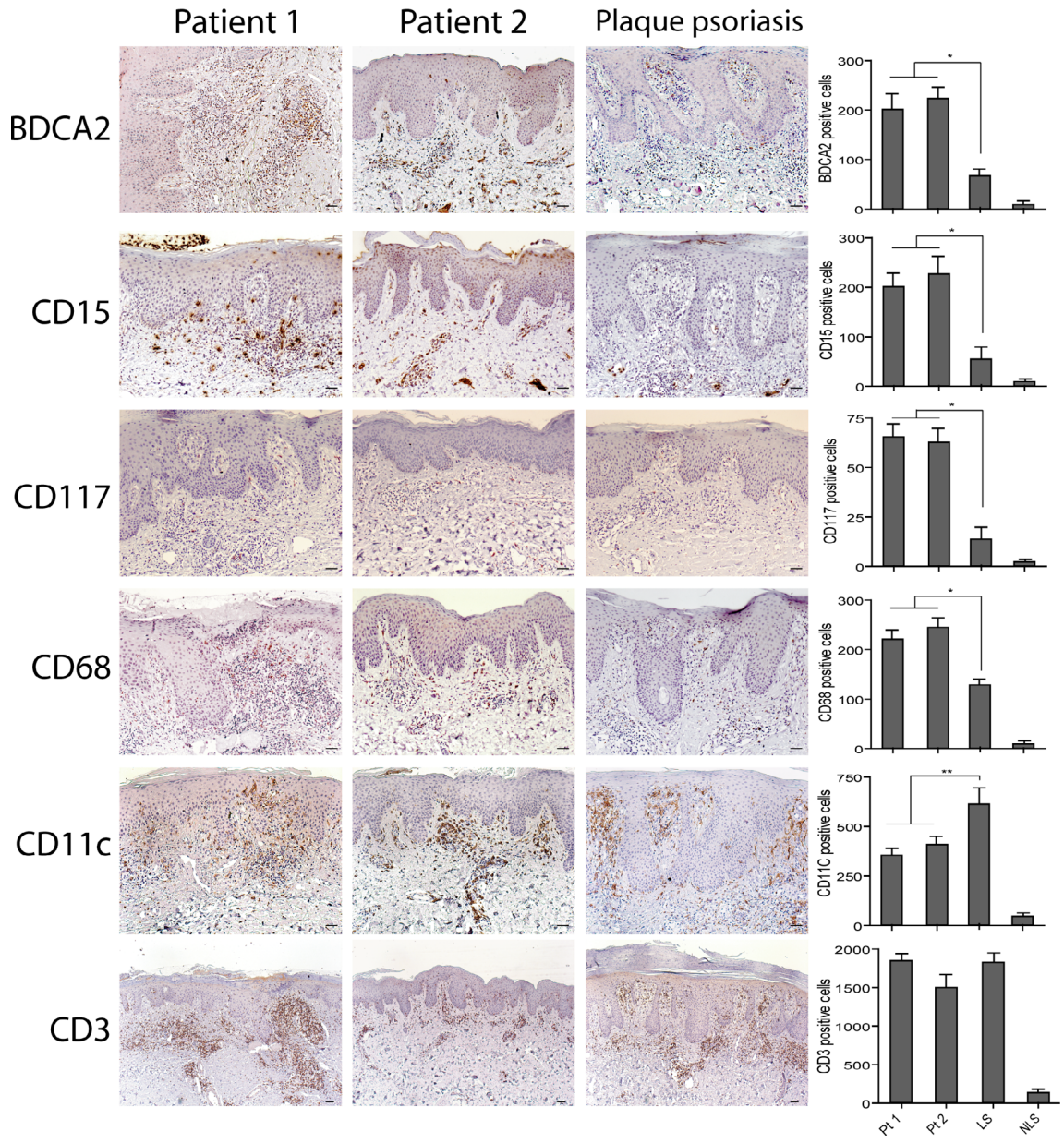

Figure 2. Innate immunity cells highly infiltrate paradoxical psoriasis skin lesions. Immunohistochemistry analysis of paradoxical skin reactions obtained from patient 1 (Pt1) and 2 (Pt2) shows an increase of BDCA2, CD15, CD117, CD68 positive cells, a reduction of CD11c cells and similar numbers of CD3 cells, when compared with psoriasis. Lesional (LS) and nonlesional (NLS) skin of the same psoriatic patient (n = 3) was analyzed. Slides were analyzed by two pathologists with experience in dermatology. Positive cells were counted in five adjacent fields at a total magnification of ×200. Graphs show the mean number of positive cells + SD *per* three sections. One out of three representative stainings is shown. *$p < 0.01$, **$p < 0.05$ versus classical psoriasis. Scale bars, 200 μM.

We next evaluated the local expression of psoriasis-related cytokines, such as IL-17A, IFN-γ, and IL-22, as well as IL-36γ which is highly released by neutrophils [27]. As shown in Figure 3, IFN-γ immunoreactivity decreased in psoriasiform reactions of patients 1 and 2, as compared to classical psoriasis, whereas IL-22 positivity was significantly augmented in the infiltrate, in particular in cells with a macrophage-like morphology (~2.1-fold increase). Due to the numerous neutrophils present in the dermis of paradoxical reactions, IL-36γ positivity was also enhanced, compared to classical psoriasis. However, IL-36γ expression in the

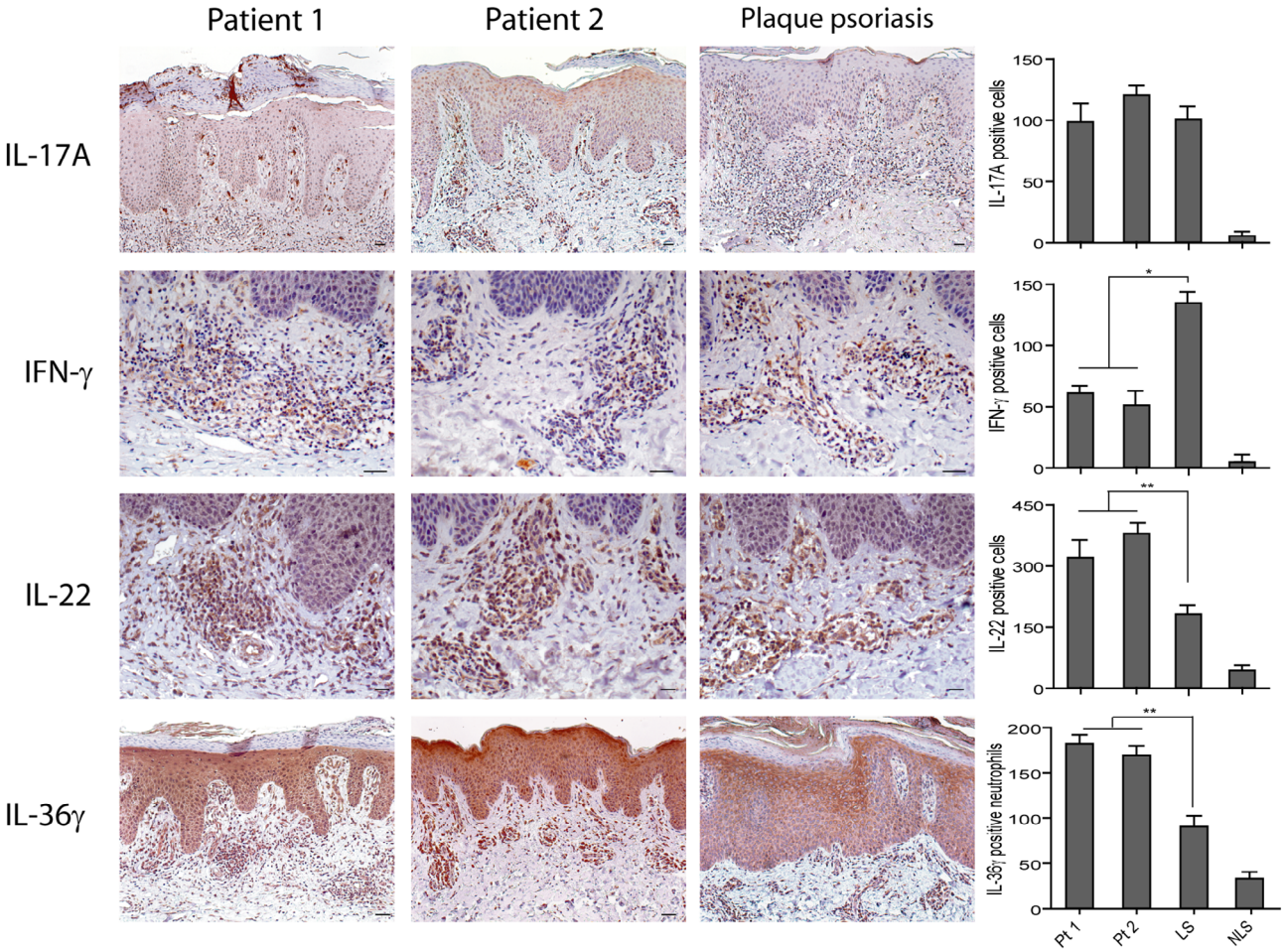

Figure 3. Expression of psoriasis-related cytokines in paradoxical psoriasiform reactions. Immunohistochemistry analysis performed on paradoxical skin lesions obtained from patients 1 (Pt1) and 2 (Pt2) shows similar values of IL-17A$^+$ cells, a reduction of dermal IFN-γ$^+$ cells and an increase of IL-22$^+$ or IL-36γ$^+$ cells, when compared with psoriatic skin lesions. LS and NLS skin of the same psoriatic patient (n = 3) was analyzed. Graphs show the mean of number of positive cells + SD *per* three sections. One out of three representative stainings is shown. *$p < 0.01$, **$p < 0.05$, versus classical psoriasis. Scale bars, 200 μM.

epidermal compartment was similar (Figure 3). FACS analysis of the T-cells isolated from skin biopsies confirmed a significant reduction of IFN-γ$^+$ CD3$^+$ cells in patients 1 and 2, when compared to CD3$^+$ cells isolated from classical psoriasis (~7- and 1.7-fold decrease, respectively) (Figure 4). The reduction of IFN-γ positivity was also observed in circulating CD3$^+$ cells of patients 1 and 2. Similarly, TNF-α positivity was lower in skin T-lymphocytes of patients 1 and 2. TNF-α positivity of circulating CD3$^+$ cells was instead lower only in patient 1, as compared to patient 2 and patients with classical psoriasis (Figure 4). Moreover, IL-17A positivity was comparable in patient 1 and psoriatic patients, whereas it was very high in T cells isolated from the skin of patient 2, where a mixed population of T cells, responsible for either the psoriasiform or eczematous reactions, is likely present (Figure 1). IL-22 was similar in T cells of psoriasiform lesions and classical psoriasis, whereas it was substantially reduced in PBMCs (Figure 4). CD3$^+$ cells from skin biopsies of HS patients were enriched in CD8$^+$, but not in CD4$^+$ cells, when compared to PBMC isolated from the same patients. CD3$^+$ skin T cells of classical psoriasis showed instead an enrichment of both CD4$^+$ and CD8$^+$ subpopulations (Figure 4).

Overexpression of innate immunity molecules in paradoxical psoriasis

Since we found that the inflammatory infiltrate pattern in paradoxical psoriasis strongly resembles that present in acute psoriasis, we next analyzed selected innate

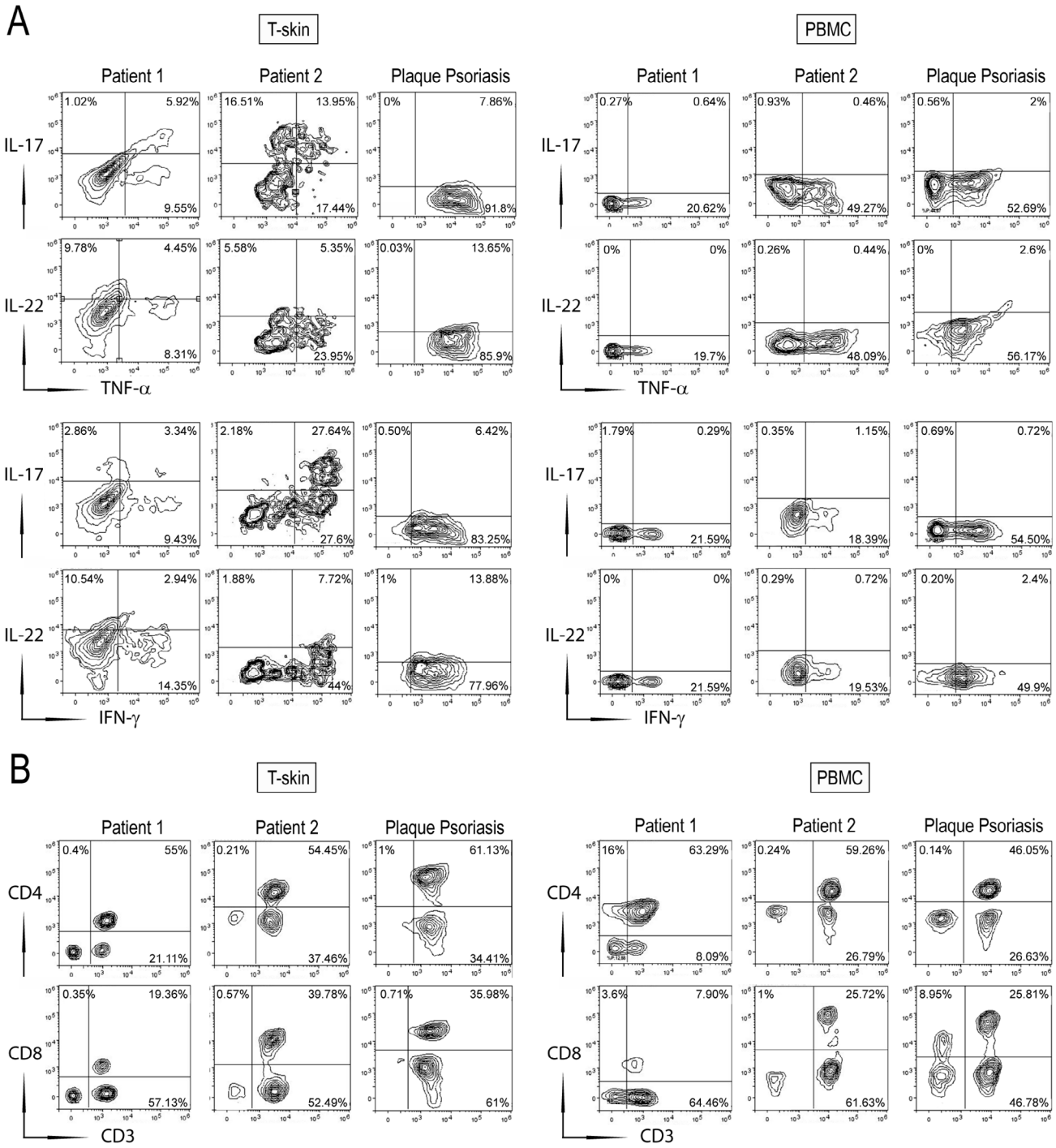

Figure 4. Immunophenotypic characterization of skin T cells (T-skin) and PBMCs isolated from HS patients with psoriasiform lesions. T-skin cells (left panel) and PBMCs (right panel) were isolated from biopsies and blood, respectively, of patients 1 and 2 and from psoriatic patients (n = 2). Co-expression of IL-17, IL-22, TNF-α, or IFN-γ on gated $CD3^+$ cells (A), and surface CD4, CD8, and CD3 (B), were analyzed by flow cytometry. The percentage of positive fluorescent cells is shown in each quadrant. The results show the mean values of data obtained for one representative experiment out of three experiments.

immunity molecules potentially involved in the triggering of psoriasis. As shown in Figure 5A, the type I IFN-β was expressed in paradoxical skin lesions, mainly in keratinocytes, at levels significantly higher than classical psoriasis (~1.9-fold increase). IFN-β expression was also detected in cells with a T-cell- and DC-like morphology, as well as in endothelial cells. The epidermis of psoriasiform reactions was also immunoreactive for IFN-κ, another keratinocyte-derived type I IFN [34]. IFN-κ expression was similar in the two psoriasis conditions, even if it showed different subcellular localization within keratinocytes, being cytoplasmic in psoriasiform lesions and membrane-bound in classical psoriasis (Figure 5A). IFN-κ staining was also present in cells with a monocyte- or DC-like morphology, at comparable levels in classical and nonclassical psoriasis (Figure 5A). Similarly to IFN-β, IFN-α2a and IFN-λ1, but not IFN-λ2 and IFN-λ3, were greatly increased in paradoxical psoriasis, as compared to plaque psoriasis (Figure 5B and data not shown). Of note, LL-37 was strongly expressed in psoriasiform skin lesions, at levels higher than classical psoriasis (Figure 5B). We finally investigated LT-α and LT-β, two members of the cytokine TNF family, also known as TNF-β and TNF-C [35], possibly deregulated by anti-TNF-α therapy. Both lymphotoxins were strongly overexpressed in paradoxical skin reactions, especially in keratinocytes of the basal layer epidermis.

As a whole, these data reveal the presence of an overexpressed innate immunity pattern in the skin of HS patients with paradoxical psoriasis.

SNP characterization in HS patients developing paradoxical psoriasis

In order to understand whether paradoxical reactions of HS patients had a genetic basis, we analyzed a number of SNPs predisposing to psoriasis. Among them, we studied SNPs frequent in the psoriatic population, such as polymorphisms of the *HLA-C* and *HLA-B* regions and the *ERAP1* gene. We also analyzed genetic variants of pathogenic cytokines, receptors and signal transducers (i.e. TNF-α, IL-17F, IL-17RA, IL-23R, IL-12B, IKBζ, A20, A20 binding protein, Tyk2), and of skin-barrier proteins (i.e. CDSN, CCHCR1) (see supplementary material, Table S1). All HS patients showed variants of *ERAP1* and the *HLA-C* region, either homozygosis or heterozygosis (Table 1). Three SNPs in *ERAP1* (rs30187/rs30186/rs26653) and nine variants in *HLA-C* (rs114395371/rs9264942/rs10484554/rs2524095/rs28383849/rs9264944/rs2853922/rs147538049/rs9264946) were, in fact, found in all patients, with SNPs distributed differently in the three patients. None of them showed the classical *HLA-Cw6* allele, even though patients 1 and 3 carried three point SNPs (rs2524095/rs2853922/rs386698994) mapping near the *HLA-Cw6* SNP position (rs17192540) (Table 1). In contrast, patient 2 mostly showed genetic polymorphisms (rs9264942/rs10484554/rs28383849/rs9264944/rs147538049/rs9264946) present in the genomic region containing a second variant of *HLA-Cw6* (Table 1). All HS patients carried SNPs in *NFKBIZ* (rs3217713) and *TNFAIP* (rs610604) genes, encoding IKBζ and A20 proteins respectively. Interestingly, patient 3 showed the higher number of psoriasis-related SNPs, and shared a number of SNPs with patient 2 (rs7637230/rs4819554/rs3132554/rs10542126/rs3130983), and rs280519 with patient 1 (Table 1). Patient 3 also carried two other SNPs in *CDSN* (rs1062470/rs707913) and three SNPs in *CCHCR1* (rs1576/rs130079/rs746647) (Table 1).

Although rs11209026 in the *IL23R* gene has been previously associated with paradoxical psoriasiform reactions to anti-TNFs [36], we could not find this SNP in any of the HS patients. Two other SNPs in *IL23R*, rs72676067, and rs1004819, were instead detected in patients 2 and 1, respectively.

Discussion

Psoriasis pathogenesis involves both innate and adaptive immunity responses, overactive in different clinical phases and characterized by specific patterns of inflammation. While innate immunity processes predominate in early/acute phase, with immune cells such as pDC, neutrophils, mast cells and macrophages being abundant in skin lesions, adaptive immune responses are typical of chronic psoriasis [10,14,15,37]. Local overproduction of IFN-α and other innate immune mediators, such as antimicrobial peptides, also characterize early psoriasis [37,38]. Conversely, during the development of chronicity, type I IFNs are no longer produced, in part due to the inhibitory effects of TNF-α, which determines the decline of innate immunity processes and the mounting of adaptive immune responses [39]. During this phase, TNF-α is important for immune activation of mDC, which, after encountering the causative antigen(s), are responsible for T-cell expansion.

In this study, we found that paradoxical psoriasis evoked by anti-TNF-α therapy in patients affected by HS strongly resembles early psoriasis. In fact, by comparing skin lesions of paradoxical psoriasis with classical psoriasis, we observed a marked dermal

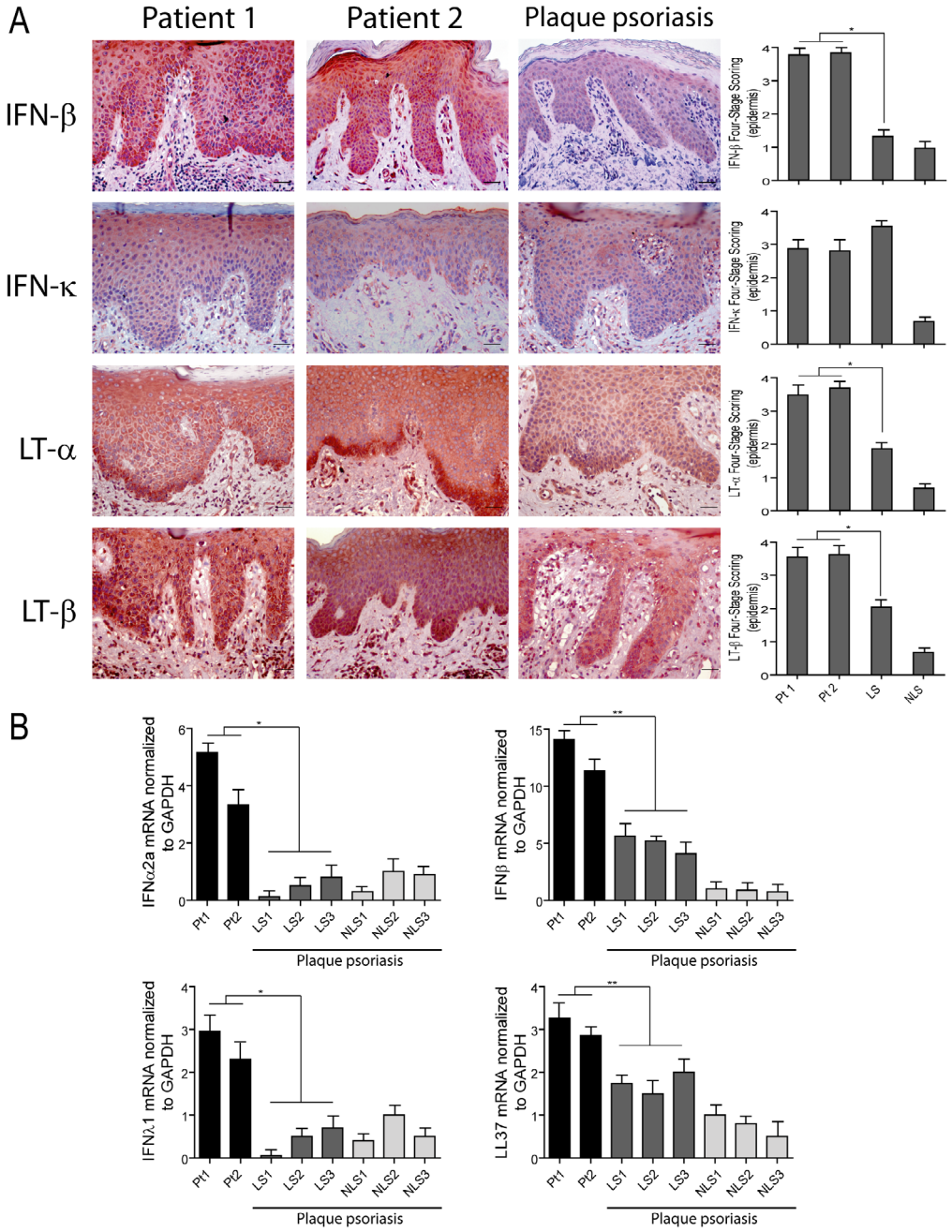

Figure 5. Innate immunity molecules are overexpressed in the skin of HS patients after TNF-α treatment. (A) Immunohistochemistry analysis of paradoxical skin reactions obtained from patients 1 (Pt1) and 2 (Pt2) shows an increase of IFN-β, LT-α, LT-β, and similar IFN-κ positivity, when compared with psoriatic skin lesions. LS and NLS skin of the same psoriatic patient ($n = 3$) was analyzed. Graphs show the mean + SD of semiquantitative, four-stage scoring, ranging from negative immunoreactivity (0) to strong immunoreactivity (4+) and relative to the epidermal expression of the indicated molecules. One out of three representative stainings is shown. *$p < 0.01$, versus classical psoriasis. Scale bars, 200 μm. (B) mRNA expression of *IFNα2a*, *IFNβ*, *IFNλ1* and *LL37* was analyzed by real-time PCR in skin lesions of patients 1 (Pt1) and 2 (Pt2) and in skin biopsies from LS and NLS skin of three psoriatic patients. mRNA values were normalized to *GAPDH* mRNA. Values obtained from triplicate experiments were averaged, and data presented as means of $2^{-\Delta\Delta CT}$ + SD. *$p < 0.01$, **$p < 0.05$.

Table 1. SNPs carried by HS patients developing paradoxical psoriasis after anti-TNF-α therapy

dbSNP ID	Gene	Patient 1	Patient 2	Patient 3
Antigen presentation				
rs30187	ERAP1	●	◑	◑
rs30186	ERAP1	●	◑	●
rs11743410	ERAP1	○	○	◑
rs26653	ERAP1	●	◑	●
rs114395371	HLA-C region	○	◑	○
rs17192540	HLA-C region (HLA-Cw6)	○	○	○
rs2524095	HLA-C region	●	○	●
rs2853922	HLA-C region	●	○	●
rs386698994	HLA-C region	●	○	◑
rs79709508	HLA-C region (HLA-Cw6 2v)	●	●	○
rs28383849	HLA-C region	○	◑	○
rs10484554	HLA-C region	○	◑	○
rs147538049	HLA-C region	○	◑	○
rs9264944	HLA-C region	○	◑	○
rs9264946	HLA-C region	○	◑	○
NF-κB pathway and T-cell activation				
rs72676067	IL23R	○	◑	○
rs1004819	IL23R	◑	○	○
rs41313262	IL23R	○	○	◑
rs11209026	IL23R	○	○	○
rs3217713	NFKBIZ	●	●	●
rs7637230	NFKBIZ	○	◑	●
rs2546890	IL12B	○	◑	◑
rs1800610	TNF-α	○	◑	○
rs2397084	IL17F	◑	○	○
rs71562288	TRAF3IP2	○	○	◑
rs33980500	TRAF3IP2	○	◑	○
rs610604	TNFAIP3	◑	◑	●
rs12720356	TYK2	○	○	◑
rs280519	TYK2	◑	○	●
rs4819554	IL17RA	○	◑	●
Skin barrier function				
rs3132554	CDSN	○	◑	◑
rs1042127	CDSN	○	◑	○
rs1042126	CDSN	○	◑	◑
rs1062470	CDSN	○	○	◑
rs707913	CDSN	○	○	◑
rs3130983	CDSN	○	◑	◑
rs1576	CCHCR1	○	○	●
rs130079	CCHCR1	○	○	◑
rs746647	CCHCR1	○	○	●
rs130075	CCHCR1	◑	○	○

DNA was obtained from blood samples of HS patients 1–3, and SNPs analyzed by next-generation sequencing technology in MiSeq system, as described in 'Material and methods' section. For each sample, a cDNA library of 44 amplicons potentially containing 71 SNPs located in genes predisposing to psoriasis was developed. SNP-carrying genes were classified accordingly to their functions (i.e. control of antigen presentation, NF-κB pathway and T-cell activation, skin barrier).
dbSNP ID, data base SNP identification number at NCBI; rs, reference *SNP* ID number; ERAP1, endoplasmic reticulum aminopeptidase 1; NFKBIZ, NF-κB inhibitor zeta; TRAF3IP2, TRAF3 interacting protein 2; TNFAIP3, TNF-α induced protein 3; TYK2, tyrosine kinase 2; IL17RA, IL-17 receptor A; CDSN, corneodesmosin; CCHCR1, coiled-coil alpha-helical rod protein 1. ●: homozygotic variant; ◑: heterozygotic variant; ○: WT. Rs17192540 and rs79709508 were relative to HLA-Cw6 and HLA-Cw6 second allelic variant (HLA-Cw6 2v).

accumulation of innate immunity cells, including pDCs, neutrophils, mast cells, and macrophages. In parallel, the expression levels of innate immunity molecules potentially involved in induction of the psoriasiform phenotype, greatly increased in paradoxical reactions. Among them, IFN-α2a, IFN-β, and IFN-λ1 are overexpressed in the skin of HS patients following anti-TNF-α therapy. Also, LT-α and LT-β, as well as LL-37, were detected at very high levels in paradoxical psoriasis, when compared to classical psoriasis. A transient IFN-α upregulation has already been described in classical psoriasis, during the early phase of disease development, as well as in paradoxical psoriasis [19,37]. Concomitantly, IFN-β is known to be expressed by pDCs in both conditions [19]. We found that IFN-β, together with IFN-κ and lymphotoxins

were impressively expressed in the epidermal compartment of paradoxical skin reaction, as well as in pDCs and leukocytes infiltrating the dermis. Type I IFNs and lymphotoxins released by keratinocytes might have a fundamental pathogenic role in paradoxical psoriasis. However, the mechanisms by which these molecules promote a psoriatic skin phenotype are not yet known, neither in paradoxical nor in classical psoriasis. On the contrary, the immunological function of IFN-α has been extensively studied, especially in classical psoriasis, where it is known to induce Th17 responses [40]. In paradoxical psoriasis, IFN-α could have a different role, with antigen-specific Th17 responses being absent. It could induce chemokines at the epidermal level, such as CXCL10 and CXCL9, responsible for the recruitment of DC and nonspecific T cells. These inflammatory cells could in turn sustain and amplify local inflammatory responses in paradoxical reactions [41].

The induction of innate immunity players in paradoxical psoriasis is dependent on the loss of TNF-α function in limiting the innate immune responses in the skin, as previously demonstrated [19]. In fact, TNF-α blockade determined the accumulation of pDCs and inhibition of their maturation. As a consequence, pDCs could release very high levels of the type I IFN-α6 and IFN-β, being, thus, responsible for paradoxical psoriasis. Together with pDCs, we found other innate immunity cells present in psoriasiform lesions of HS patients. Among them, $CD15^+$ neutrophils, c-kit/$CD117^+$ mast cells, $CD68^+$ macrophages and monocytes abundantly infiltrate the dermis of paradoxical skin reactions. This pattern of leukocyte subpopulations is very similar to that found in early psoriasis, and is consistent with the high local production of IL-36 cytokines and with the overactive innate immunity processes present during the initial phase of psoriasis, as previously shown [14,27]. Conversely, similarly to paradoxical psoriasis, adverse HS in patients affected by autoimmune disorders, including psoriasis and Crohn's disease, might be dependent on aberrant innate immunity responses evoked by TNF-α blockade [42,43]. Indeed, a number of pathogenic cytokines common to psoriasis were found in HS skin, including IL-36, together with inflammatory mediators active on neutrophils and Th17 cells largely present in the affected areas [27]. Other than pDCs, innate immune cells could also be recruited by chemokines released by keratinocytes, fibroblasts and endothelial cells (i.e. CCL20, chemerin), whose expression depends on type I IFNs produced by resident skin cells themselves. In fact, other than controlling the expression of type I IFNs in pDCs, TNF-α might negatively regulate these molecules in keratinocytes, which notoriously also contribute to the induction of innate immunity pathways in early psoriasis [10]. This hypothesis is supported by our findings that type I IFNs are induced in keratinocytes of paradoxical psoriasis and vice versa are present at low levels in chronic plaque psoriasis. It would be important to confirm the high expression of innate immunity mediators following TNF-α blockade *in vitro* in primary keratinocyte cultures, as demonstrated for cultured pDCs. It would be also relevant to analyze whether, similarly to paradoxical psoriasis, acute psoriasis shows exaggerated expression of type I IFNs and lymphotoxins in the epidermal compartment. In that case, it can be supposed that TNF-α temporally limits innate immunity processes evoked not only by pDCs but also by keratinocytes to unleash adaptive immune responses in psoriatic skin.

Concerning the unknown expression and role of lymphotoxins in paradoxical reactions and in classical psoriasis, a previous study confirmed the pivotal function of LT-α, together with TNF-α, in determining NF-κB-mediated skin inflammatory reactions in $I\kappa B\alpha^{-/-}$ mice [44]. In addition, patients affected by psoriatic arthritis treated with etanercept showed increased serum levels of LT-α [45]. Therefore, lymphotoxins might be deeply involved in psoriasis pathogenesis, and TNF-α could tightly control their expression in both keratinocytes and lymphocytes. Further studies are needed to evaluate LT-α and LT-β expression and their role in the different phases of psoriasis development, and to understand the function of keratinocyte-derived lymphotoxins.

Analysis of the T-cell infiltrate in paradoxical skin reactions demonstrated a significant reduction of IFN-γ- or TNF-α-producing $CD3^+$ cells in paradoxical psoriasis, when compared to chronic psoriasis. However, $CD8^+$ and IL-17^+ lymphocytes were present in paradoxical psoriasiform reactions, at levels comparable to psoriasis, even if it is conceivable that they were nonspecifically recruited. The absence of bursting of a type I IFN T-cell response in paradoxical skin reactions was not surprising, if we consider that it is typical of the chronic phase in classical psoriasis [10,11,15]. On the contrary, IL-22-producing cells increased in psoriasiform reactions of HS patients, even though positive cells showed mostly a macrophage-like morphology. Our findings extend previous studies showing the upregulation of IL-22 mRNA expression in paradoxical psoriasis, and identifying innate immunity cells, and not only T lymphocytes, as cellular sources of IL-22 [19]. IL-22 overexpression could be responsible for hyperproliferation and de-differentiation of keratinocytes typical of the epidermis of paradoxical psoriasiform lesions.

Finally, although an inflammatory cytokine milieu, inducing the local production of chemokines and cytokines by resident skin cells, can be effectively established in paradoxical psoriasis, it seems to be insufficient to induce the development of chronic psoriasiform reactions in HS patients, possibly through the lack of mDC and T-cell activation by the causative antigen(s) of psoriasis.

Despite the effective use of adalimumab in patients with severe HS, 2–5% of treated patients develop paradoxical psoriasis [6]. Anti-TNF-α treatment can induce paradoxical psoriasis even in patients affected by other diseases characterized by high levels of TNF-α [8,46,47]. Notably, this side effect can also occur in patients undergoing psoriasis treatment with anti-TNFs. Guttate or pustular forms in palmo-plantar/scalp areas frequently represent the subclinical types of psoriasis that develop in these reactive patients. The reason why anti-TNF induces a similar psoriatic phenotype (same subtype and localizations) only in a portion of subjects affected by different autoimmune conditions is still unknown. It is reasonable to speculate the influence of genetic factors predisposing to paradoxical psoriasis, and specifically being involved in innate immunity pathways, in particular in pDC activation and/or type I IFN and TNF-α signaling. Indeed, an association between polymorphisms in the *IL-23R*, *FBXL19*, *CTLA4*, *SLC12A8*, and *TAP1* genes and paradoxical psoriasis has been found [36]. On the other hand, there is a positive association between HS and psoriasis, with the prevalence of HS increased in patients with psoriasis, suggesting a common genetic predisposition [48]. To date, no evidence correlating the presence of SNPs and the development of psoriasiform lesions in patients affected by HS exist. In our study, we found that all HS patients carried numerous allelic variants in *HLA-C*. None of the patients showed the *HLA-Cw6* susceptibility allele, even though other SNPs in the proximity of the *HLA-Cw6* SNP and neighboring to other *HLA-C* variants were found. Concomitantly, HS patients carried allelic variants in the *ERAP1* gene. However, due to the lack of antigen-specific CD8+ T-cell responses in HS patients, the link between the presence of SNPs in the *HLA-C* region/*ERAP1* gene and susceptibility to paradoxical psoriasis is apparently missing. Indeed, other than having a role in MHC class I antigen presentation, ERAP1 is involved in the activation of innate immunity pathways, by inducing the inflammasome and production of cytokines and chemokines (i.e. IL-6, TNF-α, and CCL2) [49]. Importantly, allelic variants of *ERAP1* leading to missense mutation increase the capability of ERAP1 to induce inflammation in autoimmune diseases [50]. HS patients also carried polymorphisms in *NFKBIZ* and *TNFAIP3*, which could be responsible for NF-κB hyperactivation in HS patients, as demonstrated for other pathological conditions [51,52]. Importantly, allelic variants in *NFKBIZ* and *TNFAIP3* might determine the enhanced type I IFN expression observed in pDCs and keratinocytes of paradoxical lesions, as both IKBζ and A20 can transcriptionally regulate IFN expression, respectively, via activation and inhibition of NF-κB [53]. Finally, genetic variants in *TNFAIP3*, in particular those imparting lower A20 expression, might be responsible for uncontrolled IFN-β expression, as demonstrated by silencing *TNFAIP3* mRNA expression in a vascular model of inflammation [54]. All these SNPs in psoriasis susceptibility loci are likely genetically transmitted, as all three patients examined had a positive family history for psoriasis. In the future, it will be necessary to extend the analysis of psoriasis-related SNPs to a larger cohort of HS patients developing psoriasiform reactions, but also in a population successfully responding to anti-TNF-α treatment, to identify differences in the genetic background of the patients. The identification of genetic biomarkers correlating with an adverse response to anti-TNF-α therapy will be fundamental to predict the risk of developing paradoxical psoriasis.

In conclusion, our study shows that paradoxical psoriasis induced by anti-TNF in patients affected by HS has immunological features common to early phase psoriasis, mainly characterized by cellular and molecular players of innate immunity. Among them, LT-α and LT-β, as well as IFN-κ and IFN-λ1, have been identified as new innate mediators potentially involved in the induction of paradoxical psoriasis. Of note, we found that, in addition to pDCs, keratinocytes are also a source of type I IFNs, in particular IFN-β, likely as consequence of TNF-α inhibition. It will be important to evaluate the effects of anti-TNF-α therapy on keratinocytes in paradoxical psoriasiform reactions, especially in terms of type I IFN production, to identify new pathogenic mechanisms involved in the early phase of psoriasis.

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Author contributions statement

LF, TT, MAP, GP, and CM enrolled HS and psoriatic patients, and collected clinical data. MM, CS, LM, and SM performed immunohistochemical studies, RNA and genetic analyses. GLS performed bioinformatics analysis of SNPs. FS and CC isolated and analyzed T cells of skin biopsies and blood of HS patients. LF, MM, SM, and CA designed and interpreted the experiments. CA wrote the manuscript, and, together with MM, compiled the figures. LF, MM, GG, AC, and SM made revisions and proofread the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Table S1 List of the analyzed SNPs

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Research Article

Selective Immunomodulation of Inflammatory Pathways in Keratinocytes by the Janus Kinase (JAK) Inhibitor Tofacitinib: Implications for the Employment of JAK-Targeting Drugs in Psoriasis

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IFN-γ and IL-22 are deeply involved in the pathogenesis of psoriasis, as they boost the expression of inflammatory genes and alter proliferative and differentiative programs in keratinocytes. The JAK1/JAK2/STAT1 and JAK1/TYK2/STAT3 pathways triggered by IFN-γ and IL-22, respectively, are aberrantly activated in psoriasis, as highlighted by the peculiar STAT1 and STAT3 signatures in psoriatic skin lesions. To limit the detrimental consequences of IFN-γ and IL-22 excessive stimulation, psoriatic keratinocytes activate suppressor of cytokine signaling (SOCS)1 and SOCS3, which in turn dampen molecular signaling by inhibiting JAK1 and JAK2. Thus, JAK targeting appears to be a reasonable strategy to treat psoriasis. Tofacitinib is an inhibitor of JAK proteins, which, similarly to SOCS, impedes JAK phosphorylation. In this study, we evaluated the immunomodulatory effects of tofacitinib on epidermal keratinocytes in *in vitro* and *in vivo* models of psoriasis. We demonstrated the selectivity of tofacitinib inhibitory action on IFN-γ and IL-22, but not on TNF-γ or IL-17 proinflammatory signaling, with suppressed expression of IFN-γ-dependent inflammatory genes, and restoration of normal proliferative and differentiative programs altered by IL-22 in psoriatic keratinocyte cultures. Tofacitinib also potently reduced the psoriasiform phenotype in the imiquimod-induced murine model of psoriasis. Finally, we found that tofacitinib mimics SOCS1 or SOCS3 activities, as it impaired the same molecular pathways in IFN-γ or IL-22-activated keratinocytes.

1. Introduction

Psoriasis is an immune-mediated skin disease characterized by epidermal abnormalities and prominent inflammatory cell infiltrate [1–3]. Current opinion on the pathogenesis of psoriasis emphasizes the role of cytokine signaling to drive an inflammatory cycle, in which infiltrating dendritic cells and autoreactive T lymphocytes, mainly represented by IL-17-producing T cells, T-helper-1 (Th1), and Th22 cells, release IL-17, IFN-γ, IL-22, and TNF-α. All these cytokines induce keratinocyte expression of a plethora of immune mediator determinant for recruitment and activation of additional dendritic cells and T lymphocytes, which in turn reinforce the pathogenic cycle by perpetuating keratinocyte activation [4–6]. Proinflammatory cytokines, in particular, IL-22 and IL-17, are also responsible for hyperproliferation and altered terminal differentiation of keratinocytes, as well as impairment of the apoptotic pathways, all typical features of psoriasis [4–6]. Thus, IFN-γ and IL-22 inflammatory cytokines are deeply involved in the pathogenesis of psoriasis, and their Janus Kinase (JAK)1/JAK2/signal transducers and activators of transcription (STAT)1 and JAK1/tyrosin kinase

(TYK)2/STAT3 proximal signaling are aberrantly activated, as highlighted by STAT1 and STAT3 signatures in psoriatic skin lesions [7–10]. Also, IL-17 and TNF-α proinflammatory cytokines elicit immune responses in psoriatic keratinocytes, through molecular pathways independent on JAK/STAT and involving NF-κB, Act1, and ERK1/2 [11, 12].

To limit an excessive stimulation by inflammatory cytokines, keratinocytes express suppressors of cytokine signaling (SOCS) molecules, a family of endogenous inhibitors of cytokine-dependent signaling [6, 13–16]. SOCS1 and SOCS3 function as potent suppressors of IFN-γ and IL-22 signaling in keratinocytes, respectively. At molecular level, SOCS1 and SOCS3 inhibit JAK1–2 by functioning as pseudosubstrates, hence impeding the activation of IFN-γ and IL-22 receptors and downstream STATs. As a consequence of the loss of STAT1 activity, keratinocytes overexpressing SOCS1 can no longer express inflammatory molecules in response to IFN-γ [13–15]. Similarly, IL-22-induced proliferative and antidifferentiative effects on keratinocytes are efficiently counteracted by SOCS3-dependent STAT3 inhibition [15, 17]. On the other hand, SOCS cannot influence JAK-independent molecular pathways in keratinocytes, including TNF-γ signaling [13].

Because of the importance of inflammatory cytokines in psoriasis, JAK targeting represents a logical strategy to treat this disease. Various JAK inhibitors are in preclinical development or have been tested in clinical trials. Among them, tofacitinib is an oral JAK inhibitor with an intracellular mechanism of action against JAKs, already in use for systemic treatment of rheumatoid arthritis [18], and under evaluation for the treatment of both plaque psoriasis [19] and psoriatic arthritis [20]. Phase 3 studies in patients with moderate-to-severe chronic plaque psoriasis have demonstrated the efficacy of tofacitinib in improving clinical outcomes [21]. JAK inhibition by tofacitinib strongly reduces clinical signs of psoriasis, and, potently blocks signaling through the common γ chain-containing receptors, including IL-2, IL-4, IL-7, IL-9, and IL-15, or through canonical receptors for cytokines, such as IFN-γ, IL-21, IL-6, and to a lesser extent, IL-12 and IL-23 [22]. In preclinical models, tofacitinib was shown to affect both innate and adaptive immune responses and inhibited pathogenic T helper (Th)17 cell differentiation by suppressing IL-23 expression [23].

While the mechanisms of T-cell activity inhibition and modulation of differentiation by tofacitinib are well characterized [23–25], few information on the immunomodulatory effects on psoriasis-related pathways activated in resident keratinocytes, or on its capability to mimic SOCS inhibitory circuits exist for this drug. In this study, we evaluated the immunomodulatory effects of tofacitinib on epidermal keratinocytes in experimental *in vitro* and *in vivo* models of psoriasis. In particular, we studied the tofacitinib effect on JAK/STAT pathway and downstream inflammatory molecules in human keratinocyte cultures activated with proinflammatory molecules related to psoriasis, including IFN-γ, IL-22, IL-17, and TNF-γ, as well as *in vivo* in the imiquimod- (IMQ-) induced murine model of psoriasis. We also investigated the impact of tofacitinib on other protein targets induced by IFN-γ or IL-22 signaling in keratinocytes and to mimic SOCS1 or SOCS3 activities.

2. Materials and Methods

2.1. Keratinocyte Cultures and Treatments. Primary cultures of human keratinocytes were obtained from skin biopsies of psoriatic patients ($n = 5$) afferent to 5th Dermatology Unit at IDI-IRCCS and prepared as previously described [6]. Patients had definite plaque-type psoriasis diagnosed according to standard criteria, and they had not received any systemic or topical therapy for at least 1 month before skin donation. Skin biopsies were obtained after patient's informed written consent, with the approval of the IDI-IRCCS Local Ethics Committee (Prot. N. 474/1/2016; study: "Studio delle chinurenine in pazienti affetti da psoriasi"). Second- or third-passage keratinocytes were used in all experiments, with cells cultured in the serum-free medium KGM (Clonetics, San Diego, CA), for at least 3–5 days (at 60–80% confluence) before performing treatments. Some experiments were performed on keratinocyte cultures undergoing terminal differentiation, achieved by growing cells at 100% of confluence ($t0$) and, thus, keeping them in culture for another 4 d.

Stimulations with 200 U/ml recombinant human (rh) IFN-γ (R&D Systems, Minneapolis, MN, USA), as well as 50 ng/ml rh TNF-α, IL-22, or IL-17 (all from R&D Systems), were performed in keratinocyte basal medium (KBM, Clonetics). Tofacitinib (CP 690,550 compound) was obtained from Pfizer Inc. (Peapack, NJ) and administered by pretreating cultures for 1 h before cytokine stimulation. Cytotoxicity of tofacitinib was previously tested by measuring the activity of lactate dehydrogenase (LDH) released from keratinocyte cultures, using Cytotoxicity Detection Kit Plus-LDH (Roche Diagnostics, Milan, Italy), following the manufacturer' instructions.

2.2. Immunoprecipitation, Immunoblotting, and Densitometry. Protein extract preparation, immunoprecipitation, and immunoblotting were performed accordingly to standard procedures [6]. The Abs used for the study were as follows: anti-IFN-γRα subunit (C-20), anti-IL-22R1 (3-RE40), anti-TYK2 (C-20) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphotyrosine (clone 4G10; Upstate Biotechnologies, Temecula, CA), anti-JAK1, anti-JAK2 (Upstate Biotechnologies), anti-phosphotyrosine- (pTyr701-) STAT1 (Santa Cruz Biotechnology), anti-phosphoserine- (pSer727-) STAT1, anti-phosphotyrosine- (pTyr705-) STAT3 and anti-phosphoserine- (pSer727-) STAT3 (Cell Signaling), anti-STAT1 and anti-STAT3 (C-20) (Santa Cruz Biotechnology), anti-phospho-ERK1/2 (E4; Santa Cruz Biotechnology), anti-ERK1/2 (C-16; Santa Cruz Biotechnology), anti-phospho-p65 (Ser276), anti-IκBα, HRP-conjugated anti-c-myc (9E10), anti-p63 (4A4), anti-β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-keratin (KRT)1, and anti-loricrin (both from Covance, Meryville, CA). Filters were properly developed with anti-mouse, anti-goat, or anti-rabbit Ig Abs conjugated to HRP using the ECL-plus detection system (Amersham, Dubendorf,

Switzerland) or, otherwise, the SuperSignal West Femto kit (Pierce, Rockford, IL, USA). Immunoblots of experiments were subjected to densitometry using an Imaging Densitometer model GS-670 (Bio-Rad) supported by the Molecular Analyst Image software, and band intensities were evaluated in three independent experiments. Data are expressed as fold induction ± SD in experimental time-course relative to untreated or tofacitinib-treated samples, to which were given a value of 1.

2.3. Transient Transfection and Luciferase Assay. Cultured keratinocytes grown in six-well plates were transiently transfected with the STAT3-responsive plasmid pLucTKS3 (a generous gift of Prof. J. Turkson, University of Central Florida, Orlando, FL) or pGASLuc plasmid by using Lipofectin reagent (Invitrogen), according to the manufacturer's instructions. At 24 h post transfection, cells were pretreated with tofacitinib for 1 h and then stimulated with IL-22 or IFN-γ for 8 h. After cell lysis in an appropriate buffer (Promega Italia, Milan, Italy), *Firefly* luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega). To normalize the transfection efficiency, pRL-null plasmid encoding the *Renilla* luciferase was included in each transfection. Luciferase activity was further normalized by total cellular protein content assayed using Bradford (Sigma-Aldrich, Milan, Italy).

2.4. Intracellular Signaling Array. PathScan Intracellular Signaling Array Kit was purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA; Catalog #7323). This array allows the simultaneous detection of 18 signaling molecules when phosphorylated or cleaved. They include ERK1/2, STAT1, STAT3, Akt (Thr308 and Ser473 phosphorylation), AMPKa, S6 ribosomal protein, mTOR, HSP27, Bad, p70 S6 kinase, PRAS40, p53, p38, SAPK/JNK, PARP, caspase 3, and GSK-3b. Whole protein lysates from keratinocyte cultures treated with IFN-γ, IL-22, or TNF-α in the presence or absence of tofacitinib were prepared using lysis buffer that was provided in the kit and processed following the manufacturers' instructions. The Bio-Rad Gel Documentation System was used to take detailed pictures of the array using the Quantity One software using the ChemiDoc XRS function. Values of graphs are expressed as densitometric units and were normalized to internal positive control.

2.5. Proliferation Assays. 8×10^4 cells were seeded in 12-well plates and, the day after, starved in KBM. Culture stimulation with IFN-γ, IL-22, or TNF-α was conducted either in the presence or absence of tofacitinib. After 2 d of treatment, cells were evaluated by Trypan blue exclusion test. Crystal violet assays were also performed to evaluate proliferation. Thus, 2×10^4 cells were grown for 48 h in 96-well plates and stained with 0.5% crystal violet, whose incorporation was measured at 540 nm in an ELISA reader (model 3550 UV ELISA reader; Bio-Rad, Hercules, CA).

2.6. Apoptosis Analysis. Apoptosis of keratinocytes was evaluated using the FITC Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Milan, Italy). Viability, necrosis, and apoptosis were analysed by flow cytometry. Cells were analysed with a FACScan equipped with Cell Quest software. The percentage of Annexin V^+, PI^+, and Annexin V/PI^+ cell populations were evaluated in keratinocyte cultures left untreated or treated with IFN-γ, IL-22, or TNF-α in the presence or absence of tofacitinib.

2.7. RNA Isolation and Real-Time Polymerase Chain Reaction (PCR). Total RNA from keratinocyte cultures was extracted using the TRIzol reagent (Invitrogen); mRNA was reverse-transcribed into cDNA and analysed by real-time PCR. The expression of human SOCS3, S100A7, IL-20, HBD-2, LL-37, and HPRT-1 mRNA was evaluated in the ABI Prism SDS 7000 PCR instrument (Applied Biosystems, Branchburg, NJ), using SYBR Green PCR reagents or TaqMan PCR Master Mix. The same PCR tools were employed to analyse murine IL-17A, IL-22, IFN-γ, TNF-α, CXCL10, CCL2, CCL20, CXCL16, and IL-6 mRNAs. The forward and reverse primers employed for real-time PCR for SOCS3 were 5′-AAGGACGGAGACTTCGATTCG-3′ and 5′-AAACTT GCTGTGGGTGACCAT-3′, and for LL-37 5′-TTTTGC GGAATCTTGTACCCA-3′ and 5′-TCTCAGAGCCCAGA AGCCTG-3′. The sequences of the primers for β-defensin-(HBD-) 2 mRNA have been previously described [26]. Primers for S100A7, IL-20, and HPRT-1 were provided by Applied Biosystems (HS 00161488, HS 00218888, and HS 4333768, respectively). Primers used for the detection of murine molecules were retrieved from previous studies [27]. Human and murine mRNA level values were normalized to HPRT-1 and β-2-microglobulin mRNA, respectively. The values obtained from triplicate experiments were averaged, and data presented as mean $2^{-\Delta\Delta CT} \pm$ SD.

2.8. Multiplex Immunoassay and ELISA. Media conditioned for 48 h by psoriatic keratinocyte cultures stimulated with IFN-γ or IL-22 in the presence or absence of tofacitinib were harvested and filtered. The simultaneous quantitative measurement of cytokines/chemokines in small amounts of supernatants was achieved by using the xMAP multiplex technology (Luminex) and a BioPlex 200 System equipped with magnetic washer workstation Bio-Plex ProTM and Manager Software version 6.1 (Bio-Rad Laboratories, Milan, Italy). In particular, a Pro-Human Cytokine Panel (Bio-Plex, Pro-Human Chemokine 40-plex Panel, cat # 171AK99MR2, Bio-Rad) was used to measure the following analytes: 6Ckine/CCL21, BCA-1/CXCL13, CTACK/CCL27, ENA-78/CXCL5, Eotaxin/CCL11, Eotaxin-2/CCL24, Eotaxin-3/CCL26, Fractalkine/CX3CL1, GCP-2/CXCL6, GM-CSF, Gro-α/CXCL1, Gro-β/CXCL2, I-309/CCL1, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8/CXCL8, IL-10, IL-16, IP-10/CXCL10, I-TAC/CXCL11, MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13, MDC/CCL22, MIF, MIG/CXCL9, MIP-1α/CCL3, MIP-1δ/CCL15, MIP-3α/CCL20, MIP-3β/CCL19, MPIF-1/CCL23, SCYB16/CXCL16, SDF-1α + β/CXCL12, TARC/CCL17, TECK/CCL25, and TNF-α, following the manufacturers' instructions. In parallel, CCL5 was measured with a commercially available sandwich ELISA kit (R&D Systems) and an ELISA reader model 3550 UV (Bio-Rad). Psoriatic keratinocyte cultures were conducted in duplicate

using two different keratinocyte strains. Data were expressed as mean pg/ml or ng/ml ± SD.

2.9. IMQ-Induced Psoriasiform-Like Model. 8 weeks old female BALB/cJ mice (Harlan Laboratories, San Pietro al Natisone, Italy) were employed in all the experiments. Shaved mouse dorsal skin was treated daily for 5 consecutive days with 50 mg Aldara cream containing 5% IMQ (MEDA AB, Solna, Sweden). On day 5, full-thickness skin biopsies of the treated area were collected with an 8 mm biopsy puncher. Skin was fixed in neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for histopathological analysis. In some experiments, 50 μl Aldara cream was mixed with tofacitinib (in DMSO solution) at a final concentration of 10 and 0.5 mM. A group of 10 mice was used for each experimental condition. On day 5, full skin was fixed in neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for histopathological analysis. Otherwise, full skin was frozen in nitrogen liquid and further processed for RNA extraction, which was performed by using TRIzol reagent (Invitrogen). RNA from ten mice per experimental group were pooled, reverse-transcribed into cDNA, and analysed by real-time PCR, as previously described.

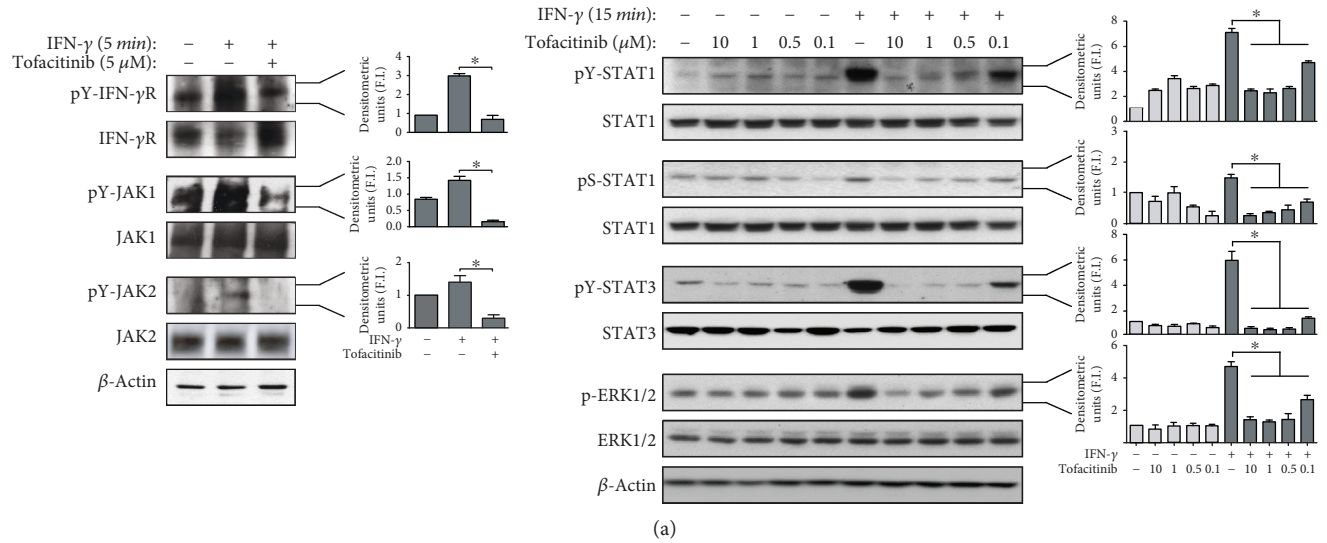
2.10. Histopathology and Immunohistochemistry. Fixed murine skin was embedded in paraffin, and tissue sections were deparaffinized and stained with H&E for histological analysis. Epidermal and scale thickness and cell infiltrate number were analysed as parameters of skin acanthosis and inflammation. Average epidermal and scale thickness was quantified by a researcher blind to the experimental groups who took five measurements per three sections for each mouse. Cells infiltrating dermis were also counted in three skin sections for each mouse. Immunohistochemistry was performed by using primary Abs against CD3 (Dako, Glostrup, Denmark), Ly6G, CD11c, and CD11b (BD Biosciences), Ki67 (Novocastra, Newcastle upon Tyne, UK), KRT10 (Covance), phospho-STAT3 (Tyr705) and phospho-STAT1 (Tyr701) (both from Cell Signaling), IL-17A (R&D Systems) and IL-22 (Novus Biologicals, Oakdille, Canada), and immunoreactivities developed with secondary biotinylated mAbs and staining kits (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with Mayer's hematoxylin and were visually analysed by two pathologists experienced in dermatology. Positivity was evaluated in 5 adjacent fields at a magnification of 200x. A semiquantitative, four-stage scoring system was applied, ranging from negative immunoreactivity (0) to strong immunoreactivity (4+) for KRT10 in the epidermis.

2.11. Stable Keratinocyte Transfectants. HaCaT cells were stably transfected with *myc*/SOCS1, *myc*/SOCS2, *myc*/SOCS3, or empty pcDNA3 (mock) plasmids as previously reported [6, 13]. HaCaT SOCS clones were treated with IFN-γ or IL-22, whereas mock clones were pretreated with tofacitinib and then stimulated with IFN-γ or IL-22 in DMEM.

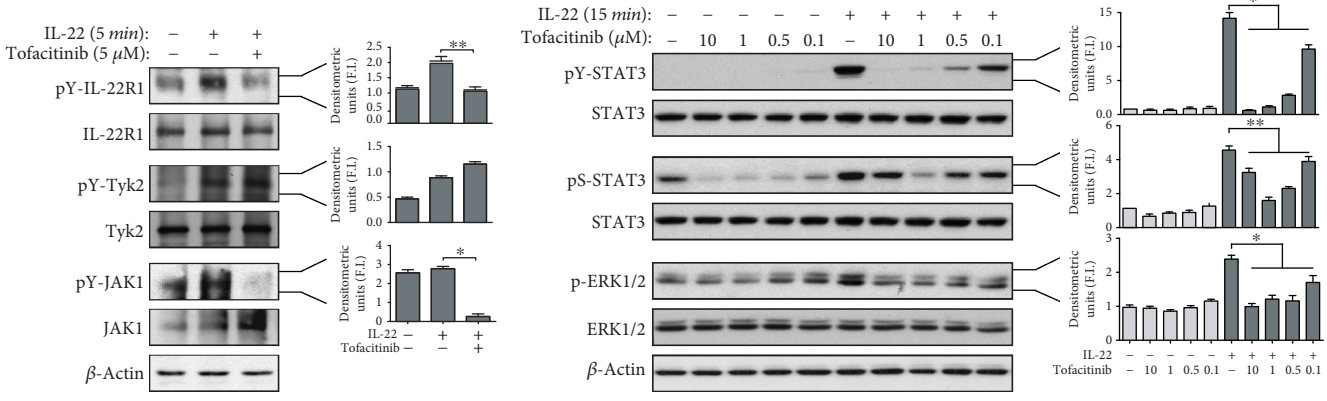
2.12. Statistical Analysis. For *in vitro* studies, statistical significance was evaluated using Wilcoxon's signed rank test (SigmaStat; Jandel, San Rafael, CA, USA). Values of $p \leq 0.05$ were considered significant. For *in vivo* experiments, the significance of differences between experimental groups (mice treated with IMQ vs. mice treated with IMQ plus tofacitinb 100 mM or 5 mM) was calculated by unpaired Student's t-test. Statistical analysis was performed with Prism v.5.0 (GraphPad Software, La Jolla, CA, USA), and values are expressed as the mean + SD of n animals. Values of $p < 0.05$ were considered significant.

3. Results

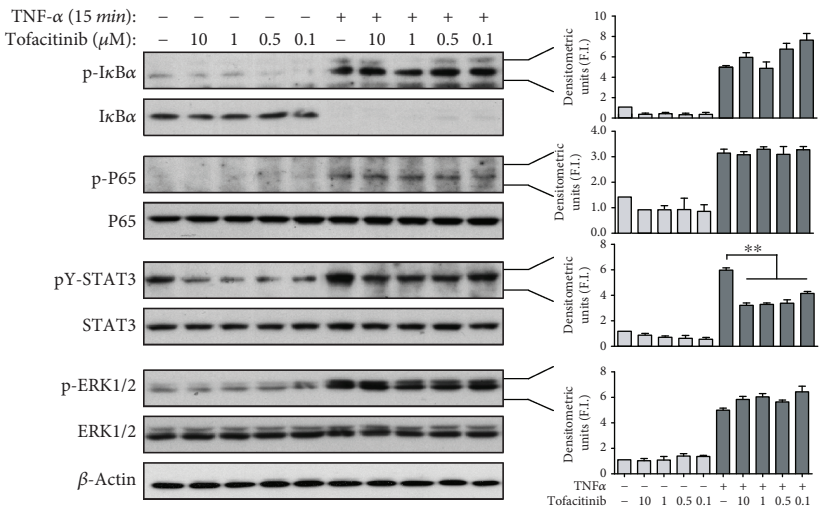
3.1. Tofacitinib Efficiently Inhibits on JAK/STAT-Dependent Pathways in IFN-γ- or IL-22-Activated Keratinocytes. We initially studied the impact of tofacitinib on intracellular pathways activated in human keratinocytes by proinflammatory cytokines with a pathogenic role in psoriasis, including IFN-γ, IL-22, TNF-α, and IL-17. To this end, primary keratinocyte cultures were established from skin biopsies of psoriatic patients ($n = 5$). The choice to employ psoriatic keratinocyte cultures raised from the fact that these strains are more responsive to triggering factors, as compared to keratinocytes obtained from healthy donors, probably due to their genetic background and different susceptibilities to proinflammatory cytokines [4, 5]. Tofacitinib had no cytotoxic effects on keratinocytes even at higher concentrations, as tested by measuring the activity of lactate dehydrogenase released by cultures (not shown). One hour pretreatment with different doses of tofacitinib (0.1–10 μM) was followed by stimulation of keratinocyte cultures with rh IFN-γ (200 U/ml), IL-22 (50 ng/ml), TNF-α (50 ng/ml), or IL-17 (50 ng/ml) for different time periods (Figure 1, data not shown). Tofacitinib efficiently inhibited IFN-γ and IL-22 proximal signaling, with reduced phosphorylation of IFN-γ R, JAK1, and JAK2, as well as IL-22R1 and JAK1, but not TYK2, respectively (Figures 1(a) and 1(b), left). As a consequence of proximal signaling inhibition, downstream STAT1 and STAT3 phosphorylation was dose dependently inhibited in IFN-γ-stimulated cultures (Figure 1(a), right). Interestingly, phospho-ERK1/2 activation was also reduced by tofacitinib, even if less potently if compared to STATs (Figure 1(a), right). Similarly to what we observed for IFN-γ, IL-22 could not induce STAT3 or ERK1/2 phosphorylation in the presence of tofacitinib (Figure 1(b), right). In order to evaluate the specificity of action of tofacitinib on pathways dependent on JAKs, we analysed its effects on the signaling of TNF-α or IL-17, cytokines which notoriously can activate NF-κB and MAP kinases but not JAKs. As shown in Figure 1(c), tofacitinib could not influence phosphorylation of IκBα or p65 NF-κB subunit, nor ERK1/2 in response to TNF-α. In contrast, tofacitinib reduced STAT3 activation induced by TNF-α, even if at a lower degree if compared to that observed for IFN-γ- or IL-22-treated samples. Of note, tofacitinib could not regulate signaling pathways activated by IL-17 in keratinocytes, including NF-κB (data not shown). Finally, tofacitinib potently reduced the IFN-γ- or IL-22-induced transactivation of STAT1- or STAT3-binding promoters in keratinocytes, as assessed in cultures transfected with the IFN-γ-inducible reporter plasmid, pGAS-Luc, or the IL-22-inducible reporter plasmid,



(a)

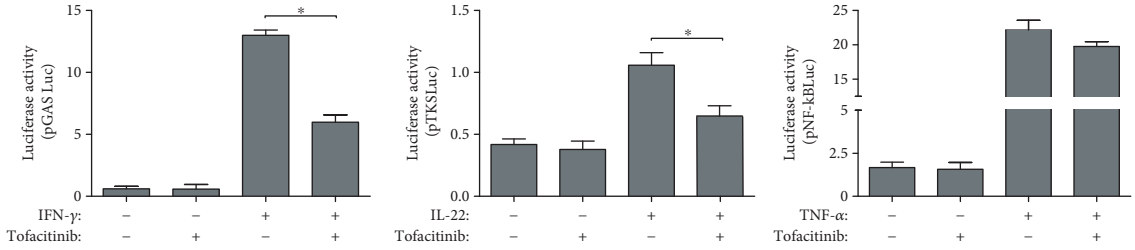


(b)



(c)

Figure 1: Continued.



(d)

Figure 1: Tofacitinib inhibits IFN-γ- and IL-22- but not TNF-α-induced molecular signaling in psoriatic keratinocytes cultures. (a) Protein extracts obtained from psoriatic keratinocytes pretreated or not with vehicle alone or the indicated doses of tofacitinib, and then stimulated or not with IFN-γ for the indicated time periods, were subjected to immunoprecipitation for IFN-γRα, JAK1, or JAK2 and Western blotting analysis by using anti-phosphotyrosine Ab to detect IFN-γRα, JAK1, or JAK2 phosphorylation. Filters were stripped and reprobed with anti-IFN-γRα, JAK1, or JAK2 Abs. Phosphorylated and unphosphorylated forms of STAT1, STAT3, and ERK1/2 were monitored in keratinocytes by WB analysis. (b) Protein extracts were obtained from keratinocytes pretreated or not with tofacitinib in the presence of IL-22 for the indicated time periods and were subjected to immunoprecipitation for IL-22R1, TYK1, or JAK2 and, then, WB analysis by using anti-phosphotyrosine Ab to detect IL-22R1, TYK1, or JAK2 phosphorylation. Filters were stripped and reprobed with anti-IL-22R1, TYK1, or JAK2 Abs. Phosphorylated and unphosphorylated forms of STAT1, STAT3, and ERK1/2 were also monitored in keratinocytes by WB analysis. (c) Protein extracts were obtained from keratinocytes pretreated or not with tofacitinib in the presence of TNF-α for 15 min and then analysed by WB analysis to detect basal or phospho-IκBα, basal or phosphor-p65 subunit of NF-κB, and phosphorylated and unphosphorylated forms of STAT3 and ERK1/2. Graphs in (a), (b), and (c) represent densitometric analyses of the indicated proteins shown in representative WB. Data are expressed as mean ± SD fold induction (F.I.) calculated relatively to the untreated samples, which were given a value of 1. $^{*}p < 0.01$, $^{**}p < 0.05$. (d) Psoriatic keratinocyte cultures transiently transfected with a STAT1-, STAT3-, or NF-κB-responsive plasmids, termed, respectively, pGAS-Luc, pLucTKS3, or pNF-κB-Luc were treated with 5 μM tofacitinib or vehicle alone for 2 h and then stimulated with IFN-γ, IL-22, or TNF-α for 8 h prior to assay Firefly luciferase activity on cellular extracts. Data are expressed as Firefly luciferase values normalized to Renilla luciferase and micrograms of proteins and are shown as mean + SD of $n = 6$ samples pooled from two experiments. $^{*}p < 0.01$.

pLucTKS3, respectively (Figure 1(d)). As a whole, these data demonstrate that tofacitinib totally abrogated JAK/STAT pathways activated by IFN-γ and IL-22 in human psoriatic keratinocytes, whereas it could not influence JAK-independent molecular pathways, such as those activated by TNF-γ or IL-17.

3.2. Analysis of the Effect of Tofacitinib on Additional Molecular Pathways Induced by IFN-γ or IL-22 in Keratinocyte Cultures. We next determined the protein phosphorylation profiles of keratinocyte cultures undergoing stimulation with IFN-γ, IL-22, or TNF-α for 20 min, pretreated or not with tofacitinib. This analysis was performed by using a commercial phospho-kinase array kit (see Materials and Methods), which detects intracellular kinases and signaling node molecules, including Akt, AMPKα, mTOR, HSP27, BAD, p53, JNK, p38, PARP, and caspase 3, other than STAT1 and STAT3, specifically activated by IFN-γ and IL-22. As shown in Figure 2, tofacitinib could significantly reduce the IFN-γ-dependent upregulation of Akt phosphorylation at both Thr308 and Ser473 residues, AMPKα, p38, PARP, and caspase 3, other than STAT1 (data not shown) and STAT3. In parallel, it decreased Akt phosphorylation at both Thr308 and Ser473 residues, AMPKα, mTOR, HSP27, p38, JNK, and STAT3, activated by IL-22 treatment in keratinocyte cultures (Figure 2). However, tofacitinib inhibition of these additional molecular pathways was weaker if compared to that observed on STAT. Again, TNF-α-induced intracellular kinase pattern could not be influenced by tofacitinib treatment, with the exception of STAT3 (Figure 2).

3.3. Effects of Tofacitinib on Keratinocyte Proliferation, Differentiation, and Apoptosis Processes. We assessed whether tofacitinib regulated keratinocyte growth and proliferation, as well as differentiation and apoptosis in psoriatic keratinocyte cultures ($n = 3$). Cells were pretreated with tofacitinib (5 μM) for 1 h and then stimulated with IFN-γ, IL-22, or TNF-α for 48 h. As previously reported [13, 17], IFN-γ decreased proliferation of keratinocyte cultures whereas IL-22 enhanced such process by inhibiting terminal differentiation. When tofacitinib was coadministered, the effects of these cytokines on keratinocyte proliferation were totally reverted (Figure 3(a)). Similarly, tofacitinib could significantly abrogate IL-22-induced inhibition of differentiation, as well as IFN-γ-induced apoptosis of keratinocytes (Figures 3(b) and 3(c)). In contrast, tofacitinib did not influence TNF-γ-induced processes in keratinocytes, in particular apoptosis, as shown in Figure 3(c).

3.4. Regulation by Tofacitinib of Expression of Psoriasis-Related Inflammatory Molecules by Keratinocytes. We then evaluated whether tofacitinib could influence keratinocyte expression of proinflammatory genes induced by IFN-γ or IL-22 via JAK/STAT pathway. To this end, the expression of a variety of molecules involved in the induction or control of skin inflammation was studied by cytofluorimetry, bioplex multiplex immunoassays, and real-time PCR analysis of psoriatic keratinocyte cultures pretreated with tofacitinib and then stimulated with rh IL-22 or IFN-γ. We found that tofacitinib substantially reduced IFN-γ-induced expression of ICAM-1, HLA-DR and MHC class I membrane molecules, and numerous inflammatory mediators, including CX3CL1,

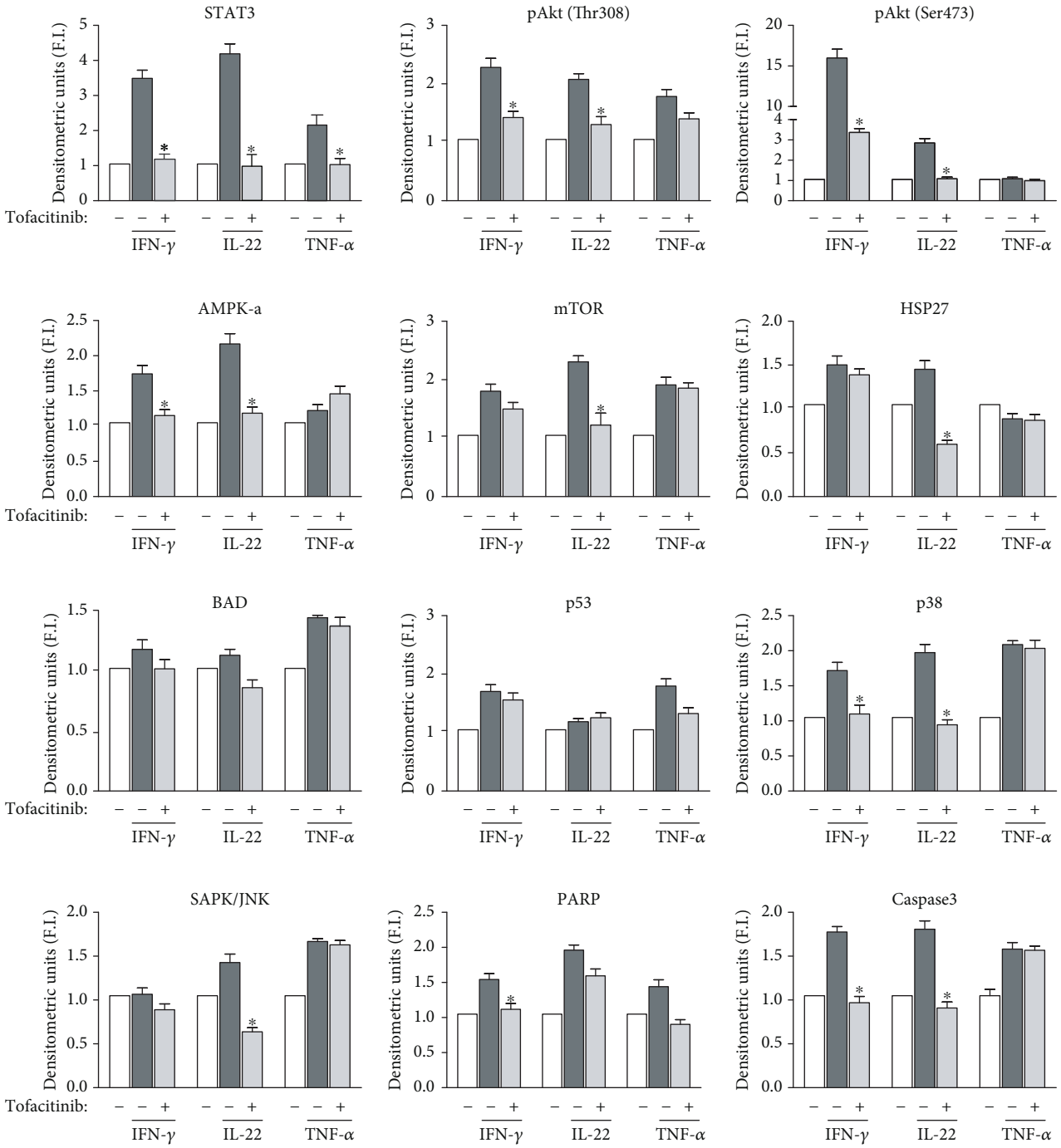


Figure 2: Tofacitinib regulates IFN-γ- and IL-22- but not TNF-α-induced signaling molecules in psoriatic keratinocytes. Protein extracts obtained from psoriatic keratinocytes pretreated with 5 μM tofacitinib or vehicle alone and then stimulated or not with IFN-γ, IL-22, or TNF-α for 20 min were used on a PathScan intracellular signaling array, which allows the simultaneous detection of 18 signaling molecules when phosphorylated or cleaved. They include ERK1/2, STAT1, STAT3, Akt (Thr308 and Ser473 phosphorylation), AMPKa, mTOR, HSP27, Bad, p53, p38, SAPK/JNK, PARP, and caspase 3. Developed slides were acquired at ChemiDoc system. Graphs represent densitometric analyses of the indicated proteins. Data are expressed as mean ± SD fold induction (F.I.) calculated relatively to the untreated samples, which were given a value of 1. Protein panel was analysed in two assays with two different keratinocyte strains. Each value was normalized to an internal positive control. $^*p < 0.05$ for samples treated with tofacitinib vs. untreated, in the presence of cytokines.

CXCL1, CXCL8, CXCL10, CCL1, CCL2, CCL5, MIF chemokines, IL-6, and SOCS3 mRNA (Table 1). Similarly, tofacitinib could downregulate IL-22-induced expression of CX3CL1, CXCL8, CXCL12, and CCL2 chemokines, as well as of IL-20 and SOCS3 in keratinocyte cultures (Table 2). Inflammatory molecules induced by TNF-α or IL-17 could not be regulated by tofacitinib (data not shown). Thus, tofacitinib treatment could influence the expression of genes that

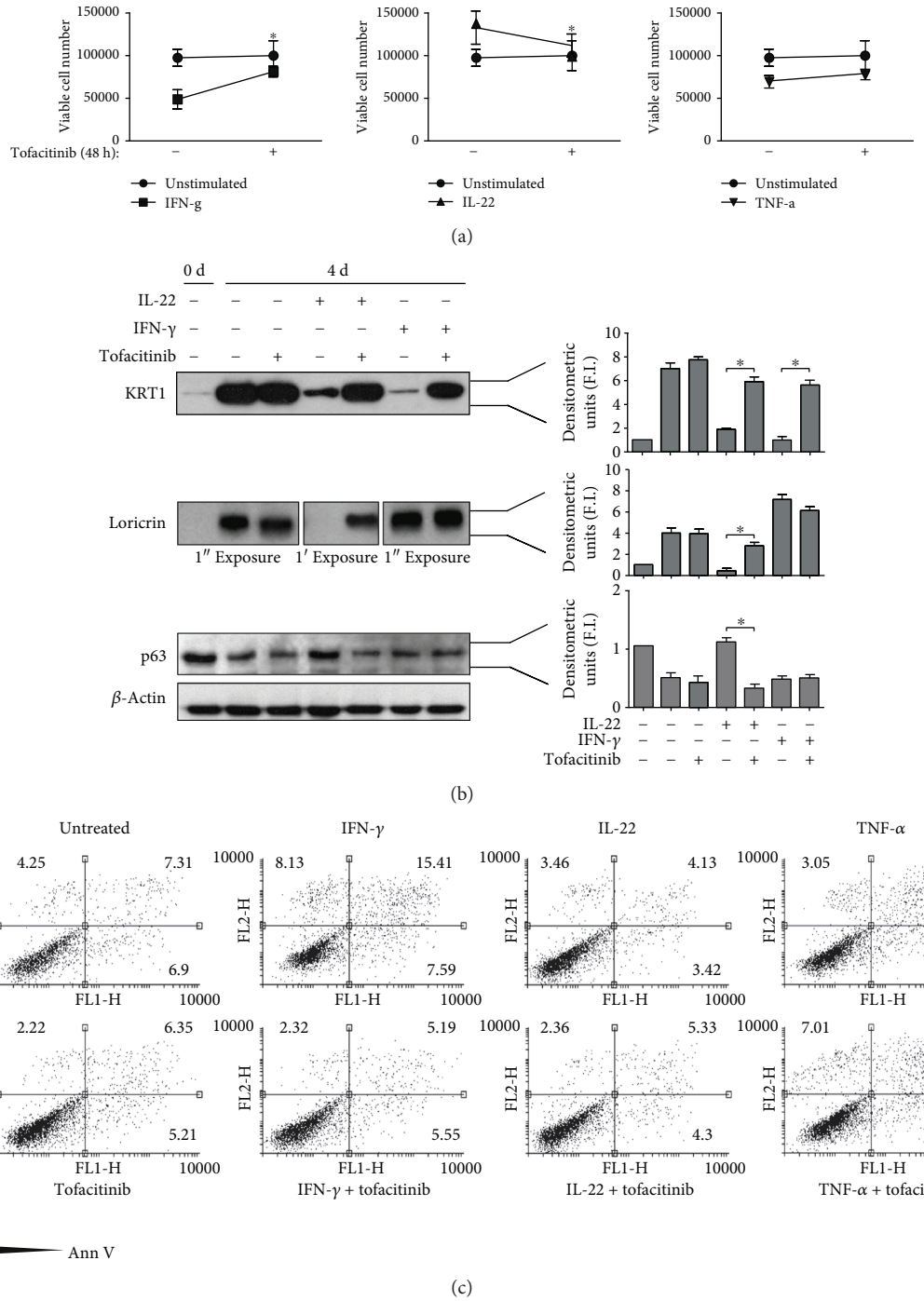


Figure 3: Tofacitinib regulates proliferation, differentiation, and apoptosis of IFN-γ- and IL-22- but not TNF-α-treated keratinocytes. Proliferation of keratinocyte cultures untreated or treated with 5 μM tofacitinib, either in the presence or absence of IFN-γ, IL-22, or TNF-α, was analysed by Trypan blue exclusion test (a), which was performed after 48 h of culture. Data are expressed as total cell number ± SD. $^*p < 0.05$. (b) Keratin 1 (KRT1), loricrin, and p63 were analysed by WB by using protein lysates obtained from keratinocyte cultures grown at 100% confluency (0 d) or for additional four days (4 d), in the presence or absence of tofacitinib and IFN-γ or IL-22. Graphs show densitometric values ± SD of KRT1, loricrin, or p63. Data are expressed as densitometric units, expressed as fold induction (F.I.) of treated vs. untreated samples, which were given a value of 1. $^*p < 0.01$. (c) Apoptosis of cultured keratinocytes treated with 5 μM tofacitinib in the presence or absence of IFN-γ, IL-22, or the proapoptotic stimulus TNF-α for 48 h was examined by measuring Annexin (Ann V)/propidium iodide (PI) fluorescence through flow cytofluorimetry. A representative experiment of three performed with two different psoriatic keratinocyte strains is shown, with numbers indicating the percentage of PI^+ (upper left), Ann V^+ (lower right), PI/Ann V^+ (upper right), or negative (lower left) cells.

Table 1: Tofacitinib effects on the expression of inflammatory molecules induced by IFN-γ in keratinocytes.

	Untreated*	TOF	IFN-γ	IFN-γ + TOF
Membrane molecules				
ICAM1	2.4 ± 0.2	2.0 ± 0.18	75.5 ± 6.3	6.8 ± 0.56†
HLA-DR	1.4 ± 0.15	1.4 ± 0.12	3.5 ± 0.25	1.4 ± 0.13†
MHC-I	72 ± 5.2	63 ± 4.5	146 ± 11.2	84 ± 7.4†
Chemokines				
CX3CL1	0.40 ± 0.05	0.36 ± 0.08	580.64 ± 55.06	26.20 ± 3.26†
CXCL1	0.80 ± 0.12	0.80 ± 0.07	69.00 ± 5.6	0.40 ± 0.08†
CXCL8	0.92 ± 0.11	2.60 ± 0.32	84.52 ± 7.45	5.04 ± 0.61†
CXCL10	29.08 ± 2.2	25.04 ± 0.14	2557.44 ± 150.2	29.08 ± 2.58†
CXCL12	107.20 ± 9.72	110.20 ± 2.43	141.52 ± 12.25	119.12 ± 10.61
CXCL16	2.92 ± 0.32	5.28 ± 0.68	18.48 ± 1.58	6.16 ± 0.81
CCL1	0.80 ± 0.1	0.80 ± 0.14	37.04 ± 2.94	0.60 ± 0.04†
CCL2	0.80 ± 0.95	0.76 ± 0.67	474.60 ± 49.46	0.80 ± 0.12†
CCL5	2.0 ± 0.4	1.9 ± 0.24	2305 ± 250.12	4.5 ± 0.6†
MIF	337.08 ± 35.71	637.04 ± 52.47	1745.88 ± 153.5	918.04 ± 85.8†
Cytokines, AMPs, SOCS				
IL-6	5.92 ± .0.79	5.28 ± 0.48	17.40 ± 1.57	5.28 ± 0.65†
IL-20	1.00 ± 0.12	0.90 ± 0.10	0.98 ± 0.10	0.58 ± 0.04†
LL-37	1.00 ± 0.11	0.89 ± 0.09	0.85 ± 0.10	0.70 ± 0.08
HBD2	1.00 ± 0.12	0.66 ± 0.08	1.11 ± 0.12	0.67 ± 0.08
S100A7	1.00 ± 0.13	1.11 ± 0.2	2.61 ± 0.0.25	2.83 ± 0.3
SOCS3	1.00 ± 0.12	1.13 ± 0.10	25.59 ± 2.65	2.14 ± 0.20†

Note: IFN: interferon; TOF: tofacitinib; ICAM: intercellular adhesion molecule; HLA-DR: human leukocyte antigen-antigen D related; MHC: major histocompatibility complex; CXCL: CXC-chemokine ligand; CL: chemokine ligand; MIF: macrophage migration inhibitory factor; IL: interleukin; LL37: antimicrobial peptide; HBD: human-defensin; S100: S100 calcium-binding protein; SOCS: suppressor of cytokine signaling. *Keratinocyte cultures were left untreated or treated with 5 μM of tofacitinib and stimulated or not with 100 U/ml of IFN-γ. After 6 hours, IL-20, LL-37, HBD2, S100A7, and SOCS3 mRNA levels were analysed by real-time PCR and normalized to β-actin mRNA levels. Results are expressed as mean $2^{-\Delta\Delta CT}$ ± SD. After 24 hours, cells were stained with ICAM1, HLA-DR, and MHC-I mAb followed by FITC-conjugated anti-mouse IgG and then analysed by flow cytometry. Data are expressed as mean ΔMFI ± SD. At the same time, supernatants were collected and, chemokines and IL-6 were measured by Bioplex, except for CCL5 which has been evaluated by ELISA. Results are expressed as mean pg/ml ± SD. †$p < 0.05$ compared to untreated or stimulated keratinocytes.

were regulated in a STAT-dependent manner at the transcriptional level.

3.5. Analysis of the Effects of Tofacitinib on the IMQ-Induced Murine Model of Psoriasis. IMQ-induced dermatitis in mice can serve as a model for the analysis of pathogenic mechanisms involved in psoriasis [28]. In this model, a major role of the IL-23/IL-17/IL-22 axis has been demonstrated, with IL-22-deficient mice being resistant to psoriasis development induced by IMQ [29]. Thus, the effect of tofacitinib was studied in this model, with the drug being administered together with IMQ for 5 days, at two different concentrations (0.5 and 10 mM). As shown in Figure 4, tofacitinib substantially reverted the psoriasiform phenotype in IMQ-treated mice (Figure 4(a)) and, even at the lower dose, reduced psoriasiform signs, including epidermal and scale thickness, as assessed by quantifying the average of these parameters on images of skin sections stained by H&E (Figures 4(b)–4(d)). Tofacitinib also reduced the widespread inflammatory infiltrate in the dermis, as compared with control (Figures 4(b) and 4(e)). Moreover, tofacitinib administration led to a dose-dependent reduction of the number of $CD3^+$ T lymphocytes, $Ly6G^+$ neutrophils, $CD11c^+$ dendritic cells, $CD11b^+$ macrophages infiltrating the dermis, and of the keratinocyte proliferation marker Ki67 (Figure 5). Conversely, compartimentalization and expression of the marker of differentiation, such as KRT10, rather weak in the suprabasal layer epidermis of IMQ-treated mice, were restored by treatment with the drug (Figure 5). Of note, tofacitinib dramatically reduced the presence of phospho-STAT3 and phospho-STAT1 in the nucleus of epidermal cells of mouse skin in a dose-dependent manner (Figure 6(a)), with the higher drug concentration responsible of almost total disappearance of phospho-$STAT3^+$ (~80% of reduction) and $STAT1^+$ cells (~95% of reduction) in IMQ-treated mice.

Since IL-17- and IL-22-producing leukocytes are implicated in the pathogenic processes associated to IMQ-induced psoriasiform reactions (28–30), and keratinocytes are actively involved in recruiting these cells in lesional skin, we investigated tofacitinib effect on the presence of IL-17- and IL-22-producing cells into mouse skin and the expression of chemokines potentially involved in their recruitment. Immunohistochemistry and real-time PCR of IL-17A and IL-22, together with IFN-γ and TNF-α mRNA analyses (Figure 6(b)), showed that tofacitinib decreased the number of $IL\text{-}17^+$ or $IL\text{-}22^+$ cells in the IMQ-treated skin, consistently

Table 2: Tofacitinib effects on the expression of inflammatory molecules induced by IL-22 in keratinocytes.

	Untreated*	TOF	IL-22	IL-22 + TOF
Membrane molecules				
ICAM1	2.4 ± 0.2	2.0 ± 0.18	2.5 ± 0.24	1.8 ± 0.16
HLA-DR	1.4 ± 0.15	1.4 ± 0.12	1.4 ± 0.12	1.4 ± 0.11
MHC-I	72 ± 5.2	63 ± 4.5	57 ± 4.8	54 ± 5.2
Chemokines				
CX3CL1	0.40 ± 0.05	0.36 ± 0.08	46.72 ± 4.57	19.72 ± 1.87†
CXCL1	0.80 ± 0.12	0.80 ± 0.07	0.80 ± 0.09	0.40 ± 0.035
CXCL8	0.92 ± 0.11	2.60 ± 0.32	83.04 ± 9.43	49.04 ± 4.85†
CXCL10	29.08 ± 2.2	25.04 ± 0.14	36.60 ± 3.57	43.28 ± 3.19
CXCL12	107.20 ± 9.72	110.20 ± 2.43	119.12 ± 12.91	111.16 ± 5.76
CXCL16	2.92 ± 0.32	5.28 ± 0.68	12.92 ± 1.39	10.52 ± 1.15
CCL1	0.80 ± 0.1	0.80 ± 0.14	10.60 ± 1.08	10.60 ± 1.10
CCL2	0.80 ± 0.95	0.76 ± 0.67	1.72 ± 0.08	0.72 ± 0.62
CCL5	2.0 ± 0.4	1.9 ± 0.24	1.95 ± 0.16	1.73 ± 0.13
MIF	337.08 ± 35.71	637.04 ± 52.47	1800.12 ± 105.9	1807.00 ± 135.7
Cytokines, AMPs, SOCS				
IL-6	5.92 ± .0.79	5.28 ± 0.48	7.24 ± 0.84	6.24 ± 0.54
IL-20	1.00 ± 0.12	0.90 ± 0.10	3.17 ± 0.17	3.16 ± 0.05
LL-37	1.00 ± 0.11	0.89 ± 0.09	0.84 ± 0.11	1.51 ± 0.13
HBD2	1.00 ± 0.12	0.66 ± 0.08	3.62 ± 0.39	3.96 ± 0.56
S100A7	1.00 ± 0.14	1.11 ± 0.2	1.73 ± 0.16	1.71 ± 0.18
SOCS3	1.00 ± 0.12	1.13 ± 0.10	8.12 ± 0.95	0.64 ± 0.05

Note: IL: interleukin; TOF: tofacitinib; ICAM: intercellular adhesion molecule; HLA-DR: human leukocyte antigen-antigen D related; MHC: major histocompatibility complex; CXCL: CXC-chemokine ligand; CL: chemokine ligand; MIF: macrophage migration inhibitory factor; LL37: antimicrobial peptide; HBD: human-defensin; S100: S100 calcium-binding protein; SOCS: suppressor of cytokine signaling. *Keratinocyte cultures were left untreated or treated with 5 μM of tofacitinib and stimulated or not with 75 ng of IL-22. After 6 hours, IL-20, LL-37, HBD2, S100A7, and SOCS3 mRNA levels were analysed by real-time PCR and normalized to β-actin mRNA levels. Results are expressed as mean $2^{-\Delta\Delta CT} \pm$ SD. After 24 hours, cells were stained with ICAM1, HLA-DR, and MHC-I mAb followed by FITC-conjugated anti-mouse IgG and then analysed by flow cytometry. Data are expressed as mean ΔMFI ± SD. At the same time, supernatants were collected and, chemokines and IL-6 were measured by Bioplex, except for CCL5 which has been evaluated by ELISA. Results are expressed as mean pg/ml ± SD. †$p < 0.05$ compared to untreated or stimulated keratinocytes.

with a significant reduction of IL-17 and IL-22 mRNA levels. In contrast, neither IFN-γ nor TNF-α mRNA expression could be significantly influenced by tofacitinib application (Figure 6(b)). Of note, both IL-17^+ and IL-22^+ cell populations had mostly a macrophage/dendritic cell-like morphology, similar to that of CD11c- and CD11b-stained cells (Figure 5). Finally, we analysed the expression of keratinocyte-derived chemokines in mouse skin biopsies by real-time PCR analysis. A significant reduction of chemokines, such as CCL2, CXCL16, CCL20, and IL-6, was detected after the application of tofacitinib (Figure 6(c)). The effect of tofacitinib was not exerted on CXCL10 or other keratinocyte chemokines whose expression was strictly dependent on IFN-γ (Figure 6(c) and data not shown).

The effect of tofacitinib was dose-dependent (Figures 4–6), and no change in all the analysed markers was observed by treating mouse skin with tofacitinib alone (not shown).

3.6. Tofacitinib and SOCS1 or SOCS3 Impair the Same Molecular Pathways in IFN-γ- or IL-22-Activated Keratinocytes. In psoriatic keratinocytes, SOCS1 and SOCS3 molecules act as endogenous repressors of cytokine signaling and function by directly inhibiting JAK1 and JAK2 proteins, thus impeding STAT activation [13, 16]. This part of the study investigated whether tofacitinib could inhibit the same molecular pathways suppressed by SOCS1 and SOCS3 in keratinocytes. To this end, a comparison of the main molecular pathways activated by IFN-γ and IL-22 was carried out in keratinocyte overexpressing SOCS1 or SOCS3 and tofacitinib-treated mock clones. SOCS2 clones were used as negative control, as in these cells, IFN-γ or IL-22 signaling is not influenced by SOCS2 transgene presence [13]. A number of keratinocyte clones stably expressing SOCS were previously generated in our lab, as previously described [6, 13–15]. SOCS1, SOCS3, and SOCS2 clones ($n = 2$ for each) were tested for their levels of transgene contents and, then, activated with IFN-γ or IL-22 (Figure 7(a)). In parallel, mock clones ($n = 2$) were treated with tofacitinib together with IFN-γ or IL-22. The expression pattern of phosphorylated STAT1, STAT3, and ERK1/2 in IFN-γ- or IL-22-treated mock clones was identical to that induced in psoriatic keratinocytes, with tofacitinib efficiently inhibiting these signal transduction pathways (Figures 7(b) and 7(c)). Similarly to tofacitinib, the presence of SOCS1 or SOCS3 transgene determined an impairment of STAT1 and STAT3 activation in response to IFN-γ, and of STAT3 in response to IL-22,

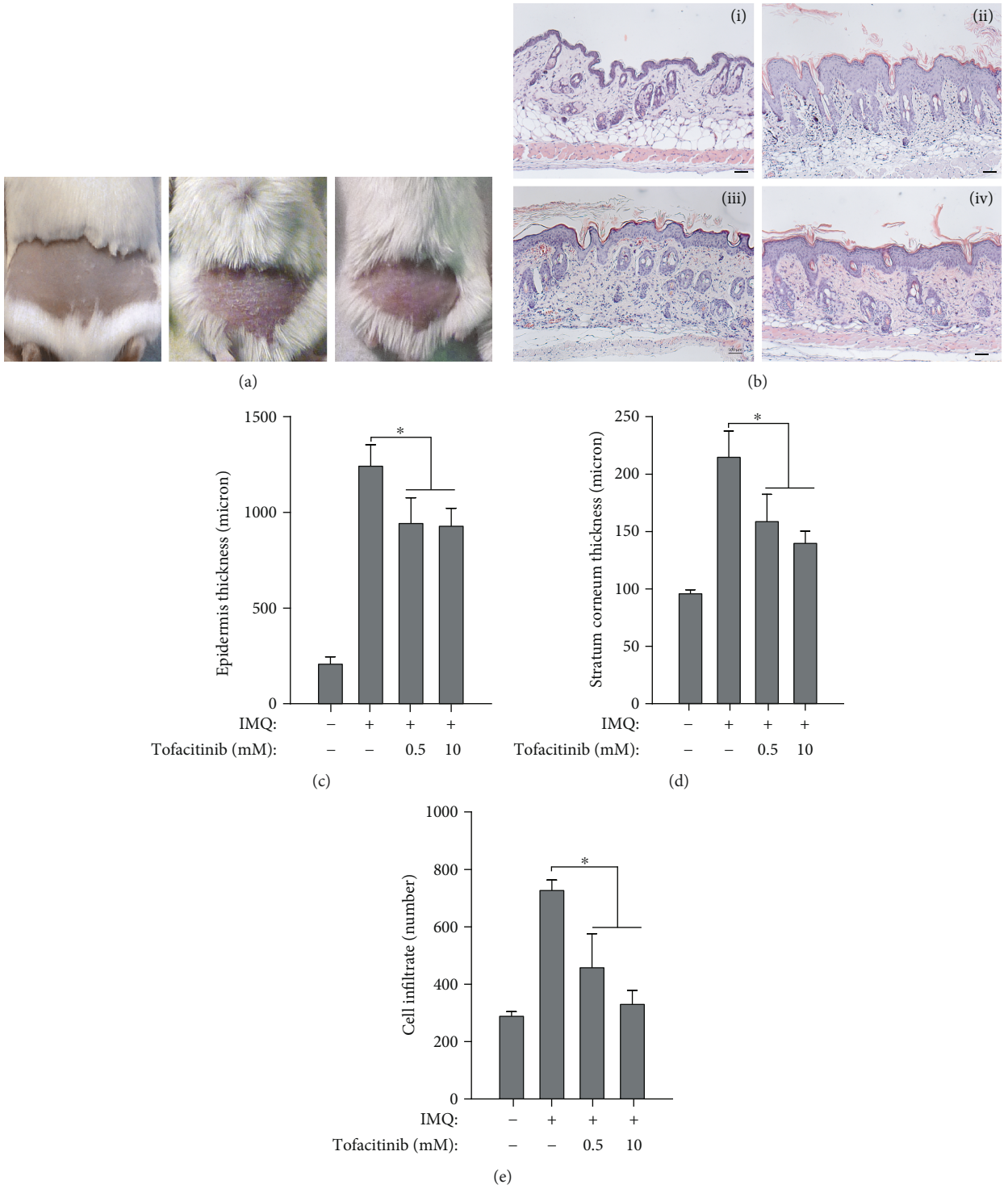


Figure 4: Tofacitinib inhibits inflammatory responses in the IMQ-induced psoriasiform mouse model. (a) Representative pictures of back-shaved mice left untreated (left), IMQ-treated (middle), or undergoing to cotreatment with IMQ and 10 mM tofacitinib. (b) Representative H&E staining of (i) untreated, (ii) treated with IMQ cream, and in the presence of 10 mM (iii) or 0.5 mM (iv) tofacitinib. Mouse skin treated with IMQ reverted their condition after tofacitinib topical application of 0.5 and 10 mM. The quantification of (c) epidermal, (d) scale thickness, and (e) cell infiltrate number was analysed as parameters of skin acanthosis and inflammation. Graphs show means of microns of epidermis and stratum corneum thickness and mean of number of cells infiltrating dermis per section, ±SD *per* group ($n = 10$ mice). $^*p < 0.001$.

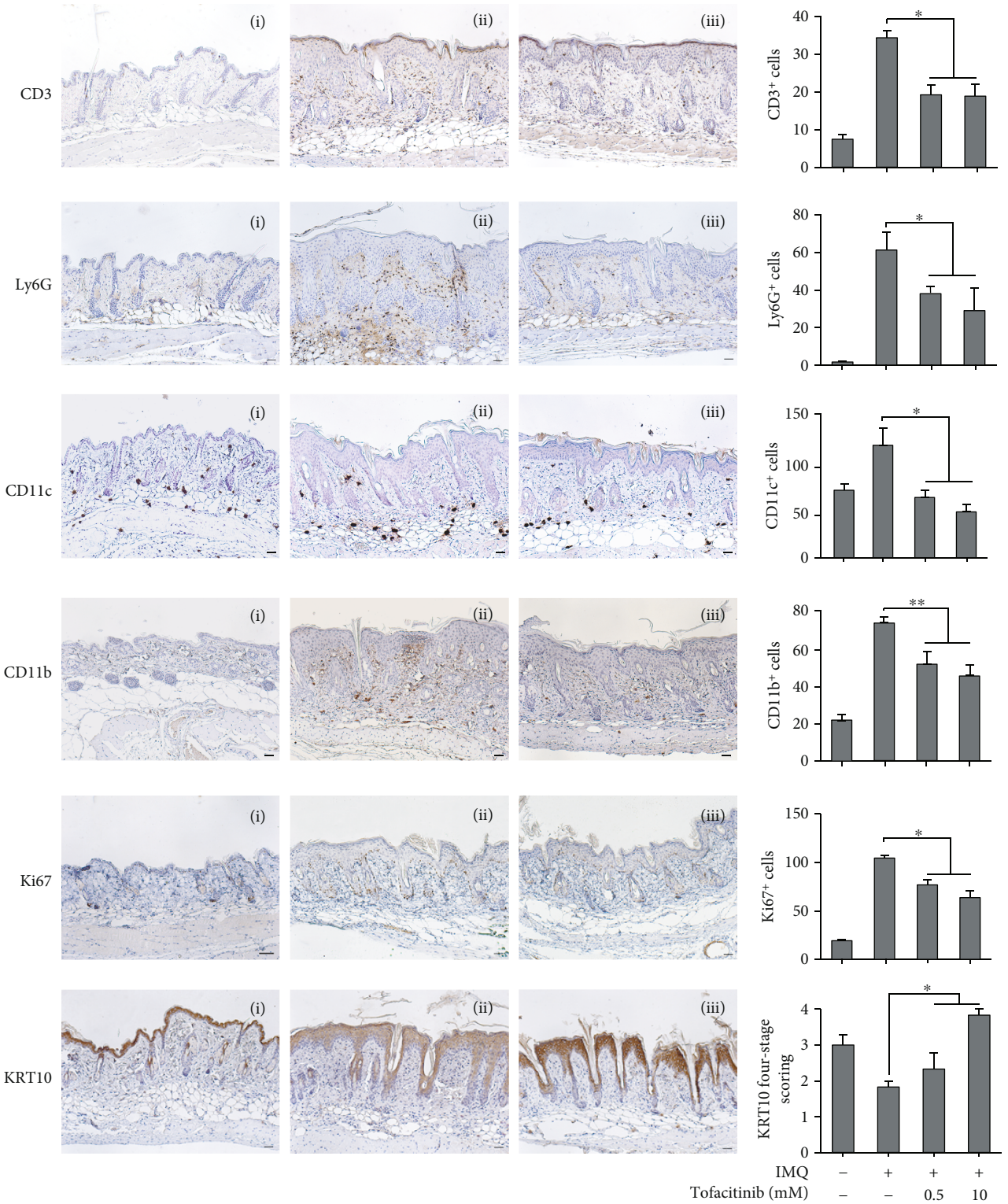


Figure 5: Tofacitinib counteracts IMQ-induced leukocyte infiltration, proliferation, and dedifferentiation in mouse skin. Immunohistochemistry analysis of mouse skin left untreated (i), IMQ-treated (ii), and IMQ-treated in the presence of tofacitinib (iii) shows reduction of positive CD3, LY6G, Ki67, CD11c, and CD11b cells and an increase of KRT10 in the epidermis after tofacitinib treatment. Sections were counterstained with Mayer's hematoxylin and were visually evaluated by a pathologist experienced in dermatology. Bars, 200 μM. One of four representative stainings is shown. Graphs show the mean of number of positive cells or of semiquantitative, four-stage scoring values for KRT10 ± SD *per* three sections *per* experimental group ($n = 10$ mice). $^{*}p < 0.01$, $^{**}p < 0.001$.

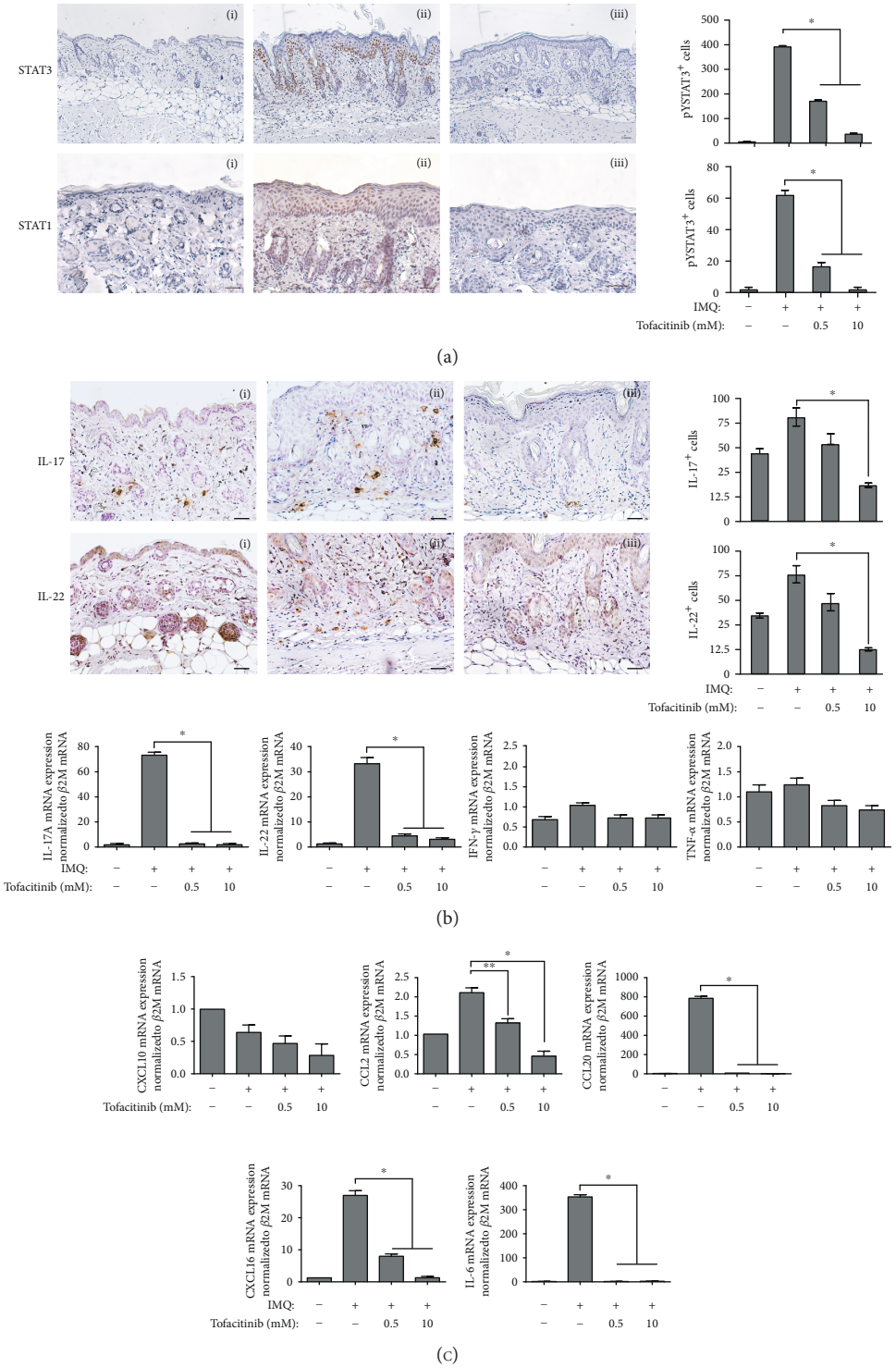


Figure 6: Tofacitinib counteracts IMQ effects in mouse skin. Immunohistochemistry analysis of mouse skin left untreated (i), IMQ-treated (ii), and IMQ-treated in the presence of tofacitinib (iii) shows reduction of STAT1-, STAT3-, IL-17A-, and IL-22-positive cells (a) and (b) after tofacitinib treatment. Sections were counterstained with Mayer's hematoxylin and were visually evaluated by a pathologist experienced in dermatology. Bars, 200 μM. One of four representative stainings is shown. Graphs show the mean number of positive cells $\pm$ SD *per* three sections *per* experimental group ($n = 10$ mice). $^{*}p < 0.01$. In (b), graphs show real-time PCR analyses of IL-17A, IL-22, IFN-γ, and TNF-α performed on pooled mRNA samples ($n = 10$) of mouse skin treated as indicated. $^{*}p < 0.01$. In (c), graphs show real-time PCR analyses of CXCL10, CCL2, CCL20, CXCL16, and IL-6 performed on pooled mRNA samples ($n = 10$) of mouse skin treated as indicated. $^{*}p < 0.01$, $^{*}p < 0.05$.

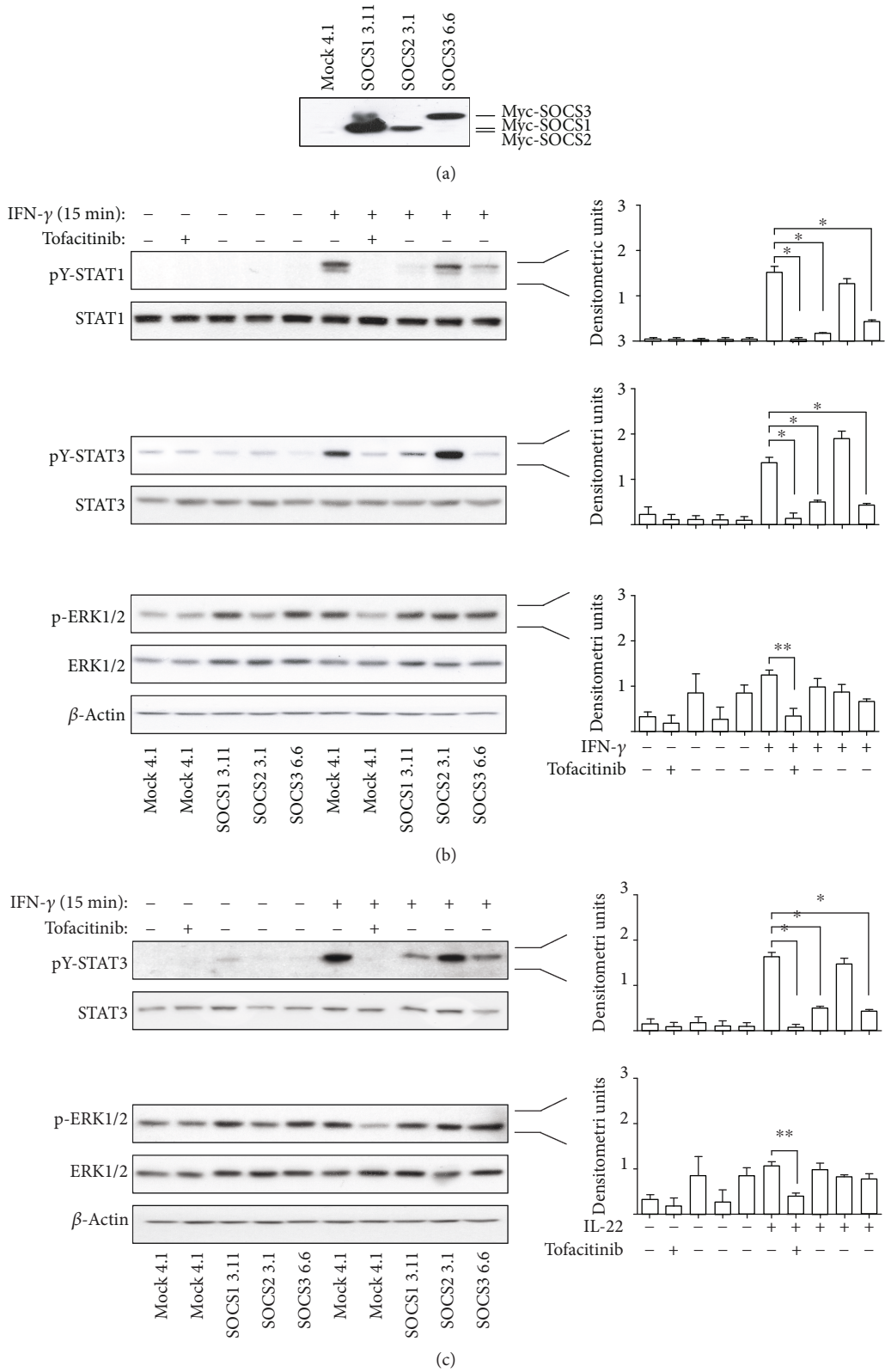


Figure 7: Tofacitinib inhibits the same IFN-γ- or IL-22-activated molecular pathways suppressed by SOCS1 or SOCS3. WB analysis was performed on protein lysates of HaCaT keratinocyte clones overexpressing SOCS1, SOCS2, or SOCS3, stimulated with IFN-γ (a) or IL-22 (b) or left untreated. Analysis was also performed on MOCK-transfected cells left untreated or treated with IFN-γ (a) or IL-22 (b), in the presence or absence of 5 μM tofacitinib. Both basal and phospho-STAT1, phospho-STAT3, and phospho-ERK1/2 were evaluated. Graphs show densitometric analysis of WB bands, and data are expressed means of densitometric units, calculated using two and four different keratinocyte clones for each transgene and mock clones to detect STATs and ERK1/2 proteins, respectively. $^{*}p < 0.01$, $^{**}p < 0.05$.

respectively (Figures 7(b) and 7(c)). Finally, the finding that phospho-ERK1/2 was efficiently inhibited in mock clones by tofacitinib was consistent with the absence of its upregulation in IFN-γ- or IL-22-treated SOCS1 and SOCS3 clones (Figures 7(b) and 7(c)). As expected, SOCS2 could not regulate molecular pathways triggered by IFN-γ and IL-22 in keratinocyte clones.

As a whole, these data demonstrate that tofacitinib similarly to SOCS1 and SOCS3, by targeting JAKs, can impair the same intracellular cytokine-dependent pathways in keratinocytes.

4. Discussion

Increasing evidence suggests that JAK proteins are a potential target for immunosuppressive drugs against psoriasis and other immune-mediated skin diseases, especially those elicited by epidermal keratinocytes exposed to massive amounts of proinflammatory cytokines, including IFN-γ and IL-22 [15, 16, 30]. In recent years, small molecule JAK inhibitors have been developed and extensively investigated for different pathological conditions [22, 24, 30]. Among them, the JAK inhibitor tofacitinib was shown to improve clinical outcomes in patients with moderate-to-severe psoriasis [19, 21, 24]. A recent study on its effects in psoriatic patients showed a dramatic and rapid shutdown of phospho-STAT1 and phospho-STAT3 and downstream-regulated genes in the epidermis and reduced pathologic T-cell and dendritic cell number in lesional skin, as well as expression of IL-17, IL-22, and IFN-γ [24].

The present study was aimed at understanding which inflammatory molecular pathway(s) activated can be specifically inhibited by tofacitinib in psoriatic keratinocytes. We found that this drug totally abrogated JAK/STAT pathways activated by IFN-γ and IL-22, as evaluated in experimental *in vitro* and *in vivo* models of psoriasis. These findings are important since IFN-γ and IL-22 inflammatory cytokines are deeply involved in the pathogenesis of psoriasis, as they stimulate keratinocyte proliferation, impair their differentiation, and promote a "feed-forward" inflammatory responses. Tofacitinib inhibition was exerted specifically on JAK1 and JAK2, but not on TYK2, and, as consequence, IFN-γ and IL-22 receptor phosphorylation, as well as the proximal cytokine signaling leading to STAT1 and STAT3 phosphorylation, were impaired. These effects were specific for IFN-γ and IL-22 and could not be observed on TNF-α or IL-17 signaling. This was not surprising, since TNF-α and IL-17 do not signal intracellularly through JAK/STAT and activate molecular pathways involving TRAF2/TRADD/NF-κB or TRAF2/TRADD/MAPK and Act1/TRAF6/NF-κB [11, 12]. In contrast, we observed that TNF-α induced STAT3 activation in keratinocytes, an effect that was partially inhibited by tofacitinib. This result could be explained by a direct action of tofacitinib on JAK-dependent signaling activated by TNF-α-induced cytokines (for instance IL-6), which could in turn activate STAT3 in an autocrine loop. Interestingly, tofacitinib also inhibited phosphorylation of ERK1/2 induced by IFN-γ or IL-22, but not that promoted by TNF-α. This dichotomy could depend by the fact that ERK1/2 activation by IFN-γ or IL-22 is mediated by JAK, whereas TNF-α-driven phosphorylation of ERK1/2 is downstream to TRAF2/TRADD [12].

A number of dysfunctional intracellular signaling pathways have been found in psoriatic keratinocytes other than STAT1 and STAT3, including NF-κB-, AP-1-, p38-, and ERK1/2 kinase-activated pathways [4]. An analysis of additional intracellular kinases and signaling node molecules demonstrated that tofacitinib also reduces the IFN-γ-dependent upregulation of Akt, AMPKα, p38, PARP, and caspase 3 and the IL-22-dependent Akt, AMPKα, mTOR, HSP27, p38, and JNK. However, tofacitinib inhibitory effects on these molecular pathways were minimal if compared to those observed on STAT1 and STAT3, indicating an ancillary or indirect action of JAK in upregulating such pathways. Again, TNF-α-induced intracellular kinase pattern could not be influenced by tofacitinib, apart from those pathways that were dependent on JAK and not by TRAF2/TRADD.

Another part of the study intended to evaluate the effects of tofacitinib on those biological processes that are profoundly altered in psoriatic epidermis (i.e., proliferation, differentiation, and apoptosis) as a consequence of the deleterious effects of IFN-γ and IL-22 [6, 17]. In this context, we demonstrated that tofacitinib reduced proliferation and dedifferentiation promoted by IL-22 in keratinocytes. These results were confirmed in the IMQ *in vivo* murine psoriasis model, in which epidermal hyperproliferation, altered differentiation, and inflammation were mainly IL-22/STAT3-dependent [29]. The concurrent treatment with tofacitinib led to reduced expression of epidermal STAT3, proliferation markers, and increased production of markers of differentiation. Interestingly, tofacitinib also counteracted the cytostatic and proapoptotic effects of IFN-γ on keratinocytes, likely *via* inhibition of STAT1, known to mediate these effects. However, inhibition of IFN-γ-dependent antiproliferative effects on keratinocytes might not be strategic in a hyperproliferative disorders, such as psoriasis, although IFN-γ can induce massive proliferation of psoriatic stem cells, and its injection into prelesional psoriatic skin causes epidermal hyperplasia and plaque development [31, 32]. IFN-γ signaling and type 1T cells were shown to participate to the expression of psoriasiform phenotype in IMQ mice only partially [28]. Nonetheless, we found phospho-STAT1 localized in the nuclei of epidermal keratinocytes of IMQ-treated skin, with tofacitinib totally inhibiting its expression. It is plausible that inhibition of STAT1, together with STAT3, is indirectly responsible for the reduction in inflammatory infiltrate, due to the decrease of STAT1- and/or STAT3-dependent gene expression of chemokines in keratinocytes, such as CXCL10, CXCL1, CXCL8, CCL2, and CCL5, and of immunomodulatory molecules, including ICAM-1 and MHC class I and II. As result, T-cell, neutrophil, dendritic cell, and macrophage subpopulations could no longer accumulate in IMQ-treated skin in the presence of tofacitinib. Also IFN-γ-induced, but not IL-22-induced IL-6 and IL-20, two psoriasis-related cytokines were inhibited by this drug. It is noteworthy that the majority of inflammatory molecules induced by IL-22 in keratinocytes could not be downregulated by tofacitinib, with the exception of CX3CL1 and CXCL8 chemokines. Also, IL-22-dependent

antimicrobial molecules could not be influenced. In contrast, SOCS3 mRNA expression was totally abrogated by tofacitinib, accordingly with our previous findings that STAT3-silenced keratinocytes were not able to upregulate SOCS3 in response to IL-22 [17].

Importantly, IL-17- and IL-22-producing cells were strongly reduced by JAK blockade in IMQ-treated skin, similarly to what observed in human psoriasis, where improvement of clinical and histologic signs by tofacitinib was associated with an inhibition of IL-17 gene expression and IL-23/Th17 pathway [24]. In contrast, neither IFN-γ nor TNF-α mRNA expression was influenced by tofacitinib. Immunohistochemistry analysis also showed that both IL-17^+ and IL-22^+ cells present in mouse skin had mostly a macrophage/dendritic cell-like morphology, accordingly with recent findings showing the presence and pathogenicity of IL-23-bearing and IL-17/IL-22-producing macrophage and dendritic cell subpopulations in the IMQ model [33]. In parallel, tofacitinib determined a strong reduction of keratinocyte-derived chemokines involved in the recruitment of pathogenic leukocyte populations *via* CCR2 or CCR6, such as CCL2 and CCL20. Although tofacitinib potently downregulated chemokine expression in keratinocytes and, in turn, leukocyte recruitment into mice skin, it is likely that its effect could be explicated directly on type 17 and 22T-cell differentiation, by interfering with IL-23R signaling and subsequent IL-17/IL-22 induction [23]. However, it is important to highlight the limited presence of IL-17- and IL-22-producing cells with a T-cell-like morphology in mouse skin at 5 days of IMQ application.

Finally, we demonstrated that tofacitinib and SOCS, in particular SOCS1 and SOCS3, by targeting identical signaling molecules, or JAK1 and JAK2, can impair the same intracellular pathways in keratinocytes. In fact, STAT1, STAT3, and ERK1/2 were not upregulated in keratinocyte clones overexpressing SOCS1 or SOCS3 in response to IFN-γ, nor STAT3 and ERK1/2 in response to IL-22, similarly to tofacitinib that abrogated cytokine-induced STAT1, STAT3, and ERK1/2 in control clones. These results are due to the fact that both tofacitinib and SOCS1/3 act on JAK with a high degree of kinome selectivity and display the same final biochemical effects of JAK inactivation. In fact, tofacitinib as well as SOCS1/3 impede auto- and trans-phosphorylation of JAK, with the first blocking ATP binding site of JAK1-2-3 and competing with ATP [34], and SOCS1/3 by interacting with the –GQM-amino acidic residues of JAK, determinant for its binding to substrates [35]. Importantly, while tofacitinib interacts with all JAKs but not with TYK2, SOCS1 and SOCS3 can bind and inactivate JAK1, JAK2, and TYK2 but not JAK3. Evidence that tofacitinib and SOCS1/3 can have the same anti-inflammatory effects on keratinocytes also comes from our recent studies performed with two small peptides mimicking SOCS1 and SOCS3 and sharing kinase inhibitory regions critical for JAK1 and JAK2 inactivation. Similar to tofacitinib, these two peptido-mimetics were able to switch off the IFN-γ- and IL-22-dependent inflammatory/immune responses of keratinocytes in cutaneous disease contexts characterized by the presence of IFN-γ-releasing Th1 and IL-22-releasing Th22 infiltrate, such as psoriasis and squamous skin cell carcinoma, respectively [15, 16].

5. Conclusions

As a whole, our study demonstrated the selectivity and specificity of tofacitinib inhibitory action on intracellular molecular pathways dependent on IFN-γ and IL-22 in keratinocytes. The blockade of IFN-γ/JAK1/JAK2/STAT1/STAT3 and IL-22/JAK1/STAT3 pathways had the important consequence to inhibit the expression of many IFN-γ-dependent inflammatory genes, as well as restore proliferative and differentiation programs altered by IL-22 in psoriatic keratinocytes. Considering that epidermal keratinocytes are the outermost component of the skin and that tofacitinib has a potent inhibitory effect on inflammatory responses evoked by these cells, it could be included in formulations for the topical therapy of psoriasis. Application of JAK inhibitors could be useful especially during the chronicization of the disease, where IFN-γ-dependent T-cell responses predominate. Indeed, the efficacy of topical therapy of tofacitinib and other JAK inhibitors in psoriasis has been extensively demonstrated [36] and is also considered for the treatment of other inflammatory skin conditions characterized by JAK hyperactivation, such as lichen planus and atopic dermatitis [37].

Importantly, tofacitinib inhibitory activity could also be explicated directly on type 17 and type 1T-cells, by impeding their differentiation and expansion. In fact, Th17 cell differentiation is abrogated in the absence of STAT3, whereas overexpression of a constitutively active STAT3 form results in greatly increased numbers of IL-17-producing cells [38, 39]. Similarly, STAT1 is abundantly activated in Th1 cells, mainly in response to IFN-γ, which is in turn critical to the generation and maintenance of Th1 immunity [23].

Due to the heterogeneity of pathogenic mechanisms operating in psoriasis, and to the variety of molecular cascades potentially activated by proinflammatory cytokines, a combination of JAK inhibitors and TNF-α or IL-17 blockers might elicit more favorable and efficacious therapeutic effects in psoriatic patients, by intercepting and blocking inflammatory responses at multiple levels.

Finally, local or systemic JAK/STAT inhibition by tofacitinib could be crucial for the development of optimized therapeutics also for the treatment of skin tumors characterized by aberrant IL-22 signaling and STAT3 activation in keratinocytes. The latter includes basalioma and squamous cell carcinoma, where IL-22-producing T cells aberrantly activate tumor growth and epithelial carcinogenesis through STAT3 (16).

Data Availability

All the data used to support the findings of this study are included within the article, with the exception of data concerning (1) tofacitinib effects on IL-17 signal transduction in cultured keratinocytes; (2) tofacitinib effect on phospho-STAT1 expression, as detected by using phospho-kinase array kit; (3) tofacitinib effects on TNF-alpha- or IL-17-

induced expression of inflammatory molecules by keratinocytes; and (4) tofacitinib effect on $CD11c^+$ dendritic cells infiltrating the dermis in IMQ-treated mouse skin. The latter data are available from the corresponding author upon request.

Conflicts of Interest

Cristina Albanesi won an Inflammation Aspire 2015 Research Award, a competitive grant program supported by Pfizer for Investigators in Europe. All other authors declare that they have no conflicts of interest.

Acknowledgments

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