Immunological consequences of cutaneous Toll-like receptor 2 signaling

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Yuliya Skabytska

aus Korsun-Schewtschenkiwsky (Ukraine)

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Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. med. Tilo Biedermann
2. Berichterstatter:	Prof. Dr. rer.nat. Hans-Georg Rammensee
3. Berichterstatter:	Prof. Dr. rer.nat. Carsten Schmidt-Weber

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1 Introduction

1.1 Skin as an interface and immunological organ

The skin is the largest organ at the interface between the environment and the host. Consequently, the skin has a central role in host defense[1]. It continuously encounters signals from the environment, which may act as triggers of inflammation. Different functional compartments of the skin translate these signals into immune responses, both of the innate and the adaptive immune system[2]. The skin displays not only a protective function as a physiological barrier, but it is also a site of initial recognition of foreign substances, where decisions about the induction or inhibition of an immune response take place[3]. The skin's innate immune system consists of three main components: anatomical/physical barrier (stratum corneum), cellular (antigen presenting cells, keratinocytes, mast cells, and PMNs) and secretory elements (antimicrobial peptides (AMPs), cytokines, and chemokines)[4]. It is now clear that the most effective anti-microbial response involves a balance between the innate and adaptive immune system[5].

The role of immune function of the skin is crucial, as immune dysfunction is implicated in the pathogenesis of a large variety of inflammatory skin disorders, including atopic and allergic contact dermatitis[6,7].

1.1.1 Atopic dermatitis is an inflammatory skin disease

Atopic dermatitis (AD) is a chronic inflammatory skin disease. It affects at least 15% of children and is characterized by cutaneous hyperreactivity to environmental triggers[6,8]. Various studies indicate that AD has a complex etiology, with activation of multiple immunologic and inflammatory pathways. Complex interactions among susceptibility genes, the host's environment, defects in skin barrier function and systemic and local immunologic responses contribute to the pathogenesis of AD[8]. As AD has increasing prevalence rates especially in western countries, a ''hygiene hypothesis'' has been generated. According to this hypothesis increased hygiene standards with less infectious diseases during early years contributes to the development of AD and other allergic diseases because of the absence of pivotal immune priming inducing immune tolerance[9,10]. Detailed characterization of AD inflammation reveals a biphasic cutaneous cytokine milieu with an initial recruitment

of IL-4-producing Th2 cells followed by a more mixed phenotype in the chronic phase[11-13].

The skin of most patients with AD is colonized with *Staphylococcus aureus (S. aureus)*. *S. aureus* can be isolated from clinically affected and unaffected skin, and both acute and chronic AD lesions are colonized. Staphylococcal colonization density is significantly lower in healthy individuals than in patients with AD and bacterial counts on unaffected skin are lower than on affected skin[14]. *Staphylococcus aureus* colonization is regarded as one of the most important initiating and exacerbating factors in AD[15,16].

Patients with AD have an increased propensity toward cutaneous viral infections. Infections by herpes simplex virus (HSV) referred to as eczema herpeticum[17]. Epidemiological data suggest that AD patients with more severe disease and with greater Th2 polarity are at greatest risk for skin infections with HSV or *S. aureus*[17,18].

1.1.2 *Staphylococcus aureus* as a skin pathogen

Staphylococcus aureus is a frequent pathogen on the human host, where it colonizes mucosal and dermal surfaces. *S. aureus* is able to cause a broad spectrum of infectious diseases from superficial cutaneous infections to the severe systemic sepsis[19]. *S. aureus* has a wide repertoire of virulence factors. For example, cell-surface proteins (including protein A) that promote adhesion to damaged tissue and to the surface of host cells[20], which is a prerequisite for colonization and disease. Virulence factors are crucial for development of staphylococcal infections, which make them important targets for the host immune system in order to generate immune responses. Some of the most important inducers of such immune responses are lipoproteins (Lpp). They belong to one of the major classes of cytoplasmic membrane-anchored proteins. Lpp are functionally important at the interface between the membrane and the cell wall. Many of them are part of ABC transporters. They are involved in nutrient uptake, in mediating antibiotic resistance and some of them have a role in protein folding[21]. One predominant staphylococcal Lpp is SitC[22], which is the binding component of the staphylococcal iron transporter SitABC. In immunological competent individuals, innate immune responses limit the establishment of the infectious disease, providing a rapid defense. Keratinocytes, which comprise 90-95% of the total epidermal cell population, play a pivotal role for the first defence. In addition to their function in the maintenance of the keratin barrier, they produce vast repertoire of cytokines, chemokines and AMPs[3]. Even a simple skin disruption or an

ultraviolet insult can initiate production of cytokines by keratinocyte[23]. The cytokines and chemokines further shape the local microenvironment by attracting and activating other immune cells. Both, keratinocytes and professional phagocytes such macrophages, neutrophils and dendritic cells, recognize *S. aureus*[19]. This recognition is managed by the binding of the bacterial surface of *S. aureus* to so called pattern recognition receptors (PRRs) on immune cells.

1.2 Pathogen associated molecular patterns (PAMPs) and pattern-recognition receptors (PPRs)

PRRs recognize highly conserved molecular patterns common to many classes of pathogens, known as pathogen associated molecular patterns (PAMP)[24]. PAMP are nucleic acids, lipids, lipoproteins, carbohydrates or peptidoglycans from bacteria, fungi or protozoa. PRRs are expressed constitutively by the host and they are germline-encoded. Both the epithelial barrier cells and resident innate immune cells in the skin express PRRs[7,25]. These innate responses occur rapidly and are efficient at killing pathogens, therefore limiting pathogen-derived tissue injury. Indeed, in the early hours after infection, activation of PRRs results in fast killing of pathogens either directly by the cells of innate immune system such as macrophages or indirectly by induction of proinflammatory responses mediated by the release of cytokines and chemokines. There are several classes of PRRs: Toll like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs).

1.2.1 Toll-Like Receptors

Among PRRs, Toll-like receptors (TLRs) are a well characterized family with distinct recognition profiles[26]. TLR1–10 are the best characterized human PRRs. The recognition of PAMPs by TLRs occurs in various cell compartments, including the cell surface (TLR1, 2, 4–6, 10) and endosomes (TLR3, 7–9). The TLR family members are expressed on the cell membranes of innate immune cells (DCs, macrophages, natural killer cells) and of adaptive immunity cells (T and B cells) and of non-immune cells (epithelial and endothelial cells)[27]. This fact emphasizes their function across the entire spectrum of innate and adaptive immunity. TLRs are believed to function as homo- or hetero-dimers. Most TLRs transduce a signal through the intracellular adapter molecule called myeloid differentiation factor 88 (MyD88), activating NF-κB

and other transcription factors, which results in the induction of pro-inflammatory cytokine genes[28].

TLR2 has emerged as a principle receptor for Gram-positive bacteria, especially *S. aureus*[29] and it is now known that staphylococcal Lpp are the major ligands for TLR2[21,30]. Purified native staphylococcal Lpp, including SitC, were shown to induce cytokines through the TLR2-MyD88 signaling pathway[31]. The use of *S. aureus* mutants deficient in maturation of lipoproteins (*Δlgt*) and improved Lpp purification methods show that TLR2 is activated by Lpp[29,30]. *In vivo*, different murine infection models showed that mice, deficient in TLR2, display increased susceptibility to staphylococcal infections with severe disease course, higher bacterial loads in tissue and/or reduced inflammation[32,33]. When compared to other TLRs, TLR2 recognizes a remarkably broad range of PAMPs. These include bacterial lipopeptides from Gram-positive bacteria and lipoarabinomannan from mycobacteria. This high diversity of ligand recognition by TLR2 comes possibly from its unique ability to homodimerize as well as heterodimerize with TLR1 and TLR6[34].

1.2.2 TLR2 heterodimers and ligands

Ozinsky et al.[35] were the first to show, that TLR2, unlike other TLRs, has to form heterodimers with TLR1 or TLR6, to be able to initiate cell activation. Studies using knockout mice identified TLR1 as the coreceptor required for the recognition of bacterial triacylated lipoproteins such as Pam3Cys[36,37]. Diacylated components such as lipoprotein FSL-1 and Pam2Cys interact with TLR2/TLR6 heterodimers[38,39]. Using fluorescence resonance energy transfer (FRET) in human primary monocytes, Triantafilou et al.[34] have shown that, a small preexisting population of TLR2 heterodimers increases rapidly upon ligand treatment. Additionally it was shown, that TLR2/6 ligand binding reduced the percentage of preformed TLR2/1 heterodimers but not vice versa. Employing lipid raft-disrupting agents[40], it was demonstrated that TLR2 heterodimers translocate to lipid rafts, depending on their interactions with specific ligands. The functional properties of *S. aureus* lipopeptides have been investigated in different cell types[39,41,42]. But data concerning functional consequences of activated different TLR heterodimers *in vivo* are sparse.

1.3 The adaptive immune response requires innate immune recognition

Recognition of PAMPs by TLRs and other pathogen receptors on skin cells initiates a signaling cascade, leading to activation of transcription factors activator protein (AP)-1 and nuclear factor (NF)-κB, which ultimately results in the production of pro-inflammatory cytokines, chemokines, AMPs and inducible enzymes in the skin. AMPs and chemokines have several effects. They repel infection by direct killing of the pathogen. Beside this, they are chemotactic for phagocytes and dendritic cells (DCs). Activation of phagocytes leads to triggering of the respiratory burst and killing of engulfed organisms[43]. DCs are the most important antigen presenting cells (APCs). Activated DCs migrate to skin draining lymph nodes and present antigens, captured in the skin, to antigen-specific T cells. This triggers the activation and proliferation of T cells[44,45]. Beside this, DCs direct the immune phenotypes of T cells, determining T cell polarization to the different Th subtypes. $CD4^+$ T cells are capable of differentiating into at least 4 distinct functional phenotypes: IFN-γ producing Th1 cells, IL-4 producing Th2 cells, IL-17 producing Th17 cells, and inducible regulatory T cells (Treg), which inhibit immune responses[46]. During this process DC-derived cytokines play the most important role. The differentiation of $CD4^+$ T cells into Th1 depends on IL-12, IL-4 induces Th2, whereas IL-23 together with IL-1β, IL-6 and with or without TGF-β induce Th17 cells. Furthermore, IL-10 is important for the inducible suppressive Tregs[47]. Activated T cells acquire effector functions and become effector T cells (Teff). They express new homing receptors, which direct their migration into the tissue where the antigen was detected. Once in this location, Teff cells produce cytokines that activate local cells, among them other immunological cells to control or eliminate the foreign material[48].

1.4 Mechanisms to limit inflammation

Overactivation of TLRs leads to the generation of strong pro-inflammatory signals with persistence of proinflammatory cytokines, such as TNFα and IL-6[49]. Probably the best known example of a dangerous inflammatory reaction during infection is the sepsis syndrome, in which generalized inflammation induced by overproduction of cytokines leads to hypotension, intravascular coagulation, multiple organ failure, which finally could lead to death[50]. Thus, mechanisms to terminate and limit cutaneous inflammation need to be effective. These regulatory feed-back

mechanisms involve induction of tolerogenic DCs, apoptosis of effector T cells, release of anti-inflammatory cytokines or activation of Treg cells[51]. In recent years Myeloid-derived suppressor cells (MDSCs) have been appreciated as one of the main cell populations responsible for regulatory immune responses, both adaptive and innate.

1.4.1 Myeloid-derived suppressor cells

MDSCs are a heterogeneous group of myeloid cells comprised of hematopoietic progenitor cells and precursors of macrophages, DCs and granulocytes[52]. In mice, MDSCs express both the myeloid lineage differentiation antigens Gr-1 (Ly6G and Ly6C) and αM integrin CD11b. In recent years several other markers have been used to describe specific subsets of these cells. Many MDSCs in tumor bearing mice coexpressed CD115 and CD124[53]. Macrophage marker F4/80[54] and costimulatory molecule CD80[55] have also been described on some subsets of MDSCs.

MDSCs were originally described as a population of cells that accumulates in the blood and lymphoid organs of tumor-bearing mice[52]. Expansion of MDSCs has been detected in almost all tumor models. However, MDSCs have been shown to regulate immune responses during other pathological situations including bacterial and parasitic infection, autoimmune pathologies and inflammation. An expansion of MDSCs was observed during infections with different microorganisms, such as Salmonella typhimurium[56], Trypanosoma cruzi[57], Candida albicans[58] or Toxoplasma gondii[59]. During a polymicrobial sepsis, MDSCs can induce the suppression and Th2-polarization of the T cell response. Among others, this activation of MDSCs is based on MyD88, an adaptor protein on different TLRs[60]. The induction of MDSCs during chronic inflammation is dependent on secretion of different interleukins like IL-1β[61] and IL-6[62].

A common feature for MDSCs is their high potential to suppress T cell responses. MDSCs from tumor-bearing animals have been shown to suppress $CD8^+$ cells[63]. Multiple mechanisms could be involved in this process. T cell apoptosis is one such mechanism[64]. MDSCs can cause immune suppression through inhibition of activation of T cells[65]. This can be achieved by TCR ζ chain downregulation[66,67] or by induction of peripheral tolerance[63] or by changes in the pattern of cytokines secreted by T cells[68]. In most cases, the effective suppression requires close cell-cell-contact[68]. This suggests that the involved cells interact either through membrane-bound molecules and/or through the release of rapidly degradable soluble mediators.

Nitric oxide (NO) is one of the main suppressive factors produced by MDSCs[68]. Many experimental settings show that T cell suppression by MDSCs depends on NO[57,69,70]. The inducible NO-synthase (iNOS) and arginase can generate NO from the amino acid L-arginine. The inhibition of iNOS and arginase abolished suppression by MDSCs. The generation of NO by MDSCs also needs cell-cell-contact[70]. NO is known to block the IL-2 signal transduction cascade in T cells[71]. Apoptosis of T cells induced by NO was also described[72]. Nagaraj et al. demonstrated a nitration of TCR leading to anergy as one mechanisms of $CD8^+$ T cell suppression by MDSCs[73].
Despite recent progress, the precise function of MDSCs in the context of inflammation and the mechanisms of MDSCs induction are not well-understood.

1.5 Aim of the thesis

The skin harbors an active immune network playing a crucial role in host defense and in shaping immune responses. The aim of this work was to investigate how the constant interaction of the skin with bacteria impairs the immune system. In particular it was important to dissect the functional consequences of TLR2 activation in the skin. Using various *in vivo* mouse models of cutaneous inflammation we mimicked different immunological situations. Cutaneous application of bacterial substances, bacterial lysates or living bacteria was used to imitate the contact between the skin and microorganisms. Investigation of AD patients, where this intense interaction of bacterial substances and the cutaneous immune networks is part of disease pathogenesis, further completed this study, providing clinical data.

2 Results

2.1 TLR2 ligands enhance Th2-mediated dermatitis

To investigate the impact of TLR2 ligands on AD inflammation in the early phase of AD was the aim of **S. Kaesler** in her work "**TLR2 ligands promote chronic atopic dermatitis through IL-4 mediated suppression of IL-10**". For this purpose a mouse model for acute AD inflammation was established. OVA-specific Th2 cells were adoptively transferred and activated in the skin of naive mice. In this model ear swelling correlates with antigen specific inflammation. Using *IL4*$^{-/-}$ cells and mice **Kaesler et al.** have shown that Th2-cell mediated dermatitis was dependent on IL-4, which is known as a dominant cytokine of human AD in the early phase of inflammation[13,74]. It is also known that *S. aureus* is a dominant trigger of AD[15,16]. Lipoproteins and lipoteichoic acid (LTA) were shown to be predominant staphylococcal TLR2 ligands[30,75]. Moreover, Travers et al demonstrated a correlation between the amount of LTA in AD lesions and AD aggravation[75]. Using LTA together with the lipoprotein Pam2 in the adoptive transfer experiments, the activation of TLR2 in combination with IL-4 enhanced and sustained cutaneous inflammation. These data suggest that in the early phase of AD, where Th2-mediated inflammation predominates, TLR2 ligands (from pathogenic *S. aureus)* cause a transformation of the cutaneous inflammation from a transient into a chronic, persistent form.

By experiments, where WT or *Tlr2*$^{-/-}$ Th2 cells were adoptively transferred into either WT or *Tlr2*$^{-/-}$ mice **Kaesler et al.** have found that this enhancement of dermatitis was independent of TLR2 on T cells. This indicates that the predominant target cells of pro-inflammatory TLR2 signals are skin resident cells, most likely DCs, because these cells are the most important APC in the skin. Searching for the underlying mechanism **Kaesler et al.** discovered that the concerted activation of TLR2 and IL-4-receptor on innate immune sentinels potently suppressed IL-10. IL-10 is the most important anti-inflammatory cytokine with immunomodulatory properties[76]. So we suggest that IL-10 suppression exacerbates Th2-mediated dermatitis and initiates the chronic phase of persistent inflammation.

Taken together, these data show that TLR2 activation on skin resident cells aggravates cutaneous inflammation through the binding of ligands from pathogen bacteria.

2.2 Non-pathogenic bacteria alleviate cutaneous inflammation

The skin is constantly colonized with bacteria, but detectable inflammation is rare in healthy individuals. This indicates that there must be mechanisms, which inhibit harmful inflammation. A recent double blind placebo controlled clinical trial, performed by the Department of Dermatology of the University of Tübingen, has given a hint about a possible mechanism. It demonstrated that the non-pathogenic microbe *Vitreoscilla filiformis* (Vf) abrogated cutaneous inflammation in AD patients when directly applied onto patients´ skin[77]. The aim of the study of **T. Volz et al. "Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells"** was to investigate the underlying molecular mechanism of this inhibition. For our *in vivo* experiments we first utilized a mouse model of AD, in which mice of the NC/Nga strain were sensitized to the allergen and hapten fluorescein isothiocyanate (FITC). NC/Nga mice are a specific strain that has been shown to develop AD-like skin lesions and clinical features most closely resembling human AD[78]. In this model ear swelling also correlates with antigen specific inflammation. The addition of Vf lysate during several episodes of allergen contact showed significantly reduced ear swelling after allergen challenge, indicating a reduction of skin inflammation. *Ex vivo* antigen specific stimulation of draining lymph node revealed a reduction in T cell proliferation in Vf-treated mice. Consistently, FITC-specific IFN-γ production by T cells, which is the hallmark cytokine of chronic AD[13,74] was also significantly reduced. Beside this, only T cells from mice previously exposed to Vf displayed antigen specific production of IL-10, whereas control mice failed to do so. IL-10 is the most important anti-inflammatory cytokine with multifunctional properties depending on cell types and settings[76]. It was known that IL-10 production by DCs contributes to the induction of tolerance[79]. *In vitro* investigations, conducted by **T. Volz**, further dissected the mechanism of this immune inhibition: Vf signals induced high levels of IL-10 and reduced the production of IL-12p70 in human and mouse DCs. Experiments with DC-T cell co-cultures demonstrated that these IL-10$^+$ DCs induced IL-10$^+$ Treg cells, which efficiently suppressed effector T cells. Investigations of innate immune pathways, activated by Vf, revealed that IL-10 production by DCs was completely dependent on TLR2.

Thus non-pathogenic bacteria could induce tolerogenic immune responses to resist the harmful inflammation. One may speculate that in the absence of a balance

between signals of non-pathogenic and pathogenic bacteria inflammation is induced due to functional dominance of signals of pathogenic bacteria. This situation could be true for inflammatory skin diseases such as AD.

2.3 Staphylococcus aureus-derived lipoteichoic acid suppresses T cells

As described before, **Kaesler et al.** found the aggravation of cutaneous inflammation by TLR2 ligand LTA. Interestingly, in another model of cutaneous inflammation we observed an opposite effect. In the model of contact hypersensitivity (CHS) to the weak hapten FITC, **Chen et al.** mimicked FITC-induced and T cell-mediated mild cutaneous inflammation described in the work **"*Staphylococcus aureus*-derived lipoteichoic acid induces temporary T cell paralysis independent of TLR2**". The cutaneous inflammation of this mouse model resembles the immune situation in non-lesional skin of AD patients. To our surprise, and in contrast to the work of **S. Kaesler**, additional exposure to LTA did not significantly amplify ear swelling. Therefore we investigated the dynamics of T cell cytokine expression in FITC CHS. Upon exposure to LTA, cutaneous IL-4 and IFN-γ mRNA expression was suppressed. Moreover, *ex viv*o T cell proliferation of draining lymph nodes was strongly inhibited. This indicated a possible direct effect of LTA on T cells. Therefore the properties of LTA were then further investigated *in vitro* in respect of their impact on T cell proliferation. We found that, in contrast to Pam2, LTA treatment led to significant suppression of T cell proliferation *in vitro*. This suppression was independent of the mode of T cell activation (antigen specific activation, unspecific activation by anti-CD3/28, activation by mitogen as PMA/Iono, activation by superantigen as SEB) and this suppression was found in both, mouse and human, T cells. Further experiments revealed that the LTA-exposed T cells were still viable and that the effect was not mediated by apoptosis. Moreover, the T cells remained fully responsive to subsequent stimulation. Interestingly, the effect was independent of TLR2 signaling.

So we found two opposing functions of LTA: **Kaesler et al.** showed an aggravation of inflammation due to LTA (pro-inflammatory effect); in contrast, the work of **K. Chen** showed clearly that LTA suppress T cell proliferation (anti-inflammatory effect). Therefore in the next two experiments we wanted clarify the conditions, under which these effects could be relevant *in vivo*. To investigate the effect of LTA on T cells *in*

vivo, we needed a model with increased frequency of antigen-specific activated (effector) T cells. This was achieved by using donors for FITC specific T cells. Mice serving as donors were sensitized with FITC and draining lymph nodes and spleens of these mice were isolated. $CD3^+$ T cells were then sorted and transferred into recipient mice, which were previously sensitized with FITC. The next day recipients were challenged with FITC or vehicle control and exposed to either LTA or PBS in addition. Interestingly, when challenged with vehicle only, in the absence of antigen, LTA elicited an ear swelling response, indicating direct pro-inflammatory effects of LTA. However, in the presence of antigen and T cell activation, LTA significantly reduced cutaneous inflammation (anti-inflammatory effect), possibly through direct inhibition of T cells.

These data indicate that LTA may function as a TLR2 ligand on skin resident cells, activating the innate immune system and leading to inflammation. In contrast, LTA suppresses T cell proliferation directly in a TLR2 independent manner. So we further hypothesize that an early innate response is mediated by pathogen recognition receptors and results in inflammation in order to fight bacteria quickly. Whereas during the later immune responses, where the T cell-mediated immune response evolves, inflammation could be harmful and should be terminated, for example by LTA which controls T cell activation. The latter is transient to avoid prolonged periods of immune suppression.

2.4 Cutaneous sensing of TLR2/6 ligands suppresses T cell immunity

2.4.1 Cutaneous exposure to TLR2/6 but not to TLR2/1 ligands ameliorates T cell-mediated recall responses

Further investigations of the role of TLR2/6 versus TLR2/1 ligands in immune response and in cutaneous immunity was one of the goals of the work of **Skabytska et al. in "Cutaneous innate immune sensing of TLR2/6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells"**. In this work human AD and murine models were used to investigate the immune consequences of a cutaneous encounter with dominant PAMPs of Gram-positive bacteria. To investigate T cell-mediated cutaneous inflammation we used a mouse model of contact hypersensitivity to FITC. Mice were sensitized with FITC, and one week later, the subsequent encounter with FITC was complemented by cutaneous application of

the lipopeptide Pam2. The functional consequences of the combined antigen and Pam2 encounter were evaluated 5 days later by applying FITC to the ear's skin. In FITC-sensitized control mice, the peak of T cell mediated CHS was determined by ear swelling thereafter. In contrast to our expectations, previous cutaneous exposure to the TLR2/6 ligands Pam2 and FSL-1 did not enhance, but almost completely abrogated FITC CHS and FITC-specific *ex vivo* T cell proliferation. Interestingly, and in contrast to Pam2, the TLR2/TLR1 ligand Pam3 failed to suppress FITC CHS and T cell proliferation. Next we wanted to know, whether bacterial lipopeptides from pathologically relevant bacteria have the same consequence for immune responses. Therefore we established a mouse model of epicutaneous bacterial colonization in the work of **Wanke at al. "*Staphylococcus aureus* skin colonization is promoted by barrier disruption and leads to local inflammation"**. This model is especially well suited for investigations of the natural route of skin colonization. In this mouse model the bacteria are not needed to be injected sub- or intra-cutaneously into the skin, but are applied onto the skin epicutaneously which resembles the natural way of skin infection. The integrity of the skin was previously affected by skin barrier disruption due to tape-stripping. This is also similar to the situation in AD, which is characterized by skin barrier defects[6,80]. The wild type (WT) mice were shaved and the skin was disrupted by tape-stripping of different strength (mild and strong), which however did not create wounds of the skin (confirmed by histological analysis). Living *S. aureus* bacteria were added to filter paper discs, placed onto the skin, and covered by Finn Chambers on Scanpor. Then the fixation was done by a stretch plaster. After overnight occlusion, Finn Chambers and plasters were removed to allow the mice to clean the skin and to ensure that the observed bacteria colonize the skin and are not only present on the skin surface. The analysis of colony-forming units (CFU) thereafter revealed that bacteria persist on the skin during at least 6 days (the longest observation time) and that the infection efficiency and the persistence of *S. aureus* was significantly higher in strongly tape stripped skin, compared to non- or mildly tape stripped skin. This suggests that epithelial barrier defects facilitate cutaneous *S. aureus* colonization, which is in accordance to further AD studies[80]. We also found an indication of enhanced cutaneous inflammation at the sites of *S. aureus* colonization: RT-PCR analysis of the infected skin revealed a significant elevation of all investigated cytokines (IL-1β, IL-6, TNF-α, IFN-γ) and induction of antimicrobial peptide (AMP) expression in comparison to non-infected skin. This

indicates that *S. aureus* application and persistence on barrier-disrupted skin induces an inflammatory cytokine response and that our model is suitable for investigation of cutaneous inflammation following bacterial colonization. Next we combined this model with the FITC CHS model to investigate whether living *S. aureus* on the skin also cause immune suppression. To clarify the role of lipoproteins in this process we additionally used lipoprotein-deficient *S. aureus* mutant (*Δlgt*) bacteria. Bacteria were applied during FITC re-exposure of FITC-sensitized mice. Similar to the TLR2/6 ligands, wt bacteria, but not lipoprotein-deficient *S. aureus* caused immune suppression.

These data show for the first time that cutaneous exposure to bacterial TLR2/TLR6 ligands is sufficient to cause systemic immune suppression.

2.4.2 Skin infection-induced immune suppression is mediated by $Gr1^+CD11b^+$ myeloid-derived suppressor cells

Investigating the cells which could mediate the immune suppression following cutaneous TLR2/6 ligands exposure, we found a strong increase of splenic $Gr1^+CD11b^+$ cells only in these experimental conditions. Immature $Gr1^+CD11b^+$ cells are known as myeloid-derived suppressor cells (MDSCs) because of their ability to suppress T cell activation. Indeed, FITC specific *ex vivo* T cell proliferation was impaired in animals previously exposed to TLR2/6 ligands. We next investigated patients with AD as a model for massive cutaneous innate sensing of Gram-positive bacteria. In humans, MDSCs are typically described as $CD11b^+CD33^+HLA\text{-}DR^-CD14^-$ cells[52]. Compared to healthy donors we observed a significant increase of MDSCs in the peripheral blood. Importantly, the upregulation of human MDSCs in peripheral blood was very impressive in patients with severe dermatitis and eczema herpeticum, which is a severe cutaneous viral infection resulting from immune suppression.

In FITC CHS, T cells migrate to the skin and elicit FITC specific dermatitis. Therefore, we wondered whether MDSCs were also recruited to the skin. Indeed, 8 h after FITC application $Gr1^+CD11b^+$ cells were significantly increased in the skin of mice exposed to Pam2. Similarly, we investigated infected human skin and found a significant increase of MDSCs in AD in comparison to healthy skin, indicating that bacterial colonization and subsequent skin inflammation induces MDSCs accumulation in the skin also in humans.

2.4.3 Suppression of T cell activation by MDSCs induced by cutaneous innate immune sensing

Recruitment of MDSCs to the skin suggested a MDSC-mediated suppression of T cell activation in the skin *in vivo*. Indeed, FACS-analysis of ear skin tissue following the final FITC-exposure revealed a significant decrease of $CD3^+$ T cells and IFN-γ-production following Pam2 exposure. To better explore, how this MDSCs induced immune suppression is mediated, we isolated MDSCs 10 days after Pam2 exposure. MDSCs are known to be a very heterogeneous cell population with at least two cell subpopulations with different suppressive properties[53,81,82]. Monocytic $Ly6C^+$ MDSCs have been described as more suppressive compared to granulocytic $Ly6G^+$ MDSCs[53,83]. Therefore we isolated these two MDSCs populations and co-cultured them with naïve splenocytes at different ratios to prove their suppressive activity. Naïve splenocytes were activated with anti-CD3/CD28 and their proliferation was analyzed. Following co-culture with $Ly6C^+$ MDSCs at a ratio of 2:1, almost complete suppression of T cell proliferation was observed, while $Ly6G^+$ cells from our model were not suppressive. MDSCs´ immunosuppressive activity was reported to be a result of the activation of inducible NOS (iNOS) and arginase 1, leading to L-arginine depletion and increased production of NO[68]. Indeed, $Ly6C^+$ MDSCs from Pam2-exposed animals produced high levels of NO. NO production and T cell suppression by $Ly6C^+$ MDSCs was completely abrogated in a transwell experiment, indicating that physical contact of MDSCs with T cells as it is likely happening in the skin and MDSC activation by T cells is a prerequisite for MDSC´s NO production and MDSC-mediated immune suppression. To collect further evidence, we investigated PBMCs of AD patients and were able to detect a distinct $iNOS^+$ population of $CD11b^+CD11c^-$ cells which most likely are NO producing MDSCs. These cells were completely absent in healthy individuals. Moreover, using three color fluorescence immunohistology in AD skin samples, we also detected $iNOS^+CD11b^+CD11c^-$ cells. To investigate the evidence of MDSCs mediated immune suppression in humans, we then analyzed peripheral blood of AD patients and found T cell receptor ζ chain significantly down-regulated, which is known to be a general characteristic of immune suppression and one of the major features of MDSC-mediated T cell inhibition[66,67]. To further investigate whether human MDSCs were suppressive, we depleted $CD11b^+$ cells from PBMCs and analyzed proliferation of T cells. In almost all healthy volunteers (7 of 8) CD11b depletion resulted in reduced T cell proliferation, on

contrary, this was only observed in one out of 7 AD patients. This finding demonstrates that MDSCs, which are present among the $CD11b^+$ population in AD patients but not in healthy individuals, are immunosuppressive.
These data, together with the findings of increased numbers of MDSCs in AD skin and elevated T cell proliferation following MDSCs depletion, indicate that MDSCs are not only increased in AD blood and skin, but also exert their suppressive activity allowing e.g. herpes viruses to spread.

2.4.4 Pam2-induced immune suppression is dependent on cutaneous TLR2

Since our data showed that cutaneously induced MDSCs are potent suppressors of T cell mediated immune responses, it was of major interest, to explore how innate immune sensing in the skin initiates MDSCs. Therefore, we next determined the role of TLR2. *Tlr2*$^{-/-}$ and wild type mice were treated with or without cutaneous Pam2 exposure. Previous Pam2 exposure inhibited FITC CHS in wt mice. Conversely, Pam2 exposure in *Tlr2*$^{-/-}$ mice failed to inhibit FITC-specific CHS and T cell proliferation. Accordingly, MDSCs accumulation and systemic reduction of T cells was not detectable in *Tlr2*$^{-/-}$ mice previously exposed to Pam2. Cutaneous innate immune sensing through TLR2 may act through resident skin cells or recruited immune cells. Thus, mouse chimeras were generated to distinguish if TLR2 sensing is managed by skin resident or by recruited hematopoietic cells. Wild type mice reconstituted with wild type bone marrow (BM) (WT + WT-BM) and wild type mice that obtained *Tlr2*$^{-/-}$ BM (WT + *Tlr2*$^{-/--}$-BM) upregulated MDSCs following Pam2 exposure. In contrast, *Tlr2*$^{-/--}$ mice reconstituted with WT-BM (*Tlr2*$^{-/-}$ + WT-BM) failed to accumulate MDSCs, similar to control *Tlr2*$^{-/--}$ mice with *Tlr2*$^{-/--}$ BM (*Tlr2*$^{-/--}$ + *Tlr2*$^{-/-}$-BM). Thus, TLR2 expression on skin resident cells is necessary and sufficient for accumulation of MDSCs.

2.4.5 IL-6 is required for Pam2-induced immune suppression

Our previous experiments have shown that cutaneous Pam2 sensing through TLR2 is sufficient to induce MDSCs and consecutive suppression of cutaneous recall responses. To identify the underlying mechanisms, we analyzed cutaneous mRNA expression following the application of FITC with or without Pam2 or Pam3 in

sensitized mice. Interestingly, both Pam2 and Pam3 unequivocally and moderately upregulated TNF and CXCL2 mRNA compared to FITC-only treated mice. Strikingly, upregulation of IL-6 mRNA in the skin was most pronounced following Pam2 exposure. In comparison with skin following FITC-only or FITC-plus-Pam3 exposure, cutaneous Pam2 exposure induced a 400-fold upregulation of IL-6 mRNA. To regulate MDSC induction in the bone marrow, cutaneous IL-6 must reach the blood stream. Indeed, IL-6 concentrations in mouse sera strongly increased one day after cutaneous Pam2 exposure. These data suggest that IL-6 plays a crucial role in Pam2 induced MDSC induction; therefore, *IL6*$^{-/-}$ mice were investigated. In contrast to WT mice, cutaneous Pam2 exposure in *IL6*$^{-/-}$ mice failed to reduce FITC-specific CHS, and no induction of MDSCs could be detected.

Taken together, these data suggest a scenario in which Pam2 is sensed by TLR2 on skin-resident cells, leading to the expression and secretion of IL-6 in such high amounts that MDSCs expand and accumulate, causing systemic immune suppression, which leads to the inhibition of cutaneous recall responses.

2.5 IL-4 abrogates T cell-mediated inflammation by the silencing of IL-23

IL-4 is another pleiotropic cytokine, similar to IL-6. In the work described above, **Kaesler et al.** have found that IL-4 is a key cytokine for AD aggravation. On the other hand, Ghoreshi et al. has shown an improvement of cutaneous inflammation in humans in another skin disease (psoriasis) by IL-4 therapy[84]. This indicates a complex way of IL-4 function and its interaction with other cytokines *in vivo*. Recently it has become evident, that IL-17 is a key cytokine in the pathogenesis of psoriasis[85,86]. Therefore the hypothesis of the work of **Guenova et al.** "**IL-4 abrogates Th17 cell-mediated inflammation by selective silencing of IL-23 in antigen-presenting cells"** was to ask, if IL-4 could affect IL-17 directly or indirectly for example by targeting the polarization of T cells. DCs and their cytokines determine the quality of an immune response[45]. In particular, they direct the phenotype of T cells[87]. *In vitro* experiments with DCs of **E. Guenova** have given a hint about the mechanism of IL-4-mediated suppression of T cell-mediated inflammation. They have shown that addition of IL-4 to different human DCs cultures caused a strong reduction of IL-23 with simultaneous induction of IL12p70, which was visible on RNA level as well as on protein level. IL-23 is crucial for the

polarization and maintenance of Th17 cells. Consequently the subsequent DC-T cell coculture revealed that IL-4 treated DCs failed to induce IL-17 producing Th17 cells. Instead, $CD4^+$ T cells developed a highly polarized Th1 phenotype with high IFN-γ production. The analysis of human skin by histological staining and RT-PCR has detected a significant enhancement of both IL-23 and IL-17 in psoriatic skin. Consistent with *in vitro* data, the IL-4 therapy caused dose-dependent reduction of these cytokines. Simultaneously, IL-12 was induced. These data indicate that IL-4 affected DCs change by re-programming their phenotype to low IL-23 and high IL-12 producers with a reduction of Th17 cells as a consequence. These *in vitro* data had to be proven in an *in vivo* mouse model. We used the 2,4,6-trinitrochlorobenzene (TNCB)-induced delayed type hypersensitivity reaction (DTHR), a suitable model for investigation of IL-17-mediated cutaneous inflammation. Systemic administration of IL-4 to TNCB-sensitized mice reduced cutaneous inflammation (detected as ear swelling). An RT-PCR analysis of the inflamed tissue, following the IL-4 therapy, showed a strong reduction of *IL23A* and of *IL17A* in the ear tissues of mice challenged with TNCB. To directly test whether IL-4 prevented inflammation and the related DTHR primarily by suppressing IL-23, we treated sensitized mice with IL-4 during a TNCB challenge, and one group of IL-4-treated mice was treated with recombinant mouse IL-23. The IL-4 therapy severely suppressed IL-23 levels and the DTHR almost to background levels. In sharp contrast, replacing the missing IL-23 fully restored the cutaneous DTHR.

The *in vitro* DC data and the results of the human study strongly suggested that DCs are the key cells, which are affected by IL-4. But we could not exclude that IL-4 also targets other cell types (for example T cells) *in vivo*. To find the mechanism of IL-4-mediated immune regulation *in vivo*, we generated bone marrow (BM) chimeric mice, in which IL-4 signaling is selectively blocked in either T cells or DCs. We generated BM chimeric mice with hematopoietic cells consisting of *Stat6*$^{-/-}$ and *T cell*$^{-/-}$cell mix (*Stat6*$^{-/-}$/T cell-deficient mice). STAT6 is an important molecule for the IL-4R signaling pathway; therefore STAT6-deficient mice could be considered as IL-4 signaling deficient[88]. After transplantation those chimeric mice only harbored T cells that are deficient of STAT6 and therefore unresponsive to IL-4 therapy. When challenging the sensitized chimeric mice, we observed a comparable ear swelling in all the chimeric mice. This demonstrates that the beneficial effect of IL-4 in cutaneous inflammation is not mediated by T cells, but rather by DCs. To prove this, we generated BM chimeric

mice with hematopoietic *Stat6*$^{-/-}$/*MhcII*$^{-/-}$ cell mix (*Stat6*$^{-/-}$/*MhcII*$^{-/-}$ mice). Those mice have STAT6 negative DCs (MHC-II^{+}), in which IL-4 signaling is impaired, whereas the STAT6^{+} T cells remained responsive to IL-4. When we treated the *Stat6*$^{-/-}$/*MhcII*$^{-/-}$ mice with IL-4 during the challenge phase, cutaneous inflammation was significantly reduced to the levels comparable to non-treated mice. These data thus indicate that the anti-inflammatory effect of IL-4 is directly mediated via DCs and not by T cells.
Taken together, the data of this study show that IL-4 inhibits inflammation by suppressing the capacity of DCs to produce IL-23. Depleted IL-23 levels then cause lower numbers of Th17 cells, which leads to increased skin inflammation and induce tissue damage in psoriasis.

In conclusion, we identified that cutaneous TLR2 signaling has multifunctional consequences on skin immunity and systemic immune responses. TLR2 activation can cause both, an amplification of cutaneous inflammation and an immune suppression due to induction of IL-10 production, of regulatory T cells and of myeloid derived suppressor cells, which finally leads to suppression of dermatitis. In addition we identified how IL-4 regulates immune responses on the level of DCs.

3 Discussion

The central aim of this work was to investigate the consequences of cutaneous TLR2 activation. We have not found one definitive answer to this very complex question. Our data suggest that TLR2 ligands can both induce and aggravate inflammation, but also that innate immune sensing of TLR2 ligands can cause opposite effects of immune suppression or even tolerance. It seems to depend on the particular immune situation, the immune phase and the strength of cutaneous inflammation, the combination with cytokines, the cell types activating and interacting and many other yet unknown factors. Therefore we used different *in vivo* and *in vitro* models to mimic various immune situations. These experimental settings were necessary to dissect the wide consequences of interactions between the host and microorganisms on the skin interface.

3.1 TLR2 signaling causes aggravation or alleviation of AD

S. aureus has been suggested as one important AD trigger and TLR2 ligands, lipoproteins and LTA, are its predominant components[16,75]. The work of **S. Kaesler et al.** confirmed this hypothesis and demonstrated how the aggravation of cutaneous inflammation due to *S. aureus* occurs: the combination of the early AD cytokine IL-4 and activation of TLR2 on skin resident cells caused an inhibition of anti-inflammatory IL-10 and consequently the aggravation and chronification of AD. Interestingly and intriguingly **T. Volz et al.** found that TLR2-dependent activation of DCs by components of non-pathogenic microbe *Vitreoscilla filiformis* resulted in an opposite effect. In this context IL-10 is not suppressed but induced and serves as key cytokine in alleviating cutaneous inflammation. Both effects are confirmed by clinical studies with AD patients. Staphylococcal TLR2 in AD lesions have been shown to correlate with AD severity[75]. The beneficial effect of non-pathogenic bacteria is well-known[9,10] and cutaneous applicated *Vitreoscilla filiformis* has been found to ameliorate AD[77]. Thus, what makes the difference? The first obvious difference of these two studies is the pathogenicity of the investigated bacteria. It is known that non-pathogenic bacteria induce tolerance[10,89] and some bacteria have evolved tolerance induction as evasion strategy[90,91]. But it is still enigmatic how the same innate signaling via TLR2 results in even opposing immune consequences. As, in a complex, physiological, immune situation, the innate immune system does not only recognize one single

TLR2 ligand, but it is rather a constellation of sensing via multiple PPRs, i.e., LPS and TLR4, RNA and TLR3, CpG DNA motifs and TLR9, etc. and thus their combination may be determining the outcome. It should also not be excluded that xenogeneic signals (delivered through a currently undefined mechanism) might synergize with microbial exposure for these effects. Another explanation could be that the strength of the innate immune signaling plays a role. A constant mild inflammation could be interpreted from the host immune system as a stimulus to counteract, similar to the phenomenon of T cell anergy due to a low affinity antigen.

The other difference between these two works is the immune situation investigated. **S. Kaesler et al.** analyzed an early AD phase with predominant IL-4, whereas the subject of **T. Volz`** work is rather advanced cutaneous inflammation, where IFN-γ is a key cytokine. Thus, the interaction of different cytokines with TLR2 ligands during immune sensing could represent another level of immune regulation.

Overall, these data suggest that the imbalance of non-pathogenic and pathogenic bacteria on AD skin contributes to skin inflammation. Non-pathogenic microorganisms tend to induce tolerance in healthy skin, thus avoiding harmful inflammation.

3.2 Lipoteichoic acid has opposing immunological functions

Interestingly, we identified one substance which displays opposing immunological functions depending on the target cell type. As described above, **S. Kaesler et al.** showed pro-inflammatory function of LTA on APCs, most likely on DCs. The anti-inflammatory function of this substance was shown by **K. Chen et al.** who investigated the function of LTA on T cells. T cells are the dominant cells in the adaptive immune system and they mediate cutaneous inflammation in the later phase of defense against bacteria[19]. Unexpectedly **K. Chen** found that LTA suppressed T cell proliferation is independent of TLR2. A similar function of LTA was shown in platelets where it inhibited platelet aggregation[92] It has also been reported that bacterial components from *S. aureus* inhibited fibroblast proliferation *in vitro*[93] and one *in vivo* study also reported inhibitory properties of LTA[94]. Several non-pathogenic microorganisms such as *Staphylococcus epidermidis* also contain LTA. Therefore the anti-inflammatory function of LTA could be explained as an evasion mechanism evolved by pathogens during the evolution. In addition, our *in vivo* experiments suggested another possible explanation for LTA functions. We hypothesize that in

the early phase of inflammation sensing of LTA by the innate immune system is beneficial to the host, acting pro-inflammatory to fight the bacterial invasion. In the later stage, where the inflammation is rather harmful, LTA acts on T cells as an anti-inflammatory agent, helping to terminate ongoing immune responses.

3.3 TLR2 heterodimers show functional differences

As mentioned before, the immune responses to microorganisms are very divers and complex, partially because of the presence of a large variety of PAMPs. In the work of **Skabytska et al.** we have taken advantages of some microbial derived molecules, which are exclusively bound by one specific TLR2 heterodimer. The result of this work is, that different TLR2 heterodimers differ in their immunological functions *in vivo*. Cutaneous exposure to TLR2/TLR6 but not TLR2/TLR1 ligands induced systemic immune suppression. With these data we have shown for the first time such distinct functional differences for ligands of the two TLR2 heterodimers *in vivo.* This suggests that the presence of certain TLR ligands, the ratio of different TLRs within a cell or a possible interaction between TLR2 and TLR1 or TLR6 defines the nature of consecutive immune responses. Variety of receptor specificity achieved by combination of different TLR receptors could be beneficial to the host cell, as, the structure of bacterial Lpp is not constant in each bacterium. It was shown recently, that the degree of Lpp-acylation depends on environmental factors and growth phase. Lipoprotein SitC was triacylated when *S. aureus* was in the exponential growth phase at neutral pH and diacylated in the post exponential phase at low pH[95]. At the situation on the skin, where pH is low and chronic *S. aureus* colonization (which is almost always found in AD) is present, a post exponential growth phase of *S. aureus* can be assumed. Consequently, Lpp from *S. aureus* on the skin are more diacylated. Based on our data, we hypothesize that diacylation of Lpp could have immune suppressive effects as a consequence. Further, one can also assume that pathogenic and non-pathogenic skin microflora may have different acylation properties and therefore different compositions of TLR2 ligands and thus overall differ in their immune consequences.

Our data further create a more detailed understanding about mechanisms functional in MDSC induction driven by infections. Signaling through MyD88 was described to be required for the complete expansion of MDSCs, however the exact cascade of

events was not investigated[60]. Data from our chimera experiment indicate that TLR2 predominantly on skin resident cells and not on hematopoietic cells is necessary and sufficient for the accumulation of MDSCs in the spleen and induction of systemic immune suppression. This underlines the decisive key role of the skin for systemic immune regulation.

3.4 Myeloid-derived suppressor cells mediate immune suppression in severe AD

We have shown for the first time a significant increase of MDSCs in the blood and skin of AD patients. This result and our further experiments with CD11b depletion of PBMCs and the presence of iNOS expressing CD11c negative cells in AD patients suggested a disease-specific induction as well as a suppressive activity of MDSCs in AD. We propose that severe AD causes an increase in the frequency of immunosuppressive MDSCs as an attempt to stop severe inflammation. This results in temporary immune suppression and increases the susceptibility for secondary infection. Indeed, immune suppression in response to strong cutaneous inflammation is a well known phenomenon in patients with AD[17,18]. These patients suffer from spreading of herpes viruses like in eczema herpeticum. So we contributed to the understanding of the complex clinical presentation of AD showing that MDSCs are responsible for immune suppression in this disease. Based on our findings, detection of MDSCs in peripheral blood could also be further developed as biomarker for immune suppression in severe AD. As perspective new therapeutic options may be developed that include the depletion (apheresis) or transfer of autologous MDSCs to regulate immune responses.

3.5 IL-6 and IL-4 have pleiotropic roles in cutaneous inflammation

IL-6 is a multifunctional cytokine with a broad spectrum of biological activities including immune regulation, hematopoiesis and inflammation[96]. Overproduction of IL-6 has been shown to play a pathological role in inflammatory autoimmune diseases such as rheumatoid arthritis[97]. And *Il6*$^{-/-}$ mice have shown impaired inflammatory responses[98]. But there are also reports about an anti-inflammatory role of IL6. In some settings IL-6 orchestrates down-regulation of pro-inflammatory cytokines as well as up-regulation of anti-inflammatory molecules[99]. We also identified IL-6 in its anti-inflammatory role, as a key factor for MDSC accumulation

following skin infection and induced immune suppression. However, we classify this mechanism rather as a negative feed-back-loop in severe inflammation, secondary to its pro-inflammatory function. Our data suggest that Pam2 activates TLR2/TLR6 on resident skin cells, which causes production of IL-6 by skin resident cells. The level of this cytokine (and a risk of dangerous tissue damage) is so high that a negative feed-back mechanism is induced, leading to the MDSC accumulation and subsequent immune suppression.

Cutaneous innate immune cells[100], keratinocytes and even melanocytes[101-103] are all capable of producing IL-6. It is especially evident in AD, where keratinocytes act as a critical first line of defense against microbial infection. Early IL-6 production was described after a direct contact of keratinocytes with *S. aureus*[104]. Moreover, IL-6 was found to be increased in AD skin[105] and especially in AD skin lesions[75], where the amount of IL-6 correlated with bacterial burden[75]. Genome wide association studies recently also identified an IL-6 receptor (IL-6R) variant as a new risk factor for AD[106] and a small case series with three patients demonstrated therapeutic efficacy of an IL-6R blockade by tocilizumab, an IL-6R antibody[107].

Immune suppression after increased IL-6 may be a common mechanism, because IL-6 has also been implicated in the progression of established tumors, a condition in which MDSCs suppress anti-tumoral immune responses[62,108].

Thus, these data highlight that IL-6, next to its well characterized pro-inflammatory properties, is also crucial for anti-inflammatory responses by orchestrating negative-feed-back-loops through MDSCs.

IL-4 is another well characterized cytokine with known pleiotropic functions. As shown above, **S. Kaesler et al.** has identified IL-4 as a key cytokine responsible for aggravation of cutaneous inflammation in early AD. We have clearly shown in human and mouse data potent immunosuppressive properties of IL-4 in another inflammatory skin disease, psoriasis, in the work of **E. Guenova et al.** . Interestingly, both works revealed that IL-4 affects APCs, most likely DCs, to exert these opposing effects. DCs and their cytokines determine the outcome and quality of immune responses, for example through polarization of T cells[45]. Consequently, the alteration of DC functions has profound impact on the quality of immune responses, not only during initiation but also for ongoing immune responses. Which of these effects predominates depends on the immunologic networks in a given immune situation. IL-4 acts as an APC modifier, directly instructing APCs to change their cytokine

repertoire. This includes the depletion of IL-23, one most important maintenance factors of Th17 response. At the same time IL-12p70 is upregulated, promoting the replacement of a Th17 response by a Th1 response, which proved to be of therapeutic use in psoriasis. Interestingly, psoriasis is characterized by the absence of IL-4, and both Th1 and Th17 cells prevail in the skin[85,86]. In configurations of the immune system, in which IL-10 is a dominant regulator, the APC modifier IL-4 shuts down IL-10 production, instructing an increase and persistence of inflammation. This was shown for AD, which, in the early phase, is dominated by a Th2 immune response and high IL-4 levels.

4 Summary

The skin plays a major protective role against pathogens, not only as physical barrier, but also as site of first recognition of exogenous substances and as orchestrator of consecutive immune responses. Moreover, it is known that immunological crosstalk between skin resident cells and immune cells is required for effective immune responses. The skin is constantly in contact to Gram-positive bacteria and consequently to different TLR2 ligands. We identified cutaneous TLR2 activation as a multifaceted pathway with various and in part opposing consequences on immune responses. In the early Th2-dominated phase of atopic dermatitis (AD), TLR2 ligands contributed to aggravation and persistence of cutaneous inflammation due to IL-4-mediated suppression of IL-10 in APC during ongoing inflammation. In contrast, a study with non-pathogenic bacterial lysates (*Vitreoscilla filiformis*) revealed that TLR2 activation suppressed AD due to induction of IL-10 and IL-10 producing regulatory T cells pointing to an anti-inflammatory role of TLR2 activation. Functional analyses of TLR2 ligands revealed that cutaneous exposure to diacylated TLR2/6 ligands, but not to triacylated TLR2/1 ligands inhibited subsequent cutaneous T cell-mediated recall responses. This was due to a systemic induction of $Gr1^+CD11b^+$ myeloid-derived suppressor cells (MDSCs) directly suppressing T cells. Investigating AD patients, where TLR2 ligands accumulate, we detected a significant increase of MDSCs in the peripheral blood and skin of AD patients in comparison to healthy individuals. Interestingly, signals through TLR2 on skin cells, but not on hematopoietic cells, as well as cutaneous IL-6 induction were necessary and sufficient for the expansion of MDSCs and their immunomodulatory effect in this context. Investigating the underlying mechanism of IL-4-mediated therapy of psoriasis we found that IL-4 predominantly suppressed the IL-23/Th17 immune axis in this disease. These investigations demonstrate that the same signaling molecules within a complex immune network can be involved in the signaling of opposite immune reactions like inflammation or suppression of inflammation. Thus, single linear activation pathways have to be integrated in a network to understand the final immune outcome. This consideration of the whole signaling network is not only necessary to understand the pathogenesis of immunological diseases and their therapeutic strategies, but also to unravel possible side effects and restrictions of current immune therapies.

5 Zusammenfassung

Unsere Haut ist ständig Bakterien und Antigenen der Umwelt ausgesetzt. Deswegen hat das Immunsystem der Haut die Aufgabe, nicht nur die Fremdsubstanzen zu erkennen, sondern auch die darauf entsprechenden Immunantworten zu generieren und deren Verlauf zu steuern. Vor allem ist die Haut mit Gram positiven Bakterien und somit mit TLR2-Liganden im ständigen Kontakt. Wir fanden unterschiedliche und zum Teil gegensätzliche Auswirkungen der kutanen TLR2-Aktivierung auf das Immunsystem. In der frühen Th2-dominierenden Phase der atopischen Dermatitis (AD) führten TLR2-Liganden zu einer Verschlechterung und Chronifizierung der Hautentzündung. Die wurde durch die Hemmung der IL-10-Produktion in den APCs durch IL-4 bewerkstelligt. Eine weitere Arbeit mit den Lysaten von nicht-pathogenen *Vitreoscilla filiformis* zeigte dagegen, dass die Aktivierung von TLR2 zu einer Hemmung der kutanen Entzündung und somit zu einer Besserung der AD führte. Der hierfür zugrundeliegende Mechanismus war die Induktion von IL-10 und IL-10-produzierenden regulatorischen T-Zellen. Funktionale Analysen von TLR2-Liganden ergaben, dass eine kutane Applikation von TLR2/6- und nicht TLR2/1-Liganden in einer systemischen Immunsuppression der T-Zell-Antworten resultierte. Wir identifizierten weiter, dass $Gr1^+CD11b^+$ myeloide Suppressorzellen (MDSCs) diese Wirkung vermittelten. Untersuchungen bei AD Patienten zeigten einen höheren Level an MDSCs im Blut im Vergleich zu Kontrollgruppen. Interessanterweise war die Präsenz der TLR2 Rezeptoren auf den Hautzellen und nicht auf den hämatopoetischen Zellen notwendig und ausreichend für die MDSC-Induktion. Darüber hinaus identifizierten wir kutanes IL-6 als das Schlüsselzytokin für die MDSC-vermittelte Immunsuppression. Auf der Suche nach dem Mechanismus der IL-4-Therapie in Psoriasis fanden wir, dass IL-4 überwiegend die IL-23/Th17-Achse in dieser Erkrankung hemmt. Diese Ergebnisse zeigen, wie ein komplexes immunologisches Netzwerk, wie es in der Haut vorherrscht, durch teilweise überlappende Signale so eingestellt und verändert werden kann, dass es zu völlig unterschiedlichen Immunantworten kommt. Um das finale Ergebnis der Immunantwort zu verstehen, müssen nicht nur einzelnen Signalwege, sondern deren Interaktion betrachtet werden. Diese Betrachtungsweise hilft bei dem Verständnis der Pathogenese der immunologischen Erkrankung sowie bei der Entwicklung therapeutischer Ansätze.

6 Abbreviations

AD	atopic dermatitis
AMPs	antimicrobicidal peptides
APC	antigen presenting cells
BM	bone marrow
CHS	contact hypersensitivity model
CFU	colony forming units
DC	dendritic cells
FITC	fluorescein isothiocyanate
IL	interleukin
Lgt	diacylglyceryl transferase gene
Lpp	lipoproteins
NO	nitric oxide
LTA	lipoteichoic acid
MAMPs	microorganism-associated molecular patterns
MDSC	myeloid-derived suppressor cells
MyD88	myeloid differentiation factor 88
Pam3Cys	tri-palmitoyl cysteinyl lipopeptide (Pam3CSK4) adapted from the *Escherichia coli* Braun's lipoprotein
Pam2Cys	di-palmitoyl cysteinyl lipopeptide – Pam2Cys as well as Pam3Cys mimic the proinflammatory properties of bacterial lipoproteins
PAMPs	pathogen-associated molecular patterns
PPRs	pattern recognition receptors
SitC	predominant lipoprotein in *Staphylococcus*, part of the iron transporter SitABC;
S.	*Staphylococcus*
TCR	T cell receptor
Teff	effector T cells
TLR	Toll-like receptor
Treg	regulatory T cells
Vf lysate	lysate of the non-pathogenic bacterium *Vitreoscilla filiformis*.
WT	wild type

7 References

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8 Publications

8.1 Original publications

Skabytska Y, Wölbing F, Günther C, Köberle M, Kaesler S, Chen K, Guenova E, Demircioglu D, Kempf W, Volz T, Rammensee H, Schaller M, Röcken M, Götz F, Biedermann T.
Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. Accepted, DOI: 10.1016/j.immuni.2014.10.009, Immunity.

Skabytska Y, Peschel A, Biedermann T, Köberle M.
Immunosuppression after Gram-positive *Staphylococcus auresus* sepsis is mediated by induction of myeloid-derived suppressor cell via pleiotropic inflammatory signaling. In preparation

Guenova E*, **Skabytska Y***, Hoetzenecker W*, Weindl G, Sauer K, Tham M, Kim K, Park J, Seo J, Levesque M, Volz T, Köberle M, Kaesler S, Thomas P, Mailhammer R, Ghoreschi K, Schäkel K, Amarov B, Eichner M, Schaller M, Röcken M, Biedermann T. *These authors contributed equally to this work.
IL-4 abrogates Th17 cell-mediated inflammation by selective silencing of IL-23 in antigen-presenting cells. In revision, PNAS

Chen K, **Skabytska Y**, Kaesler S, Hein U, Röcken M, Biedermann T.
Staphylococcus aureus-derived lipoteichoic acid induces temporary T cell paralysis independent of TLR2. In preparation

Kaesler S, Volz T, **Skabytska Y**, Hein U, Chen K, Guenova E, Röcken M, Biedermann T. (2014)
TLR2-ligands promote chronic dermatitis through IL-4 mediated suppression of IL-10. J Allergy Clin Immunol. 2014 Jul;134(1):92-9.
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Volz T, **Skabytska Y**, Guenova E, Chen KM, Frick J, Kirschning C, Kaesler S, Röcken M, Biedermann T. (2014)
Nonpathogenic Bacteria Alleviating Atopic Dermatitis Inflammation Induce IL-10-Producing Dendritic Cells and Regulatory Tr1 Cells. J Invest Dermatol. 134(1):96-104

Wanke I, **Skabytska Y**, Kraft B, Peschel A, Biedermann T, Schittek B. (2013)
Staphylococcus aureus skin colonization is promoted by barrier disruption and leads to local inflammation. Exp Dermatol. 22(2):153-5.

Ghashghaeinia M, Cluitmans J, Akel A, Dreischer P, Toulany M, Köberle M, **Skabytska Y**, Saki M, Biedermann T, Duszenko M, Lang F, Wieder T, Bosman GJ. (2012)
The impact of erythrocyte age on eryptosis. Br J Haematol. 7(5):606-14.

8.2 Congress abstracts

8.2.1 Talks

Yuliya Skabytska, Florian Wölbing, Claudia Günther, Martin Köberle, Susanne Kaesler, Emmanuella Guenova, Thomas Volz, Tilo Biedermann

Immune suppression in severe atopic dermatitis is mediated by myeloid-derived suppressor cells

41. Tagung der Arbeitsgemeinschaft Dermatologische Forschung (ADF), Köln, 2014.

Yuliya Skabytska, Susanne Kaesler, Ko-Ming Chen, Florian Wölbing, Tilo Biedermann

TLR activation on the skin causes systemic immune suppression mediated by myeloid-derived suppressor cells

25. Mainzer Allergie Workshop, Deutsche Gesellschaft für Allergologie und klinische Immunologie, Mainz, 2013

Yuliya Skabytska, Ko-Ming Chen, Susanne Kaesler, Tilo Biedermann

Cutaneous recruitment of myeloid-derived suppressor cells following innate immune signaling limits T cell mediated skin inflammation

42nd Annual Meeting of Europian Society for Dermatological Research (ESDR), Venice, 2012

Yuliya Skabytska, Ko-Ming Chen, Susanne Kaesler, Tilo Biedermann

Cutaneous TLR2-6 ligands limit T cell mediated skin inflammation by IL-6 dependent induction of myeloid-derived suppressor cells

72nd Annual Meeting of Society for Inversitgative Dermatology (SID), Raleigh, 2012

Yuliya Skabytska, Martin Köberle, Ko-Ming Chen, Susanne Kaesler, Tilo Biedermann

Cutaneous exposure to TLR2 ligands is sufficient to induce systemic immune regulation mediated by myeloid-derived suppressor cells

41nd Annual Meeting of Europian Society for Dermatological Research (ESDR), Barcelona, 2011

Yuliya Skabytska, Ko-Ming Chen, Susanne Kaesler, Tilo Biedermann

Cutaneous pathogen associated molecular pattern (PAMP) induce systemic immune regulation mediated by myeloid-derived suppressor cells
38. Tagung der Arbeitsgemeinschaft Dermatologische Forschung (ADF), Tübingen, 2011

Yuliya Skabytska, Koming Chen, Susanne Kaesler, Ulrike Hein, Thomas Volz, Tilo Biedermann
Allergy prevention by stabilization of the immunological skin barrier by means of bacterial PAMPs
Doktorandenkolloquium der Landesstiftung Baden-Württemberg, 2009

8.2.2 Poster presentations

Yuliya Skabytska, Tilo Biedermann, Martin Köberle
Post-septic immune-suppression following Gram positive sepsis is mediated by TLR dependent induction of myeloid derived suppressor cells.
40. Tagung der Arbeitsgemeinschaft Dermatologische Forschung (ADF), Dessau, 2013, Poster prize

Yuliya Skabytska, Martin Köberle, Ko-Ming Chen, Susanne Kaesler, Tilo Biedermann
Cutaneous TLR2 ligands induce systemic immune regulation mediated by myeloid derived suppressor cells
71nd Annual Meeting of Society for Inversitgative Dermatology (SID), Phoenix, 2011

Yuliya Skabytska, Martin Köberle, Ko-Ming Chen, Susanne Kaesler, Tilo Biedermann
Cutaneous innate immune sensing of TLR2 ligands induces myeloid derived suppressor cells and potently suppresses cutaneous immune responses to limit and terminate skin inflammation
40. Tagung der Arbeitsgemeinschaft Dermatologische Forschung (ADF), Marburg, 2012

Yuliya Skabytska, Ko-Ming Chen, Susanne Kaesler und Tilo Biedermann
Bakterien auf der Haut: schaden oder helfen sie uns?
Forschungstag der Lebenswissenschaften, Baden-Württemberg Stiftung, Stuttgart, 2011

9 Erklärung zum Eigenanteil

For the study "**TLR2 ligands promote chronic atopic dermatitis through IL-4-mediated suppression of IL-10**" (Susanne Kaesler, Thomas Volz, Yuliya Skabytska, Ulrike Hein, Ko-Ming Chen, Emmanuella Guenova, Martin Röcken, and Tilo Biedermann) most of work was done by S. Kaesler. Y. Skabytska assisted in one experiment for *in vitro* generation of Th2 cells and participated in an adoptive transfer experiment. T. Biedermann supervised the project.

For the study **"Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells"** (Thomas Volz, Yuliya Skabytska, Emmanuella Guenova, Ko-Ming Chen, Julia-Stefanie Frick, Carsten J. Kirschning, Susanne Kaesler, Martin Röcken and Tilo Biedermann, 2013, *J Invest Dermatol*) T. Volz has done the most part of the work. Y. Skabytska performed *in vivo* experiments and subsequent *ex vivo* analyses. T. Biedermann supervised the project.

For the study **"*Staphylococcus aureus*-derived lipoteichoic acid induces temporary T cell paralysis independent of TLR2"** (Ko-Ming Chen, Yuliya Skabytska, Susanne Kaesler, Ulrike Hein, Martin Röcken, Tilo Biedermann) fundamental *in vivo* and *in vitro* experiments was done by K. Chen. Y. Skabytska continued the project by repeating and completing the essential experiments. T. Biedermann supervised the project.

For the study **"Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells"** (Yuliya Skabytska, Florian Wölbing, Claudia Günther, Martin Köberle, Susanne Kaesler, Ko-Ming Chen, Emmanuella Guenova, Doruk Demircioglu, Wolfgang Kempf, Thomas Volz, Hans-Georg Rammensee, Martin Schaller, Martin Röcken, Friedrich Götz, Tilo Biedermann) all experiments, except immunhistological staining of skin, were done by Y. Skabytska. T. Biedermann supervised the project.

For the study "**Staphylococcus aureus skin colonization is promoted by barrier disruption and leads to local inflammation"** (Ines Wanke, Yuliya Skabytska, Beatrice Kraft, Andreas Peschel, Tilo Biedermann and Birgit Schittek, 2013, *Exp*

Dermatol) I. Wanke and Y. Skabytska performed *in vivo* experiments together. *Ex vivo* analyses were done by I. Wanke. B. Schittek supervised the project.

For the study **"IL-4 abrogates T cell mediated inflammation in mice and humans by the selective silencing of IL-23"** (Emmanuella Guenova, Yuliya Skabytska, Kamran Ghoreschi, Wolfram Hoetzenecker, Karin Sauer, Günther Weindl, Manuela Tham, , Thomas Volz, Peter Thomas, Reinhard Mailhammer, Knut Schäkel, Boyko Amarov, Martin Eichner, Martin Schaller, Martin Röcken, Tilo Biedermann) Y. Skabytska performed *in vivo* experiments with IL-23 treatment and bone marrow chimera experiments. E. Guenova et al. did the rest of experiments. T. Biedermann supervised the project.

10 Acknowledgement

Die Arbeit in der AG Biedermann und insgesamt im Forschungslabor Hautklinik hat mir große Freude gemacht. Es war mir eine große Ehre bei so vielen interessanten Projekten mitzumachen. Die Erfahrungen der AG, eine Vielfalt etablierter Methoden, ein guter Bezug zu der Klinik, Möglichkeiten immer wieder neue Modelle und Methoden zu erlernen und nicht zuletzt eine gute finanzielle Unterstützung waren die Voraussetzungen für diese Arbeit. Susanne Kaesler und Ko-Ming Chen haben die wichtigsten Mausmodelle der kutanen Entzündung etabliert. Die Ärzte Florian Wölbing und Thomals Volz waren gute Helfer in den klinischen Fragen. Martin Köberle hat bei den Infektionsversuchen geholfen; er war immer eine große Hilfe in IT- und Statistik-Angelegenheiten. Der Leiter der AG, Prof. Biedermann ist ein großes Vorbild. Seine Ideen, Motivation und das Glaube an diese Arbeit sind unglaublich und waren Grundlagen dafür. Und natürlich bedanke ich mich bei allen TAs der AG, die ein reibungsloses Arbeiten ermöglicht haben. Allen Kolleginnen und Kollegen des Labors Röntgenweg und Liebermeisterstrasse danke ich für die ständige Unterstützung.

Diese Arbeit hat mir wahnsinnig Spaß gemacht. Es war nicht nur ein „Job“, sondern eine Lebenserfahrung, eine „sich selbst kennenlernen“. Diese Arbeit hat mir gezeigt, dass alles möglich ist und bestätigt, dass „hartes“ Arbeiten belohnt wird.

12 Curriculum vitae

Personal details

Name	Yuliya Skabytska
Date of Birth	28th April 1980
Place of Birth	Konsun-Schewtschenkiwsky (Ukraine)

Education and Qualification

1996 - 2000 Biology studies at the Kiev-Mohyla Acadamy, Kiew, Ukraine

2003 Start of the molecular medicine studies at the Friedrich-Alexander University Erlangen

2008 Diploma at the Friedrich-Alexander University Erlangen

Subjects: Immunology
Cell biology
Pathogenic mechanisms
Pharmacology

2008 Diploma thesis „The anti-DNA antibodies in the MFG-E8 deficient mice are somatically mutated”

2008 Start of doctor thesis “Immunological consequences of cutaneous TLR2 signaling" at the Eberhard Karls University Tuebingen at the laboratory of Prof. Tilo Biedermann

13 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit zum Thema “Immunological consequences of cutaneous TLR2 signaling" eigenständig, ohne unerlaubte Hilfe und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Alle sinngemäß und wörtlich übernommenen Textstellen aus Veröffentlichungen oder aus anderweitigen fremden Äußerungen habe ich als solche kenntlich gemacht.

Tübingen, den 21.10.2014 Yuliya Skabytska

Dr. Tilo Biedermann
Technische Universität Munich
Department of Dermatology
Biedersteinerstr. 29
Munich 80802
GERMANY

Oct 17, 2014
IMMUNITY-D-14-00217R2

Dear Dr. Biedermann,

We have reviewed your revised manuscript, "Cutaneous innate immune sensing of toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells"(IMMUNITY-D-14-00217R2), and I am delighted to be able to accept the paper for publication in the next available issue of the journal.

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Bruce Koppelman, Ph.D.
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immunity@cell.com
Phone: 617 397 2851

Cutaneous innate immune sensing of toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells

Yuliya Skabytska[1], Florian Wölbing[1], Claudia Günther[2], Martin Köberle[1,3], Susanne Kaesler[1], Ko-Ming Chen[1], Emmanuella Guenova[1,4], Doruk Demircioglu[5], Wolfgang E. Kempf[1,3], Thomas Volz[1,3], Hans-Georg Rammensee[6], Martin Schaller[1], Martin Röcken[1], Friedrich Götz[5], Tilo Biedermann[1,3]

[1]Department of Dermatology, Eberhard Karls University, Liebermeisterstr. 25, 72076, Tübingen, Germany
[2]Department of Dermatology, Technical University Dresden, Mommsenstr. 11, 01069 Dresden, Germany
[3]Department of Dermatology and Allergy, Technische Universität München, Biedersteinerstr. 29, 80802, Munich, Germany
[4]Department of Dermatology, University Hospital Zurich, Gloriastr. 31, CH-8091 Zurich, Switzerland
[5]Department of Microbial Genetics, Eberhard Karls University, Waldhäuser Straße 70/8, 72076, Tübingen, Germany
[6]Department of Immunology, Institute of Cell Biology, and German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ) Partner Site Tübingen, Eberhard Karls University, Auf der Morgenstelle 15, 72076 Tübingen, Germany

Corresponding author:
Prof. Dr. med. Tilo Biedermann
Biedersteinerstr. 29, 80802 Munich
Tel.: +49 89-4140-3170 Fax.: +49 89-4140-3171
E-Mail-Address: Tilo.Biedermann@tum.de

Running Title: Skin infection-induced MDSCs suppress T cell immunity

Summary

Skin is constantly exposed to bacteria and antigens, and cutaneous innate immune sensing orchestrates adaptive immune responses. In its absence, skin pathogens can expand, entering deeper tissues leading to life-threatening infectious diseases. To characterize skin-driven immunity better, we applied living bacteria, defined lipopeptides, and antigens cutaneously. We found suppression of immune responses due to cutaneous infection with Gram-positive *S. aureus*, which was based on bacterial lipopeptides. Skin exposure to toll-like receptor (TLR)2-6- but not TLR2-1-binding lipopeptides potently suppressed immune responses through induction of $Gr1^{+}CD11b^{+}$ myeloid-derived suppressor cells (MDSCs). Investigating human atopic dermatitis, in which Gram-positive bacteria accumulate, we detected high MDSC amounts in blood and skin. TLR2 activation in skin resident cells triggered interleukin-6 (IL-6), which induced suppressive MDSCs, which are then recruited to the skin suppressing T cell-mediated recall responses such as dermatitis. Thus, cutaneous bacteria can negatively regulate skin-driven immune responses by inducing MDSCs via TLR2-6 activation.

Introduction

The skin is the largest organ at the interface between the environment and the host. The skin plays a major protective role not only as physical barrier but also as the site of first recognition of microbes and orchestrates consecutive immune responses (Naik et al., 2012; Swamy et al., 2010; Volz et al., 2011).

Staphylococcus aureus (*S. aureus*) is one of the most potent skin pathogens and is found to colonize skin of about 30 to 50% of healthy adults, among them 10-20% persistently (Lowy, 1998). Coming from the skin *S. aureus* can infect any tissue of the body and cause life-threatening diseases, particularly because of the widespread occurrence of antibiotic-resistant strains, known as methicillin-resistant Staphylococcus aureus (MRSA) (Saeed et al., 2014). In atopic dermatitis (AD) patients, there is an approximately 200-fold increase of *S. aureus* colonization with more than 90% of AD patients displaying *S. aureus* in comparison to the healthy skin (Leung and Bieber, 2003).

Microbes are first sensed by the innate immune system through pattern recognition receptors (PRRs), which recognize microbe-associated molecular patterns (MAMPs) (Kawai and Akira, 2010). Both epithelial cells and resident innate immune cells in the skin express PRRs (Kupper and Fuhlbrigge, 2004; Lai and Gallo, 2008). Among PRRs, Toll-like receptors (TLRs) are a well-characterized family with distinct recognition profiles (Kawai and Akira, 2010). TLR2 has emerged as a dominant receptor for Gram-positive bacteria, especially *S. aureus* (Biedermann, 2006; Lai and Gallo, 2008; Mempel et al., 2003). Among TLR2 ligands, lipoproteins seem to be especially important because the lipoprotein diacylglyceryl transferase (*lgt*) deletion mutant of *S. aureus* induces much less proinflammatory cytokines in human cell lines (Stoll et al., 2005) and less TLR2-MyD88 adaptor protein-mediated inflammation in a mouse model of systemic infection (Schmaler et al., 2009). It is now established that there are different classes of lipopeptides that all bind TLR2 (Müller et al., 2010; Schmaler et al., 2009). However, how these TLR2 ligands differ in regard to functional consequences has not

been thoroughly investigated. TLR2 is known to form heterodimers with TLR1 and TLR6 to interact with this broad spectrum of ligands (Kang et al., 2009). TLR1 is required as a co-receptor for recognition of triacylated lipopeptides, such as Pam3Cys (Buwitt-Beckmann et al., 2006; Jin et al., 2007), while diacylated lipopeptides, such as FSL-1 or Pam2Cys, interact with TLR2-TLR6 heterodimers (Mae et al., 2007; Mühlradt et al., 1997). Functional properties of *S. aureus* lipopeptides in respect to TLR2 heterodimers have been investigated in several cell types (Buwitt-Beckmann et al., 2006; Hajjar et al., 2001), but evidence demonstrating specific functional consequences for the activation of different heterodimers *in vivo* is lacking.

Sustained activation of TLRs causes persistent production of proinflammatory cytokines, such as tumor necrosis factor (TNF) or interleukin-6 (IL-6), leading to tissue damage (Kawai and Akira, 2010; Kupper and Fuhlbrigge, 2004; Lai and Gallo, 2008). Consequently, to reconstitute the integrity of the surface organ, mechanisms to limit cutaneous inflammation must be effective (Lai et al., 2009). In recent years $Gr1^+CD11b^+$ myeloid-derived suppressor cells (MDSCs) have been identified as one cell population responsible for modulating immune responses (Bronte, 2009; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009). The most characteristic functional property of MDSCs is to suppress T cell responses (Gabrilovich et al., 2001; Kusmartsev et al., 2000). In the context of inflammation the precise function of MDSCs and the mechanisms of MDSC induction are not well-understood; but in a sepsis model with Gram-negative bacteria their induction has been shown to depend on TLR4-MyD88 activation (Delano et al., 2007), and in tumor models, different innate cytokines, such as IL-6, induce MDSC accumulation (Bunt et al., 2007; Chalmin et al., 2010). However, the suppression of IL-6 also increases susceptibility to bacterial and fungal infections, indicating pleiotropic effects of IL-6 (Hoetzenecker et al., 2011).

In this study we have identified a pathway of immune regulation that operates in the skin. We mimicked intense cutaneous contact to bacteria in different *in vivo* mouse models by using

living bacteria and lipopeptides. We investigated AD as a model for massive cutaneous immune sensing of Gram-positive bacteria in humans. We found that cutaneous infection with *S. aureus* caused immune suppression. The exposure to TLR2-6 ligands was sufficient to cause an almost complete reduction of consecutive cutaneous recall responses. This skin exposure induced accumulation of MDSCs, allowing MDSC recruitment to the skin, and suppression of T cell-mediated recall responses. Signals through TLR2 on skin resident cells but not on recruited hematopoietic cells, as well as cutaneous IL-6 induction, were necessary and sufficient for the expansion of MDSCs and consecutive immune suppression. These data demonstrate that cutaneous recognition of TLR2-6 ligands orchestrates a unique pathway of cutaneous immune modulation mediated by MDSCs, indicating a yet unknown level of immune counter-regulation.

Results

Cutaneous *Staphylococcus aureus* induces immune suppression.

We aimed to characterize the consequences of intense cutaneous innate immune sensing as in the case of colonization or infection with Gram-positive bacteria. We established a mouse model of epicutaneous colonization with pathologically-relevant *S. aureus* (Wanke et al., 2013). Mimicking *S. aureus* skin infection by applying living *S. aureus* bacteria onto the skin with disrupted skin barrier we found a distribution of the bacteria not only in the skin, but also in the internal organs (spleen and kidney) (Figure 1A), indicating the importance of the skin as an effective defense immune organ with the potential to impact the whole immune system. To investigate how bacterial infection influences consecutive immune responses, we combined this model of bacterial colonization and the murine T cell-mediated contact hypersensitivity (CHS) to FITC, in which bacteria were applied epicutaneously during FITC re-exposure of FITC-sensitized mice (see protocol in Figure S1A). The application of FITC onto the ear led to FITC-specific dermatitis as determined by ear swelling which corresponded to the strength of the FITC-specific immune response. The cutaneous application of *S. aureus* 7 days previous to the FITC challenge did not enhance, but significantly reduced ear swelling and immune cell infiltration (Figure 1B, C). This immune suppression was completely dependent on immune sensing of bacterial lipoproteins, as lipoprotein-deficient *S. aureus* mutant (*Δlgt*) (Stoll et al., 2005) failed to induce immune suppression. Injecting *S. aureus* into the subepithelial dermis (intracutaneous route) also induced consecutive immune suppression, which, however, tended to be weaker compared to effects of *S. aureus* application onto the epithelium (Figure S1B). To identify underlying mechanisms of *S. aureus*-induced cutaneous immune suppression we analyzed skin-draining lymph nodes. Only exposure to wild-type (WT) *S. aureus* bacteria and not the lipoprotein-deficient *Δlgt S. aureus* reduced *ex vivo* FITC-specific T cell proliferation (Figure 1D). In the

spleen, $CD4^+$ and $CD8^+$ T cells were also reduced in mice cutaneously exposed WT *S. aureus* but not in mice exposed to lipoprotein-deficient *Δlgt S. aureus* (Figure 1E). Only in mice displaying suppressed T cells we detected a strong increase of $Gr1^+CD11b^+$ so called myeloid-derived suppressor cells (Figure 1E). In contrast to this, accumulation of $Gr1^+CD11b^+$ was not detected in the liver (Figure S1D). At d3 after FITC challenge, MDSCs were also slightly increased in draining lymph nodes due to cutaneous WT *S. aureus* infection, corresponding the decrease of proliferating $Ki67^+$ T cells (Figure S1E). Further experiments investigating other suppressive cell populations showed no alterations in the number of regulatory T (Treg) cells and IL-10 producing cells (Figure S1F); the numbers of Langerhans cells (LC, defined as $CD11c^{lo}CD205^{hi}$) and $CD11c^+$MHC-II^+ cells were also unchanged, dermal dendritic cells (dDCs, defined as $CD11c^{hi}CD205^{lo}$) were slightly increased (Figure S1E). These data indicate that MDSCs function independent of Treg cells and do not inhibit migration of DCs into lymph nodes.

In order to further emphasize the functional and clinical relevance of these findings, we investigated atopic dermatitis (AD) patients. AD is a perfectly suited model disease for investigations on immune consequences of skin exposure to bacteria, as AD is an inflammatory skin disease that is nearly always covered with and triggered by *Staphylococci*. In humans, MDSCs are typically described as $CD11b^+CD33^+$HLA-DR^-CD14^- cells (Gabrilovich and Nagaraj, 2009). We observed a significant increase of MDSCs in the peripheral blood mononuclear cells (PBMCs) of AD patients (Figure 1F). The upregulation of human MDSCs was especially consistent in patients, in which severe AD was complicated by eczema herpeticum, which is a severe cutaneous viral infection resulting from immune suppression (Figure 1F**,** red squares) (Beck et al., 2009; Wollenberg et al., 2003), suggesting suppressive properties of MDSCs also in AD patients.

These data show that cutaneous *S. aureus* is sufficient to induce MDSCs and to cause immune suppression.

Cutaneous exposure to TLR2-6 but not to TLR2-1 ligands ameliorates T cell-mediated recall responses

Next, we investigated the intriguing finding that lipoprotein-deficient *S. aureus* failed to induce immune suppression in our model (Figure 1B). As lipoproteins are sensed by different TLR2 heterodimers (Henneke et al., 2008), we have taken advantage of microbial-derived molecules which are exclusively bound by one specific TLR2 heterodimer. We selected three lipopeptides for our studies: TLR2-6 ligands diacyl lipopeptides FSL-1 and Pam2Cys and the triacylated lipopeptide Pam3Cys that is often used as a reference compound for TLR2-1 activation. As in our previous model, lipopeptides were applied to the skin during re-exposure of FITC-sensitized mice to FITC (see protocol Figure S1A). Similarly to the living *S. aureus*, the cutaneous exposure to TLR2-6 ligand FSL-1 almost completely abrogated consecutive FITC-specific recall responses (Figure 2A, B), FITC-specific *ex vivo* T cell proliferation (Figure 2C) and orchestrated splenic reduction of $CD4^+$ and $CD8^+$ T cells together with MDSC accumulation (Figure 2D). This result was confirmed with another TLR2-6 ligand, Pam2Cys (Figure 2E-H). In contrast to Pam2Cys, the TLR2-TLR1 ligand Pam3Cys failed to suppress FITC-specific dermatitis and T cell proliferation (Figure 2E-G). Accordingly, no reduction of $CD4^+$ and $CD8^+$ T cells and no induction of $Gr1^+CD11b^+$ cells could be detected (Figure 2H).

These data show that cutaneous exposure to bacterial TLR2-TLR6 ligands is sufficient to cause immune suppression and that activation of TLR2-TLR6 heterodimers differs in regard to functional consequences from activation of TLR2-TLR1 heterodimers.

Further, in order to control whether the presentation of the antigen FITC is directly influenced by Pam2Cys exposure, we analyzed the number of FITC positive DCs 14 hours after cutaneous FITC application and Pam2Cys exposure. There were no differences in the numbers of FITC positive $CD11c^+MHC\text{-}II^+$ cells and other dendritic cell populations (dDC, LC) in draining lymph nodes (Figure S1G). Similarly, the analysis of other cell populations at this early stage of the response revealed comparable numbers of T cells ($CD4^+$, $CD8^+$), activated T cells ($CD4^+CD25^+$) and proliferating cells ($Ki67^+$) (Figure S1H), IL-10 producing cells and Treg cells (Figure S1I) in both mouse groups. The treatment of mice with cyclophosphamide for Treg cell depletion failed to reverse Pam2Cys-induced immune suppression (Figure S1J-L), further indicating that Treg cells are not involved in this type of immune suppression.

Skin infection-induced immune suppression is mediated by $Gr1^+CD11b^+$ myeloid-derived suppressor cells

Next, as proof of concept that MDSCs are the responsible cells for the observed immune suppression upon cutaneous Pam2Cys exposure, we depleted $Gr1^+$ cells. This depletion caused an abrogation of immune suppression (Figure 3A right). Inversely, the adoptive transfer of MDSCs, isolated from mice previously exposed to Pam2Cys, resulted in reduction of both FITC-specific dermatitis and T cell proliferation (Figure 3B,C). To investigate whether human MDSCs in AD patients with intense cutaneous exposure to lipoproteins were suppressive, we depleted $CD11b^+$ cells from PBMCs and analyzed proliferation of activated T cells. The $CD11b^+$ population among PBMCs consists of antigen presenting cells and, in addition, contains MDSCs in AD but not healthy individuals. Consequently, in 7 of 8 healthy volunteers CD11b depletion resulted in reduced T cell proliferation (Figure 3D, left)**.** On the contrary, this was only observed in one out of 7 AD patients (Figure 3D, right).). These results demonstrate that MDSCs, which are present among the $CD11b^+$ population in AD

patients but not in healthy individuals, are immunosuppressive. Indeed, T-cell receptor ζ-chain was significantly down-regulated in AD patients (Figure 3E), which is known to be one of the major features of MDSCs-mediated T cell inhibition (Zea et al., 2005)

Taken together, these data revealed that skin infection-induced immune suppression is mediated by MDSCs.

Myeloid-derived suppressor cells are recruited to the skin in mice and humans

Detecting MDSCs in human blood and mouse spleen following cutaneous innate immune sensing indicates systemic MDSC expansion. Therefore, we next monitored the kinetics of MDSC induction in mice a) in the bone marrow (BM), its primary source (Figure 4A, left), and b) in one site of MDSC enrichment, the spleen (Figure 4A, right) at different time points after cutaneous Pam2Cys exposure. Starting on day 2, $Gr1^+CD11b^+$ cells in the bone marrow increased and peaked at day 7 with about 75% of cells being $Gr1^+CD11b^+$. In the spleen, both $CD4^+$ and $CD8^+$ T cells were strongly reduced. $Gr1^+CD11b^+$ cells increased starting at day 4 with up to 7-fold induction on day 11 following cutaneous Pam2Cys exposure (Figure 4A).

In FITC-CHS, T cells migrate to the skin and elicit dermatitis. Therefore, we analyzed whether MDSCs were also recruited to the skin. Indeed, 8 h after FITC challenge $Gr1^+CD11b^+$ cells were significantly increased in the skin of mice previously exposed to Pam2Cys (Figure 4B). Similarly, we compared healthy skin with lesional skin from AD patients colonized or infected with *S. aureus*. Flow cytometry analysis confirmed a significant increase of MDSCs in the skin of AD patients compared to healthy skin (Figure 4C), indicating that presence of bacteria and subsequent skin inflammation induce MDSC accumulation in the skin also in humans.

Suppression of T cell activation by MDSCs is induced by cutaneous innate immune sensing

Recruitment of MDSCs to the skin suggested MDSC-mediated suppression of T cell activation in the skin *in vivo*. As first indication we found that the depletion of $CD11b^+$ cells of isolated skin cells caused a stronger T cell proliferation following stimulation with anti-CD3-D28 in comparison to cells not depleted of $CD11b^+$ cells (Figure S2A), confirming a suppressive function of skin MDSCs *ex vivo*. Moreover, flow cytometry analysis of ear skin tissue following the FITC challenge revealed a significant decrease of $CD3^+$ T cells (Figure 5A, right) and IFN-γ production (Figure 5A, left) in previously Pam2Cys-exposed mice. Expression analysis of other cytokines revealed a significant decrease of the Th2 cell cytokine IL-4 (a target for a systemic AD treatment (Beck et al., 2014)), IL-10 and a tendency for IL-17 inhibition (Figure 5B). The investigation of cutaneous chemokines in the skin showed a down-regulation of most analyzed chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL17, CCL20, CCL27). Only T cell attracting CCL22 (a CCR4 ligand) and CCL28 (CCR3 and CCR10 ligand) were significantly upregulated (Figure 5C). The corresponding chemokine receptors were expressed on the MDSCs in the skin, blood and bone marrow (Figure 5D), which further indicates that MDSCs are attracted to the site (and by similar mechanism) of T cell migration.

To explore the mechanisms mediating MDSC-induced immune suppression, we isolated MDSCs 10 days after Pam2Cys exposure. Flow cytometry analysis revealed the presence of both $Ly6C^+$ and $Ly6G^+$ MDSCs. Morphological evaluation of isolated MDSCs confirmed that $Ly6G^+$ MDSC were granulocytic, whereas $Ly6C^+$ MDSCs were monocytic (Figure S2B). In the skin $Gr1^+CD11b^+$ cells were further characterized as CD11c-, CD15-, MHC-II-, B220-negative and positive for CD16-32, partly positive for F4-80 (Figure S2C) and splenic $Ly6C^+$ cells had a similar phenotype (Figure S2C). Next, we isolated $Gr\text{-}1^{dim}Ly6G^-Ly6C^+CD11b^+$ ($Ly6C^+$) and $Gr\text{-}1^{high}Ly\text{-}6G^+CD11b^+$ ($Ly6G^+$) MDSCs from Pam2Cys-exposed mice and co-cultured them with naïve splenocytes (responder cells) activated with anti-CD3-CD28 antibodies (Abs) at different ratios. Following co-culture with $Ly6C^+$ MDSCs at a ratio of 2:1,

almost complete suppression of T cell proliferation was observed, while $Ly6G^+$ cells were not suppressive (Figure 5E, left). Investigating the suppressive activity more thoroughly revealed that $Ly6C^+$ MDSCs inhibited Th0 $CD4^+$ T cells as well as of Th1-, Th2- and Th17-polarized cells (Figure S2D). MDSCs´ immunosuppressive activity is reported to be a result of the activation of inducible NOS (iNOS), leading to increased production of nitric oxide (NO) (Gabrilovich et al., 2001). Indeed, we found an increased iNOS expression in the skin after FITC challenge in Pam2Cys-exposed mice (Figure S2E) and $Ly6C^+$ MDSCs from Pam2Cys-exposed animals produced high concentration of NO (Figure 5E, middle). NO production and T cell suppression by $Ly6C^+$ MDSCs was completely abrogated in a transwell experiment (Figure 5E middle, Figure S2F), indicating that MDSC activation is a prerequisite for MDSC NO production and MDSC-mediated suppression. Flow cytometry analysis of the co-culture confirmed higher expression of iNOS by $Ly6C^+$ cells (with a very low expression of arginase and IL-10 by both MDSC subsets) (Figure S2G). In addition, the inhibition of iNOS by L-NMMA or L-NIL completely abrogated MDSC-mediated suppression of T cell proliferation (Figure 5E, right). Similarly, in PBMCs of AD patients we detected a distinct $iNOS^+$ population of $CD11b^+CD11c^-$ cells. These cells were completely absent in healthy individuals (Figure 5F). Importantly, we also detected $iNOS^+CD11b^+CD11c^-$ cells in AD skin (Figure 5G, Figure S2H).

All together, the above results indicate that skin infection-induced MDSCs are present in the skin in mice and humans, where they inhibit T cell proliferation by means of cell-to-cell contact and iNOS.

Pam2Cys-induced immune suppression is dependent on cutaneous TLR2

Next, we investigated underlying mechanisms how innate immune sensing in the skin initiates MDSCs. Therefore we determined the role of TLR2. *Tlr2*$^{-/-}$ and WT mice were treated as

shown in Figure S1A with or without cutaneous Pam2Cys exposure. In contrast to WT mice (Figure 6A left), *Tlr2*$^{-/-}$ mice failed to inhibit FITC-specific CHS (Figure 6A right) and T cell proliferation (Figure 6B) and no reduction of $CD4^+$ and $CD8^+$ T cell numbers and accumulation of MDSCs (Figure 6C) was observed following Pam2Cys exposure. Cutaneous innate immune sensing through TLR2 may act through skin resident cells or recruited circulating blood immune cells. Thus, mouse chimeras were generated to distinguish between TLR2 sensing of skin resident or recruited hematopoietic cells, as depicted in Figure S3A. Chimerism was confirmed by PCR of bone marrow cells (Figure S3B). The percentage of MDSCs was analyzed following the protocol shown in Figure S1A. WT mice, reconstituted with WT BM (WT + WT-BM) and WT mice, that obtained *Tlr2*$^{-/-}$ BM (WT + *Tlr2*$^{-/-}$-BM), upregulated MDSCs following Pam2Cys exposure (Figure 6D, top). In contrast, *Tlr2*$^{-/-}$ mice reconstituted with WT BM (*Tlr2*$^{-/-}$ + WT-BM) failed to accumulate MDSCs, similar to control *Tlr2*$^{-/-}$ mice with *Tlr2*$^{-/-}$ BM (*Tlr2*$^{-/-}$ + *Tlr2*$^{-/-}$-BM) (Figure 6D, bottom). Thus, TLR2 expression on skin resident cells, which next to keratinocytes includes radiation resistant skin resident Langerhans or mast cells, is necessary and sufficient for MDSC accumulation.

Next, we investigated a functional role of TLR2 on MDSCs. Chimeric mice were generated by reconstitution with 50% CD45.1 WT and 50% CD45.2-*Tlr2*$^{-/-}$ BM (Figure S3C). Following Pam2Cys exposure, approximately 20% of spleen cells were MDSCs irrespective whether WT CD45.1 or *Tlr2*$^{-/-}$CD45.2 cells were analyzed (Figure S3D), demonstrating that TLR2 is dispensable on MDSC precursor cells for MDSC induction and accumulation.

Cutaneous IL-6 is critically required for MDSC induction

Our previous experiments showed that cutaneous Pam2Cys sensing through TLR2 is sufficient to induce MDSCs and consecutive suppression of cutaneous recall responses. To identify underlying mechanisms we first analyzed which cells in the skin could be responsible

for sensing Pam2Cys. Immunofluorescence staining of TLRs after exposure of mice to Pam2Cys or Pam3Cys showed an upregulation of the corresponding TLR on keratinocytes (Figure 7A). Similar analyses of human skin samples showed pronounced TLR2 expression in human skin albeit at lower amount in AD compared to healthy skin (Figure S4A), as known from other studies (Kuo et al., 2013). Next, we analyzed the functional consequences of the TLR upregulation. We exposed mice to different TLR ligands (Pam2Cys, Pam3Cys, CpG and LPS) and analyzed cutaneous mRNA expression of cutaneous cytokines. All TLR ligands moderately upregulated TNF and the chemokine CXCL-2 was most dominantly induced by Pam2Cys and Pam3Cys (Figure 7B). Upregulation of IL-6 mRNA in the skin was most pronounced only after Pam2Cys exposure. In comparison to skin following FITC-only or FITC-plus-other TLR-ligands exposure, cutaneous Pam2Cys exposure induced a 400-fold upregulation of IL-6 mRNA (Figure 7B, right). On the protein level we detected increased IL-6 production by CD45 negative cells (which were also MHC-II negative, Figure S4B) (Figure 7C). To confirm these data, we stimulated primary human keratinocytes with TLR ligands and detected upregulation of IL-6 production exclusively following Pam2Cys treatment (Figure 7D).

To regulate MDSC induction in the bone marrow (Figure 4A), cutaneous IL-6 needs to reach the blood stream (Chalmin et al., 2010). Indeed, IL-6 concentrations in mouse sera strongly increased one day after cutaneous Pam2Cys exposure (Figure 7E). These data suggest that IL-6 plays a crucial role in Pam2Cys-induced MDSC induction; therefore, *Il6*$^{-/-}$ mice were investigated. In contrast to WT mice, cutaneous Pam2Cys exposure in *Il6*$^{-/-}$ mice failed to suppress FITC-CHS (Figure 7F), and no induction of MDSCs could be detected (Figure 7G). Consequently, the injection of IL-6 into the mice caused an increase of MDSCs in the spleen (Figure S4C, D), suggesting that IL-6 is responsible for MDSC induction and expansion. To investigate whether IL-6 plays a role in MDSC migration to the skin, we applied anti-IL-6 antibody shortly before challenge and analyzed MDSC numbers in the skin. We found a

335 significant and unequivocal increase of $Gr1^{+}CD11b^{+}$ cells in both conditions (Figure S4E)
336 and the adoptive transfer of MDSCs into *Il6*$^{-/-}$ mice showed a suppression of immune
337 responses, comparable to what is observed in WT mice (Figure S4F). To investigate whether
338 IL-6 plays a role for MDSC development, we analyzed MDSCs generation *in vitro*. BM-
339 derived MDSCs (see suppl. Methods) were treated with IL-6 during development and their
340 suppressive function was investigated in a suppression assay with responder cells. As shown
341 in Figure 7H, the exposure of MDSCs to IL-6 during generation enhanced their suppressive
342 function. These data indicate that IL-6 supports induction and development of suppressive
343 MDSCs, but not their migration to the skin.
344 Taken together, these data suggest a scenario in which Pam2Cys is sensed by TLR2 on skin
345 resident cells, leading to the expression and secretion of IL-6 in such high amounts that
346 MDSCs expand and accumulate, leading to the inhibition of cutaneous recall responses.

Discussion

In this study we found that cutaneous exposure to bacteria and bacterial substances known to act as potent MAMPs induced a strong immune suppression mediated by MDSCs. These findings highlight that certain classes of bacterial molecules are able to orchestrate unique pathways that, even after limited cutaneous exposure, are sufficient to induce immune suppression. We found that cutaneous exposure to TLR2-TLR6 but not to TLR2-TLR1 ligands induced MDSCs and consecutive cutaneous immune suppression. Bacteria differ in the acylation patterns of their lipoproteins (Kurokawa et al., 2012b). Our results suggest that they may differ in their potential to activate different TLR2 heterodimers and to regulate immune responses as well. Consequently, acylation properties may characterize bacteria as pathogens or commensals. It was shown recently, that the degree of lipoprotein-acylation depends on environmental factors and growth phase. Lipoprotein SitC was triacylated when *S. aureus* was in the exponential growth phase at neutral pH and diacylated in the post exponential phase at low pH (Kurokawa et al., 2012a). At the situation on the skin, where pH is low and chronic *S. aureus* colonization (which is almost always found in AD) is present, a post exponential growth phase of *S. aureus* can be assumed. Consequently, lipoproteins from *S. aureus* on the skin are more diacylated. Based on our data, we hypothesize that diacylation of lipoproteins induces acute inflammation followed by immune suppression as a consequence. Further, one can also assume that pathogenic and non-pathogenic skin microflora may have different acylation properties and therefore different compositions of TLR2 ligands and thus overall differ in their immune consequences.

Previous data using a systemic sepsis model with Gram-negative bacteria derived from the gut described the MyD88 and TLR4 pathway to be most relevant for MDSC expansion (Delano et al., 2007). However, the exact cascade of events was not investigated (Arora et al., 2010; Delano et al., 2007). Our data investigating the common route of cutaneous infection with Gram-positive bacteria show that TLR2 activation on skin resident cells mediates MDSC

accumulation and consecutive immune suppression. Induction of MDSCs by activation of cutaneous TLR2-6 most dominantly involves IL-6. Cutaneous innate immune cells (Blander and Medzhitov, 2004), keratinocytes and even melanocytes (Stadnyk, 1994; Takashima and Bergstresser, 1996) are all capable of producing innate cytokines, such as IL-6. Indeed, in AD, where keratinocytes act as a critical first line of defense against microbes, early IL-6 production has been described after direct contact of keratinocytes with *S. aureus* (Sasaki et al., 2003). Moreover, IL-6 has been found to be increased in AD skin (Fedenko et al., 2011) and especially in AD skin lesions (Travers et al., 2010), in which the amount of IL-6 correlates with bacterial burden (Travers et al., 2010). Genome wide association studies recently also identified an IL-6 receptor (IL-6R) variant as a risk factor for AD (Esparza-Gordillo et al., 2013) and a small case series with three patients has demonstrated therapeutic efficacy of an IL-6R blockade by tocilizumab, an IL-6R antibody (Navarini et al., 2011). These observations confirm the importance of IL-6 production by skin cells in response to microbes; however the precise immune consequences of cutaneous IL-6 induction had not been elucidated. Our data allow to propose a model, how the cutaneous innate immune network functions: diacylated lipopeptides activate TLR2-TLR6 on skin resident cells followed by marked IL-6 production leading to the MDSC accumulation, which is a prerequisite of subsequent immune suppression by MDSCs. Our data also indicate that these TLR2-6-induced MDSCs are prototypic MDSCs as characterized in other settings. Moreover, our data further has identified that skin infection-induced MDSCs suppressed immune responses in mice and humans.

In conclusion, our study reveals a consequence of cutaneous innate immune sensing for adaptive immune functions. The presence of certain lipoproteins on the skin may serve not only as danger signal for the initiation of effective immune responses, but may also be able to counter-regulate inflammation and potently control and suppress immune responses.

Experimental Procedures

Animals

Specific-pathogen-free, WT BALB/c mice were purchased from Charles River (Sulzfeld, Germany). *Tlr2*$^{-/-}$ mice (C57BL/6) were from C. Kirschning (Institute of Medical Microbiology, University Duisburg-Essen) and were backcrossed to BALB/c for 10 generations. *Il6*$^{-/-}$-BALB/c mice were from Dr. M. Kopf (Swiss Federal Institute of Technology, Switzerland). All mice were kept under specific pathogen-free conditions in accordance with FELASA (Federation of European Laboratory Science Association) in the University of Tübingen. The experiments were performed with the approval of the local authorities (Regierungspräsidium Tübingen HT1/10, HT3/11, HT7/11, HT5/13, HT8/13). Age-matched female mice were used in all experiments.

Epicutaneous mouse skin infection model

The experimental model is based on epicutaneous application of the *S. aureus* on shaved skin of mice (Wanke et al., 2013). Mice were sensitized with FITC following the protocol as shown in Figure S1A. At days 7 and 10 3x10^8 WT or lgt-mutant *S. aureus* Newman in 30µl PBS or PBS control were added to filter paper discs placed onto the prepared skin and covered by Finn Chambers on Scanpor (Smart Practice, Phoenix, USA). Before application to the skin, barrier was disrupted by tape-stripping.

FITC contact hypersensitivity and exposure to TLR2 ligands

Mice were sensitized by administration of 80 µl of a 0.37% FITC solution (dissolved in 1:1 acetone:dibutyl phthalate, Sigma Aldrich, Taufkirchen, Germany) on the shaved abdomen on days -8, -7. TLR2 ligands were applied intracutaneously together with the second epicutaneous application of FITC on days -1 and 0 (Figure S1A) in the following concentrations per mouse: Pam2Cys: 2 µg, Pam3Cys: 4 µg, FSL-1: 40 µg. Control mice obtained PBS instead of TLR2 ligands. At d7 mice were challenged by epicutaneous

application of 0.37% FITC solution on both sides of the ears. Ear thickness was measured with a micrometer (Oditest®, Kroeplin, Germany) as previously described (Volz et al., 2014), and data are expressed as change in ear thickness compared to thickness before treatment. In some experiments mice were treated with 0.3µM CpG 1668 (0.2µM, Eurofins Genomics, Ebersberg, Germany), 1µg/mouse LPS (from Salmonella minnesota R595, Alexis Biochemicals, Lausen, Switzerland), cyclophoshamide (2mg/mouse, Sigma-Aldrich, Taufkirchen, Germany), 20µg/mouse rmIL-6 (20µg/mouse) or 50µg/mouse anti-IL-6 (Biolegend, San Diego, USA).

Human MDSCs

The study was approved by the local ethics committee of the University of Tübingen, Germany, and written informed consent was obtained from all subjects (project number 344/2011BO2, 345/2011BO2, 396/2011BO2, 040/2013BO2, 180/2013BO2). PBMCs were obtained from heparinized blood by centrifugation (800g for 30min) using Ficoll-Histopaque (Biochrom, Berlin, Germany). MDSCs in the blood or skin of either healthy volunteers or non-AD-controls or atopic dermatitis patients were analyzed by flow cytometry and characterized as $CD11b^+CD33^+HLA\text{-}DR^-CD14^-$ cells.

Bone marrow chimeras

Recipient mice were lethally irradiated at 7.0 cGy and next day bone marrow cells (10^6 cells per recipient) were i.v. injected into recipient mice. To confirm the chimerism of mice, genotyping of bone marrow cells by PCR for the WT and the mutated *Tlr2* gene was conducted (Figure S3B).

Depletion of $CD11b^+$ cells

$CD11b^+$ cells were depleted from PBMCs using the $CD11b^+$ Beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol.

Statistical analysis

Unless otherwise stated, quantitative results are expressed as means +/- standard deviations and differences were compared by unpaired, two-tailed Student's t-test ($p < 0.05$ was regarded as significant).

Contribution:

T.B. and Y.S. designed the study, analyzed the data and wrote the manuscript; Y.S. performed the experiments; C.G. performed histological staining of human samples; F.W., E.G and T.V. cooperated in regard to human samples and participated in the manuscript preparation; M.K. assisted with data analysis and bacteria preparation; D.D and F.G provided WT and *Δlgt S. aureus*; S.K., M.S. H-G.R and M.R. contributed to project development by fruitful discussions; K.C., W.K., D.D, F.G. participated in the manuscript preparation.

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668 **Figure legends**

669

670 **Figure 1. Cutaneous *Staphylococcus aureus* induces immune suppression in mice and**
671 **humans.** (A-E) FITC-sensitized wild-type (WT) mice were treated following the protocol in
672 Figure S1A (with living WT or lipoprotein mutant *(Δlgt) S. aureus*). Bacterial load as colony
673 forming units (CFU) (mean +/- standard deviation SD, *n*=5) (A), ear swelling (mean +/- SD,
674 *n*=5) (B), histology (H&E staining) (C), proliferation of skin-draining lymph node (LN) cells
675 stimulated *ex vivo* with FITC (detected as counts per minute (cpm) of ^{3}H-thymidine
676 incorporation) (mean +/- SD of triplicates) (D), and the percentage of cell populations in the
677 spleen (mean +/- SD, *n*=5) (E) were investigated. *: $P < 0.05$. (F) PBMCs from atopic
678 dermatitis (AD) patients (*n*=33) and healthy volunteers (*n*=30) were analyzed for MDSCs,
679 defined as $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}CD14^{-}$ cells. The dots represent individual values and the
680 horizontal bar is the group mean. Red squares represent MDSCs of patients with severe AD
681 and eczema herpeticum. *: $P < 0.05$ (Mann-Whitney test). Data are representative of at least
682 two independent experiments. See also Figure S1.

683

684 **Figure 2. Cutaneous exposure to TLR2-6 but not TLR2-1 ligands ameliorates T cell-**
685 **mediated recall responses of the skin.** WT mice were treated following the protocol shown
686 in Figure S1A. Mice were cutaneously exposed to FSL-1 in (A-D) and Pam2Cys or Pam3Cys
687 in (E-H). Ear swelling response (mean +/- SD, *n*=5) (A, E), histology (H&E staining) (B, F),
688 proliferation of skin-draining LN cells stimulated *ex vivo* with FITC (mean +/- SD of
689 triplicates) (C, G) and the percentage of cell populations in the spleen (mean +/- SD, *n*=5) (D,
690 H) are shown. Data are representative of at least two independent experiments. Experiments
691 shown in (A) were performed with FSL-1 from two different providers. *: $P < 0.05$.

692

Figure 3. Myeloid-derived suppressor cells are responsible for skin infection-induced immune suppression. (A) WT mice were treated with FITC with or without cutaneous Pam2Cys exposure following the protocol in Figure S1A. The mice were additionally treated with Gr1 depleting (right) or with an isotype control antibody (left) at day 2 and 4. Ear swelling response (mean +/- SD, *n*=5, left) was evaluated. Data are representative of two independent experiments. (B-C) WT mice were treated following the protocol shown in Figure S1A (without Pam2Cys exposure). One group of mice received Ly6C-Ly6G positive cells from donors that were sensitized with FITC and exposed to Pam2Cys. The control group received spleen cells from naïve mice. The ear swelling response (mean +/- SD, *n*=5) (B) and the FITC-specific proliferation of LN cells (as cpm, mean +/- SD of triplicates) (C) were evaluated. (D) $CD11b^+$ cells of PBMCs from healthy volunteers (*n*=8, left) and AD patients (*n*=7, right) were depleted, stimulated with anti-CD3-CD28-mAbs and analyzed for proliferation. *: $P < 0.05$ (Mann-Whitney test). (E) PBMCs from healthy donors (*n*=8) and AD patients (*n*=7) were analyzed for TCR ζ-chain expression (mean fluorescence intensity, MFI, $CD3^+$ Gate of living cells) by intracellular flow cytometry. Each dot represents an individual value, the horizontal bar is the group mean. *: $P < 0.05$ (Mann-Whitney test). See also Figure S2.

Figure 4. Skin infection-induced MDSCs accumulate in the skin in mice and humans. (A) WT mice were treated following the protocol in Figure S1A. The percentage of $CD4^+$, $CD8^+$ or $Gr1^+CD11b^+$ cells in Pam2Cys-exposed mice were analyzed by flow cytometry at indicated time points after Pam2Cys exposure in bone marrow (left) and spleen (right) (mean +/- SD, *n*=3). Asterisks show significant differences compared with t=0 determined by one-way analysis of variance (ANOVA) followed by Dunnet's post test. *: $P < 0.05$ Data are representative of two independent experiments. (B) Cells from ear skin, isolated 4 h or 8 h after FITC challenge, were analyzed by flow cytometry (gate: living cells). A representative

flow cytometry plot (left), means +/- SD (n=5) (middle), and total numbers of $Gr1^+CD11b^+$ cells (mean +/- SD, n=5) (right) are shown. Data are representative of three independent experiments. (C) Cells isolated from skin samples of AD patients (n=9) and non-AD-controls (n=9) were analyzed by flow cytometry (gate: living cells) for MDSCs, defined as $CD11b^+CD33^+HLA\text{-}DR^-CD14^-$ cells. A representative flow cytometry plot with the gating strategy first for $CD11b^+CD14^-$ (top) and then $CD33^+HLA\text{-}DR^+$ (bottom) and the percentage of the $CD11b^+CD33^+HLA\text{-}DR^-CD14^-$ cells (left) and cumulative analysis (right) is shown. Each of the dots represents an individual value and the horizontal bar the group's mean. *: $P < 0.05$ (Mann-Whitney test). n.s., not significant.

Figure 5. Skin infection-induced MDSCs suppress T cell activation through mechanisms requiring NO production. (A) WT mice were treated following the protocol in Figure S1A. 24 h (A, B) or 8 h (C, D) after FITC challenge ear tissue cells were analyzed. (A) Flow cytometry for $CD3^+$ cells (top) and IFN-γ production (bottom). A cumulative result (means +/- SD, n=5) is shown. (B-C) Quantitative RT-PCR analysis for cytokines (B) or chemokines (C) (normalized to housekeeping genes β-actin-APDH) and means +/- SEM (n=5) are shown. Expression of the skin of FITC only-exposed mice was set as 1. *: $P < 0.05$. (D) Cells isolated from bone marrow, blood and skin of Pam2Cys-treated mice were analyzed for chemokine receptor expression by flow cytometry (gate: $Gr1^+CD11b^+$ of living cells), shown as percentage of $Gr1^+CD11b^+$ (means +/- SD, n=5). (E) Spleen cells were co-cultured *in vitro* with $Ly6C^+$ or $Ly6G^+$ MDSCs as indicated, stimulated by anti-CD3-CD28-mAbs and analyzed for proliferation (left); supernatants (ratio 2:1) were analyzed for NO production by Griess reaction (mean +/- SD of experimental triplicates) (middle), iNOS inhibitors L-NMMA and L-NIL were added to the co-culture (right). Significant differences between experimental conditions were assessed by one-way ANOVA followed by Tukey's post-hoc test (*: $P < 0.05$). Data are representative of at least two independent experiments. (F) PBMCs from

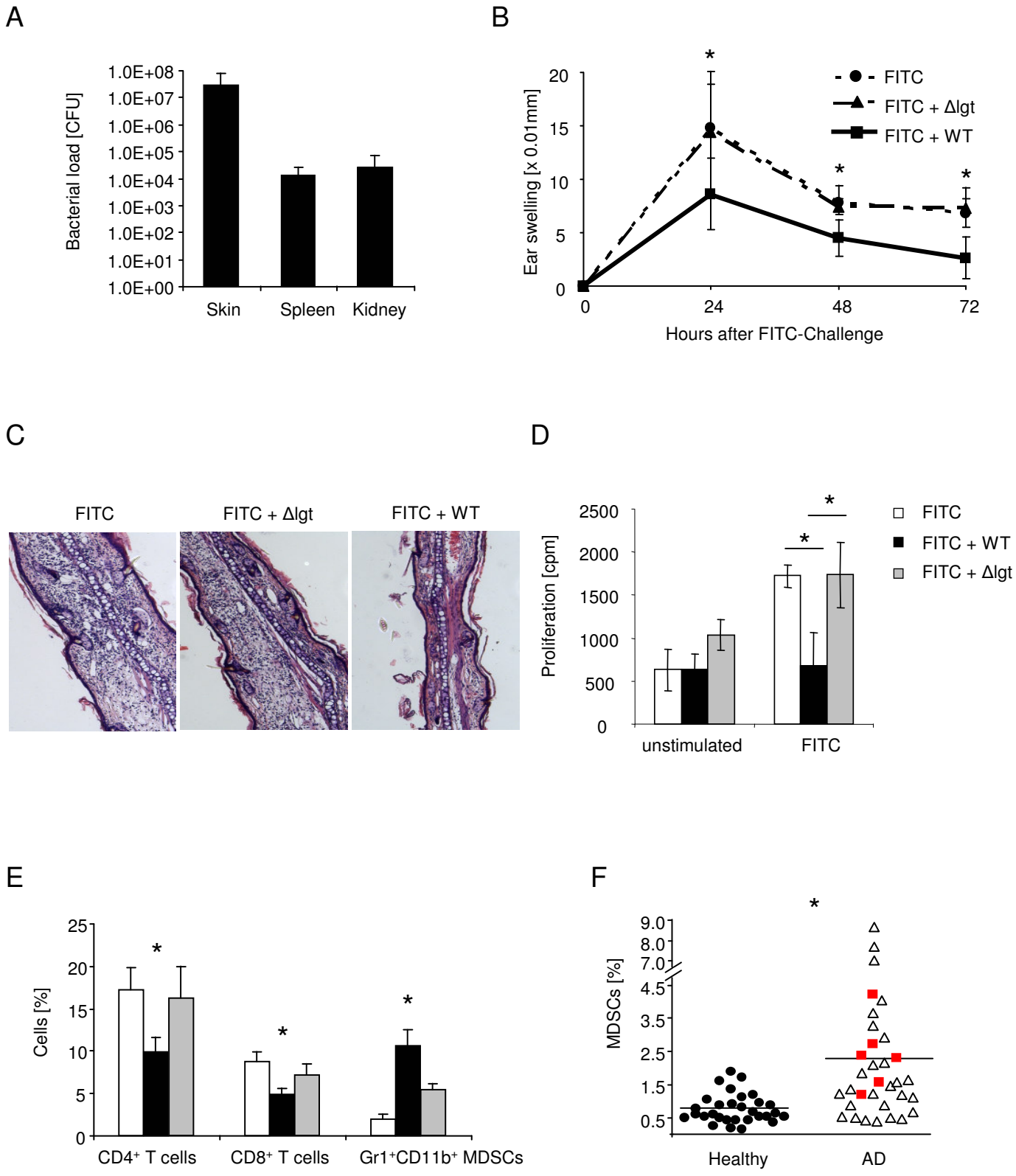
healthy donors and AD patients were analyzed by intracellular flow cytometry (iNOS$^+$ in CD11b$^+$CD11c$^-$Gate of living cells). A representative result out of 7 individuals is shown. (G) Skin tissue of AD patients was analyzed by immunofluorescence. Arrows indicate cells positive for CD11b and iNOS and negative for CD11c. Scale bar represents 25 μm. See also Figure S2.

Figure 6. Pam2Cys-induced immune suppression is dependent on TLR2. (A-C) WT and *Tlr2*$^{-/-}$ mice were treated following the protocol shown in Figure S1A and ear swelling (mean +/- SD, *n*=5) after FITC challenge (A), proliferation of lymph node cells after FITC stimulation *ex vivo* (mean +/- SD of triplicates) (B), and the percentage of spleen cell populations (mean +/- SD, *n*=5) (C) were analyzed. (D) WT mice or *Tlr2*$^{-/-}$ were irradiated and reconstituted with WT or *Tlr2*$^{-/-}$ bone marrow cells (see Figure S3A). 7 weeks later, the chimeric mice were treated following the protocol shown in Figure S1A and their spleen cells were analyzed by flow cytometry. The percentage of Gr1$^+$CD11b$^+$ cells is shown (mean +/- SD, *n*=5). Data are representative of three independent experiments. *: $P < 0.05$, n.s., not significant. See also Figure S3.

Figure 7. IL-6 is required for induction of Gr1$^+$CD11b$^+$ cells and Pam2Cys-induced immune suppression. (A-C) WT mice were treated following a protocol similar to that shown in Figure S1A. 24 h after cutaneous exposure to TLR ligands or PBS (control), immunoflourescence for TLR2 (red), TLR6 or TLR1 (blue) and nuclei (green) was done in A, a representative picture (n=3) is shown. Scale bar represents 30 μm (B) The skin was evaluated for the expression of TNF, CXCL-2, and IL-6 mRNA by quantitative RT-PCR analysis (normalized to housekeeping gene β-actin). Expression in the skin of untreated mice (naïve) was set as 1 (mean +/- SD, *n*=5). (C) Skin cells were isolated and analyzed for IL-6 production by intracellular flow cytometry, a cumulative analysis (mean +/- SD, n=5) is

771 shown. (D) Primary human keratinocytes were isolated, treated with TLR ligands for 24h and
772 the production of IL-6 was measured by ELISA (mean +/- SD of triplicates). (E) WT mice
773 were treated following a protocol similar to that shown in Figure S1A and IL-6 concentrations
774 in the sera were analyzed by ELISA (mean +/- SD of triplicates). (F, G) WT and *Il6*$^{-/-}$ mice
775 were treated following the protocol shown in Figure S1A and ear swelling (mean +/- SD, *n*=5)
776 (F) and the percentage (mean +/- SD, *n*=5) of Gr1^{+}CD11b^{+} cells (G) were analyzed. (H) Bone
777 marrow-derived MDSCs were treated with IL-6 (in indicated concentrations) during
778 generation and their suppressive activity was measured in a co-culture with activated spleen
779 cells (responder cells) in ratio 1:4. Proliferation of responder cells without MDSCs was set as
780 100%. Data are representative of two independent experiments. *: $P < 0.05$. See also Figure
781 S4.

Figure 1


A
Bacterial load [CFU]
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Skin
Spleen
Kidney
B
Ear swelling [x 0.01mm]
20
15
10
5
0
0
24
48
72
Hours after FITC-Challenge
FITC
FITC + Δlgt
FITC + WT
C
FITC
FITC + Δlgt
FITC + WT
D
Proliferation [cpm]
2500
2000
1500
1000
500
0
unstimulated
FITC
FITC
FITC + WT
FITC + Δlgt
E
Cells [%]
25
20
15
10
5
0
CD4+ T cells
CD8+ T cells
Gr1+CD11b+ MDSCs
F
MDSCs [%]
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8.0
7.0
4.5
3.5
2.5
1.5
0.5
Healthy
AD

Figure 2

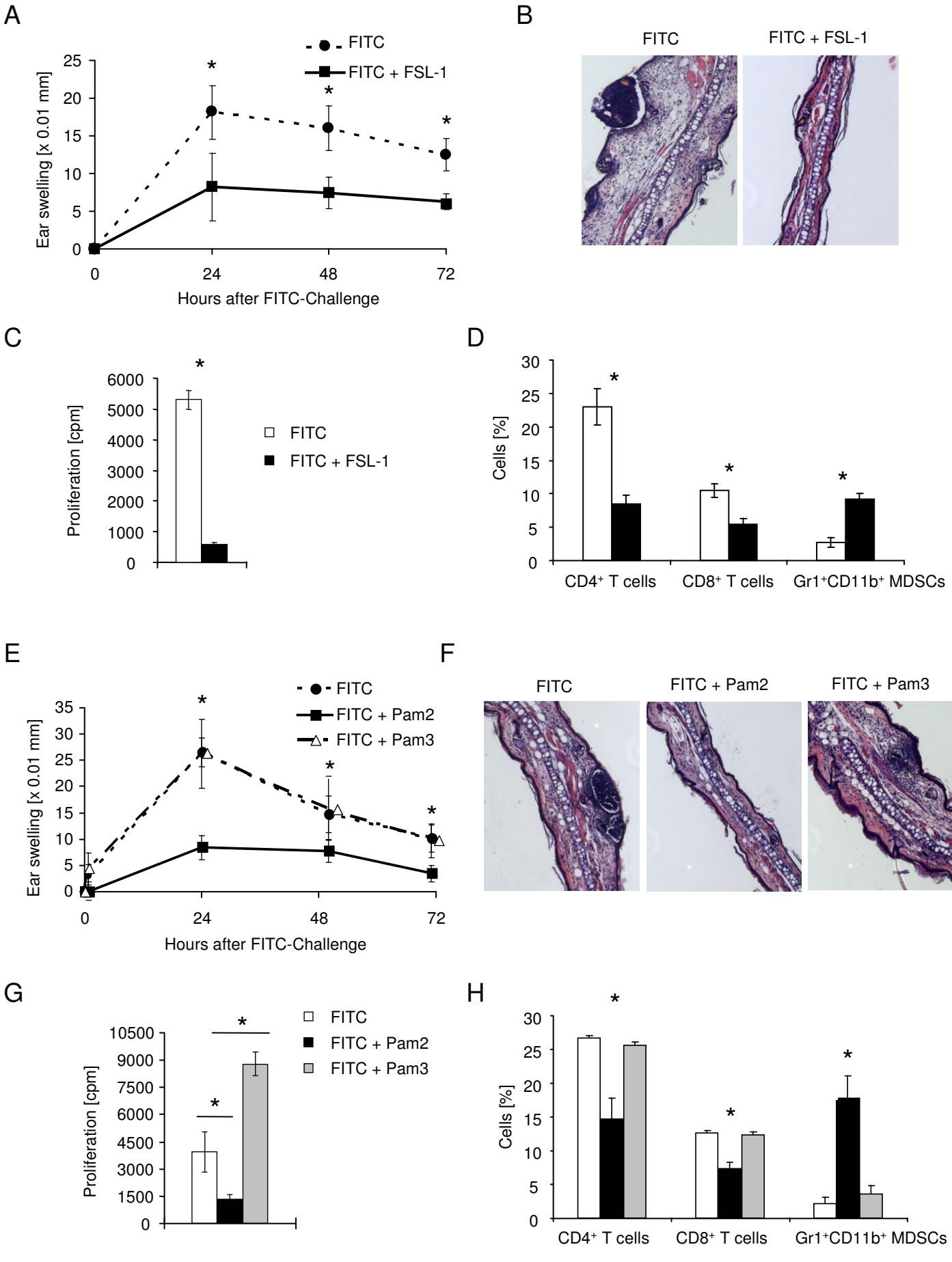

A
FITC
FITC + FSL-1
Ear swelling [x 0.01 mm]
Hours after FITC-Challenge
B
FITC
FITC + FSL-1
C
Proliferation [cpm]
FITC
FITC + FSL-1
D
Cells [%]
CD4+ T cells
CD8+ T cells
Gr1+CD11b+ MDSCs
E
FITC
FITC + Pam2
FITC + Pam3
Ear swelling [x 0.01 mm]
Hours after FITC-Challenge
F
FITC
FITC + Pam2
FITC + Pam3
G
Proliferation [cpm]
FITC
FITC + Pam2
FITC + Pam3
H
Cells [%]
CD4+ T cells
CD8+ T cells
Gr1+CD11b+ MDSCs

Figure 3

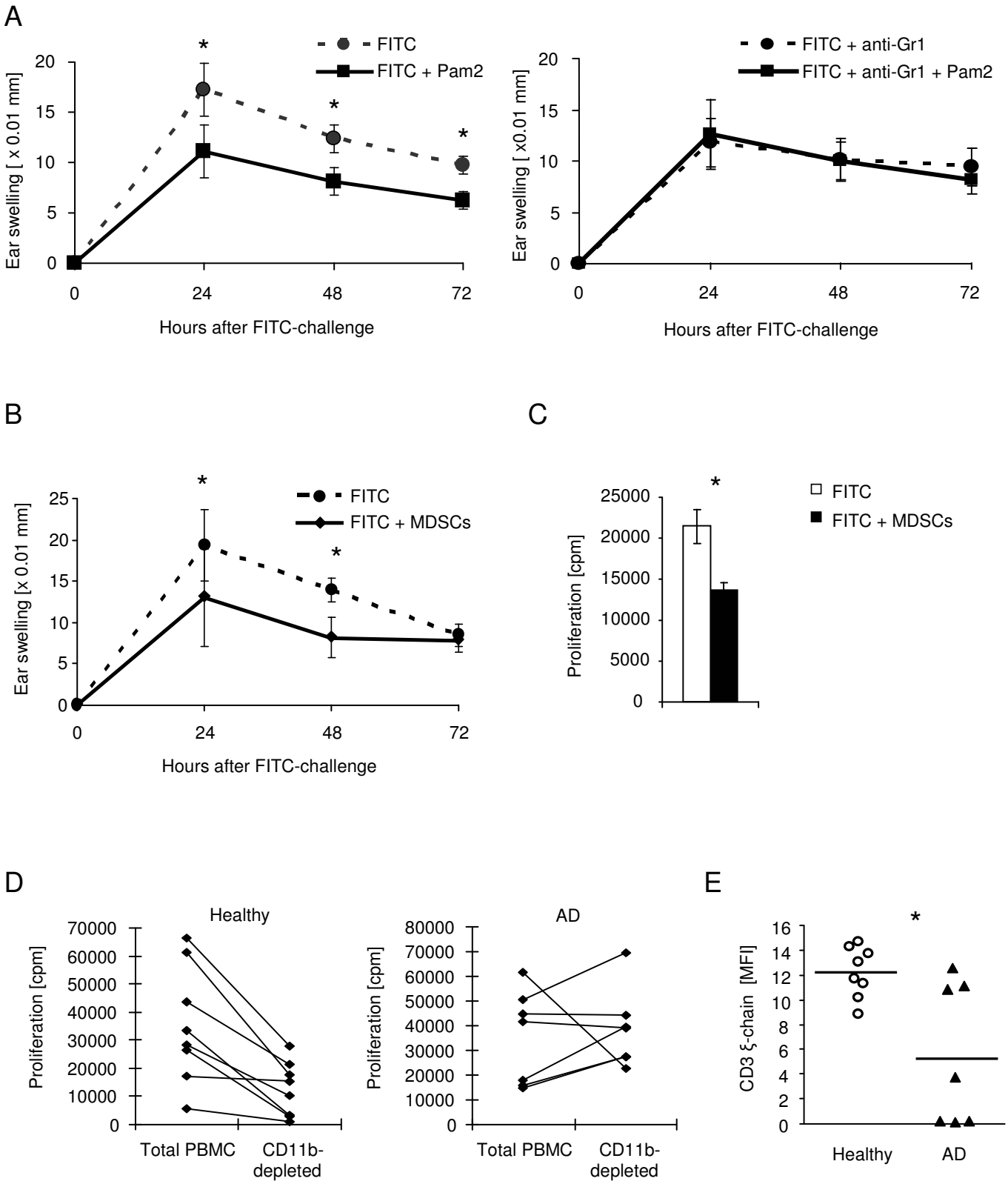

A
FITC
FITC + Pam2
Ear swelling [x 0.01 mm]
Hours after FITC-challenge
FITC + anti-Gr1
FITC + anti-Gr1 + Pam2
Ear swelling [x0.01 mm]
Hours after FITC-challenge
B
FITC
FITC + MDSCs
Ear swelling [x 0.01 mm]
Hours after FITC-challenge
C
FITC
FITC + MDSCs
Proliferation [cpm]
D
Healthy
AD
Proliferation [cpm]
Total PBMC
CD11b-depleted
E
CD3 ξ-chain [MFI]
Healthy
AD

Figure 4

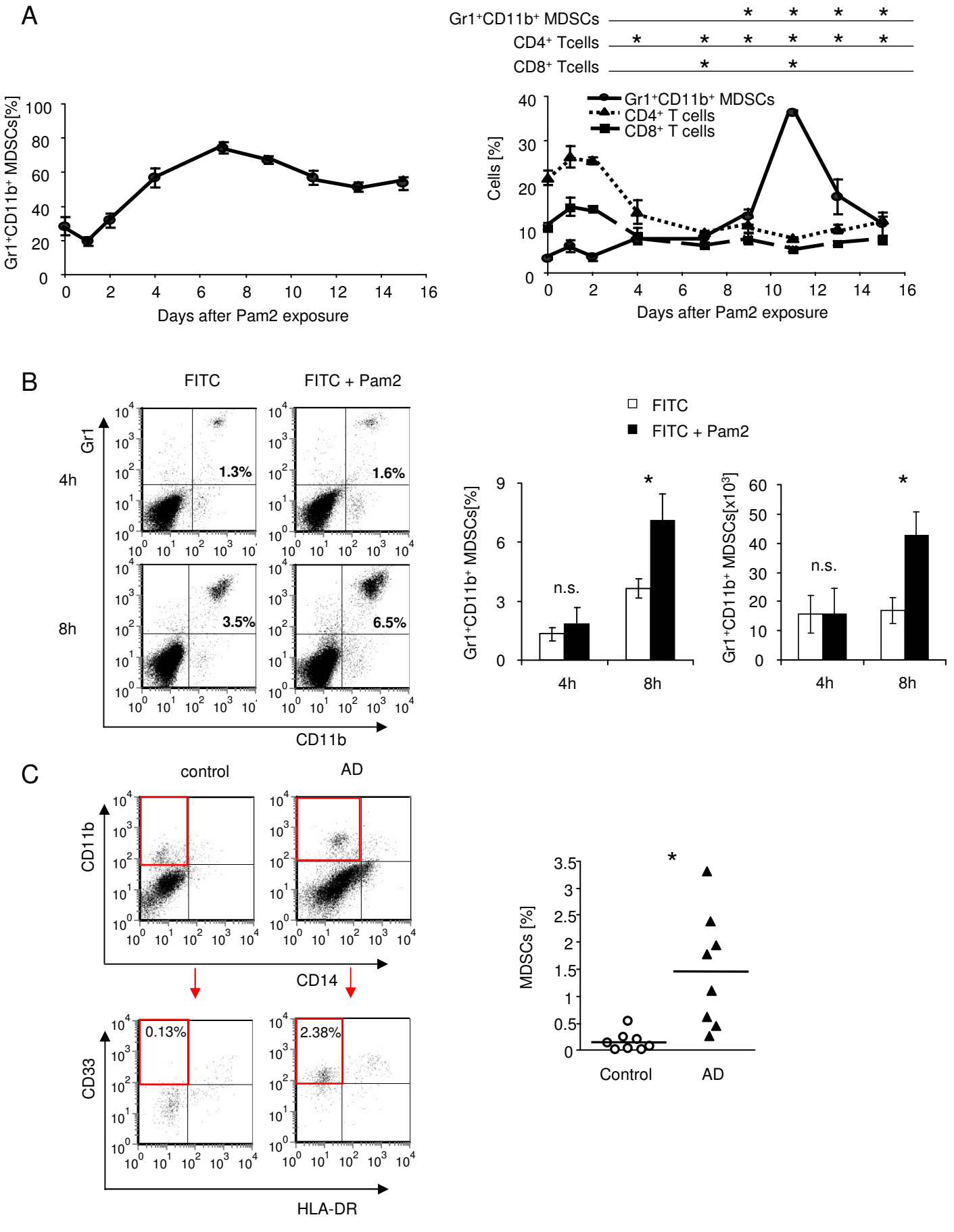

A
Gr1+CD11b+ MDSCs
CD4+ Tcells
CD8+ Tcells
Gr1+CD11b+ MDSCs[%]
Days after Pam2 exposure
Gr1+CD11b+ MDSCs
CD4+ T cells
CD8+ T cells
Cells [%]
Days after Pam2 exposure
B
FITC
FITC + Pam2
Gr1
4h
8h
1.3%
1.6%
3.5%
6.5%
CD11b
FITC
FITC + Pam2
Gr1+CD11b+ MDSCs[%]
n.s.
4h
8h
Gr1+CD11b+ MDSCs[x10³]
n.s.
4h
8h
C
control
AD
CD11b
CD14
CD33
0.13%
2.38%
HLA-DR
MDSCs [%]
Control
AD

Figure 5

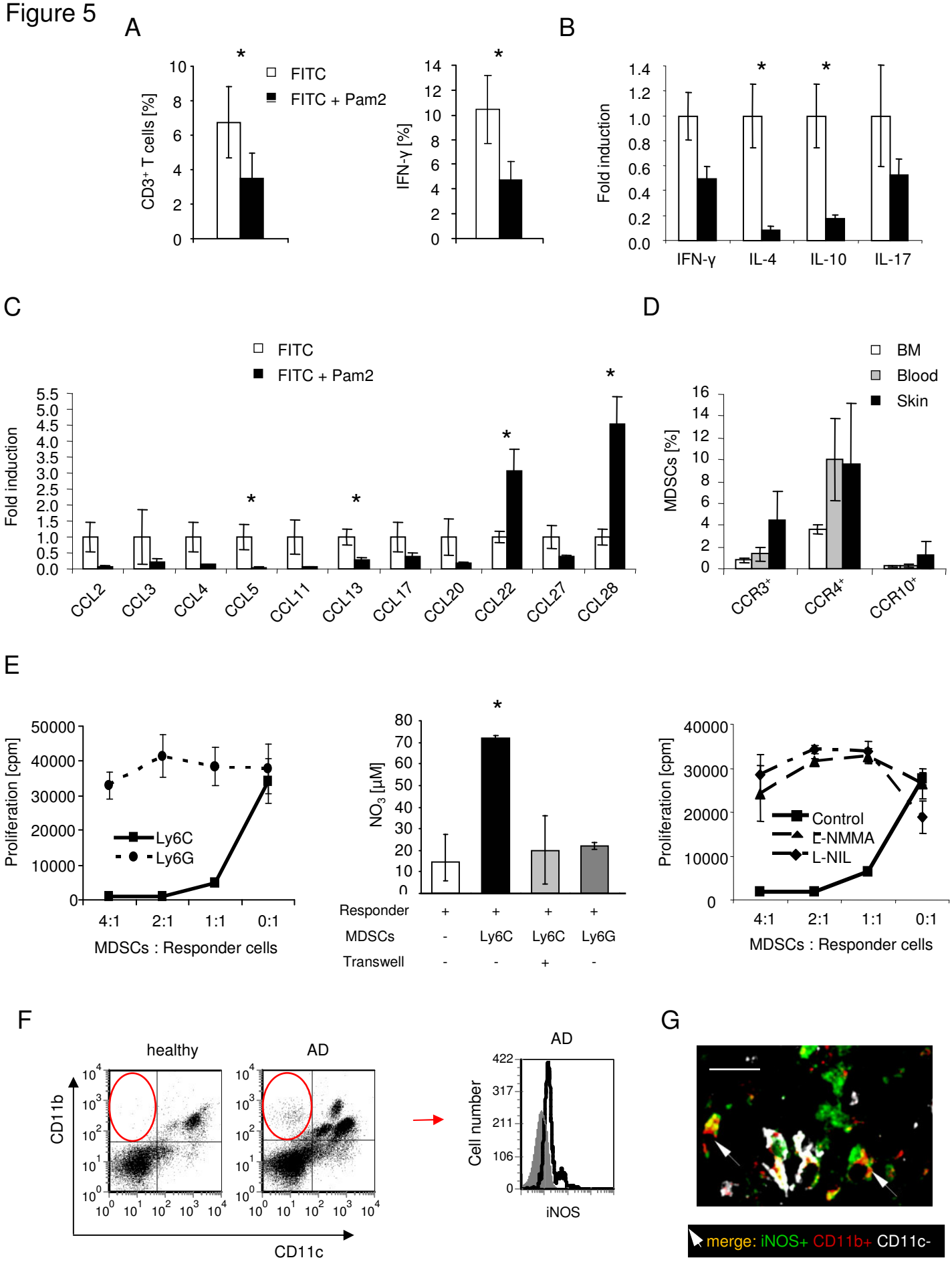

A
B
C
D
E
F
G
FITC
FITC + Pam2
CD3+ T cells [%]
IFN-γ [%]
Fold induction
IFN-γ
IL-4
IL-10
IL-17
CCL2
CCL3
CCL4
CCL5
CCL11
CCL13
CCL17
CCL20
CCL22
CCL27
CCL28
BM
Blood
Skin
MDSCs [%]
CCR3+
CCR4+
CCR10+
Proliferation [cpm]
Ly6C
Ly6G
MDSCs : Responder cells
NO3 [µM]
Responder
MDSCs
Transwell
Control
L-NMMA
L-NIL
healthy
AD
CD11b
CD11c
Cell number
iNOS
merge: iNOS+ CD11b+ CD11c-

Figure 6

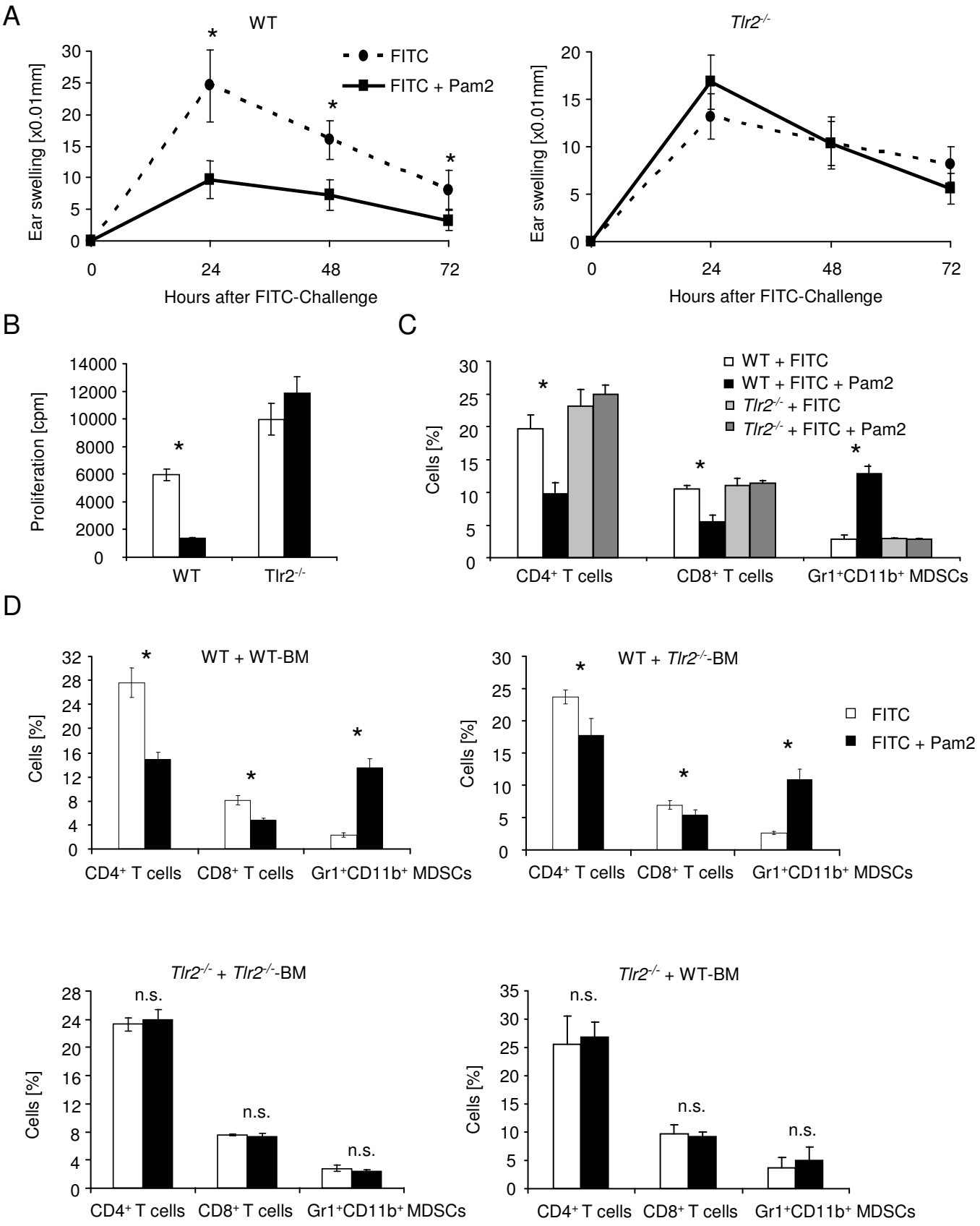
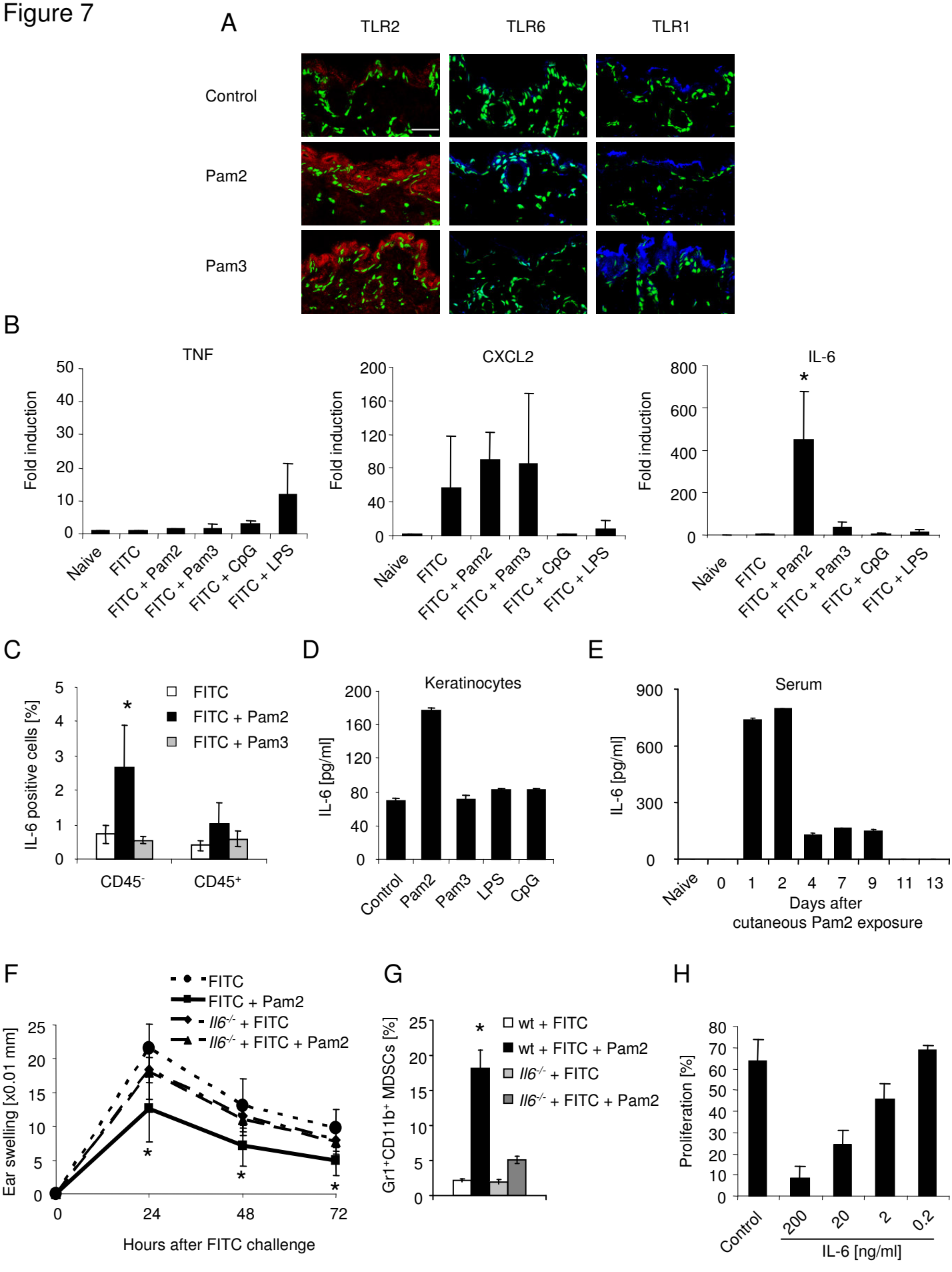

A
WT
FITC
FITC + Pam2
Ear swelling [x0.01mm]
Hours after FITC-Challenge
Tlr2-/-
B
Proliferation [cpm]
WT
Tlr2-/-
C
WT + FITC
WT + FITC + Pam2
Tlr2-/- + FITC
Tlr2-/- + FITC + Pam2
Cells [%]
CD4+ T cells
CD8+ T cells
Gr1+CD11b+ MDSCs
D
WT + WT-BM
WT + Tlr2-/--BM
FITC
FITC + Pam2
Tlr2-/- + Tlr2-/--BM
Tlr2-/- + WT-BM
n.s.

Figure 7


A
TLR2
TLR6
TLR1
Control
Pam2
Pam3
B
TNF
CXCL2
IL-6
Fold induction
Naive
FITC
FITC + Pam2
FITC + Pam3
FITC + CpG
FITC + LPS
C
IL-6 positive cells [%]
FITC
FITC + Pam2
FITC + Pam3
CD45-
CD45+
D
Keratinocytes
IL-6 [pg/ml]
Control
Pam2
Pam3
LPS
CpG
E
Serum
IL-6 [pg/ml]
Naive
Days after cutaneous Pam2 exposure
F
FITC
FITC + Pam2
Il6-/- + FITC
Il6-/- + FITC + Pam2
Ear swelling [x0.01 mm]
Hours after FITC challenge
G
wt + FITC
wt + FITC + Pam2
Il6-/- + FITC
Il6-/- + FITC + Pam2
Gr1+CD11b+ MDSCs [%]
H
Proliferation [%]
Control
IL-6 [ng/ml]

Toll-like receptor 2 ligands promote chronic atopic dermatitis through IL-4–mediated suppression of IL-10

Susanne Kaesler, PhD,[a]* Thomas Volz, MD,[a]* Yuliya Skabytska, MSc,[a] Martin Köberle, PhD,[a] Ulrike Hein, MD,[a] Ko-Ming Chen, MD,[a] Emmanuella Guenova, MD,[a,b] Florian Wölbing, MD,[a] Martin Röcken, MD,[a] and Tilo Biedermann, MD[a,c] *Tübingen and Munich, Germany, and Zurich, Switzerland*

Background: Atopic dermatitis (AD) is a T cell–mediated inflammatory skin disease, with T_H2 cells initiating acute flares. This inflamed skin is immediately colonized with *Staphylococcus aureus*, which provides potent Toll-like receptor (TLR) 2 ligands. However, the effect of TLR2 ligands on the development of T_H2-mediated AD inflammation remains unclear.

Objective: We investigated the progression of T_H2 cell–mediated dermatitis after TLR2 activation.

Methods: Using models for acute AD with T_H2 cells initiating cutaneous inflammation, we investigated the consequences of TLR2 activation. Dermatitis, as assessed by changes in ear skin thickness and histology, was analyzed in different BALB/c and C57BL/6 wild-type and knockout mouse strains, and immune profiling was carried out by using *in vitro* and *ex vivo* cytokine analyses.

Results: We show that T_H2 cell–mediated dermatitis is self–limiting and depends on IL-4. Activation of TLR2 converted the limited T_H2 dermatitis to chronic cutaneous inflammation. We demonstrate that the concerted activation of TLR2 and IL-4 receptor on dendritic cells is sufficient for this conversion. As an underlying mechanism, we found that the combinatorial sensing of the innate TLR2 ligands and the adaptive T_H2 cytokine IL-4 suppressed anti-inflammatory IL-10 and consequently led to the exacerbation and persistence of dermatitis.

Conclusion: Our data demonstrate that innate TLR2 signals convert transient T_H2 cell–mediated dermatitis into persistent inflammation, as seen in chronic human AD, through IL-4–mediated suppression of IL-10. For the first time, these data show how initial AD lesions convert to chronic inflammation and provide another rationale for targeting IL-4 in patients with AD, a therapeutic approach that is currently under development. (J Allergy Clin Immunol 2014;134:92-9.)

Key words: Staphylococcus aureus, *Toll-like receptor 2, innate immunity, IL-4, T_H2, atopic dermatitis, IL-10*

Atopic dermatitis (AD) is a frequent inflammatory skin disease characterized by reduced skin barrier function, intracutaneous T-cell activation, itchy dermatitis, and susceptibility to cutaneous microbial and viral infections, and its prevalence has markedly increased during the past 3 decades. AD is thought to be based on (1) the genetic trait causing susceptibility and (2) environmental factors.[1-5] A detailed characterization of AD inflammation has revealed a biphasic cutaneous cytokine milieu with initial recruitment of IL-4–producing T_H2 cells, followed by a mixed phenotype in the chronic phase.[6,7] Although cutaneous barrier dysfunction also contributes to T_H2 cell polarization, the T_H2 cell cytokine IL-4 further reduces the cutaneous barrier. Additionally, IL-4 suppresses antimicrobial peptide production and immune function, allowing cutaneous microbes to expand and persist.[2,8-10] Next to this prominent communication between the epithelium and T cell–derived cytokines, professional antigen-presenting cells, especially activated dendritic cells (DCs), are also found in AD lesions and are thought to promote AD initiation and persistence by recruiting and activating T cells.[11]

In contrast to the cascade of steps driving adaptive immunity during AD, much less is known about the role of innate immune activation. In the skin rapid activation of innate sentinels drives the first-line response to microbes. The key event in this process is the recognition of microbial pathogen-associated molecular patterns (PAMPs) by specific receptors, such as Toll-like receptors (TLRs), on sentinel cells.[12] After activation by innate immune signals, immune sentinels, such as DCs, orchestrate adaptive immune responses during infections, autoimmunity, allergy, and tolerance.[13,14] To this end, pathogen recognition receptors on DCs recognize a broad spectrum of different

From [a]the Department of Dermatology, Eberhard Karls University, Tübingen; [b]the Department of Dermatology, University Hospital Zurich, Zurich; and [c]the Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich.
*These authors contributed equally to this work.

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Corresponding author: Tilo Biedermann, MD, Department of Dermatology, Eberhard Karls University, Liebermeisterstr, 25, 72076 Tübingen, Germany. E-mail: tilo.biedermann@med.uni-tuebingen.de.

0091-6749/$36.00

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Abbreviations used
AD: Atopic dermatitis
DC: Dendritic cell
IL-4R: IL-4 receptor
LTA: Lipoteichoic acid
OVA: Ovalbumin
Pam2: Pam2Cys
PAMP: Pathogen-associated molecular pattern
TLR: Toll-like receptor
WT: Wild-type

PAMPs.[13] These innate immune sentinels detect more than 1 signal at a time, but only recently has it been appreciated that the combination of incoming signals is crucial for the outcome of immune responses.[15] This is of special interest in the context of AD because AD skin lesions are heavily colonized with gram-positive *Staphylococcus aureus* and PAMPs from *S aureus* predominantly bind TLR2.[2,16] Interestingly, levels of cutaneous TLR2 ligands strongly correlate with AD severity.[17] Increased understanding of combinative innate immune sensing is required to comprehend the pathogenesis of chronic inflammatory diseases at interface organs, and studying such diseases can serve as a model to establish general mechanisms of innate inflammation.

In the present work we analyzed the consequences of innate immune sensing through TLR2 for T_H2 cell–mediated cutaneous inflammation. We used T_H2 cell–mediated dermatitis models because IL-4–producing T_H2 cells are found in early AD lesions when the skin encounters increasing innate TLR2 signals derived from *S aureus.* We found that a single exposure to TLR2 ligands converts T_H2-mediated transient dermatitis to chronic persistent cutaneous inflammation. These findings mimic the development of skin lesions in patients with AD. As the underlying mechanism, we demonstrated that the concerted activation of TLR2 and IL-4R on innate immune sentinels potently suppressed IL-10, thereby exacerbating T_H2-mediated dermatitis and initiating chronic inflammation. The most important immune skin sentinels are DCs, and we show that the concerted activation of TLR2 and IL-4R on DCs is sufficient to convert limited dermatitis into aggravated and persisting inflammation. Thus the combined sensing of innate immune signals together with the hallmark adaptive cytokine of early inflammation, IL-4, determined the outcome of this chronic immune response in patients with AD. For the first time, these data show how initial AD lesions are converted to chronic inflammation. In addition, these findings also provide another rationale for targeting IL-4 in patients with AD, a therapeutic approach that is currently under development.

METHODS

Mice

BALB/c, C57BL/6, DO11.10, OT-II, and signal transducer and activator of transcription 6 (Stat6)–deficient mice were purchased from Charles River (Sulzfeld, Germany) or the Jackson Laboratory (Bar Harbor, Me). $Tlr2^{-/-}$ BL/6 mice were provided by C. Kirschning (Duisburg, Germany) and backcrossed to the BALB/c background, $MHCII^{-/-}$ mice were provided by L. Klein (Munich, Germany), and $Il4ra^{-/-}$ mice were provided by A. Gessner (Erlangen, Germany). All mice were kept and bred under specific pathogen-free conditions in accordance with the guidelines of the Federation of European Laboratory Science Association. All animal experiments were in compliance with both European Union and German law and were approved by local authorities (Regierungspräsidium Tübingen, HT4/03, HT2/11, HT9/13).

Ovalbumin sensitization and adoptive T-cell transfer

Ovalbumin (OVA)–specific T cells were obtained from transgenic mice (DO11.10, OT-II) or from OVA-sensitized mice.[18] $CD4^+$ T cells were isolated with microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and expanded *in vitro*. For adoptive transfer, 1×10^6 OVA-specific T_H2 cells with or without 5 μg of OVA protein (Hyglos, Regensburg, Germany) or 1.5 μg of $OVA_{323\text{-}339}$ peptide (EMC, Tübingen, Germany) were intracutaneously injected into the ear. *S aureus* lipoteichoic acid (LTA; 10 μg; obtained from T. Hartung, Konstanz, Germany) or Pam2Cys (Pam2; 2 or 4 μg; EMC) were included, where stated. DCs were exposed to 10 μg/mL Pam2 for 5 hours, washed twice with PBS, pulsed with 1 mg/mL OVA peptide for 1 hour, and washed twice with PBS. Controls were only pulsed with OVA. Where stated, DCs were incubated with 10 μg of IL-4/mL over night before Pam2 exposure. A total of 2×10^5 DCs were applied intradermally together with 1×10^6 T_H2 cells. For IL-10 supplementation in Il4ra knockout mice, 2 μg of IL-10 (PeproTech, Heidelberg, Germany) was added, and an additional 2 μg was injected 6 and 24 hours later. Control animals received PBS. For induction of endogenous OVA-specific T_H2 cells, DO11.10 mice were sensitized as previously described[19] and challenged with OVA protein, followed by Pam2 injection. Ear thickness was measured with a micrometer (Kroeplin, Schlüchtern, Germany) and expressed as a change in ear thickness after treatment.

Cell culture

T-cell culture was performed, as previously described.[20] OVA-specific T_H2 cells were expanded by the addition of 1.5 ng/mL IL-4 (PromoCell, Heidelberg, Germany) and 10 μg of OVA or 2.5 μg/mL $OVA_{323\text{-}339}$ peptide. For T_H phenotyping, T cells were stimulated with 0.5 μg/ml phorbol 12-myristate 13-acetate (Sigma, Taufkirchen, Germany) and 1 μmol/L ionomycin (Sigma). Bone marrow–derived DCs were generated and cultured, as previously described,[21] and stimulated with 10 μg of Pam2/mL or 10 μg of *S aureus* LTA/mL with or without 10 ng of IL-4/mL. Quantification of IL-4, IFN-γ, IL-10, IL-12p70 (BD PharMingen, Heidelberg, Germany), and IL-13 (eBioscience, San Diego, Calif) in the supernatant was performed by means of ELISA. For real-time analysis, DCs were cultured with the indicated additives with or without α-CD3/α-CD28–activated T_H2 cells in transwell plates for 6 hours.

Real-time quantitative PCR

Total RNA was extracted from *in vitro*–cultured DCs or from ears after adoptive transfer by using the RNA Kit (Machery & Nagel, Düren, Germany). RNA was reverse transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany), according to the manufacturer's instruction. Quantitative real-time PCR was carried out with a LightCycler LC480 (Roche, Basel, Switzerland) by using SYBR Green Supermix (Roche). Data were presented normalized to the housekeeping gene β-actin and calculated as the difference from the value obtained after transfer of OVA alone, which was set as 1.

Statistical analysis

All data are presented as means ± SEMs and representative of at least 2 experiments. Statistical analysis was performed with the unpaired Student *t* test (2-tailed) or with 2-way repeated-measures ANOVA and the Bonferroni posttest. *P* values of less than .05 were considered statistically significant.

Additional information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Self-limited allergen-induced dermatitis is mediated by IL-4

Early immune reactions in AD skin are dominated by T_H2 cells and cytokines and are believed to predispose the AD skin for colonization by bacteria, such as *S aureus*. As a model for

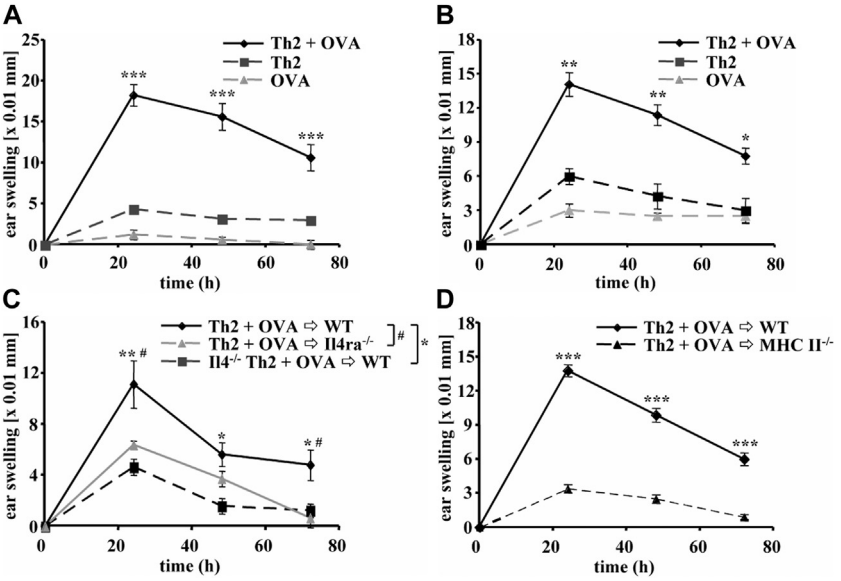

FIG 1. T_H2-mediated dermatitis is self-limited and dependent on IL-4 and MHCII. **A** and **B,** Intracutaneous OVA-specific T_H2 cells mediate dermal inflammation in the ears of naive BALB/c (Fig 1, *A*) or C57BL/6 (Fig 1, *B*) mice quantified as changes in ear thickness. **C,** Transfer of WT or $Il4^{-/-}$ OVA-activated T_H2 cells in WT or $Il4ra^{-/-}$ mice (BALB/c background) demonstrated a strict dependence on IL-4. **D,** Complete abrogation of inflammation in $MHCII^{-/-}$ mice (C57BL/6 background). n = 4-10. #**P* < .05, ***P* < .005, and ****P* < .0005.

the early phase of AD inflammation, OVA-specific IL-4–producing T_H2 cells were intracutaneously transferred with or without OVA into the ears of naive mice. Ear swelling was determined as the change in skin thickness to quantify dermatitis. Transfer of T_H2 cells with OVA provoked strong but self-limiting cutaneous inflammation, with a peak at 24 hours in both T_H2-prone BALB/c (Fig 1, *A*) and C57BL/6 mice (Fig 1, *B*). OVA-specific T_H2 cells from wild-type (WT) or IL-4–deficient mice were generated to identify the underlying mechanisms. T_H2 polarization of $Il4^{-/-}$ T cells was determined based on IL-13 production, which was identical to cytokine secretion by WT T_H2 cells, whereas IL-4 production was lacking, as expected (see Fig E1 in this article's Online Repository at www.jacionline.org). Intracutaneous transfer of WT T_H2 cells and antigen resulted in a significant increase in ear thickness in WT mice that was dependent on T_H2 cell–derived IL-4 and host IL-4R. $Il4^{-/-}$ T_H2 cells did not induce dermatitis in WT mice, and similarly, WT T_H2 cells did not induce cutaneous inflammation in mice lacking the IL-4 receptor ($Il4ra^{-/-}$; Fig 1, *C*). Importantly, IL-4–mediated dermatitis was also strictly dependent on MHCII on recipient cells because it was completely abrogated in recipients deficient for MHCII (Fig 1, *D*). Thus our data demonstrate that antigen-activated T_H2 cells induce self-limited cutaneous inflammation, which is dependent on IL-4 signaling.

TLR2 ligands enhance and sustain T_H2-mediated dermatitis through activation of skin-resident cells

S aureus is a dominant trigger of AD, providing high levels of different TLR2 ligands. One such ligand, LTA, was recently isolated from AD skin lesions and identified as a PAMP that correlates with AD severity.[17] Therefore we analyzed the role of innate immune sensing of *S aureus*–derived PAMPs by exposing the skin to those TLR2 ligands. LTA significantly enhanced dermatitis induced by OVA-specific T_H2 cells in BALB/c (Fig 2, *A*) and C57BL/6 (Fig 2, *B*) mice, indicating that innate immune sensing of *S aureus*–derived LTA contributes to AD inflammation. LTA was initially described as a ligand for TLR2,[22] although it now appears that lipoproteins in LTA preparations mediate the TLR2-dependent effects of LTA.[23,24] Therefore we next exposed the skin to the lipoprotein Pam2, another TLR2 ligand.[25] Like LTA, Pam2 significantly enhanced and sustained T_H2-mediated dermatitis in BALB/c and in C57BL/6 mice, as shown by ear swelling (Fig 2, *C* and *D*, respectively). In addition, hematoxylin and eosin staining of skin sections at 48 hours demonstrated epidermal thickening (Fig 2, *E*, yellow arrow) and a massive dermal cellular infiltrate in these Pam2 conditions, exceeding findings in self-limited T_H2 dermatitis (Fig 2, *E*, white arrows). Interestingly, IFN-γ mRNA was upregulated in skin undergoing Pam2-exposed exacerbated inflammation, although not in self-limited T_H2 dermatitis skin (Fig 2, *F*). This upregulation could be due to endogenous T-cell bystander recruitment in addition to the transfer of T_H2 cells. Therefore we extended our analyses to a model in which endogenous T_H2 cells induced by OVA sensitization are recruited to the skin after antigen challenge.[19] In this model, OVA-activated T_H2 cells led to short-lived dermatitis as well. In agreement with the results obtained with Pam2 in the transfer model, a single exposure to Pam2 in actively sensitized mice also induced severe and ongoing dermatitis, as assessed by measuring the increase in ear thickness (Fig 2, *G*) and by epidermal thickness and cellular infiltrates in histology (Fig 2, *H*). Next, we adopted our transfer model, in which T_H2 cells and OVA were administered first followed by exposure to Pam2 24 hours later because this might be closer to the situation in human AD. Exposure to Pam2 24 hours after T_H2 cell activation also significantly enhanced and prolonged

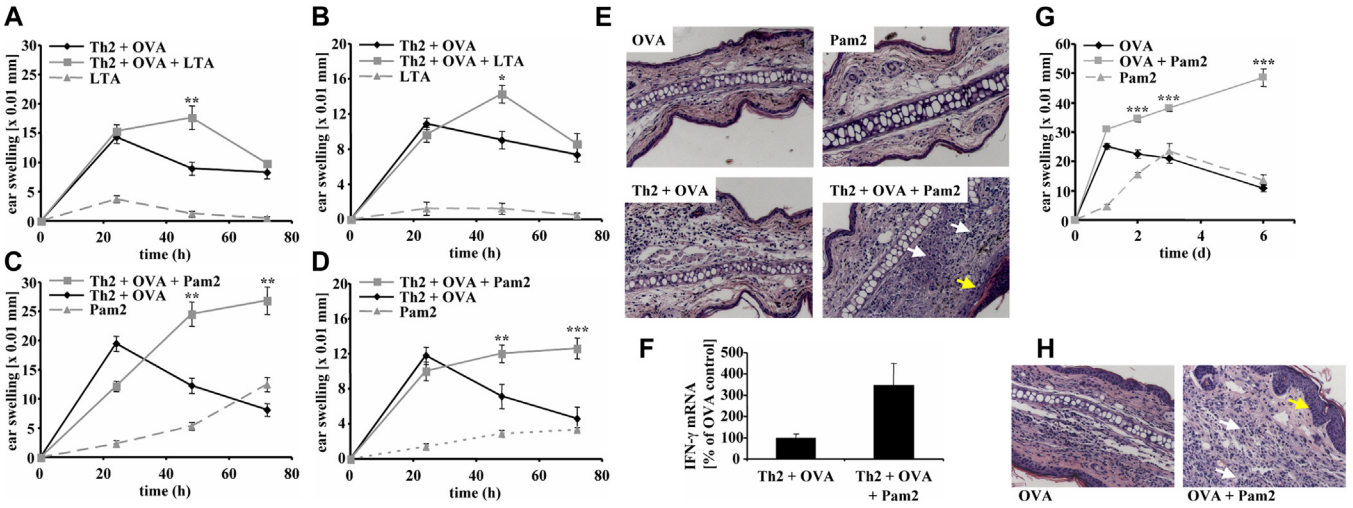

FIG 2. TLR2 ligands convert T_H2-mediated acute dermatitis into exaggerated and persistent inflammation. **A-D,** Progression of T_H2-induced dermatitis after exposure to TLR2 ligands in BALB/c (Fig 2, *A* and *C*) and C57BL/6 (Fig 2, *B* and *D*) mice is shown. LTA temporarily enhanced dermatitis (Fig 2, *A* and *B*). Pam2 converted dermatitis into persistent inflammation (Fig 2, *C* and *D*). **E,** Representative hematoxylin and eosin staining of histologic sections from C57BL/6 mice 48 hours after intracutaneous transfer. **F,** Relative IFN-γ mRNA level in ear skin 12 hours after transfer. **G** and **H,** OVA-sensitized DO11.10 mice (BALB/c background) challenged intracutaneously with OVA with or without Pam2. Cutaneous inflammation after OVA activation of endogenous T_H2 cells is shown in Fig 2, *G*, and histology (day 6) is shown in Fig 2, *H*. n = 4-20. $*P < .05$, $**P < .005$, and $***P < .0005$.

cutaneous inflammation, followed by significantly increased recruitment of $CD45^+$ leukocytes (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus innate immune sensing through TLR2 enhanced and sustained skin inflammation caused by IL-4–producing T_H2 cells.

One possible target of TLR2 ligands are T cells because TLR2 can act as a costimulatory molecule for T-cell activation.[26] WT or $Tlr2^{-/-}$ T_H2 cells were adoptively transferred into either WT or $Tlr2^{-/-}$ mice, and skin inflammation was monitored thereafter to identify the dominant target cells for TLR2 ligands. In WT mice Pam2 enhanced and sustained dermatitis induced by OVA-activated T_H2 cells, irrespective of whether the T cells were TLR2 deficient (Fig 3, *A*). In contrast, TLR2 ligands completely failed to enhance and sustain T_H2-induced dermatitis in $Tlr2^{-/-}$ mice, and the ear-swelling pattern in these mice was indistinguishable from the control transfer without TLR2 ligands (Fig 3, *B*). These data demonstrate that TLR2 ligands most likely target skin-resident antigen-presenting cells. Among these, the most important are DCs, which function as crucial sentinels of the immune system and link innate and adaptive immunity also in patients with AD.[11] Accordingly, we modified our protocol and transferred OVA-pulsed DCs with (Pam2-DC_{OVA}) or without (DC_{OVA}) Pam2 exposure together with OVA-specific T_H2 cells. Indeed, the transfer of OVA-pulsed DCs resulted in a limited antigen-induced dermatitis, whereas Pam2-DC_{OVA} caused significantly enhanced and prolonged inflammation (Fig 3, *C*). Aggravated skin inflammation was dependent on TLR2 activation of DCs because WT but not $Tlr2^{-/-}$ Pam2-DC_{OVA} exacerbated dermatitis in C57BL/6 mice (Fig 3, *D*) and BALB/c mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Together, these data demonstrate that activation of DCs through TLR2 converts limited T_H2-mediated dermatitis to an aggravated and persisting inflammation.

IL-4–induced suppression of IL-10 causes exacerbation and persistence of T_H2-mediated dermatitis by TLR2 ligands

Given the critical role of IL-4 during early skin inflammation at the time of Pam2 encounter and of DCs for Pam2-induced dermatitis exacerbation, we next investigated the combined immune sensing of IL-4 and the TLR2 ligand Pam2 by DCs. To this end, DCs were activated with Pam2 with or without additional exposure to IL-4, as in AD skin. Cytokine levels were analyzed 24 hours later. The quality and functional consequences of immune responses are determined by the cumulative effect of differentially acting cytokines. Therefore we analyzed the main representatives of the proinflammatory and anti-inflammatory cytokines IL-12 and IL-10, respectively, to anticipate the immune consequences of such DCs. We found that Pam2-mediated activation of DCs induced IL-12 and IL-10. However, when mimicking the T_H2-dominant milieu of early AD by adding IL-4, IL-12p70 levels were upregulated further in activated DCs, as expected.[14,27] More importantly, IL-10 levels were significantly suppressed (Fig 4, *A*). This resulted in a proinflammatory shift of the cytokine milieu, as represented by the IL-10/IL-12 ratio (Fig 4, *B*). This suppression of IL-10 was indeed mediated by IL-4 because it was completely abolished in DCs with defective IL-4 signaling (see Fig E4 in this article's Online Repository at www.jacionline.org).

On the basis of these *in vitro* data, we asked whether this change of cytokine expression was also associated with the observed conversion of self-limited dermatitis into aggravated inflammation *in vivo* (Fig 2). To do this, we performed quantitative real-time PCR of ear skin 12 and 24 hours after the initiation of AD-like inflammation. As expected, Pam2 exposure upregulated IL-12 levels in the skin of recipient mice, with the highest levels detected 24 hours after challenge (Fig 5, *A*). Remarkably, and consistent with our *in vitro* results, cutaneous IL-10 expression

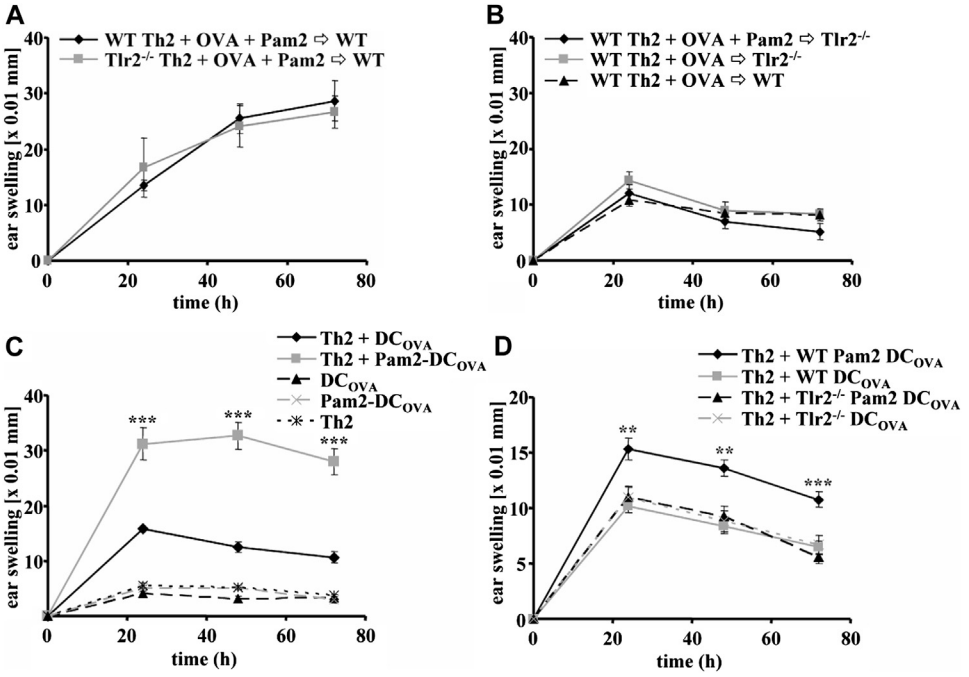

FIG 3. TLR2 activation of skin cells converts T_H2-mediated acute dermatitis to enhanced and prolonged inflammation. **A** and **B,** Crossover experiment for persistent inflammation. Fig 3, *A*, shows Pam2-induced aggravated dermatitis in BALB/c mice after intracutaneous activation of either WT or $Tlr2^{-/-}$ T_H2 cells. Fig 3, *B*, shows self-limited acute dermatitis, as in OVA-treated control animals, despite exposure to Pam2 in $Tlr2^{-/-}$ recipients (done in parallel with Fig 3, *A*). **C,** Exacerbation and prolongation of dermatitis after transfer of T_H2 cells and Pam2-exposed OVA-pulsed DCs in C57BL/6 mice. **D,** Pam2-exposed WT but not $Tlr2^{-/-}$ DCs promote exacerbated inflammation in $Tlr2^{-/-}$-C57BL/6 mice. n = 4-10. $^{**}P < .005$ and $^{***}P < .0005$.

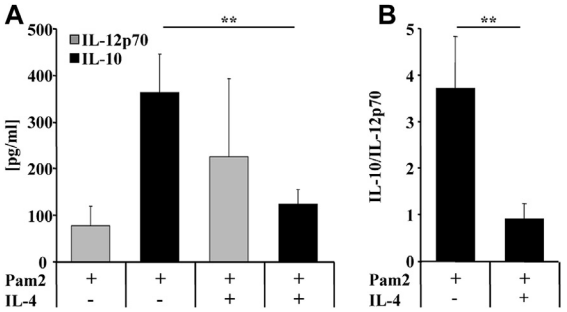

FIG 4. Suppression of TLR2 ligand–induced IL-10 by IL-4. **A,** DCs exposed to Pam2 produced intermediate levels of IL-12p70 and high levels of IL-10 *(left)*. Coactivation of Pam2-exposed DCs with IL-4 significantly suppressed IL-10 secretion (Fig 4, *A*, right), resulting in a low IL-10/IL-12p70 ratio (**B;** average of 4 experiments). $^{**}P < .005$.

appeared earlier and was highest 12 hours after Pam2 exposure (Fig 5, *A*). This opposing regulation is best reflected by the cutaneous IL-10/IL-12 mRNA ratio: in mice with self-limited T_H2-mediated dermatitis, IL-10 dominated skin inflammation at 24 hours (Fig 5, *B*, left). In contrast, the IL-10/IL-12 mRNA ratio was markedly reduced in dermatitis lesions that showed enhanced and sustained inflammation after TLR2 ligand exposure (Fig 5, *B*, right). Importantly, amplified IL-4 production, as detected by means of cutaneous IL-4 mRNA expression, was found in both self-limited T_H2 dermatitis and enhanced and sustained inflammation after Pam2 exposure (see Fig E5 in this article's Online Repository at www.jacionline.org). Therefore after innate immune sensing of TLR2 ligands, T_H2-derived IL-4 suppressed IL-10 levels, allowing TLR2 ligands to enhance and sustain AD inflammation. Indeed, IL-10 expression in Pam2-treated DCs was significantly reduced on coculture with IL-4–secreting T_H2 cells comparable with the suppression seen after direct addition of IL-4 (Fig 5, *C*). Intracutaneously transferred T_H2 cells and Pam2-DC_{OVA} were reconstituted with IL-10 to prove that IL-10 is indeed critical for the IL-4–mediated conversion of acute AD flares into persistent AD-like inflammation. Supplementation with IL-10 completely abrogated Pam2-induced dermatitis, which decreased to the level of inflammation without Pam2 activation (Fig 5, *D*). This demonstrated IL-10 to be a key regulator in the orchestration of adaptive T_H2-mediated immune responses. Next, we carried out long-term analyses of Pam2-exposed dermatitis. Impressively, after a single exposure to Pam2, T_H2-induced dermatitis increased over 5 days and remained significantly enhanced for more than 3 weeks (Fig 5, *E*). In summary, our data demonstrate that TLR2 ligands convert acute self-limited inflammation to chronic and persistent dermatitis through IL-4–mediated suppression of IL-10.

DISCUSSION

AD is based on a complex genetic trait, with skin barrier defects being among the most frequent functional abnormalities.[3,28] The majority of patients with AD have increased IgE levels toward environmental antigens and T_H2-biased T-cell immunity, which is based on both cutaneous barrier defects and an inherent immune bias toward T_H2 immunity.[2] A prerequisite for the

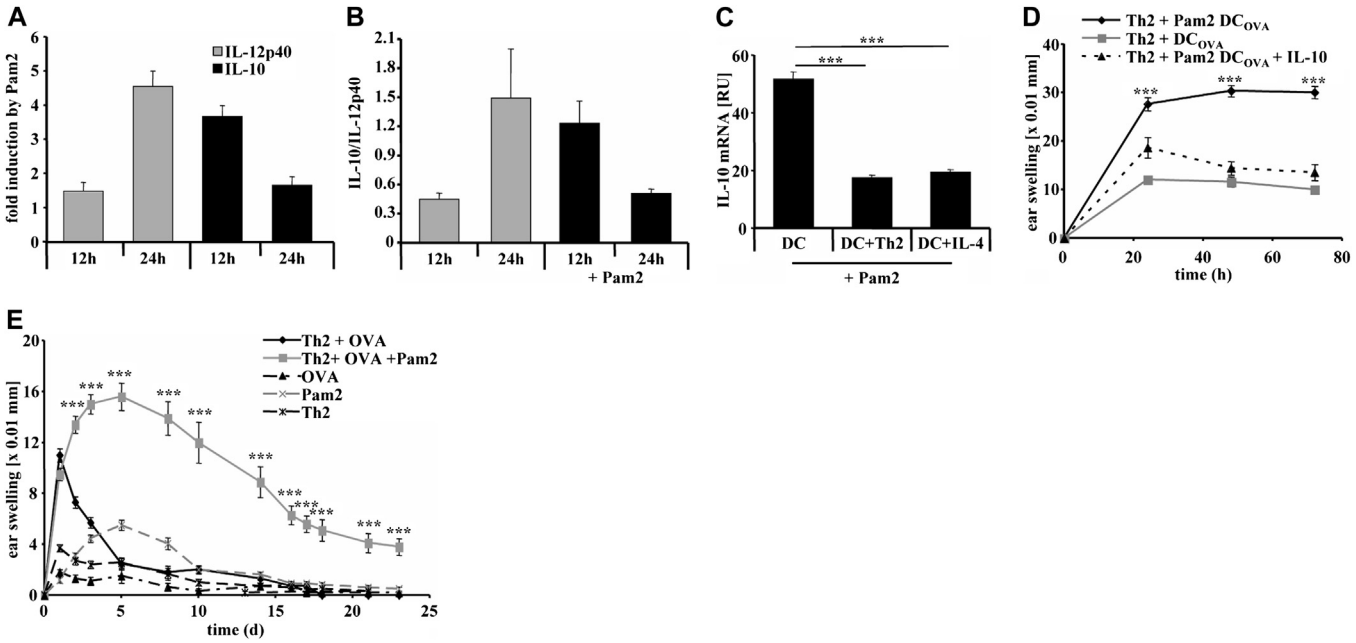

FIG 5. Suppression of IL-10 by coactivation of IL-4R and TLR2 converts acute dermatitis to chronic inflammation. **A** and **B,** Quantitative real-time PCR of cutaneous cytokines during conversion to persistent Pam2-induced dermatitis. Fig 5, *A*, shows Pam2-induced IL-12p40 and IL-10 expression. Fig 5, *B*, shows reduced IL-10/IL-12p40 ratio in persistent dermatitis. **C,** Suppression of IL-10 mRNA in Pam2-exposed DCs by means of coculture with activated T_H2 cells or the addition of IL-4. **D,** Reconstitution of IL-10 abrogated Pam2-induced aggravation of dermatitis after intracutaneous transfer of T_H2 cells, DCs, and OVA in $Il4ra^{-/-}$ mice. **E,** A single Pam2 exposure converted T_H2-mediated dermatitis into exacerbated chronic inflammation in C57BL/6 mice. n = 6-10. ***$P < .0005$.

development of AD inflammation is the initial recruitment of IL-4–producing T_H2 cells to the skin on acute triggering factors, among them exposure to environmental allergens.[29-31] However, the switch from acute AD flares to chronic cutaneous inflammation is not understood, and functional human *in vivo* studies are difficult to perform. Cutaneous colonization or infections with *S aureus* are found in almost all patients with AD, demonstrating a positive correlation between bacterial density and the severity of AD.[32] Moreover, this correlation with AD severity is also valid for TLR2 ligands found on the skin.[17] Therefore we hypothesized that TLR2 ligands play a major role in the conversion of acute AD inflammation to chronic dermatitis.

TLR2 has been associated with AD pathogenesis,[2] with the most recent work demonstrating that TLR2 contributes to skin barrier repair when acting on the epithelium.[33] To investigate how TLR2 ligands orchestrate cutaneous inflammation in patients with AD, we used a mouse model in which IL-4 is the dominant and functionally relevant cytokine, as in early AD. This allowed us to specifically focus on the effect of innate TLR2 signals in a T_H2-dominant adaptive immune response in the skin. Previous analyses concentrated on the role of TLR2 ligands for cutaneous T_H1 responses,[34] for which IFN-γ had been shown to mediate dermal thickening.[35] We showed that TLR2 ligands, as provided by *S aureus*, convert T_H2-mediated self-limited skin dermatitis into persistent and aggravated chronic inflammation. These analyses provide a rationale for the conversion of acute AD flares to chronic skin inflammation, which is observed in patients with AD. This conversion was driven by innate and adaptive signals that simultaneously activated immune sentinels of the skin. In general, TLR2 ligands are known to coinduce rather high levels of IL-10.[36] This, under homeostatic conditions, might contribute to microbiota tolerance because gram-positive bacteria persist on the skin in the absence of inflammation. However, in the setting of IL-4–dominated inflammation, this upregulation is counterregulated by IL-4, which suppresses IL-10, leading to TLR2-mediated exacerbation of inflammation. Thus the combinative sensing of adaptive IL-4 together with innate TLR2 signals directly drives skin inflammation by suppressing IL-10. These newly identified consequences of coactivating signals shed light onto a hitherto neglected effect of combinatorial immune sensing.[15] Moreover, the identification of IL-10 as a target cytokine of this immune orchestration confirms its dominant role as an important anti-inflammatory cytokine with immunomodulatory properties, limiting otherwise excessive immune responses.[37] It is well established that IL-10 regulates a variety of immune cells, including T_H2 cells.[38] Furthermore, IL-10 has even been used as a therapeutic agent for inflammatory diseases of the skin, such as psoriasis,[39] and the gut, such as Crohn disease.[40] Interestingly, an AD-like skin disease in NcNgA mice was effectively treated with IL-10,[41] and IL-10 was also relevant for the amelioration of canine AD.[42] These analyses support the concept that the adaptive cytokine IL-4 suppresses innately induced IL-10, which is responsible for the conversion of acute dermatitis to chronic and persistent inflammation.

These findings are highly relevant to patients with AD because *S aureus* is found on the skin of almost all such patients.[32] In addition to *S aureus*, *Malassezia* species have been shown to be triggers of head-and-neck variants of AD, and herpes virus infections lead to severe AD complications.[43-45] Importantly, in the context of our findings, innate signals from *Malassezia*

species and herpes viruses activate TLR2.[46,47] This indicates that our findings on *S aureus*–derived TLR2 ligands might be of general importance and also functional in other settings in patients with AD.

Our findings also further emphasize that IL-4 is a promising target for AD therapy. It has long been known that IL-4 is the hallmark cytokine of T_H2 cells and early AD and that IL-4 downregulates antimicrobial peptides and cutaneous barrier function.[6,7,48] We have now added a crucial new role for IL-4 in AD pathogenesis: IL-4 suppresses cutaneous IL-10 induced by innate signals and therefore promotes chronic AD. Until recently, however, IL-4 was targeted in patients with severe asthma[49] but not in those with AD. Now clinical trials report significant improvement of AD-related inflammation by subcutaneous application of a human mAb directed against IL-4Rα.[50-53] Together with our data, this indicates that inhibiting IL-4R signaling also prevents IL-4–mediated suppression of IL-10, which is pivotal for persistent and chronic inflammation in patients with AD after the simultaneous activation of IL-4R and TLR2.

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Key messages

- **T_H2 cell–mediated dermatitis is self-limiting and strictly depends on IL-4.**
- **TLR2 ligands convert self-limited T_H2-mediated dermatitis to chronic inflammation by activating skin-resident sentinels.**
- **TLR2 ligand–induced chronic dermatitis results from IL-4–mediated suppression of IL-10.**

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METHODS

Skin cell analysis

Mouse ear tissue was incubated with Dispase II (Sigma-Aldrich) for 2 hours at 37°C. The dermis and epidermis were separated; digested for 30 minutes at 37°C in collagenase A (Serva, Heidelberg, Germany) or trypsin-EDTA (Biochrom Berlin, Germany), respectively; and pooled again. Samples were given twice through a cell strainer to obtain single-cell suspension. After washing, cells were counted, stained for 30 minutes at 4°C, and analyzed by means of flow cytometry with an LSRII flow cytometer and FACSDiva Software (BD Biosciences, Heidelberg, Germany).

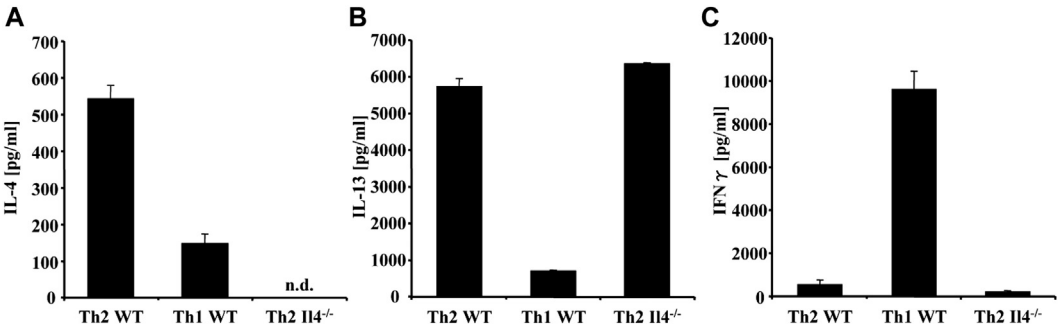

FIG E1. Phenotypic characterization of T_H2 cells. Characterization of WT and IL-4–deficient T_H2 cells used for intracutaneous transfer, as shown in Fig 2. Secretion of IL-4 **(A)**, IL-13 **(B)**, and IFN-γ **(C)** was determined by means of ELISA. For comparison, cytokine secretion by T_H1 cells is shown. *n.d.*, Not detectable.

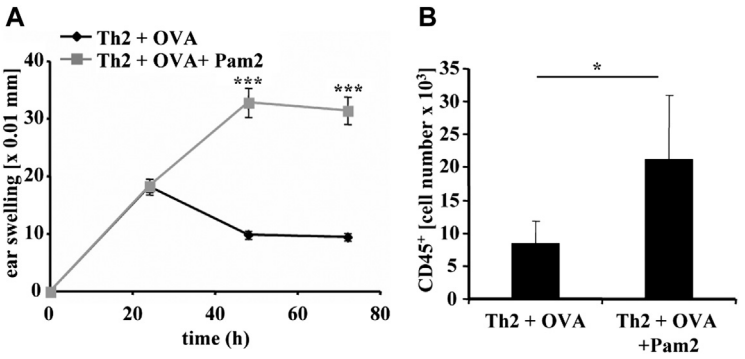

FIG E2. Pam2-induced exacerbated inflammation after T_H2 cell activation. **A,** T_H2 cells and OVA were transferred into the skin of BALB/c mice. Twenty-four hours later, some mice were additionally exposed to Pam2, which resulted in aggravated inflammation comparable with that seen after simultaneous application of T_H2, OVA, and Pam2 (see Fig 2, *C*; n = 8). **B,** Ear skin analysis from the experiment depicted in Fig E2, *A*, showing increased numbers of $CD45^+$ cells in the Pam2-exposed condition. $*P < .05$ and $***P < .0005$.

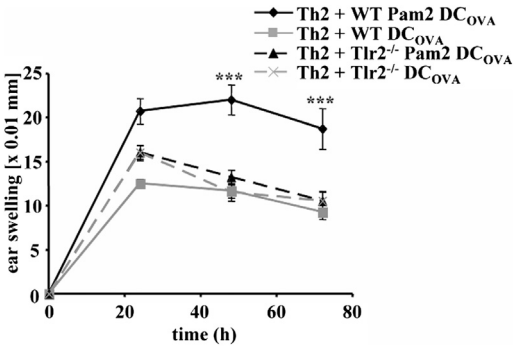

FIG E3. TLR2 activation of DCs converts T_H2-mediated acute dermatitis to enhanced and prolonged inflammation. Comparable with Fig 3, *D*, T_H2 cells and OVA-pulsed WT or $Tlr2^{-/-}$ DCs with or without Pam2 exposure were transferred into $Tlr2^{-/-}$ BALB/c mice, and ear swelling was measured thereafter. Pam2-exposed WT but not $Tlr2^{-/-}$ DCs promoted exacerbated dermatitis (n = 10). ***$P < .0005$.

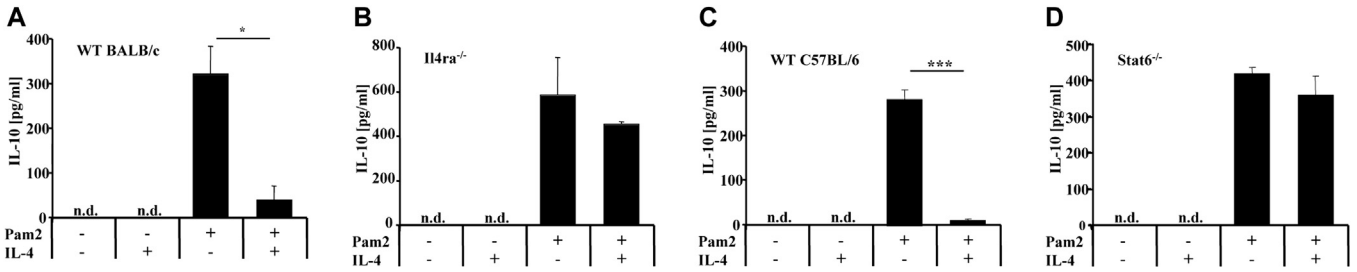

FIG E4. Suppression of IL-10 by IL-4 in Pam2-activated DCs. IL-10 secretion by bone marrow–derived DCs either from a BALB/c background **(A** and **B)** or a C57BL/6 background **(C** and **D)**, as determined by means of ELISA. Addition of IL-4 significantly suppressed Pam2-induced IL-10 in WT DCs (Fig E4, *A* and *C*) but not in DCs with defective IL-4 signaling pathways (Fig E4, *B* and *D*). *n.d.*, Not detectable. $*P < .05$ and $***P < .0005$.

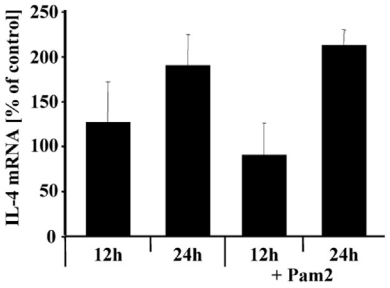

FIG E5. IL-4 expression in mouse ear skin. Relative IL-4 mRNA levels in mouse ear skin 12 and 24 hours after transfer of T_H2 cells and OVA with or without Pam2, corresponding to expression shown in Fig 5, *A* and *B*. Values were determined by using real-time PCR, normalized to β-actin, and shown relative to values obtained after transfer of OVA alone (n=6).

Nonpathogenic Bacteria Alleviating Atopic Dermatitis Inflammation Induce IL-10-Producing Dendritic Cells and Regulatory Tr1 Cells

Thomas Volz[1], Yuliya Skabytska[1], Emmanuella Guenova[1], Ko-Ming Chen[1], Julia-Stefanie Frick[2], Carsten J. Kirschning[3], Susanne Kaesler[1], Martin Röcken[1] and Tilo Biedermann[1]

The beneficial effects of nonpathogenic bacteria are increasingly being recognized. We reported in a placebo-controlled study with atopic dermatitis (AD) patients that cutaneous exposure to lysates of nonpathogenic bacteria alleviates skin inflammation. To now unravel underlying mechanisms, immune consequences of sensing nonpathogenic bacterium *Vitreoscilla filiformis* lysate (Vf) were characterized analyzing (1) differentiation of dendritic cells (DCs) and, consecutively, (2) effector functions of DCs and T helper (Th) cells *in vitro* and in a murine model of AD in NC/Nga mice *in vivo*. Topical treatment with Vf significantly reduced AD-like inflammation in NC/Nga mice. Importantly, cutaneous exposure to Vf in combination with the allergen FITC significantly also reduced subsequent allergen-induced dermatitis indicating active immune modulation. Indeed, innate sensing of Vf predominantly induced IL-10-producing DCs, which was dependent on Toll-like receptor 2 (TLR2) activation. Vf-induced IL-10+ DCs primed naive CD4+ T helper cells to become regulatory IFN-γ^{low} IL-10^{high} Tr1 (type 1 regulatory T) cells. These IL-10^{high} Tr1 cells were also induced by Vf *in vivo* and strongly suppressed T effector cells and inflammation. In conclusion, we show that innate sensing of nonpathogenic bacteria by TLR2 induces tolerogenic DCs and regulatory Tr1 cells suppressing T effector cells and cutaneous inflammation. These findings indicate a promising therapeutic strategy for inflammatory skin diseases like AD.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with increasing prevalence rates, affecting up to 10–20% of the children in western countries (Bieber, 2008; Boguniewicz and Leung, 2010). Although the detailed mechanisms underlying inflammation of AD skin are not fully understood, a defect in skin barrier function as well as an immune dysbalance play a crucial role, leading to T helper cell type 2 (Th2)–biased immune responses (Palmer *et al.*, 2006; Bieber, 2008; Irvine *et al.*, 2011). Increased hygiene standards, less infectious diseases, and lowered family sizes are claimed to lead to microbial deprivation during early years of life that facilitate misdirected effector immune responses rather than the induction of immune tolerance (''hygiene hypothesis''), contributing to increase and development of allergies and atopic diseases (Strachan, 1989; Bach, 2002; von Mutius and Vercelli, 2010). Surface organs like the skin are constantly colonized with bacteria in the absence of detectable inflammation, but the mechanisms that inhibit inflammation or even induce tolerance to the local microbiota are still enigmatic (Grice *et al.*, 2009). A reduced genetic diversity of Gram-negative gammaproteobacteria in the environment of atopic individuals was observed that profoundly influenced the skin microbiota, leading to a decrease in Gram-negative bacterium *Acinetobacter* and the anti-inflammatory cytokine IL-10, demonstrating that resident microbes shape cutaneous immune homoeostasis (Hanski *et al.*, 2012). These data ideally complement analyses in mouse models that first demonstrated the potential of Gram-negative bacterium *Acinetobacter* to prevent allergies (Debarry *et al.*, 2007; Conrad *et al.*, 2009). In contrast to prevention of allergic sensitizations, most therapeutic strategies reporting the oral use of nonpathogenic or the so-called ''probiotic'' bacteria failed to show significant effects in the treatment of AD (Lee *et al.*, 2008; Boyle *et al.*, 2009). Recently, we performed a proof-of-concept study in patients demonstrating that immune recognition of the nonpathogenic microbe *Vitreoscilla filiformis* is a promising strategy to treat AD when directly applied onto patients' skin and not orally (Gueniche *et al.*, 2008). Based on these clinical findings we hypothesized that immune recognition of Gram-negative

[1]*Department of Dermatology, Eberhard Karls University Tübingen, Tübingen, Germany;* [2]*Institute for Medical Microbiology and Hygiene, University Hospital of Tübingen, Tübingen, Germany and* [3]*Institute of Medical Microbiology, University Duisburg-Essen, Essen, Germany*

Correspondence: Tilo Biedermann, Department of Dermatology, University Hospital Tübingen, Liebermeisterstrasse 25, 72076 Tübingen, Germany. E-mail: Tilo.Biedermann@med.uni-tuebingen.de

Abbreviations: AD, atopic dermatitis; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; LPS, lipopolysaccharide; Th cell, T helper cell; TLR, Toll-like receptor; Tr1, type 1 regulatory T cell; Vf, Vitreoscilla filiformis lysate

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nonpathogenic bacterium *V. filiformis* exploits an important mechanism of microbial immune sensing, finally alleviating inflammation by induction of tolerance.

Pattern recognition receptors such as Toll-like receptors (TLRs) play a key role in detecting ''pathogen-associated molecular patterns'' (Takeuchi and Akira, 2010; Volz *et al.*, 2010; Volz *et al.*, 2012). Especially within surface organs, dendritic cells (DCs) are equipped with numerous pattern recognition receptors and act as sentinels to sense microbes, leading to DC maturation and cytokine production (Reis e Sousa, 2004; Joffre *et al.*, 2009). Activated DCs are the most potent directors of immune phenotypes in T cells, determining T-cell polarization to the different Th subtypes. During this process of Th cell differentiation, DC cytokines are most important possibly also shaping regulatory T cells (Kapsenberg, 2003; Bettelli *et al.*, 2008; Volz *et al.*, 2012). Intriguing studies demonstrated that these pathways are also critical for induction of tolerance to the microbiota (Round and Mazmanian, 2010; Geuking *et al.*, 2011).

Given the positive results from our proof-of-concept study in AD patients, we investigated the underlying mechanisms. AD-prone NC/Nga mice with Th2-dominated cutaneous hypersensitivity to FITC showed, similar to AD patients, alleviated dermatitis when their skin was exposed to *V. filiformis* signals. Importantly, this therapeutic effect was even more pronounced when the skin was exposed to *V. filiformis* signals previous to allergen challenge, indicating effective immune modulation. Indeed, *V. filiformis* signals induced high levels of IL-10 in DCs via TLR2. These DCs orchestrated the induction of IL-10^{high}, IFN-γ^{low}-producing Tr1 (type 1 regulatory T) cells. This regulation was also detected in AD mice after cutaneous exposure to Vf with dominant IL-10 production by T cells from skin-draining lymph nodes and consecutively reduced T-cell proliferation and proinflammatory cytokine production.

Thus, immune recognition of the Gram-negative nonpathogenic bacterium *V. filiformis* by DCs induces IL-10-producing DCs and regulatory Tr1 cells. This pathway may generally be functional when discriminating between ''pathogenic'' and ''nonpathogenic'' bacteria and could be exploited to alleviate cutaneous inflammation such as in AD.

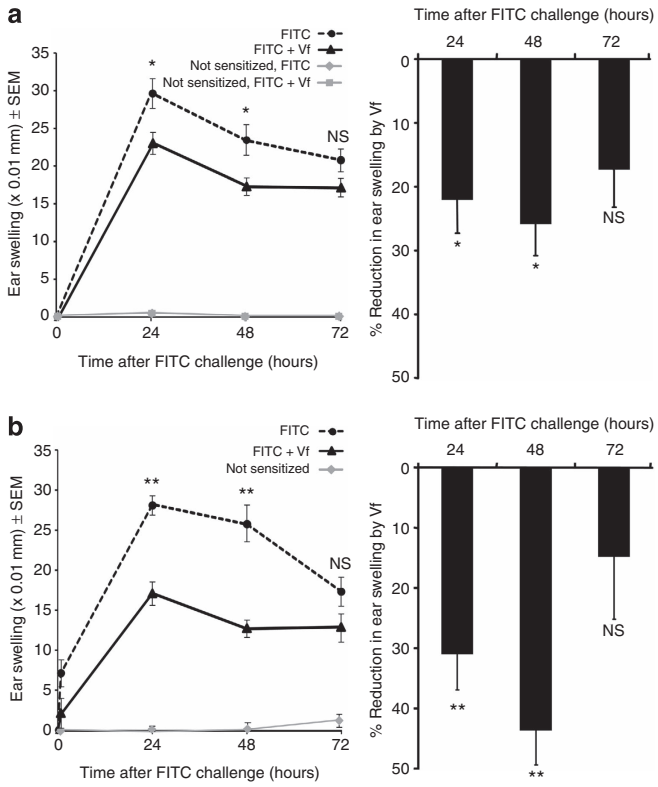

Figure 1. Cutaneous exposure to *Vitreoscilla filiformis* (Vf) attenuates skin inflammation. (**a**) FITC-sensitized NC/Nga mice showed ear swelling responses peaking at 24 hours after challenge and declining thereafter. Cutaneous exposure of mice to Vf exclusively during challenge significantly reduced ear swelling at 24 and 48 hours. Not sensitized but FITC-challenged mice (gray lines) did not display marked ear swelling responses irrespective of exposure to Vf. Percentage of reduction of ear swelling of FITC-sensitized Vf-exposed mice compared with FITC-challenged mice not receiving Vf is shown at the right. (**b**) Cutaneous exposure of mice to FITC together with Vf only previous to the final challenge significantly reduced ear swelling 24 and 48 hours after FITC challenge compared with the positive control group. Reduction of ear swelling was up to 50% compared with mice not being exposed to Vf, as depicted in the right panel. One out of two independent experiments is shown, mean ± SEM, $n = 5$ mice per group. NS, not significant. *$P < 0.05$ and **$P < 0.01$.

RESULTS

Exposure to nonpathogenic bacteria attenuates cutaneous inflammation in a murine model of AD

We recently reported effective treatment of AD lesions by topical treatment of *V. filiformis* lysate (Vf) in a double-blind, placebo-controlled clinical trial (Gueniche *et al.*, 2008). Thus, we first asked whether Vf solely suppressed cutaneous inflammation in AD at the time and site of application. To this end, mice of the NC/Nga strain that have been shown to develop AD-like skin lesions and clinical features most closely resembling human AD were investigated (Matsuda *et al.*, 1997). NC/Nga mice sensitized to the allergen FITC clearly developed dermatitis lesions as measured by a strong increase in ear thickness following allergen challenge (Figure 1a). All appropriate controls without sensitization and subsequently challenged with FITC with or without Vf exposure showed ear swelling responses that were always <0.03 mm. Dermatitis was reduced by $>25\%$ in mice that received topical treatment of ear skin with Vf during FITC challenge, indicating direct anti-inflammatory properties of Vf (Figure 1a). However, these effects remained somewhat limited. Therefore, we next asked whether exposure to Vf could also orchestrate immune modulation or tolerance mediating long-term effects in addition. Therefore, one group of FITC-sensitized mice was exposed to Vf in combination with FITC and the effects of this exposure were determined 1 week later by challenge with the allergen only. Compared with the FITC-sensitized control group, mice previously exposed to Vf together with FITC showed significantly reduced ear swelling after allergen challenge alone ($P < 0.01$), with almost 50% reduction at the peak of skin inflammation (Figure 1b). Importantly, we determined antigen-specific immunoglobulin levels in all mice sensitized to FITC with or without exposure to Vf and

found elevated antigen-specific immunoglobulin levels when compared with naive control mice. We detected no difference in FITC-specific IgG1 and IgG2a levels when comparing Vf exposed with unexposed mice and only a slight reduction in antigen-specific IgE (Supplementary Figure S1 online). Thus, Vf attenuated cutaneous T cell–mediated inflammation in a murine model of AD by modes of immune modulation, indicating that innate sensing of nonpathogenic *V. filiformis* regulates even already established adaptive immunity.

Signals from nonpathogenic bacterium *V. filiformis* induce maturation of human and murine DCs

Immune modulation or tolerance can be induced by immature or semimature DCs not fully activated. To investigate DC activation, murine bone marrow–derived dendritic cells (BMDCs) and human monocyte-derived dendritic cells were stimulated with either lipopolysaccharide (LPS) as positive control or Vf for 24–48 hours. DC maturation was assessed by FACS analysis.

Both Vf and LPS unequivocally induced maturation of BMDCs as detected by upregulation of maturation markers such as CD80, CD86, CD83, and major histocompatibility complex class II as compared with untreated cells (Figure 2a). After activation of human monocyte-derived dendritic cells with Vf or LPS, CD83, the most reliable surface marker for detection of human DC maturation, was also clearly upregulated (Supplementary Figure S2a online). This shows that signals of *V. filiformis* orchestrate the development of phenotypically mature DCs as determined by FACS analysis.

Signals from nonpathogenic bacterium *V. filiformis* orchestrate the induction of IL-10^{high} DCs

Next we analyzed cytokine production of DCs after activation with either Vf or LPS. As expected, LPS induced high amounts

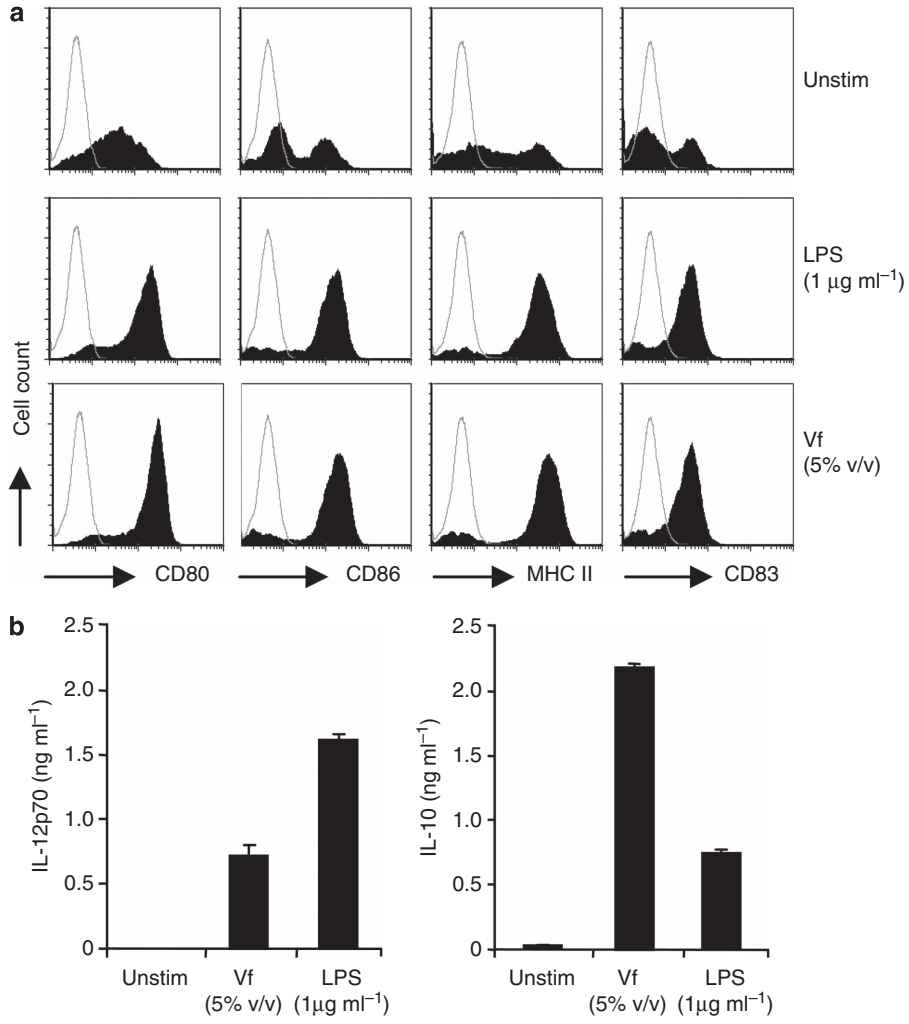

Figure 2. Signals of *Vitreoscilla filiformis* (Vf) predominantly induce IL-10-producing mature dendritic cells (DCs). DCs were exposed to Vf or lipopolysaccharide (LPS). (**a**) Vf and LPS unequivocally stimulated murine bone marrow–derived dendritic cells (BMDCs) to upregulate CD80, CD86, major histocompatibility complex class II (MHC II), and CD83 (filled area) compared with untreated cells indicating full DC maturation. Gray lines indicate isotype controls. Unstim, unstimulated. (**b**) BMDCs activated with Vf produced high levels of IL-10 but only low amounts of IL-12p70, characteristic for IL-10+ DCs. In contrast and, as expected, LPS induced high IL-12p70 but only low IL-10 levels in BMDCs. Representative data from one out of four independent experiments are shown.

of IL-12p70 and low levels of IL-10. In sharp contrast, stimulation with Vf led to a DC cytokine profile dominated by the anti-inflammatory cytokine IL-10, whereas IL-12p70 levels remained low for all *V. filiformis* strains investigated (Figure 2b and Supplementary Figure S3 online). This IL-10 production induced by Vf was dose dependent in both human and mouse DCs, indicating ligand(s) that trigger pattern recognition receptors (Supplementary Figure S2b online). Moreover, investigating numerous synthetic and bacterial-derived TLR2 ligands, Vf was always superior in the induction of IL-10 in DCs (Supplementary Figure S4 online). These data demonstrate that innate immune sensing of nonpathogenic bacteria governs DCs to predominantly produce the anti-inflammatory and potentially tolerogenic cytokine IL-10.

Innate immune pathways for DC activation and IL-10 production following encounter of signals from nonpathogenic bacterium *V. filiformis*

To gain further insight into innate immune pathways activated by Vf, we first investigated WT and MyD88$^{-/-}$ DCs. DC maturation was induced in WT DCs in response to TLR2 ligand Pam2Cys (data not shown), TLR4 ligand LPS, and Vf as detected by upregulation of major histocompatibility complex class II, CD80, CD83, and CD86 (Figure 3a). In contrast, but as expected, Pam2Cys was unable to induce maturation of MyD88$^{-/-}$ DCs (data not shown) and DC maturation in response to LPS was partially hampered. Strikingly, lack of MyD88 did not affect DC maturation in response to Vf, indicating that MyD88-independent pathways are at least in part functional for DC maturation induced by nonpathogenic bacteria (Figure 3a).

In sharp contrast to DC maturation, production of IL-10 and IL-12p70 was almost completely abolished in MyD88$^{-/-}$ DCs (data not shown). Thus, DC maturation and cytokine production operate via distinct innate immune pathways.

To investigate the pathways that lead to DC cytokine production following contact to nonpathogenic bacteria, the consequences of Vf exposure were investigated in BMDCs lacking either TLR2 or TLR4. Strikingly, IL-10 production was almost completely dependent on TLR2, as IL-10 was

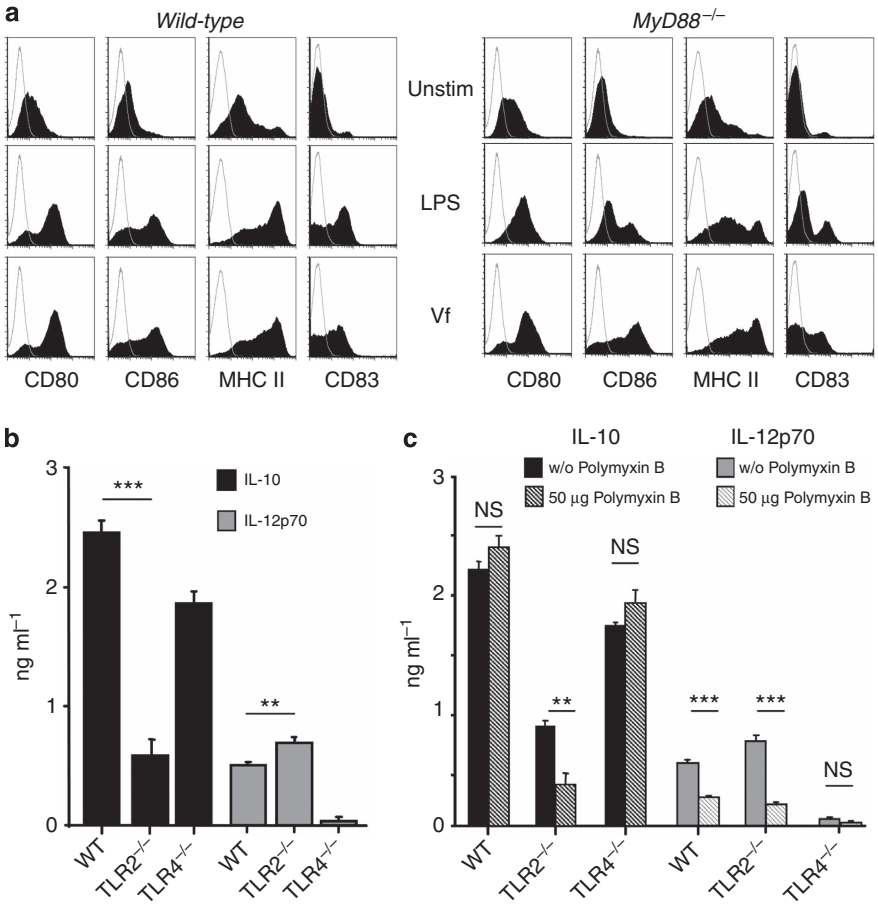

Figure 3. Distinct innate immune pathways control dendritic cell (DC) maturation and cytokine production induced by *Vitreoscilla filiformis* lysate (Vf). (**a**) Exposure to Vf also induced DC maturation in DCs lacking Toll-like receptor (TLR) adaptor protein MyD88, whereas it partially reduced lipopolysaccharide (LPS)-induced DC maturation. (**b**) Vf-induced IL-10 secretion was significantly reduced in TLR2$^{-/-}$ but only marginally affected in TLR4$^{-/-}$ DCs. In contrast, IL-12p70 production is almost completely abrogated in TLR4$^{-/-}$. (**c**) Sustained Vf-induced IL-10 production in wild-type (WT) or TLR4$^{-/-}$ DCs following pretreatment with LPS-scavenger Polymyxin B but reduced Vf-induced IL-12p70 production in WT and TLR2$^{-/-}$ DCs. Representative data (mean ± SD) from one out of three independent experiments are shown. MHC II, major histocompatibility complex class II; NS, not significant; Unstim, unstimulated. $^{**}P<0.01$ and $^{***}P<0.001$.

significantly reduced in TLR2$^{-/-}$ DCs (Figure 3b). This indicates that ligand(s) of TLR2 within Vf is/are responsible for induction of high IL-10 levels. In sharp contrast to TLR2$^{-/-}$ DCs, IL-10 production was only marginally reduced in TLR4$^{-/-}$ DCs (Figure 3b). The low IL-12p70 levels induced by Vf were also further investigated. Lack of TLR2 significantly increased IL-12p70 production, presumably because of the diminished IL-10 levels, whereas the absence of TLR4 abrogated the induction of the proinflammatory cytokine IL-12p70. Thus, we conclude that during DC exposure to Vf, at least two different dominant pathogen associated molecular patterns are functional: TLR2 ligand(s) inducing high IL-10 levels and TLR4 ligand(s) responsible for induction of IL-12p70. The latter is presumably LPS.

To further characterize the nature of the different pathogen associated molecular patterns inducing either IL-10 or IL-12p70 WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs were activated in the presence or absence of Polymyxin B that neutralizes LPS. Low IL-12p70 levels that were detected in WT and TLR2$^{-/-}$ DCs following exposure to Vf were nearly completely suppressed after preincubation of Vf with Polymyxin B, demonstrating that *V. filiformis* LPS is responsible for IL-12p70 induction (Figure 3c). Importantly, Polymyxin B treatment did not alter IL-10 production in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs compared with DCs stimulated with Vf only (Figure 3c). Thus, we conclude that innate immune signals from nonpathogenic bacteria like *V. filiformis* activate TLR2 and induce high IL-10 levels independently from LPS and the TLR4 pathway.

Priming of IL-10-producing CD4+ Tr1 cells by Vf-activated DCs

To assess the consequences on the adaptive immune system of the innate immune sensing of Vf, cocultures of DCs with Th cells were set up. First, DCs were activated with different doses of Vf, pulsed with ovalbumin, and subsequently cocultivated with naive CD4+ CD62L+ OT-II Th cells. Increasing concentrations of Vf in the previous DC culture reduced Th cell proliferation as determined by [^{3}H]-thymidine incorporation in a dose-dependent manner. This indicates direct suppressive effects on Th cells mediated by DCs activated with Vf (Figure 4a), which is in accordance to the observed direct immunosuppressive effect *in vivo* (Figure 1a). To determine immunomodulatory consequences on Th cell polarization, DCs were activated with either LPS or Vf and subsequently cocultivated for priming with Th cells as described before. Primed Th cells were then expanded with IL-2 for 10 days, stimulated, and analyzed for cytokine production. Th cells primed by DCs that were activated with LPS produced high IFN-γ levels and no IL-4, indicating Th1 polarization (Figure 4b). In contrast, IFN-γ levels secreted by Th cells primed with Vf-exposed DCs were markedly reduced compared with LPS-DCs and, again, IL-4 production was undetectable (Figure 4b). Most importantly, Vf-exposed DCs primed Th cells to secrete several fold higher levels of IL-10 compared with controls (Figure 4b). IL-10 production in T cells was dependent on DC-derived IL-10 and TLR2 signaling as both IL-10$^{-/-}$ and TLR2$^{-/-}$ DCs failed to induce high IL-10 levels in T cells (Figure 4c). Thus, innate immune sensing of Vf induced IL-10^{high}, IFN-γ^{low} IL-4^{-} Th cells in an IL-10-dependent manner, indicating the induction of Tr1 cells.

Regulatory function of Tr1 cells induced by Vf-exposed DCs

To assess the regulatory function of Tr1 cells primed by Vf-exposed DCs, suppression assays with proliferating CD4+ effector T cells were carried out. To this end, effector CD4+ Th cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and activated with anti-CD3/28 in the presence of unlabeled Tr1 cells previously primed with Vf-exposed DCs. To balance cell numbers, control experiments were performed by adding unlabeled unpolarized CD4+ Th cells to CFSE-labeled CD4+ effector Th cells. CFSE dilution was analyzed 72 hours after activation. Although addition of unpolarized Th cells could not alter proliferation of CFSE-labeled effector Th cells, Tr1 cells previously primed with Vf-exposed DCs suppressed Th cell proliferation in a cell number–dependent manner (Figure 4d and e).

Cutaneous exposure to signals of nonpathogenic bacterium *V. filiformis* leads to enhanced T-cell IL-10 and inhibits T-cell proliferation *in vivo*

To assess functional consequences of innate immune sensing of nonpathogenic bacterium *V. filiformis* and consecutive shaping of the adaptive immune system *in vivo*, NC/Nga mice were again investigated. As described in Figure 1b, NC/Nga mice were sensitized to FITC and were or were not exposed to Vf. At 1 week after the last application, mice were challenged at the ear skin with FITC alone. Whole lymph node cells from the ear-draining lymph nodes isolated 8 hours after challenge were restimulated with antigen (FITC) or anti-CD3/CD28 antibodies *ex vivo*. Only T cells from mice previously exposed to Vf displayed antigen-specific production of IL-10, whereas control mice failed to do so (Figure 5a). Using anti-CD3/CD28 antibodies, immune modulation and induction of IL-10 production in T cells *in vivo* by previous exposure to *V. filiformis* signals was confirmed (Figure 5b). Consistently, exposure to *V. filiformis* signals reduced T-cell proliferation in draining lymph nodes *in vivo* as measured by [^{3}H]-thymidine uptake *ex vivo* following FITC restimulation (Figure 5c). To analyze the effect of enhanced IL-10 production on effector T-cell responses *in vivo*, IFN-γ, the hallmark cytokine of chronic AD, was analyzed *ex vivo* (Grewe *et al*., 1995; Biedermann, 2006). Consistent with the reduced T-cell proliferation, antigen-specific IFN-γ production by T cells was significantly reduced in mice previously exposed to *V. filiformis* signals (Figure 5d).

Together, these data show that signals of nonpathogenic bacterium *V. filiformis* induce IL-10^{high} T cells *in vivo* and inhibit antigen-specific T-cell proliferation and cytokine production, thus demonstrating the immunomodulatory role of nonpathogenic innate immune signals *in vivo*.

DISCUSSION

Modulating immune responses at surface organs using nonpathogenic bacteria is a promising strategy to treat inflammatory diseases as reported in clinical trials on inflammatory

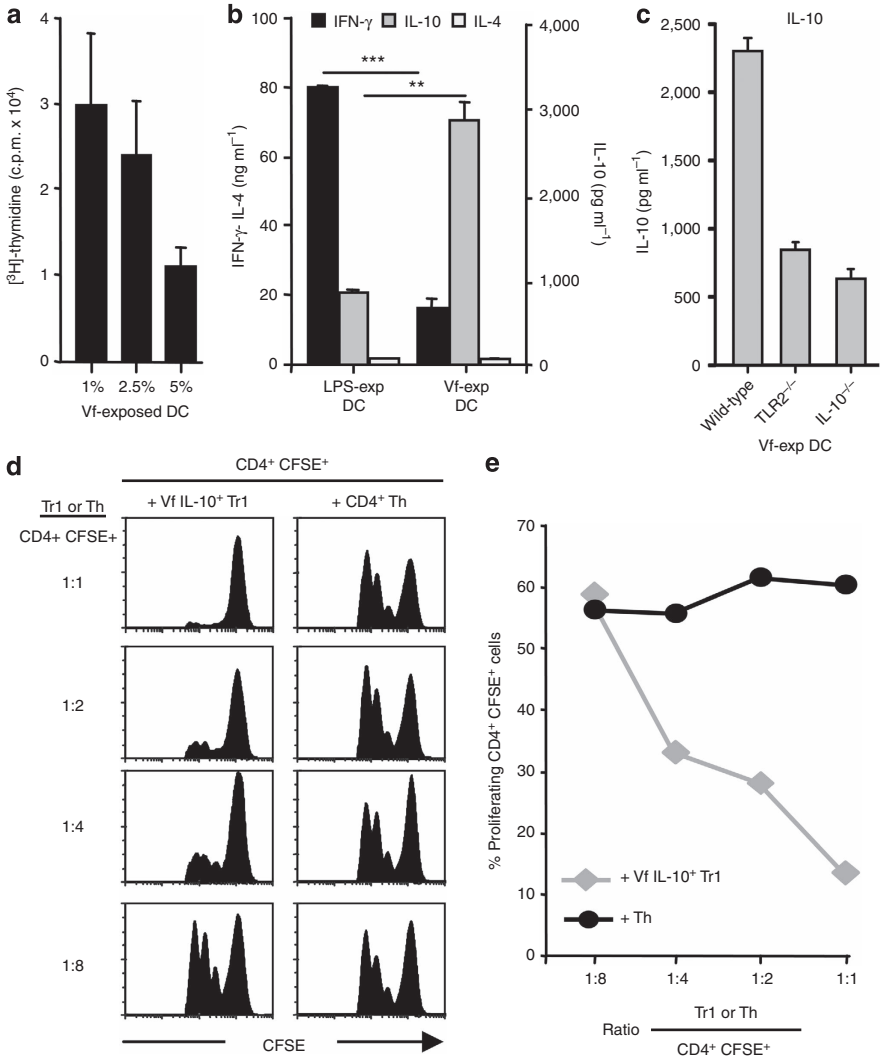

Figure 4. *Vitreoscilla filiformis* (Vf)–exposed dendritic cell (DCs) orchestrate induction of IL-10 + Tr1 (type 1 regulatory T) cells effectively suppressing T effector cells. (**a**) Direct and Vf-mediated dose-dependent reduction of antigen-specific CD4 + Th cell proliferation after activation with Vf-exposed DCs. (**b**) T-cell cytokine profile following previous stimulation with Vf-exposed (Vf-exp) or lipopolysaccharide (LPS)-exposed DCs. Induction of either Tr1 cells producing high IL-10 and low IFN-γ levels or Th1 cells with high IFN-γ and low IL-10 levels, respectively. (**c**) Vf-exposed IL-10 deficient and Toll-like receptor 2 (TLR2)–deficient DCs failed to induce high IL-10 levels in CD4 + T cells. (**d**) In contrast to T helper (Th) control cells, Vf-induced IL-10^{high} Tr1 cells inhibited proliferation of carboxyfluorescein succinimidyl ester (CFSE)–labeled CD4 + T cells in a cell ratio–dependent manner. (**e**) Quantifying proliferating CFSE + cells, shown at each cell-to-cell ratio. Representative data (mean ± SD) from one out of three independent experiments are shown. $^{**}P<0.01$ and $^{***}P<0.001$.

bowel disease (Rembacken *et al.*, 1999). We recently demonstrated that nonpathogenic bacteria are also functional when applied to the skin, alleviating cutaneous inflammation in AD patients (Gueniche *et al.*, 2008). To analyze the underlying mechanism and to elucidate how nonpathogenic bacteria may shape and modulate immune responses, we investigated consequences of innate immune sensing of *V. filiformis in vitro* and *in vivo*. NC/Nga mice sensitized to FITC are characterized by a predominance of Th2 cells and high IgE levels and develop AD-like skin inflammation as measured by increase of ear thickness after FITC challenge (Matsuda *et al.*, 1997; Dearman and Kimber, 2000; Matsuoka *et al.*, 2003). Cutaneous treatment of these NC/Nga mice with Vf during elicitation of skin inflammation significantly decreased allergen-specific dermatitis, indicating direct immunosuppressive effects of Vf. Importantly, cutaneous exposure to Vf and FITC antigen before the final allergen challenge resulted in even more pronounced suppression of cutaneous inflammation demonstrating immunomodulatory properties of Vf. This indicates that cutaneous treatment with Vf in AD patients is effective by direct and immunomodulatory pathways (Gueniche *et al.*, 2008). Indeed, *in vitro* analyses demonstrated that signals of *V. filiformis* induced a DC phenotype dominated by IL-10 already suggesting regulatory and tolerogenic properties (Lutz and Schuler, 2002; Frick *et al.*, 2010). In fact, DCs activated by signals of *V. filiformis* effectively induced regulatory T cells.

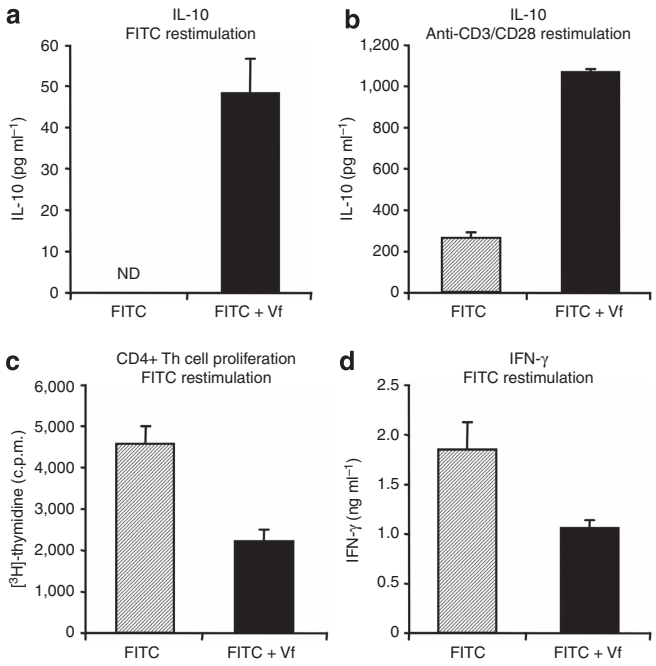

Figure 5. Cutaneous exposure to signals of *Vitreoscilla filiformis* (Vf) enhances T-cell IL-10 and inhibits T-cell proliferation *in vivo*. Induction of antigen-specific IL-10 production in draining lymph nodes was only detected in (**a**) mice previously exposed to Vf and (**b**) could be mapped to T helper (Th) cells as detected following anti-CD3/CD28 activation. (**c**) Vf-mediated immune modulation in previously Vf-exposed mice also reduced antigen-specific T-cell proliferation and (**d**) IFN-γ production as detected in lymph node cells 72 hours after *in vivo* challenge.

It was known that IL-10 production by DCs contributes to the induction of tolerance in various settings (Akbari *et al.*, 2001; Corinti *et al.*, 2001; Yu *et al.*, 2009). However, we demonstrate according to our knowledge the previously unreported finding that nonpathogenic Gram-negative bacteria induce this tolerogenic DC phenotype characterized by acquisition of a mature phenotype and a cytokine profile dominated by the production of IL-10, and Tr1 induction. The Gram-negative pathogen *Bordetella pertussis* has been shown to exploit a similar strategy to subvert host immunity and protective immune responses by induction of IL-10 and Tr1 cells (McGuirk *et al.*, 2002). In accordance with previously published results, we show that TLR adaptor molecule MyD88 is required for induction of both IL-10 and IL-12p70 production (Boonstra *et al.*, 2006). Surprisingly, however, DC maturation was independent of MyD88, indicating activation of other innate immune pathways (Medzhitov, 2009; Takeuchi and Akira, 2010). The dependence of IL-10 production on TLR2 confirms and extends data from previous reports demonstrating a dominant role for this pattern recognition receptor in the induction of IL-10 in DCs (Dillon *et al.*, 2006; Depaolo *et al.*, 2008). Lipoproteins have been isolated and identified from Gram-negative *E. coli* initially and lipopeptides derived thereof have been shown to activate TLR2 (Braun and Wu, 1994; Buwitt-Beckmann *et al.*, 2005). TLR2 activation by bacterial or synthetic ligands can result in either inflammatory or tolerogenic immune responses, but detailed mechanisms are still to be deciphered (Oliveira-Nascimento *et al.*, 2012). Lipoproteins from *Staphyloccocus aureus* binding to TLR2 have been shown to be crucial in induction of inflammation and clearance of bacteria (Schmaler *et al.*, 2009). In contrast, host immunity is subverted by *Yersinia pestis* inducing Tr1 cells triggered by TLR2/6 binding of LcrV subsequently inducing IL-10 production (Depaolo *et al.*, 2008). Porin B from *Neisseria meningitidis* is also a TLR2 ligand inducing DC activation (Singleton *et al.*, 2005). As both *V. filiformis* and *N. meningitidis* are Gram-negative bacteria belonging to the family of Neisseriaceae, it is tempting to speculate that the TLR2-activating ligand of *V. filiformis* is a bacterial porin with predominant anti-inflammatory properties (Strohl, 2005). In contrast to Gram-negative pathogenic bacteria such as Salmonella spp. eliciting proinflammatory immune responses, innate immune sensing of nonpathogenic Gram-negative bacteria like *V. filiformis* is not dominated by TLR4 signaling over TLR2 signaling and induction of inflammation, but is characterized by a more dominant TLR2 signaling and the induction of tolerance. One may speculate that the functional dominance of tolerogenic signals may be a general principle of how nonpathogenic bacteria and their hosts organize their coexistence in the absence of inflammation. Previously, it has been shown that the exposure to Gram-negative gammaproteobacterium *Acinetobacter* prevents allergic disease in mouse models and correlates with IL-10 production from healthy but not from AD individuals (Debarry *et al.*, 2007; Conrad *et al.*, 2009; von Mutius and Vercelli, 2010; Hanski *et al.*, 2012). This already demonstrates that contact to Gram-negative nonpathogenic bacteria leads to active immune recognition resulting in tolerogenic cytokine production (Hanski *et al.*, 2012). Strikingly, atopic individuals harbor significantly lower amounts of *Acinetobacter* on the skin and show diminished IL-10 production (Hanski *et al.*, 2012). In light of these findings and our data, the pathways utilized by nonpathogenic Gram-negative bacteria to induce tolerance seem to be promising targets for therapeutic strategies.

DCs have been shown to play a central role in transmitting innate immune signals into various types of adaptive immune responses (Kapsenberg, 2003; Joffre *et al.*, 2009). ''Tolerogenic DCs'' can prime Th cells to become regulatory T cells. Among these, Tr1 cells are characterized by the production of low amounts of IFN-γ but high levels of IL-10 (Groux *et al.*, 1997; O'Garra and Vieira, 2004; Shevach, 2006). Moreover, it has been reported that immune modulation by Tr1 cells reduces antigen-specific IgE but not antigen-specific IgG1 and IgG2a levels, which is similar to our findings following exposure to Vf (Cottrez *et al.*, 2000), but in contrast to, e.g., low-zone tolerance that has been reported to tremendously reduce specific immunoglobulin levels (Steinbrink *et al.*, 1996).

We could demonstrate that DCs activated by signals of nonpathogenic *V. filiformis* are potent inducers of Tr1 cells producing these signature cytokines. Moreover, these Tr1 cells strongly inhibited Th cell responses demonstrating functionality. This is in accordance with previously published results demonstrating that Tr1 cells are equally potent to FoxP3^{pos} natural regulatory T cells in controlling effector T-cell

responses (Vieira *et al.*, 2004). However, in contrast to FoxP3^{pos} natural regulatory T cells, Tr1 cells are inducible, indicating a feasible therapeutic strategy.

Identifying nonpathogenic bacteria with tolerogenic potential, crucial active microbial components within these bacteria, and activation pathways mediating active tolerance—such as TLR2 signaling—points to very promising therapeutic strategies in the treatment of inflammatory and allergic diseases, especially of the surface organs such as AD.

MATERIALS AND METHODS

FITC induced antigen-specific contact hypersensitivity

NC/Nga mice (5 mice per group) were sensitized by administration of 0.25% FITC solution (dissolved in 1:1 acetone/dibutyl phthalate) on the shaved abdomen on days 0, 7, and 14. At 7 days after the last sensitization, mice were challenged by application of 0.25% FITC solution on both sides of the ears. To determine the direct immunosuppressive effects of Vf, one group of mice was treated with Vf (20% v/v) on the ear skin during the allergen challenge period. Ear thickness was measured with a micrometer (Oditest; Kroeplin, Germany) and data are expressed as change in ear thickness as compared with that before treatment. The immunomodulatory effects of Vf were investigated by coadministration of Vf (20% v/v) on the abdominal skin at days −1, 7, 14, and 21, and not during challenge. At 7 days after the last FITC contact, all mice were challenged by application of 0.25% FITC solution onto the ears in the absence of Vf. Draining lymph nodes were collected either 8 or 72 hours after challenge and whole lymph node cells were cultivated with FITC or anti-CD3/CD28 antibodies for another 3 days. Cell culture supernatants were subjected to ELISA.

Generation and activation of human monocyte-derived DCs

DCs were generated from adherent peripheral blood mononuclear cells as previously described (Guenova *et al.*, 2008). To induce DC maturation, day 6 immature DCs (CD11c$^+$ CD14$^-$ HLA-DR$^+$ CD86$^+$ CD83$^-$) were cultured for an additional 24 or 48 hours in the presence of LPS R595 or Vf.

Generation and stimulation of murine BMDCs

Murine BMDCs were generated as described previously (Lutz *et al.*, 1999). At day 8, cells were collected, washed, and seeded in 1×10^6 ml per well in 24-well-plates. DCs were stimulated with LPS (1 μg ml^{-1}) or Vf (5% v/v) for 24 hours unless otherwise specified.

DC/T-cell coculture

Immature BMDCs were activated in the presence of ovalbumin (50 μg ml^{-1}) with LPS or Vf. Subsequently, DCs were washed extensively and cultivated together with naive CD4$^+$CD62L$^+$ OT-II Th cells in a ratio of 1:5 for 3 days. CD4+ T cells were then expanded using IL-2 (50 U ml^{-1}) for another 12 days. Resting T cells were washed and restimulated with plate-bound anti-CD3 (2 μg ml^{-1}) and anti-CD28 (5 μg ml^{-1}) in 96-well plates. Cell culture supernatants were harvested after 72 hours and subjected to ELISA.

Proliferation assays

Naive OT-II CD4$^+$CD62L$^+$ T cells (2×10^5) were activated with ovalbumin-pulsed and Vf-stimulated DCs (4×10^4) in 96-well flat-bottom plates in a total volume of 200 μl. After 5 days 0.25 μCi [^{3}H]-thymidine was added and cells were harvested after another 10 hours. Incorporated [^{3}H]-thymidine was measured using a micro beta counter (Perkin Elmer, Wiesbaden, Germany).

To asses suppressive capacity of Vf-induced IL-10+ Tr1 cells, unprimed CD4+ Th cells were isolated using magnetic cell isolation as described. These CD4+ cells were labeled with 1 μM CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's protocol and 2×10^5 CFSE+ CD4+ T cells were activated with plate-bound anti-CD3 (2 μg ml^{-1}) and soluble anti-CD28 (5 μg ml^{-1}) in the presence of activated Vf-induced IL-10+ Tr1 cells at the indicated cell ratios. Controls were set up using CFSE+ CD4+ cells cultivated together with unlabeled Th cells to ensure balanced cell numbers. Proliferation was determined 72 hours after activation.

All mice were maintained under specific pathogen free conditions at the animal facilities of the University of Tübingen according to local and federal guidelines.

Additional methods concerning animals, reagents, antibodies, bacterial lysates, Th cell isolation, and description of FACS analysis can be found online.

Statistical analysis

All data are presented as means ± SD or SEM (where indicated) of one representative experiment. Experiments were repeated at least three times if not indicated otherwise and revealed comparable results. Statistical analysis was performed with Student's *t*-tests (two tailed). Values of $P<0.05$ were considered as statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Staphylococcus aureus skin colonization is promoted by barrier disruption and leads to local inflammation

Ines Wanke[1], Yuliya Skabytska[1], Beatrice Kraft[1], Andreas Peschel[2], Tilo Biedermann[1] and Birgit Schittek[1]

[1]Department of Dermatology, Eberhard-Karls-University Tübingen, Tübingen, Germany; [2]Interfaculty Institute of Microbiology and Infection Medicine, Cellular and Molecular Microbiology, Eberhard-Karls-Universität Tübingen, Tübingen, Germany
Correspondence: Birgit Schittek, Tilo Biedermann, Liebermeisterstr. 25, 72076 Tübingen, Germany. Tel.: 0049 7071 2980832; Fax: 0049 7071 295187; e-mails: birgit.schittek@med.uni-tuebingen.de; tilo.biedermann@med.uni-tuebingen.de

Abstract: Experimental mouse models of bacterial skin infections that have been described show that pathogenic microorganisms can readily invade the epidermis and dermis to produce localized infections. We used an epicutaneous mouse skin infection model to determine how the level of barrier disruption by tape-stripping correlates with persistence of *Staphylococcus aureus* skin colonization, concomitant induction of cutaneous inflammation and infection. Furthermore, we investigated how murine skin responds to *S. aureus* colonization in a physiologic setting by analysing proinflammatory cytokines and antimicrobial peptides in mouse skin. We show that previous cutaneous damage allows skin inflammation to develop and favours *S. aureus* persistence leading to cutaneous colonization, suggesting an interdependence of cutaneous bacteria and skin. Our study suggests that skin barrier defects favour *S. aureus* skin colonization, which is associated with profound cutaneous inflammation.

Key words: antimicrobial peptides – inflammation – mouse model – *S. aureus* – skin colonization

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Background

In vivo skin infection models in which the pathogens need to be injected subcutaneously into the skin of mice have the limitations that this does not resemble the natural way of skin infection. Moreover, they are not suited to investigate processes underlying skin colonization such as *Staphylococcus aureus* colonization on skin of atopic dermatitis patients. Therefore, we used a mouse model of staphylococcal skin infection based on the epicutaneous inoculation of *S. aureus* onto mouse skin whose integrity was previously affected by tape-stripping. In previous studies, this model was used to analyse the degree of subsequent bacterial invasion into subcutaneous tissues and the dissemination of the microorganisms to other organs or to determine the effectiveness of topical antibiotic treatment for pathogen infection in skin wounds induced by extensive tape-stripping (1–3).

Questions addressed

We extended this model system for a new experimental approach determining how the extent of barrier disruption by tape-stripping correlates with the efficiency of infection, pathogen persistent skin colonization, concomitant induction of cutaneous inflammation and induction of antimicrobial peptide (AMP) expression in mouse skin.

Experimental design and results

The experimental model is based on epicutaneous application of the *S. aureus* strain 113 onto shaved skin of C57BL/6 mice. Before application, we either left the skin untreated or disrupted the skin barrier to different levels by either mild tape-stripping (3 times) or strong tape-stripping (7 times). Mild and strong tape-stripping left part of the epidermis intact and did not create a wound in contrast to another published study (1) (Figs 1a, b, S1). An inoculum of 10^7 *S. aureus* 113 in 0.015 ml of phosphate-buffered saline (PBS) or PBS control was added to 7-mm filter paper discs placed onto the prepared skin and covered by Finn Chambers on Scanpor (Smart Practice, Phoenix, AZ, USA). Fixation occurred via Fixomull stretch plaster as in patients undergoing epicutaneous patch testing (Fig. 1a).

After overnight occlusion, Finn Chambers and plasters were removed in all experimental groups, and one to 6 days after *S. aureus* application, the number of colony-forming units (CFUs) on mouse skin was quantified. In addition, biopsy samples from the application site were taken for further molecular analyses. Time titration experiments revealed that at least 1×10^6 bacteria were recovered 1 day after inoculation using epidermal scrapes to gather bacteria colonizing the epidermis. We detected 1×10^5 bacteria in the skin wash fraction, which harvests bacteria loosely attached to the skin surface (Fig. 1b, c). Interestingly, in strongly tape-stripped skin, the infection efficiency (day 1) as well as the persistence of *S. aureus* 113 as detected by scrapes was significantly higher compared with non- or mildly tape-stripped skin. This suggests that epithelial barrier defects facilitate cutaneous *S. aureus* colonization, probably by allowing bacterial adhesion, providing enhanced nutrition, possibly supported by an inflammatory response or reduced antibacterial defense.

Indeed, in strongly tape-stripped skin, *S. aureus* application induced high expression of IL-1β and IL-6 in mouse epidermis 1 and 3 days later and low but significant levels of TNF-α at 1 day after infection persisting until day 6. In contrast, *S. aureus* application in non- or mildly tape-stripped skin led to only mild, but significant, induction of IL-1-β 1 day after infection, but failed to induce IL-6 (Fig. 2). IFN-γ was only slightly induced in all cases, but started to become upregulated specifically in the experimental group with strong barrier disruption on day 6 after infection, indicating possible sensitization and migration of IFN-γ-producing lymphocytes under this condition (Fig. 2). Strong tape-stripping

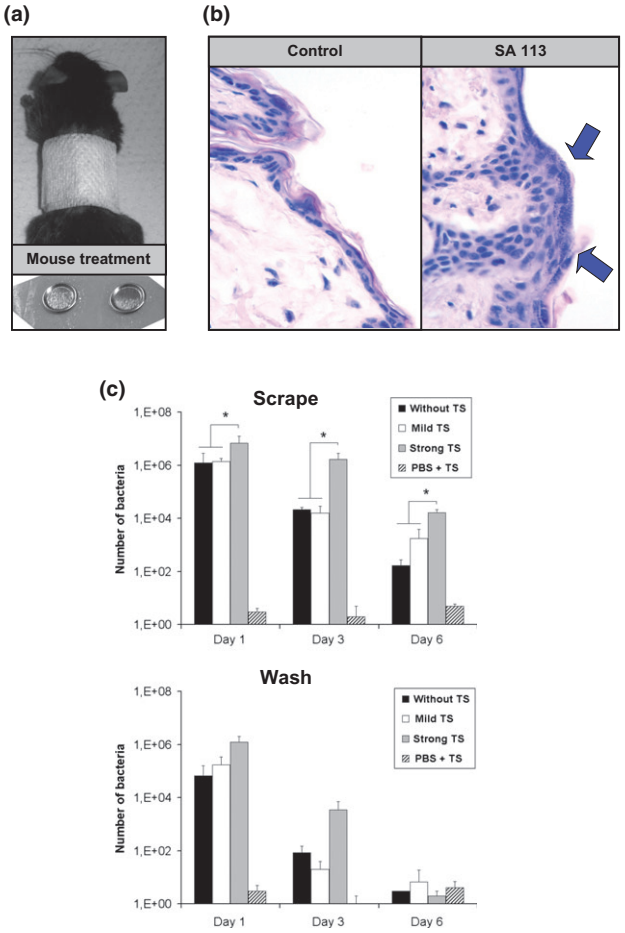

Figure 1. Epicutaneous application of *Staphylococcus aureus* onto barrier-disrupted skin leads to cutaneous bacterial persistence. (a) Shown are 8-week-old female C57BL/6 mice directly after epicutaneous *S. aureus* 113 application. The Finn Chambers on Scanpor adaptors are shown in the increment. (b) HE staining of *S. aureus* 113-challenged mouse skin reveals bacteria on the uppermost layer of infected mouse skin, but not on uninfected mouse skin (arrows mark the bacteria). (c) CFU assay shows the persistence of viable *S. aureus 113* over 6 days after the application of the bacteria onto mouse skin without or after mild or strong tape-stripping (TS) in scraped or washed skin samples. We do not find significant differences in the colonization efficiency one day after infection between the three different *S. aureus* strains 113, Newman and ATCC 25923 (data not shown). Control: Without *S. aureus* application but with tape-stripping. Four skin samples were plated in duplicate for scraped skin ($n = 8$) or on a single plate for washed skin ($n = 4$). Asterisks mark significant differences (*P*-values $\leq$ 0.05 in Student's *t*-test). There is a statistically significant difference between strong and mild tape-stripping and between strong and no tape-stripping in the scrapes. The efficiency of skin colonization is defined by the recovery of viable colony-forming units (CFU) from scraped skin of previously mild or strong tape-stripped skin compared with untaped skin.

alone induced only low expression of IL-1β and IL-6 one day after cutaneous damage, but failed to regulate TNF-α or IFN-γ at any time point. These data indicate that *S. aureus* application and persistence on barrier-disrupted skin induce an inflammatory cytokine response lasting at least 3–6 days.

Staphylococcus aureus infection of human keratinocytes grown *in vitro* and experimental barrier disruption in humans and mice are known to induce AMPs (4–7). Interestingly, in our mouse model of cutaneous bacterial persistence, we found that *S. aureus*

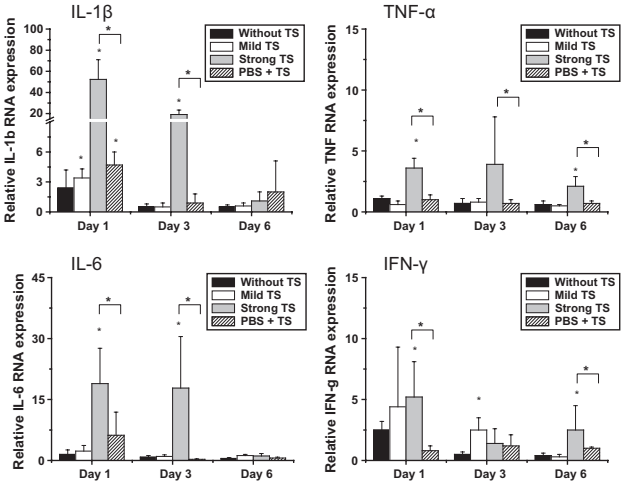

Figure 2. Epicutaneous application of *Staphylococcus aureus* induces mRNA expression of inflammatory cytokines especially in strongly tape-stripped skin. Shown is the relative RNA expression of the proinflammatory cytokines Il-1β, Il-6, TNF-α and IFN-γ in skin biopsies 1, 3 and 6 days after application of *S. aureus* 113 bacteria onto mouse skin without or after mild or strong tape-stripping (TS). Effects of tape-stripping alone are shown in the PBS + TS group. mRNA expression levels were analysed by real-time PCR. PBS-treated skin without tape-stripping was used as a control and set as 1. Four samples were analysed in duplicate ($n = 8$). Asterisks mark significant differences to the control or between labelled groups (*P*-values $\leq$ 0.05 in Student's *t*-test).

induced the expression of the mouse β-defensins mBD3, mBD4 (both are murine orthologs of human HBD-2) and mBD14 (murine ortholog of human HBD-3) in the epidermis. The highest induction levels of mBD3, mBD4 and mBD14 were seen 1 day after *S. aureus* application in the strongly tape-stripped skin, which lasted at least 3 days (Figure S2). Strong tape-stripping alone induced only a transient induction of these AMPs at day 1. Furthermore, mBD2 expression was only transiently induced 3 days after *S. aureus* application in strongly tape-stripped skin (Figure S2).

Conclusions

We describe a mouse model for cutaneous colonization and persistence of bacteria such as *S. aureus*, which resembles the natural route of how skin colonization and infection take place. *Staphylococcus aureus* colonizes nasal epithelia, is found on skin in high amounts, mainly in the case of skin barrier defects as in atopic dermatitis, and is thought to contribute actively to skin inflammation (6,8,9). Our study confirms these assumptions by demonstrating that skin barrier defects favour *S. aureus* skin colonization and that prolonged colonization is associated with profound cutaneous inflammation, suggesting an interdependence of cutaneous bacteria and skin. Thus, this mouse model is ideally suited for unravelling the role and molecular mechanisms of barrier disruption and skin inflammation in pathogen infection and for evaluating strategies to prevent skin inflammation and infection.

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Author contributions

IW, YS and BK performed the research; IW, YS, BK and BS analysed the data; AP contributed essential reagents and tools; TB and BS designed the research study; and BS wrote the manuscript.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mild and strong tape-stripping does not remove the epidermal layer.

Figure S2. Epicutaneous application of *S. aureus* induces mRNA expression of antimicrobial peptides especially in strongly tape-stripped skin.

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Letter to the Editor

Photoprotective effect of libanoridin isolated from *Corydalis heterocarpa* on UVB stressed human keratinocyte cells

Byul-Nim Ahn[1], Jung-Ae Kim[1], Chang-Suk Kong[2], Youngwan Seo[3] and Se-Kwon Kim[1,2,4]

[1]Department of Chemistry, Pukyoung National University, Busan, Korea; [2]Department of Food and Nutrition, College of Medical and Life Science, Silla University,Busan, Korea; [3]Division of Marine Environment and Bioscience, Korea Maritime University, Busan, Korea; [4]Marine Bioprocess Research Center, Pukyong National University, Busan, Korea

Correspondence: Se-Kwon Kim, Department of Chemistry, Pukyoung National University, Busan 608-737, Korea Tel.: +82-51-629-7094, Fax: +82-51-629-7099, e-mail: sknkim@pknu.ac.kr

Abstract: Ultraviolet-B (UVB) irradiation acts primarily on the epidermal basal cell layer of the skin, inducing harmful biological effects. In this study, we have investigated the effect of libanoridin isolated from *Corydalis heterocarpa* against UVB-induced damage in human keratinocyte (HaCaT) cells and the molecular mechanism underlying those effects. Treatment with libanoridin inhibited the cell cytotoxicity and LDH induced by UVB exposure at 40 mJ/cm^2. Additionally, expression levels of type IV collagenases (MMP-2, MMP-9) were decreased by libanoridin. Furthermore, MMP tissue inhibitors were enhanced followed by treatment with libanoridin. Moreover, UVB-induced activation of phosphorylation of three MAPKs such as JNK, ERK, p38 and AP-1 transcription factor were decreased by treatment with libanoridin.Our present study demonstrates that libanoridin has the abilities to inhibit UVB-induced cellular damage via ASK1-MAPK and AP-1 signalling pathways. Therefore, libanoridin may be used as an effective natural compound to prevent skin damage due to UVB exposure.

Key words: AP-1 – libanoridin – MAPK – MMP – UVB exposure

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Background

Recent studies have indicated that various physical phenomena are caused by exposure to UVB irradiation (1,2). Thus, searching for sun-blocking agents may be a basic step in the prevention of skin damage by UVB irradiation. UVB (290–320 nm) irradiation is known to be a major cause for the epidermal damage in human skin (3). Keratinocytes constitute for 90% of the cells found in the epidermis and are capable of producing cytokines in response to external stimuli (4). Human keratinocyte (HaCaT) cells were reported to be the appropriate experimented model to study the biological changes in human epidermis (5). Therefore, to study the inhibitory effects of libanoridin on cell damage induced by UVB exposure, HaCaT keratinocytes were chosen.

Halophytes are salt-tolerant plants that are adapted primarily to an ionic imbalance and hyperosmotic stress. The effect of imbalance or disruption in homoeostasis occurs at the cell level that causes molecular damage and growth arrest. Moreover, these plants commonly suffer from serious *in vivo* photodynamic damage (6). Although their cells are equipped with protective mechanisms to reduce photodynamic damage, biological activities of their secondary metabolites have been little investigated to date (7).

A kind of halophyte, *Corydalis heterocarpa*, has been used as a traditional medicine for dysentery in Korea. It has been reported

IL-4 abrogates TH17 cell-mediated inflammation by selective silencing of IL-23 in antigen-presenting cells

Emmanuella Guenova[1,11,‡,*], Yuliya Skabytska[1,‡], Wolfram Hoetzenecker[1,11,‡], Günther Weindl[1,2], Karin Sauer[1], Manuela Tham[1], Kyu-Won Kim[3], Ji-Hyeon Park[3], Ji Hae Seo[3,4], Mitchell P. Levesque[5], Thomas Volz[1], Martin Köberle[1], Susanne Kaesler[1], Peter Thomas[6], Reinhard Mailhammer[7], Kamran Ghoreschi[1], Knut Schäkel[8], Boyko Amarov[9], Martin Eichner[10], Martin Schaller[1], Rachael A. Clark[11], Martin Röcken[1,*], Tilo Biedermann[1,12*]

[1]Department of Dermatology, Eberhard Karls University Tübingen, 72076 Tübingen, Germany [2]Institute of Pharmacy, Department of Pharmacology and Toxicology, Freie Universität Berlin, 14195 Berlin, Germany [3]NeuroVascular Coordination Research Center, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742 , Republic of Korea [4]Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, Republic of Korea [5]Department of Dermatology, University Hospital of Zürich, Gloriastr. 31, 8091 Zürich, Switzerland [6]Department of Dermatology and Allergology, Ludwig-Maximilians University Munich, 80337 Munich, Germany [7]Institute of Clinical Molecular Biology and Tumor Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, 81377 Munich, Germany [8]Department of Dermatology, Heidelberg University Hospital, Voßstr. 2, 69115Heidelberg, Germany [9]Institute of Statistics and Econometrics, Freie Universität Berlin, 14195 Berlin, Germany [10]Department of Medical Biometry, Eberhard Karls University Tübingen, 72076 Tübingen, Germany [11]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA. [12]Department of Dermatology and Allergy, Technische Universität Munich, Munich, Germany.

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Interleukin (IL-) 4 can strongly suppress delayed type hypersensitivity reactions (DTHR), including organ-specific autoimmune diseases in mice and in humans. Despite the broadly documented anti-inflammatory effect of IL-4 the underlying mode of action remains unknown, as IL-4 promotes IL-12 production by dendritic cells (DC) and interferon-γ-producing TH1 cells in vivo. Studying the impact of IL-4 on the polarization of human and mouse DC, we surprisingly found that IL-4 exerts strictly opposing effects on the production of either IL-12 or IL-23. While promoting IL-12-producing capacity of DC, IL-4 completely abrogates the capacity of DC to produce IL-23. Bone marrow chimeras directly proved that IL-4-mediated suppression of DTHR strictly relies on the STAT6-dependent abrogation of IL-23 in antigen-presenting cells. In line with this, IL-4 therapy severely attenuated DTHR by selective, STAT6- and ATF3-dependent suppression of the IL-23/TH17 responses despite simultaneous enhancement of IL-12/TH1 responses. As IL-4 therapy also improves psoriasis in humans and selectively suppresses IL-23/TH17 responses, without affecting the IL-12/TH1 responses, selective IL-4-mediated IL-23/TH17-silencing is promising as treatment against harmful inflammation, while sparing the IL-12-dependent TH1 responses.

IL-4 | TH17 | IL-23

Introduction

IL-4 is a pleiotropic cytokine produced by $CD4^+$ T_H2 cells but also $CD8^+$ T cells, natural killer T cells, eosinophils, basophils, innate lymphoid and mast cells (1-6). IL-4 is a canonical type 2 immune cytokine known for its capacity to induce IgE isotype switching in B cells and to initiate and sustain T_H2 cell differentiation (2, 7). IL-4 provides protective immune responses to helminthes (8), and excessive IL-4 production is linked to T_H2-dominated allergic asthma and atopic dermatitis (9). IL-4 produced by malignant T cells further promotes a T_H2 bias and T cell immunosuppression in leukemic cutaneous T cell lymphoma (10). *In vivo*, IL-4 can suppress organ-specific autoimmune and delayed type hypersensitivity reactions (DTHR). In line with this, IL-4 is absent in naturally occurring DTHR, such as experimental autoimmune encephalomyelitis (EAE), multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD) or psoriasis (11-15). Systemic IL-4 immunotherapy improves EAE (16), experimental colitis (5), non-obese diabetes (17), collagen-induced arthritis (18), and hapten-induced contact hypersensitivity (19) in mice, and psoriasis in humans (20). The inhibitory effect of IL-4 on the autoimmune DTHR however failed to be explained by the redirection of the T_H1 immune responses towards IFN-γ-deficient type 2 immune responses. To the contrary, the number of peripheral IFN-γ^+CD4^+ T_H1 cells or serum IFN-γ even increases after IL-4 administration in mice with EAE (16), haemophagocytic lymphohistiocytosis (21) or hapten-induced contact hypersensitivity (19), and in humans with psoriasis (20); mice with transgenic overexpression of IL-4 exhibit T_H2-driven allergic-like inflammatory disease with elevated IFN-γ levels (22), and IL-4 can even directly instruct protective T_H1 immunity in mice with *Leishmania major* infection (23). Thus, although IL-4 might be an important natural inhibitor of many DTHR, the mode of action by which IL-4 suppresses inflammatory autoimmune disease and DTHR remains enigmatic. Functional and genetic data now revealed that a significant number of DTHR that have long been associated with IFN-γ-producing T_H1 cells and IL-12p70-producing APC, are mediated by IL-17/IL-22-producing Th17 cells and IL-23-producing APC, rather than by T_H1/IL-12 responses (24-28). Consistently, recent reports have correlated the level of disease activity and the absence of IL-4 with the

Significance

IL-4 has been shown to have a highly beneficial clinical outcome in delayed type hypersensitivity, autoimmune and autoinflammatory reactions in mice and humans, but its mode of action has remained controversial and has failed to be explained solely by redirection of the pathologic Th1/Th17- towards a Th2-type immune response. Here, we identify a new immunoregulatory role of IL-4 on cells of the innate immune system, describe its therapeutic mode of action in Th17-mediated autoimmune inflammation, and a new physiologically highly relevant approach to selectively target IL-23/Th17-dependent inflammation while sparing IL-12 and Th1 immune responses.

Reserved for Publication Footnotes

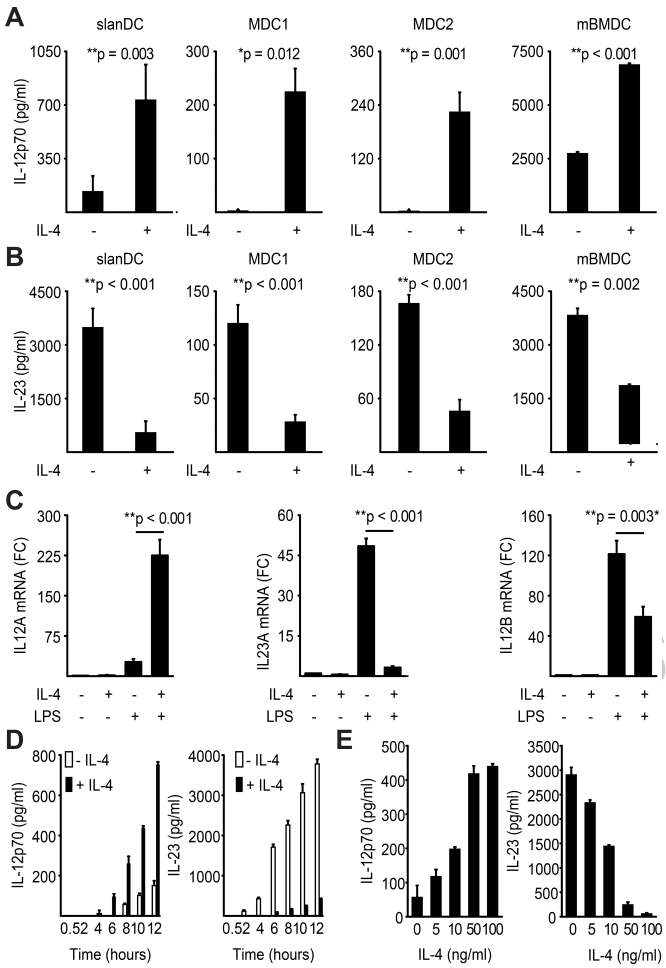

Fig. 1. Strictly opposing effects of IL-4 on either IL-12 or IL-23 secretion by DC.(A and B) Different subsets of human conventional myeloid DC and mouse BMDC were pre-incubated with 100ng/ml IL-4 and then stimulated with LPS. The IL-12 (A) and IL-23 (B) levels in the culture supernatants were determined by an ELISA. The data shown are from at least three independent experiments, and the results are expressed as the means ± SD. (C) The expression levels of transcripts encoding IL23A, IL12A, and IL12B were determined by quantitative real-time PCR in slanDC treated as described in (A). The values from three independent experiments were calculated relative to the expression levels of the housekeeping gene G6PD and were normalized to the unstimulated control. FC = fold change. (D and E) SlanDC were treated as described in (A), and IL-12p70 and IL-23 secretion was analyzed at the indicated time-points (D) or as a function of IL-4 (E). The data are expressed as means ± SD of triplicates and are representative of five independent experiments.

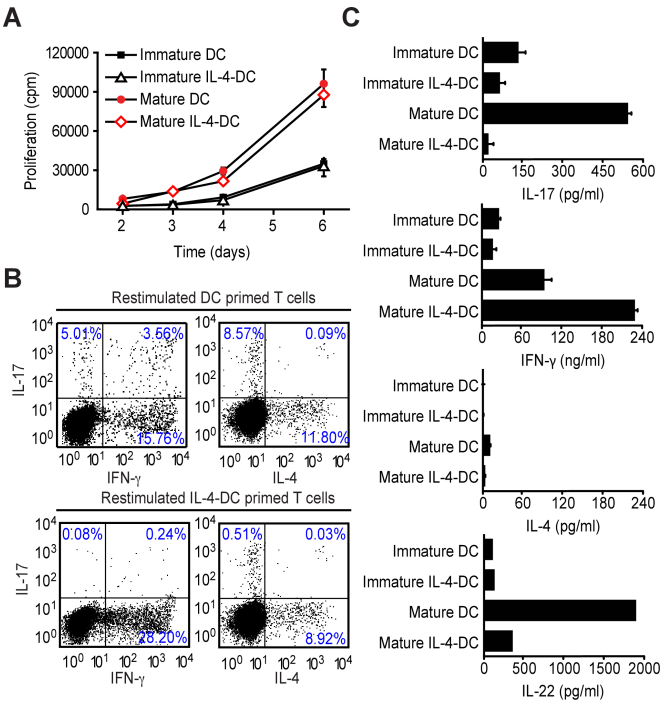

Fig. 2. IL-4 selectively abrogates the TH17 cell-inducing capacity of DC(A) DC stimulated with LPS in the presence or absence of 100ng/ml IL-4 or control DC were co-cultured with autologous naïve T cells in the presence of SEB, and proliferation was determined by 3H-thymidine uptake. (B) DC stimulated with LPS in the presence or absence of 100ng/ml IL-4 and control DC were co-cultured with autologous naïve T cells over 12 days. Cytokine production by CD4+ T cells was determined by flow cytometry following re-stimulation with PMA and ionomycin. (C) T cells co-cultured with DC stimulated as indicated in (B) were reactivated on day 12 with anti-CD3/CD28 for an additional 48 h, and cytokines were analyzed by ELISA. The results are expressed as means ± SD, and the data shown represent independent experiments from three different donors.

presence of IL-23 producing APC and IL-23-dependent T_H17 cells (29-31).

We analyzed the impact of IL-4 on the regulation of IL-23 and T_H17 in DTHR in mice and in human psoriasis. Unexpectedly, IL-4 abolished the capacity of APC to produce IL-23, while promoting IL-12p70. This selective inhibition impaired the induction and maintenance of pathogenic T_H17 cells. Bone marrow chimeras with either STAT6-deficient APC or STAT6-deficient T cells proved that IL-4 suppressed the development of T_H17 cells by abrogating the IL-23 production in APC. IL-4 therapy of psoriasis also dose-dependently suppressed IL-23-production by APC and T_H17 cells, while largely preserving IL-12 and T_H1-immunity in humans. This may open an entirely new approach for a targeted abrogation of harmful IL-23/Th17-immune reactions without affecting potentially protective IL-12/T_H1 in mycobacteria and parasite immunity (23, 32) and perhaps cancer (32).

Results

Strictly opposing effects of IL-4 on either IL-12 or IL-23 secretion by DC

To dissect the pro- and anti-inflammatory effects of IL-4 on DC, we stimulated, with TLR ligands in the presence or absence of IL-4, four distinct DC populations: BDCA-1-expressing DC (MDC1), BDCA-3-expressing DC (MDC2), 6-sulfo-LacNAc-expressing DC (slanDC), and murine bone-marrow derived DC (mBMDC). IL-4 strongly and significantly induced IL-12p70 production in all four DC subsets, in human DC 10- to 100-fold and in murine BMDC about 3-fold (Fig. 1A). Surprisingly, IL-4 simultaneously and almost completely abrogated TLR-triggered IL-23 production in all human and mouse DC populations (Fig. 1B). The opposing effects of IL-4 on the production of either IL-12 or IL-23 were transcriptionally regulated. IL-4 significantly suppressed the TLR-driven induction of *il23a* mRNA ($P=0.001$), while strongly inducing *il12a* mRNA expression ($P<0.001$; Fig. 1C). IL-4 also suppressed TLR-induced expression of the common IL-12/23p40 (*il12b*) subunit in most APC ($P<0.003$; Fig. 1C). The opposing effects on either IL-12p70 or IL-23 production seemed to be unique to IL-4, as other T_H2 cytokines, including IL-13, failed to abrogate IL-23 secretion (Fig. S1). To determine whether IL-4 affects the dynamics of IL-12 or IL-23 induction rather than the total production, we performed time-course studies over 12 hours in slanDC. We observed that, following LPS stimulation, slanDC started to produce IL-12 and IL-23 after 2-4

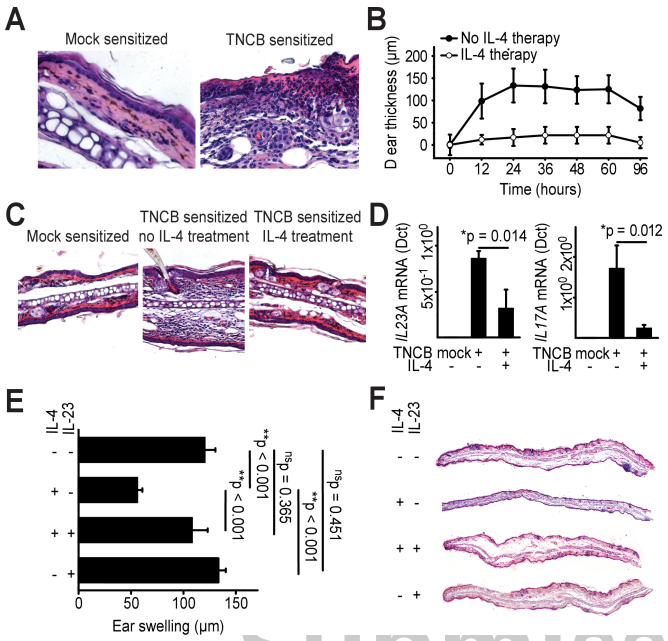

Fig. 3. IL-4-induced immune-suppression strictly depends on direct suppression of IL-23. (A) Representative H&E stains (x40) from skin inflammation after challenge with TNCB in TNCB- sensitized C57BL/6 mice (B and C). Time course (B) of the ear swelling and representative H&E stains (x20) (C) after TNCB-challenge in sensitized C57BL/6 mice, treated intraperitoneally with either PBS or IL-4 during challenge. The results in (B) are means ± SD. (D) Expression of transcripts encoding IL23A and IL17A in DTHR ear samples in mice treated with IL-4 as described in (B). Quantitative real-time PCR was performed, and the data are expressed in relative units [Δct] compared to the housekeeping gene. The results are the means ± SD. (E and F) Ear swelling (E) and representative H&E stains (F) 24 hours after challenge with TNCB in sensitized C57BL/6 mice. Mice were treated intraperitoneally with either PBS or IL-4 as in (B). Additionally, IL-23 was applied to some groups where indicated. The data are expressed as means ± SD.

hours, and IL-23 levels increased more than 100-fold during the first 12 hours (Fig. 1D). IL-4 simultaneously suppressed IL-23 but enhanced IL-12p70 production over the entire study period, showing that IL-4 did not alter the dynamics of either IL-12 or IL-23 production. Moreover, the opposing effects of IL-4 were dose-dependent and reached saturation at 100 ng IL-4/ml (Fig. 1E). IL-4 also reduced the secretion of other innate cytokines, such as IL-1β and IL-6 (Fig. S1B).

IL-4 selectively abrogates the T_H17 cell-inducing capacity of DC

The hallmark of DC function is their ability to prime naïve T cells, and steer T_H cell differentiation into either T_H1, T_H17, or T_H2 cells. To address whether IL-4 affects the capacity of DC to drive proliferation of naïve T cells, we first used either immature or *in vitro*-matured DC to stimulate naïve autologous $CD4^+CD45RA^+$ T cells. As expected, immature DC were less efficient than mature DC in inducing the proliferation of naïve $CD4^+$ T cells (Fig. 2A). IL-4 did not affect T-cell proliferation of either DC population (Fig. 2A). Based on our observation that IL-4 affected the cytokine pattern secreted by DC, we tested whether IL-4 also affected their capacity to prime naïve T cells for either T_H1, T_H17 or T_H2 differentiation. To test this, we matured DC in the presence or absence of IL-4, used them to prime naïve $CD4^+$ T cells, expanded the cells and re-stimulated such primed $CD4^+$ T cells for cytokine production. Maturation of DC in the absence of exogenous IL-4 resulted in a DC phenotype that induced both T_H1 and T_H17 cells, which produced large amounts of either IL-17 and IL-22 or IFN-γ (Fig. 2B), as previously reported (33, 34). Maturation of DC in the presence of IL-4 resulted in a DC phenotype that failed to induce T_H17 cells (≤0.5%) (Fig. 2B);

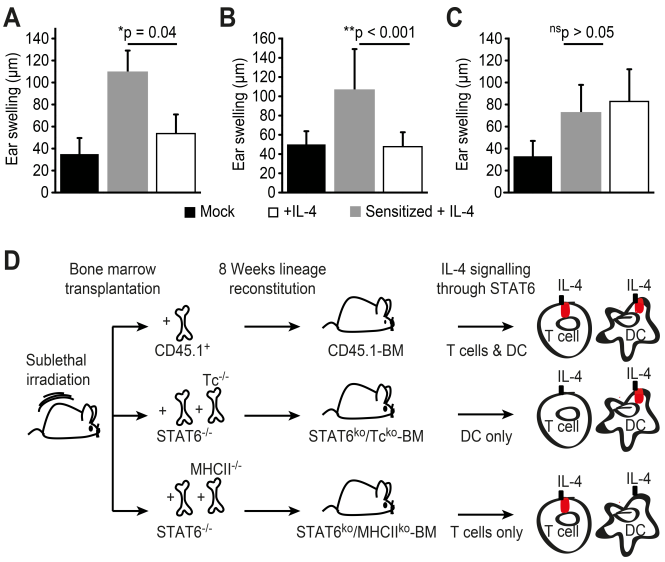

Fig. 4. IL-4 responsive APC orchestrate IL-4-induced suppression of T_H17 responses.(A to C) Ear swelling after TNCB-challenge in sensitized C57BL/6 mice bone marrow (BM) chimeric mice. IL-4 treatment during challenge was administered in some of the groups as indicated. Data from control CD45.1+-BM chimeric mice on wild type non-hematopoietic background are presented in (A). Data from STAT6-/-/Tc-/--BM chimeric mice are presented in (B), and data from STAT6-/-/MHCII-/--BM chimeric mice are presented in (C). Data are expressed as mean ± SD and represent two independent experiments. At least six mice per each group have been analyzed (D) Schematic presentation of the experimental approach for the generation of bone marrow chimeric mice.

instead the percentage of IFN-γ-producing T_H1 cells increased (Fig. 2C).

IL-4-induced immune-suppression strictly depends on direct suppression of IL-23

To analyze the biological relevance of this IL-4-mediated suppression of IL-23 and of the subsequent maintenance of T_H17 cells *in vivo*, we first studied IL-4-induced immune suppression in 2,4,6-trinitrochlorobenzene (TNCB)-induced DTHR in C57BL/6 mice. Challenging sensitized mice with TNCB resulted in pronounced ear swelling and skin inflammation characterized by epidermal hyperplasia, sub-corneal neutrophilic infiltrates, and angiogenesis (Fig. 3A). Systemic administration of IL-4 during the challenge significantly reduced the ear swelling in TNCB-sensitized mice (Fig. 3B and C), abrogated the infiltration of polymorphonuclear (PMN) cells, and normalized skin morphology (Fig. 3C). The TNCB challenge caused a strong induction of *il23a* and of *il17a* mRNA in ear tissues of mice challenged with TNCB (Fig. 3D), and IL-4 treatment during the TNCB challenge suppressed *il23a* and of *il17a* mRNA about 10-fold (Fig. 3D). To directly test whether this IL-4-mediated suppression of IL-23 also suppressed inflammation, we treated sensitized mice with recombinant mouse (rm) IL-4 during the TNCB challenge. Subsequently, we aimed, in half of the IL-4-treated mice, to prevent suppression of inflammation via systemic administration of rmIL-23, rmIL-6 or PBS. Neither PBS nor rmIL-6 restored the rmIL-4-mediated suppression of IL-23 and DTHR to almost background levels (Fig. S2A and B). In contrast, rmIL-23 fully rescued the cutaneous DTHR, as determined by the ear swelling responses (Fig. 3E and F).

IL-4-responsive APC orchestrate IL-4-induced suppression of T_H17 responses

We next determined whether IL-4 suppresses T cell-mediated inflammation by its action on $CD4^+$ T cells or through the suppression of IL-23-production of APC *in vivo*. Therefore we first generated bone marrow (BM) chimeric (BMC) mice with

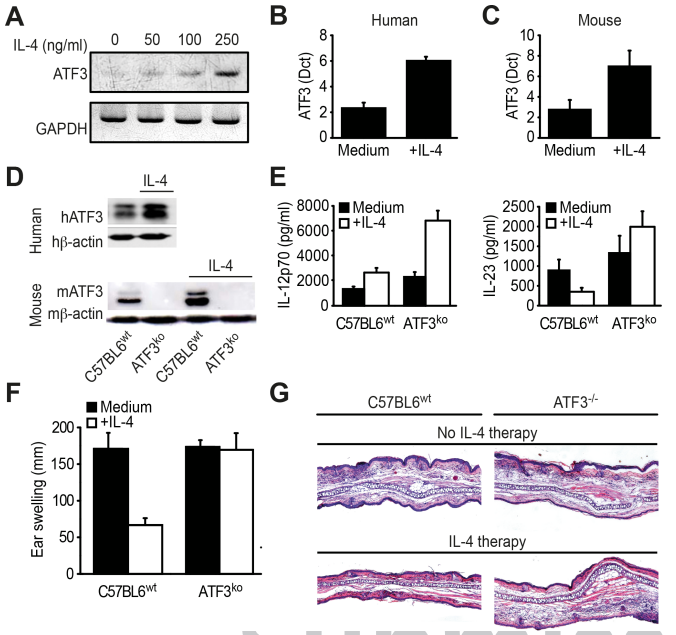

Fig. 5. IL-4 mediated suppression of IL-23 is partly mediated through activating transcription factor 3 (ATF3). (A) Representative data from a semi-quantitative PCR for ATF3 mRNA expression in murine RAW264.7 cells, analyzed as a function of IL-4. (B and C) Quantification of ATF3 mRNA expression in LPS-stimulated human (B) and murin (C) DC. Data are expressed in relative units [Δct] compared to the housekeeping gene. The results are the means ± SD. (D) Human DC and BMDC from either C57BL6wt mice or ATF3-/- mice were pre-incubated with IL-4 and then stimulated with LPS. ATF3 protein was analyzed semi-quantitatively by western blotting. Representative data from three independent experiments are shown. (E) BMDC from either C57BL6wt mice or ATF3-/- mice were treated as in (D). IL-12 and IL-23 levels in the culture supernatants were determined by ELISA. The data shown are from three independent experiments, and the results are expressed as the means ± SD. (F and G) Ear swelling (F) and representative H&E stains (G) 48 hours after challenge with TNCB in sensitized C57BL/6 and ATF3-/- mice. Mice were treated intraperitoneally with either PBS or IL-4 as in Fig. 3B. The data are expressed as means ± SD.

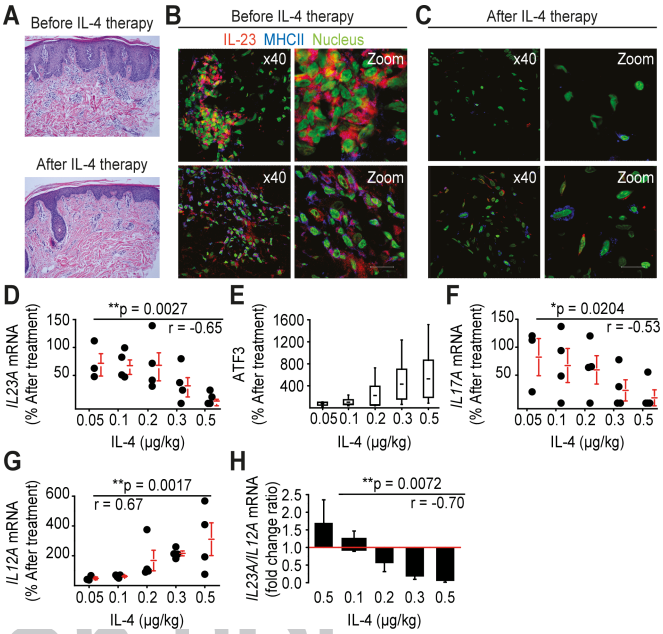

Fig. 6. IL-4 therapy of psoriasis abrogates intralesional IL-23 and IL-17 in human skin. (A) Representative H&E stains from co-localized biopsies of psoriatic skin before (A) and after systemic IL-4 treatment. (B and C) Visualization of co-localized IL-23 (red) and MHC II (blue) and IL-17 (red) and CD3 (blue) in human psoriatic skin lesions before (B) and after (B) IL-4 therapy. The nuclei are stained with YO-PRO. For colorblind-accessible images, please refer to Supplementary Fig. 9. (D to G) RT-PCR expression of transcripts encoding IL23A, ATF3, IL17A, and IL12A in psoriatic skin samples before and after different doses of IL-4 therapy. The expression of the target gene within psoriasis tissue before treatment (relative to the housekeeping gene G6PD) was set to 100%, and the expression after treatment is presented as a percentage of this value. Each dot represents one pair of specimens from a single study patient; the horizontal bars indicate the means ± SEM. *P < 0.05. **P < 0.01. (H) Ratio of *IL23A* [% after treatment] to *IL12A* [% after treatment] as detected by quantitative real-time PCR in the skin samples from the study patients treated with different doses of IL-4. The data are expressed as the means ± SEM. *P < 0.05. **P < 0.01. r = Pearson correlation coefficient.

$CD45.1^+$ hematopoiesis on a $CD45.2^+$ background ($CD45.1^+ \rightarrow CD45.2^+$ mice). Eight weeks after transplantation, engraftment efficiency and lineage reconstitution were >90%, and residual host $CD45.2^+$ BMC was <3% (Fig. S3 and S4). The chimeric mice developed a typical DTHR showing that the BMC mice could be sensitized normally; in addition, rmIL-4 suppressed cutaneous DTHR in such $CD45.1^+ \rightarrow CD45.2^+$ mice as in previous experiments (Fig. 4A). To distinguish the effects of IL-4 on either APC or on T cells, we selectively blocked IL-4 signaling in either T cells or APC of the BMC mice. This was achieved first by generating BMC chimeric mice deficient for STAT6 in the T cell lineage ($STAT6^{-/-}/Tc^{-/-} \rightarrow$ WT mice). In detail, by transplanting BM of $STAT6^{-/-}$ mice into lethally irradiated recipient mice, the T cell repertoire of $STAT6^{-/-}$ mice was established in BMC mice. By co-transplantation of BM devoid of any T cells from $Tc^{-/-}$ mice into these BMC mice, those chimeric mice had normal IL-4 sensitive STAT6 expressing APC from the $Tc^{-/-}$ donor organism, but only harbored $STAT6^{neg}$ T cells unresponsive to IL-4 therapy from the $STAT6^{-/-}$ mice. A TNCB challenge in sensitized $STAT6^{-/-}/Tc^{-/-} \rightarrow$ WT chimeric mice resulted in a similar ear swelling response as compared to the control chimeric mice ($CD45.1^+ \rightarrow$ WT $CD45.2^+$ mice) (Fig. 4A and B). Of note, IL-4 significantly reduced ear swelling and abrogated cutaneous inflammation in these sensitized $STAT6^{-/-}/Tc^{-/-} \rightarrow$ WT chimeric mice (Fig. 4B), highlighting a key role for APC in mediating the beneficial effect of IL-4 therapy. Next, to study the exclusive role of APC in this process, we extended our experiments and generated BM chimeric mice with a mixed $STAT6^{-/-}/MHCII^{-/-}$ hematopoiesis on a wild type background ($STAT6^{-/-}/MHC\ II^{-/-} \rightarrow$ WT mice), in which IL-4 signaling was completely abrogated in functional $MHCII^{pos}$ APC. TNCB challenge after sensitization of those chimeric mice resulted in an increased ear swelling comparable to what we observed in the other two chimeric mouse models (Fig. 4A to C). However, when we treated the $STAT6^{-/-}/MHC\ II^{-/-} \rightarrow$ WT mice with IL-4, cutaneous inflammation failed to improve, and the ear swelling was not reduced but remained comparable to that of $STAT6^{-/-}/MHC\ II^{-/-} \rightarrow$ WT mice not treated with IL-4 (Fig. 4C). This demonstrates the indispensable role for APC in the regulatory anti-inflammatory therapeutic effect of IL-4 in DTHR. Fig. 4D presents schematically the experimental approach for the generation of the bone marrow chimeric mice.

IL-4-mediated suppression of IL-23 is partly mediated through ATF3

ATF3 is a repressor of *il6*, but also *tnf* and *il23b* transcription in TLR4-stimulated macrophages (35, 36). ATF3 blocks *il23b* transcription by binding to repressive promotor elements near the genes coding for the *il23b* subunit in macrophages and possibly other APC, such as DC (35, 37). Because IL-4 significantly suppresses *il23b* transcription (Fig. 1C), we assessed whether the effects of IL-4 could be at least partially mediated through ATF3. Indeed, IL-4 markedly upregulated ATF3 mRNA expression and protein production in murine and human dendritic cells, and

in murine RAW264.7 cells (Fig- 5A to D). To determine the functional relevance of ATF3 on IL-23 production, we stimulated DC from either wt or ATF3$^{-/-}$ mice with LPS, and assessed mRNA expression and production of IL-23. Even in the absence of IL-4, ATF3-deficient DC produced higher amounts of IL-23 than wt-DC, and IL-4 significantly further suppressed transcription and production of IL-23 only in the ATF3-competent, but not in the ATF3-deficient DC (Fig. 5E). In line with this, IL-4 significantly reduced the TNCB-mediated DTHR in wt mice but not in ATF3$^{-/-}$ mice (Fig. 5F).

IL-4 therapy of psoriasis abrogates intralesional IL-23 and IL-17 in human skin

IL-4 suppresses IL-23 production in mouse and human DC, and abrogates their capacity to induce/maintain T_H17 responses. Moreover, rmIL-4 suppresses DTHR by suppressing IL-23 and downstream IL-17 during contact hypersensitivity in mice. We therefore asked whether this mode of immune suppression also translates to human autoimmune diseases, namely psoriasis, which is a disease that is strongly improved by IL-4 therapy or the mAb-mediated blockade of either IL-17 or IL-23 (38-40). To this end, we studied a unique population of psoriasis patients who had successfully been treated with increasing doses of systemically applied IL-4. Consistent with recent data (41, 42), *il23a* and *il17a* mRNA were both increased in psoriasis skin lesions (Fig. S5A). Confocal laser scanning microscopy co-localized the abundant IL-23 protein with HLA-DR-expressing APC, and the IL-17 protein with CD3$^+$ T cells (Fig. S5B and fig. S6A and B) in psoriasis plaques, but not in healthy skin (Fig. S7 and fig. S8). In addition to T_H1 and T_H17 cells, the psoriasis plaques contained numerous polymorphonuclear cells and, thus, share many similarities with the TNCB-induced DTHR. Because IL-4 therapy strongly improves psoriasis without suppressing IFN-γ-expressing T cells in the peripheral blood (20), we asked whether IL-4 therapy might improve psoriasis by suppressing IL-23- and IL-17, driving the T_H17-response. We examined the effect of IL-4 therapy on the expression and production of IL-17/IL-23 in a cohort of 22 psoriasis patients (i.e. 19 patients, 3 drop-outs). The study was designed as a dose-escalation study, where patients were treated for psoriasis systemically with increasing doses of IL-4 over 6 weeks. The therapy was initiated with either 0.05, 0.1, 0.2, 0.3 or 0.5 μg/kg of IL-4, and increased to the next level after three weeks, except in the last group (20). Systemic IL-4 therapy significantly improved psoriasis in a dose-dependent manner and normalized the skin morphology (20) (see also Fig. 6A). Cryopreserved tissue sections of these study patients revealed that untreated psoriasis plaques contained abundant IL-23 that co-localized with HLA-DR-expressing APC and abundant IL-17 protein that co-localized with CD3$^+$ T cells (Fig. 6B and fig. S9A). After 6 weeks of IL-4 therapy, both IL-23 and IL-17 protein were almost undetectable (Fig. 6C and fig. S9B), suggesting that IL-4 therapy suppressed IL-23 and IL-17 production also in human skin. We further analyzed the tissue samples from the study patients for the expression of *il17a*, atf3, *il23a* or *il12a* mRNA. The dose-escalation design of the study allowed us to correlate local mRNA changes for each of the three cytokines (i.e. *IL17a*, *IL23a* and *IL12a*) and of the transcription factor ATF3 with the IL-4 treatment dose. IL-4 therapy suppressed *il23a* mRNA expression in a dose-dependent manner, with 20% suppression at 0.05 μg/kg IL-4 and almost 90% suppression at 0.5 μg/kg of IL-4 (Fig. 6D). Similarly, we detected a dose-dependent upregulation of ATF3 expression in the analyzed tissue (Figure 6E). Consistent with the *il23a* mRNA suppression, IL-4 therapy dose-dependently suppressed *il17a* mRNA expression (Fig. 6F). As predicted by the *in vitro* and animal data shown above, IL-4 therapy increased *il12a* mRNA expression in human skin during the 6 weeks of IL-4 therapy (Fig. 6G). Finally, at low concentrations, IL-4 induced an IL23A/IL12A ratio of >1 (1.7 at 0.05 μg/kg IL-4), but at high IL-4 concentrations, IL-4 therapy induced a very low IL23A/IL12A ratio (0.05 at 0.5 μg/kg IL-4), a finding that could be important for the design of future IL-4 treatment regimens in humans (Figure 6H).

Discussion

IL-4 reverts both T_H1 and T_H17 cell-mediated pathology and that this effect is associated with the induction of IL-4-producing T_H2 cells in mice and humans (5, 16-20). The underlying mechanism was attributed to inhibition and replacement of pathologic T_H1 and T_H17 cells and their respective cytokines by T_H2 cells and IL-4. However, this concept fails to completely explain the therapeutic effects observed, because IL-4 exerts opposing regulatory effects on T cells and DC; IL-4 abrogates IFN-γ induction upon direct interaction with T cells (43-45). In contrast, IL-4 promotes IL-12 production by DC, thus indirectly promoting IFN-γ production in mice and humans (46-48). Importantly, these effects are not exclusive; IL-4 and IL-4-producing T_H2 cells efficiently improve established T_H1/T_H17 mediated inflammation in mice and humans while enhancing both IL-12 and IFN-γ (19, 20). These phenomena are highly suggestive of a regulatory mechanism whereby IL-4 selectively prevents T_H17 immunity, while sparing IL-12/T_H1 immunity.

We addressed this question by analyzing in detail the effect of IL-4 on the regulation of IL-23 and T_H17 cells. Starting with human DC subsets, we found that IL-4 had exactly opposing effects on IL-12 and IL-23. While IL-4 induced IL-12, it abolished the induction of IL-23 and abrogated the capacity of DC to maintain T_H17 but not T_H1 cells. Our observations are in line with reports suggesting different roles of IL-4 on DC-derived IL-12 and IL-23 (49-52), but go far beyond the former studies. We confirmed the biological relevance of this regulation in an *in vivo* experimental setting and demonstrated that IL-4 therapy could abrogate cutaneous inflammation in the elicitation phase of DTHR. In extensive bone marrow reconstitution experiments we elucidated the effects of IL-4 on the different immune cells and could demonstrate the selective mode of action of IL-4 on APC. This is important because our data show for the first time that antigen presenting innate immune cells are indispensable for the immunosuppressive effect of IL-4 therapy. Activation of APC in an IL-4-deprived or IL-4-dominated inflammatory milieu dictated their capacity to orchestrate T_H17 induction, which supports previous data suggesting that the amount of IL-4 ultimately determines whether immune responses promote or attenuate inflammatory autoimmune diseases (46, 53).

Psoriasis is characterized by the absence of IL-4, and both T_H1 and T_H17 cells prevail in the skin (11). However, the exact roles of either T_H1 or T_H17 cells remain to be defined. Our data have identified a sequence of immunological events, triggered by IL-4 that selectively impaired the IL-23/IL-17 axis and relieved T_H17-mediated pathology, while promoting IL-12 and T_H1 cytokines. This is relevant since IL-23, but not IL-12, also mediates inflammation in the absence of T cells (15), at least under experimental conditions, and via intracutaneous injection induces a psoriasis-like skin disease in mice (43). Our data further showed that IL-4 indirectly prevented the maintenance of IL-17-producing T_H17 cells by abrogating the expression and production of the T_H17 cell-associated cytokine IL-23 in APC. Together with our data, the high degree of efficacy of the anti-IL-12/IL-23p40 monoclonal antibody in the treatment of psoriasis further emphasizes the crucial role for IL-23 in disease progression (54, 55). These findings do not exclude a role for IL-12, T_H1 cells or T_H1 cytokines in psoriasis but rather confirm that the therapeutic silencing of IL-23 (for example by IL-4 or newly engineered IL-4 superkines, currently under investigation) (56) is promising for psoriasis and other T_H17/IL-23-associated autoimmune diseases.

Materials and Methods

Reconstitution experiments.

Tcrb$^{-/-}$*Tcrd*$^{-/-}$ (Tc$^{-/-}$) mice, STAT6$^{-/-}$ mice and CD45.2^{+}C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine 04609 USA). MHCII$^{-/-}$ mice were a gift from Ludger Klein, Institute of Immunology, Ludwig Maximillian University, Munich, Germany. Recipient mice were lethally irradiated at 7.0 Gy and bone marrow cells (10^6 cells per recipient) of donor mice were intravenously injected into recipient mice. Donor hematopoietic cells were either bone marrow cells from CD45.1^{+} mice, a 1:1 mixture of bone marrow cells from STAT6$^{-/-}$ and Tc$^{-/-}$ mice, or a 1:1 mixture of STAT6$^{-/-}$ and MHCII$^{-/-}$ mice. To confirm the chimerism of mice, flow cytometry was made for analysis of CD45.2^{+} (recipient mice) and CD45.1^{+} (donor mice). TNCB sensitization experiments were performed eight weeks after irradiation. A detailed description of all experimental procedures and the statistical analysis is given in SI Materials and Methods.

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Staphylococcus aureus-derived lipoteichoic acid induces temporary T cell paralysis independent of TLR2

Short title: LTA induces T cell paralysis

Ko-Ming Chen[1], Yuliya Skabytska[1], Susanne Kaesler[1], Thomas Volz[1,2], Martin Köberle[1,2], Florian Wölbing[1], Ulrike Hein[1], Carsten Kirschning?, Martin Röcken[1], Tilo Biedermann[1,2]

[1] Department of Dermatology, Eberhard Karls University, Liebermeisterstr. 25, 72076 Tübingen, Germany

[2]Department of Dermatology and Allergy, Technische Universität München, Biedersteinerstr. 29, 80802, Munich, Germany

Corresponding author:

Prof. Dr. med. Tilo Biedermann

Biedersteinerstr. 29, 80802 Munich

Tel.: +49 89-4140-3170 Fax.: +49 89-4140-3171

E-Mail-Address: Tilo.Biedermann@tum.de

Abbreviations

Abs, antibodies; AD, atopic dermatitis; FITC, fluorescein isothiocyanate; IL, Interleukin; Ig, immunoglobulin; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; LTA, lipoteichoic acid; LNs, lymph nodes; OVA, ovalbumin; PRR, pattern recognition receptor; PAMP, pathogen associated molecular pattern; *Staphylococcus aureus, S. aureus*; TLR, Toll-like receptor; TGF-β, transforming growth factor-β

Abstract

The interplay between microbes and surface organs such as skin shapes a complex immune system with several checks and balances. The first line defense is mediated by innate immune pathways leading to inflammation. The second phase consists of specific T cells invading the infected organ amplifying inflammation and defense. Consecutively, termination of inflammation is crucial to avoid chronic inflammation triggered by microbes such as in atopic dermatitis. Here we aimed to elucidate how *Staphylococcus*-derived cell wall component lipoteichoic acid (LTA) governs the second phase of immune responses when high levels of LTA on a disrupted skin barrier allow T cells direct exposure to LTA. Surprisingly we found that LTA potently suppressed T lymphocyte activation in a TLR independent manner. LTA-exposed T cells failed to proliferate and to produce cytokines. Importantly, these T cells remained completely viable and were susceptible to consecutive activation signals in the absence of LTA. Thus, LTA exposure of T cells resulted in temporary functional T cell paralysis. *In vivo* experiments revealed that T cell cytokine production and cutaneous recall responses were significantly suppressed by LTA. Thus, we identified a new mechanism of how bacterial compounds temporarily and directly modulate adaptive immune responses.

Introduction

Surface organs such as the skin developed under the pressure of constant exposure to microbes and microbial products. As a consequence, a highly complex and regulated immune system evolved conserving the integrity of both the surface organ as well as of the whole organism.

Cutaneous infection, on the one hand, was shown to very effectively induce adaptive immune responses even allowing the first vaccination strategy to successfully eradicate vaccinia virus (Tian *et al.*, 2009). On the other hand, chronic inflammatory diseases of the gut such as Crohn's disease or of the skin such as atopic dermatitis (AD) are believed to be triggered by microbes (Bieber, 2008; Kaesler *et al.*, 2014; Lipinski and Rosenstiel, 2013) and to be mediated by T cells, which migrate into the gut or skin to initiate and maintain the inflammatory process (Akdis *et al.*, 2006; Boguniewicz *et al.*, 2003; Kapp *et al.*, 2002; Mosli *et al.*, 2014). Interestingly, clinically unaffected skin of AD patients already contains a sparse perivascular T cell infiltrate in the absence of detectable signs of dermatitis (Leung *et al.*, 1983; Mihm *et al.*, 1976). In addition, analyses of biopsy samples from clinically unaffected skin of AD patients, as compared with normal non-atopic skin, demonstrate an increased number of type 2 helper T cells (Th2) expressing IL-4 and IL-13 mRNA (Hamid *et al.*, 1994).

S. aureus most commonly elicits skin, wound, and also systemic infections being a major pathogen in both community-acquired and nosocomial infections (Fournier and Philpott, 2005; Myles and Datta, 2012). These and even minor infections lead to specific elicitation of *S. aureus*-specific adaptive immune responses in most people inducing also long-lived immune memory (Broker *et al.*, 2014). More than 90% of patients with AD show *Staphylococcus aureus* colonization or infection, *S. aureus*

and S. aureus-derived substances were shown to trigger skin inflammation, and effective treatment protocols for these patients include antiseptics (Leung and Bieber, 2003). The presence of microbes on the skin may elicit or increase an immune response and constituents of *S. aureus* may function as a pro-inflammatory adjuvant. Pathogen-associated molecular patterns (PAMPs) may activate resident skin cells such as dendritic cells of the skin (Nakamura *et al.*, 2013; Volz *et al.*, 2012). Such PAMPs have been identified for *S. aureus*. Lipoteichoic acid (LTA), lipoprotein, and peptidoglycan (PG) are part of the bacterial cell wall of *S. aureus* and bind Toll-like receptor (TLR) 2, which is expressed by several immune cells also within the skin (Akira *et al.*, 2006; Biedermann, 2006; Lipinski and Rosenstiel, 2013; Tian *et al.*, 2009).

Recent researches showed that *S. aureus* found in infected dermatitis lesions leads to the presence of LTA in the majority of specimens with levels of LTA positively correlating with the concentration of *S. aureus* (Travers *et al.*, 2010; Zhang *et al.*, 2005). We recently showed in a mouse model of AD that TLR2 ligands potently exacerbate inflammation leading to chronic dermatitis (Kaesler *et al.*, 2014). These findings are in line with data from other models of AD, in which the development of skin lesions often depends on conventional housing conditions for the animals. Animals fail to develop dermatitis lesions when kept in specific pathogen free (SPF) conditions also indicating a role for microbes and PAMPs for AD (Akdis *et al.*, 2006). On the other hand, we showed that cutaneous microbes were able to ameliorate AD inflammation by inducing regulatory T cells and IL-10 (Volz *et al.*, 2013). Moreover, our group discovered recently that activation of TLR2/6 on the skin led to immune suppression mediated by myeloid-derived suppressor cells (MDSCs) (Skabytska et al.). Thus, different mechanisms to terminate and limit inflammation can be

developed to avoid tissue damage or as immune escape of microorganisms. Interestingly, among TLR2 ligands, exacerbation and persistence of the early phase of cutaneous inflammation was most potently induced by lipopeptides such as Pam2Cys with LTA showing significant but much weaker effects on skin inflammation (Kaesler *et al.*, 2014). LTA is a surface-associated adhesion macroamphiphile molecule of Gram-positive bacteria. It is released from the bacterial cells after bacteriolysis and as a consequence of bacterial cell wall turn over (Ginsburg, 2002). The physiochemical properties of LTA were postulated to be similar to those of lipopolysaccharide (LPS) in Gram-negative bacteria (Fournier and Philpott, 2005). Most prominently, concentrations of about 10 µg/ml LTA from *S. aureus* and more were shown to stimulate the production of multiple pro-inflammatory cytokines and chemokines in different leukocytes, especially in macrophages and monocytes (Cleveland *et al.*, 1996; Kapp *et al.*, 2002; Standiford *et al.*, 1994; von Aulock *et al.*, 2003). Peptidoglycan from *S. aureus* has been shown to provoke secretion of pro-inflammatory cytokines and chemoattractants (TNF-α, IL-1β, IL-6, and CXCL8) from monocytes, macrophages as well as dendritic cells (Heumann *et al.*, 1994; Timmerman *et al.*, 1993; Volz *et al.*, 2010). Thus, it is clear that LTA can activate the innate immune system, but direct effects on the adaptive immune system were not studied in detail. The purpose of this research was to study the properties of Gram-positive cell wall component LTA in regard to T lymphocyte function.

In this study, we show that limited exposure to LTA during initial contacts to fluorescein isothiocyanate (FITC) significantly suppressed lesional T cell cytokine expression. The proliferation of T cells from the draining lymph nodes of LTA-exposed skin was also significantly reduced. Moreover, LTA also significantly suppressed T cell proliferation *in vitro*, which was independent of TLR2 signaling.

Those LTA-exposed T cells were viable and LTA exposure did not induce T cell apoptosis. To investigate whether T cell suppression by LTA is a pathway with *in vivo* relevance, FITC contact hypersensitivity (CHS) and ovalbumin (OVA)-induced dermatitis as models for Th2-mediated cutaneous inflammation in AD patients were investigated. LTA potently suppressed both FITC CHS and OVA-induced dermatitis independent of TLR2. To summarize, T cell-mediated immune responses are susceptible to LTA-induced immune suppression. These newly discovered consequences of LTA exposure may be functional following effective immune defense of *S. aureus* allowing the termination of inflammation or represent another way of immune escape of *S. aureus*. Importantly, this LTA induced T cell paralysis is temporary in nature avoiding prolonged periods of immune suppression.

Results

LTA exposure during mild cutaneous inflammation suppressed T cell cytokine levels and T cell proliferation

Chronic inflammatory diseases of the skin such as AD are believed to be triggered by microbes. We wondered whether TLR2 ligands are capable to transform non-lesional skin with a detectable but sparse T cell infiltrate as found in AD patients (Leung *et al.*, 1983; Mihm *et al.*, 1976) into overt dermatitis. Contact hypersensitivity to the hapten FITC following up to six repetitive sensitizations with FITC is associated with FITC-specific immunoglobulin (Ig) E and Th2 cells (Supplementary Figure S1). We now established a model, in which the weak hapten FITC was applied only twice to the shaved abdomen of mice before a FITC challenge at the ear skin was carried out. In this model, no obvious ear swelling was detectable following the last FITC challenge

147 (Supplementary Figure 2). However, T cell recruitment to the skin can be identified
148 with T cell cytokines significantly upregulated already 4-8 hours after challenge (data
149 not shown). Moreover, FITC-specific T cells were readily detectable in these mice.
150 Thus, this model of mild T cell inflammation is ideal to identify microbial constituents
151 that trigger and therefore amplify cutaneous inflammation. However and much to our
152 surprise, exposure to up to 40 µg LTA could not significantly amplify ear swelling in
153 mildly FITC-sensitized animals. Therefore we investigated the dynamics of T cell
154 cytokine expression in animals exposed to either LTA or PBS (Figure 1a). At 8 hours
155 after challenge, cutaneous IL-4 mRNA expression was suppressed by >10 fold
156 following exposure to LTA, IFN-γ mRNA levels were reduced by a factor of 3, and IL-
157 10 expression was unchanged compared to controls (Figure 1b). To identify
158 functional consequences of LTA exposure *in vivo*, draining lymph nodes were
159 prepared at several time points following FITC +/- LTA exposure and subjected to
160 further analyses. *Ex vivo* stimulation of T cells with anti-CD3 and anti-CD28
161 antibodies (Abs) as well as with FITC demonstrated significantly reduced T cell
162 proliferation (Figure 1c) indicating consequences of LTA exposure on both FITC-
163 specific T cells and T cell bystanders.

164

165 **LTA directly suppressed CD4$^+$ T cell proliferation and cytokine production *in***
166 ***vitro***

167 In order to identify whether LTA mediated immune suppression by directly targeting T
168 cells, CD4$^+$ T cells were isolated from untreated BALB/c mice and activated by anti-
169 CD3/anti-CD28 Abs *in vitro*. These T cells were then treated with TLR2 ligands
170 Pam2Cys, staphylococcal LTA, or control medium. Pam2Cys dose dependently
171 acted as potent T cell co-stimulator reaching a maximum at 10 µg/ml confirming data

previously published (Okusawa *et al.*, 2004) (Figure 2a). In contrast, at the same concentration of 10 µg/ml LTA significantly suppressed T cell proliferation (Figure 2a). Moreover, increasing LTA concentrations further increased T cell suppression, almost completely abrogating T cell proliferation at 100 µg/ml (Figure 2a). As these experiments were performed with murine T cells, we next investigated human T cells. Human $CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) and then stimulated under several conditions including different concentrations of staphylococcal LTA for 3 days. LTA most potently inhibited human T cell activation elicited by TCR activation as with anti-CD3 and anti-CD28 Abs and superantigen (Figure 2b). Interestingly, LTA also blocked human T cell activation induced by mitogens albeit with less potency (Figure 2b). Importantly, LTA not only affected T cell proliferation but most potently also inhibited T cell cytokine production, such as IFN-γ and IL-4 in murine (Figure 2c) and human T cells (data not shown).

These data indicate that *S. aureus*-derived LTA can potently block T cell activation and thereby represent one mechanism of immune evasion. However, skin microbiota such as *S. epidermidis* that is tolerated by the host in most circumstances also contains large quantities of LTA. Therefore, for comparison, LTA was isolated from both pathogenic *S. aureus* and non-pathogenic *S. epidermidis* and analyzed in regard to T cell suppression. LTA from both, *S. aureus* and *S. epidermidis* potently suppressed T cell activation indicating a general effect of this class of PAMPs (Figure 2d).

Paralysis but not apoptosis or necrosis in LTA exposed Th cells

One possible explanation for LTA-mediated reduction of Th cell cytokine production or proliferation could be apoptosis of T cells following LTA exposure. To this end, Th cells were stained with Annexin V and propidium iodide (PI) following exposure to LTA. Surprisingly, LTA exposed Th cells exhibited even less Annexin V staining and PI uptake (Figure 3a) and no increase in trypan blue staining (data not shown) compared to control T cells. Thus, these Th cells seemed to be completely viable following LTA exposure. To further demonstrate Th cell viability, $CD4^+$ T cells were activated *in vitro* by anti-CD3/anti-CD28 and exposed to LTA for 4 days. Thereafter Th cells were cultured with low concentration of IL-2 until resting and then stimulated in the absence of bacterial constituents. In contrast to the primary stimulation, Th cells, previously exposed to LTA, readily responded to anti-CD3/anti-CD28 Abs treatment. These data demonstrate that these Th cells completely recovered from the exposure to LTA (Figure 3b). Thus, Th cells exposed to LTA were viable and remained fully responsive to subsequent stimulation. This indicated that LTA-mediated suppression could be only temporary reflecting a status of transient Th cell paralysis.

LTA-induced suppression of Th cell activation independent of TLR2 and MyD88

Next, we analyzed whether LTA functions only during incoming TCR signals as it is expected for TLR2 ligands acting as T cell costimulators. Therefore, Th cells were activated by anti-CD3/anti-CD28 Abs and LTA or Pam2Cys were added subsequently up to 3 days following activation. As expected, the costimulatory role of Pam2Cys was only functional in parallel to T cell TCR activation (Figure 4a). In sharp contrast, LTA was functional up to 48 hours following Th cells activation indicating a mechanism of action independent of TCR signaling (Figure 4a). Consequently, we

assumed that LTA is functional via structures different from TLRs. To this end, wild type, TLR2$^{-/-}$, and MyD88$^{-/-}$ CD4^{+} T cells were isolated from untreated mice and analyzed. LTA suppressed activation of Th cells from all three strains, whereas costimulation by Pam2Cys was dependent on TLR2 and MyD88 (Figure 4b).

LTA induced cell cycle arrest in activated T cells

Transforming growth factor-β (TGF-β) inhibits T cell proliferation (Kehrl *et al.*, 1986) through mechanisms directly targeting cell cycle regulators (Datto *et al.*, 1995; Hannon and Beach, 1994; Polyak *et al.*, 1994). The normal cell cycle is composed of the gap 1 phase (G1), the synthesis phase (S), the gap 2 phase (G2), and the mitosis phase (M). In the S phase, DNA replication occurs and cells at G2 and M phases of the cell cycle have double the DNA content of those at G0 and G1 phases. DNA content of cells at S phase is between that of cells in G2/M and G0/G1 phases. To better understand the underlying mechanisms of LTA mediated Th cell suppression, consequences of LTA exposure on cell cycle regulation were investigated. In fact, only 6% of LTA-treated CD4^{+} T cells were found at the S or G2/M phases (Figure 5a), whereas 25% of the control cells were. In contrast, Pam2Cys-stimulated T cells were prone to divide. As control, we also treated Th cells with TGF-β and most of these TGF-β-treated Th cells also remained at G1 phase as previously published (Kehrl *et al.*, 1986) (Figure 5b). Thus, these data further consolidated the suppressive effect of LTA exposure on Th cells and provided an explanation why this suppressive effect of LTA i) was also functional when adding LTA at later time points, ii) was functional in the absence of apoptosis and necrosis, and iii) was transient allowing normal activation of T cells following a period of resting.

LTA suppressed T cell-mediated FITC-induced inflammation and OVA-dermatitis *in vivo*

To investigate whether LTA-mediated suppression of T cells is also functionally relevant *in vivo*, we next analyzed models of T cell-mediated cutaneous inflammation. To this end, we adoptively transferred $CD3^+$ T cells from FITC-sensitized mice into mice treated as indicated in Figure 1a to increase FITC-specific T cells. The next day, recipients were challenged with FITC or vehicle control and exposed to either LTA or PBS in addition. Interestingly, when challenged with vehicle only, in the absence of antigen, LTA elicited an ear swelling response indicating direct pro-inflammatory effects of LTA, however, in the presence of antigen and T cell activation, LTA significantly reduced cutaneous inflammation (Figure 6a), confirming our data on inhibition of T cell activation *in vivo*. These data indicate that LTA may function as a TLR2 ligand on skin resident cells activating the innate immune system and leading to inflammation. In contrast, T cell-dependent contact hypersensitivity is significantly suppressed, possibly by a TLR2 independent effect. However, pro-inflammatory and immune suppressive consequences of LTA exposure may partly compensate for each other in this model. To be able to dissect TLR2-dependent from TLR2-independent effects of LTA and innate from T cell-mediated immune consequences, we used another AD-like mouse model as recently published (Kaesler *et al.*, 2014). In this model, we adoptively transferred OVA-specific Th2 cells and antigen to the ear skin of previously untreated wild type and $TLR2^{-/-}$ mice and monitored the T cell driven ear swelling response in the presence or absence LTA. Interestingly and confirming our hypothesis, in the absence of TLR2 LTA profoundly suppressed OVA-specific dermatitis (Figure 6b). These data elucidate how the host immune system

fine tunes the response to microbial PAMPs. The early innate response is mediated by pathogen recognition receptors and results in inflammation, whereas the later and T cell-mediated immune response is susceptible to immune suppression allowing the termination of inflammation. Importantly, the latter is transient in nature avoiding prolonged periods of immune suppression.

Discussion

The concentrations of LTA encountered by host cells during bacterial infections are likely to be quite high at local sites of infection. For example, the 50% lethal dose of live *S. aureus* in an intraperitoneal infection model in mice is 10^9 CFU (Dziarski *et al.*, 2003), and this amount of *S. aureus* contains about 200 µg of LTA in the peritoneal cavity fluid that is <0.1 ml. While LPS in the ng/ml range is sufficient to trigger even severe inflammatory responses (Hoetzenecker *et al.*, 2011; Kusunoki *et al.*, 1995), it has been shown that relatively large amounts of LTA (about 1~10 µg/ml) are required to elicit cellular responses *in vitro* (Fournier and Philpott, 2005). However, LTA is a major component of Gram-positive bacterial cell wall, whereas bacterial cell walls of Gram-negative harbor much less LPS. The active concentrations of LTA (10 µg or 10^7 to 10^8 CFU) as well as of LPS (20 ng or 10^7 CFU) are comparable when they are transposed to bacterial cell equivalents (von Aulock *et al.*, 2003).

LTA from *Staphylococcus aureus* is a potent stimulus for neutrophil recruitment through stimulating the production of cytokines and chemokines in macrophages and monocytes (Standiford *et al.*, 1994; von Aulock *et al.*, 2003). Following the induction of innate immune signals activation of the adaptive immunity is functional (Medzhitov, 2007). Previous studies showed that LTA merely played the pro-inflammatory role on

293 the host immune system, especially on the innate immunity, and thus the notion that
294 LTA was the ‘’LPS’’ of Gram-positive bacteria was proposed. However, as a result of
295 thousands of years of coexistence with human, *S. aureus* is a well adapted pathogen
296 that has developed several evasion mechanisms. This organism secretes proteins
297 that inhibit complement activation and neutrophil chemotaxis or that lyse neutrophils,
298 neutralizes antimicrobial defensin peptides, and its cell surface is modified to reduce
299 effectiveness of immune responses (Lowy, 2011). *S. aureus* can survive in
300 phagosomes, express polysaccharides and proteins that inhibit opsonization by
301 antibody and complement, and its cell wall is resistant to lysozyme. Moreover, *S.*
302 *aureus* expresses several types of superantigen, which disturb the normal humoral
303 immune response, resulting also in anergy, apoptosis, and immunosuppression
304 (Foster, 2005; Kaesler *et al.*, 2012; Rooijakkers *et al.*, 2006). It has also been
305 reported that bacterial components from *S. aureus* inhibited fibroblast proliferation *in*
306 *vitro* (Edds *et al.*, 2000). In addition, LTA isolated from *S. aureus* was demonstrated
307 to inhibit platelet function and platelet-monocyte aggregation (Sheu *et al.*, 2000). Not
308 only pathogens like *S. aureus*, but also parts of the normal microbiome like *S.*
309 *epidermidis* contain LTA. Furthermore, *S. aureus* sometimes lives as a commensal of
310 the human skin. Recently, it was shown that a unique LTA produced by *S.*
311 *epidermidis* inhibits uncontrolled skin inflammation during skin injury (Lai *et al.*, 2009).
312 After skin injury, the host RNA from damaged cells activates TLR3 in the
313 keratinocytes, which accounts for the release of inflammatory cytokines, resulting in
314 inflammation. Staphylococcal LTA inhibits both inflammatory cytokine release from
315 keratinocytes and inflammation triggered by injury through a TLR2-dependent
316 mechanism (Lai *et al.*, 2010). However, how the known pro-inflammatory
317 macroamphiphile molecule works directly on adaptive immune cells was still

318 unknown. Functional TLR2 expression on T cells were demonstrated by other groups
319 (Komai-Koma *et al.*, 2004; Reynolds *et al.*, 2010), and *Bacteroides fragilis* signals
320 through TLR2 on Foxp3^{+} regulatory T cells promoting immunologic tolerance (Round
321 *et al.*, 2011). It is therefore reasonable to postulate that LTA can exert its modulatory
322 effect directly on adaptive immunity.

323 In our current study, we demonstrate that the application of *Staphylococcus aureus*
324 derived cell wall component LTA most potently suppressed T lymphocyte activation,
325 the late phase of defense. This suppression was independent of TLR signaling and
326 functional by means of transient cell cycle arrest. Thus, we identified a novel
327 mechanism of how bacterial compounds temporarily directly modulate the adaptive
328 immune system. This new mechanism of T cell paralysis may be functional to
329 terminate inflammation such as in Gram-positive infection or during immune evasion
330 of pathogenic bacteria representing another level of regulation of the complex
331 interplay between microbes and the host.

332

333 **Materials and Methods**

334 **Animals**

335 Pathogen-free, 6- to 12-week-old wild type BALB/c mice and C57BL/6 mice were
336 purchased from Charles River (Sulzfeld, Germany). MyD88$^{-/-}$ BL/6 were from Akira's
337 group (Osaka, Japan), TLR2$^{-/-}$ mice were from C. Kirschning (Institute of Medical
338 Microbiology, University Duisburg-Essen). All wild type and knockout mice were kept
339 and bred under specific pathogen free conditions in accordance with the guidelines of
340 FELASA (Federation of European Laboratory Science Association) in the animal
341 facility of Eberhard Karls University. Age-matched female mice were used in all

experiments. All animal experiments were in compliance with both European Union and German law and approved by the local authorities (HT1/10).

Reagents

Fluorescein isothiocyanate (FITC), 2-mercaptoethanol, phorbol 12-myristate 13-acetate (PMA), ionomycin, concanavalin A (con A), and staphylococcal enterotoxin B (SEB) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Purified LTA from *Staphylococcus aureus* was obtained from InvivoGen (San Diego, USA). Purified LTA from *Staphylococcus epidermidis* was a gift from Porf. Hartung (Konstanz, Germany). Pam2Cys and Pam3Cys were from EMC microcollections (Tübingen, Germany). DMEM, RPMI 1640, penicillin/streptomycine, L-glutamine, sodium-pyruvate, MEM-amino acids (50X) and HEPES were from Biochrom (Berlin, Germany). Fetal calf serum (FCS) was from PAA (Cölbe, Germany). PBS (w/o Ca^{2+}, Mg^{2+}) were from Gibco/Invitrogen (Karlsruhe, Germany). ACK lysis buffer was from Cambrex (Walkersville, USA). Endotoxin-free ovalbumin was purchased from Hyglos (Bernried, Germany). Streptavidin-horseradish peroxidase and TMB-substrate solution were from BD Biosciences (Heidelberg, Germany).

FITC-induced antigen-specific contact hypersensitivity

6- to 12-week-old wild type female BALB/c mice (5 to 6 mice per group) were sensitized by administration of 0.5% FITC solution (dissolved in 1:1 acetone:dibutyl phthalate) on the shaved abdomen on days 0, 1. Six days after the last sensitization all mice were challenged by application of 0.5% FITC solution on both sides of the ears. Ear thickness was measured thereafter with a micrometer (Kroeplin, Germany)

and data are expressed as change in ear thickness compared to before treatment. Draining lymph node cells were cultured with FITC or anti-CD3 and anti-CD28 antibodies for another 3 days. Cell culture supernatants were collected and subjected to ELISA.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from mouse ears was isolated using the Macherey-Nagel isolation kit (Düren, Germany). Total RNA was reverse-transcribed to cDNA by using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). The oligonucleotide primer sequences were as follows: IFN-γ forward primer, 5'-CTCTGAGACAATGAACGCTAC-3', and IFN-γ reverse primer, 5'-TCTTCCACATCTATGCCACTT-3'; IL-4 forward primer, 5'-GACGGCACAGAGCTATTGATG-3', and IL-4 reverse primer, 5'-ACCTTGGAAGCCCTACAGACG-3'; IL-10 forward primer, 5'-CAACATACTGCTAACCGACTC-3', and IL-10 reverse primer, 5'-CATTCATGGCCTTGTAGACAC-3'. Real-time PCR assay was carried out with iCycler (Bio-Rad) by using iQ SYBR Green Supermix (Bio-Rad). Data are presented as normalized to housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT).

Proliferation assays

Whole lymph node cells or $CD4^+$ T cells (250000 cells/well) were activated with different antigens/antibodies in 96 well flat bottom plates (Greiner, Frickenhausen, Germany) in a total volume of 200 µl. After 3-4 days 0.25µCi [^{3}H] thymidine (GE

390 Healthcare, Freiburg, Germany) was added per well and cells were harvested after
391 another 16 hours. Incorporated [^{3}H] thymidine was measured using a microbeta
392 counter (Perkin Elmer, Wiesbaden, Germany).

393

394 **CD4$^+$ T cell isolation and stimulation for *in vitro* experiments**

395 Lymph nodes and spleens of untreated mice were isolated and lymphocytes were
396 prepared by homogenizing organs through a nylon mesh cell strainer. Red blood
397 cells were lyzed by incubating with ACK lysis buffer for 5 minutes. Cells were washed
398 twice and CD4$^+$ T cells were purified using a CD4$^+$ isolation kit from Miltenyi Biotech
399 (Bergisch Gladbach, Germany). Human CD4$^+$ cells were isolated from peripheral
400 blood mononuclear cells, which were obtained from heparinized blood samples from
401 healthy volunteers by density gradient centrifugation (800 g for 30 min). CD4$^+$ T cells
402 were resuspended in DMEM containing 10% heat-inactivated FCS, penicillin (100
403 U/mL), streptomycine (100 µg/mL), 0,5 mM sodium-pyruvate, 5 mM HEPES, 1%
404 MEM-amino acids (50X) and 2-mercaptoethanol (50 µM). T cell were stimulated with
405 presence of 4 µg/ml (murine) or 0.5µg/ml (human) anti-CD3 and 2 µg/ml (murine) or
406 0.5µg/ml (human) anti-CD28 (Biolegend, San Diego, USA). For some assays
407 PMA/Iono (1 ng/ml; 500 ng/ml), Con A (2 µg/ml), SEB (4 µg/ml) or TGF-β (2ng/ml)
408 were added.

409

410 **Measurement of cytokines**

411 IFN-γ and IL-4 (BD Biosciences) levels in culture supernatants were assayed using
412 ELISA kits according to the manufacturer's instructions.

413

Apoptosis detection

Detection of apoptotic cells and necrotic cells was performed by using the Annexin V Apoptosis Detection Kit (BD Biosciences).

Cell cycle analysis

Cell cycle analyses were performed by using the APC BrdU Flow Kits (BD Biosciences) according to the manufacturer's instructions.

Adoptive transfer

FITC CHS model: Naïve mice serving as donors were sensitized by FITC on day -15, -14, -8, -7, -1, and 0, and on day 6, axillary lymph nodes (LNs), inguinal LNs, and spleens of these mice were isolated. $CD3^+$ cells were then isolated from secondary lymphoid organs with Pan T Cell Isolation Kit (Miltenyi Biotec) and transferred into recipient mice, which were previously sensitized twice with FITC. The next day (day 7) recipients were injected with LTA or PBS and challenged with FITC or vehicle control.

OVA-dermatitis model: OVA-specific T cells were harvested from OVA-sensitized mice and expanded *in vitro* under Th2-promoting conditions. For adoptive transfer $1x10^6$ OVA-specific Th2 cells and 5 µg OVA protein with or without 10 µg *S. aureus* LTA were intracutaneously injected into the ear skin of wild type and $TLR2^{-/-}$ BL/6 recipient mice.

Ear thickness was measured with a micrometer and expressed as change in ear thickness following treatment.

Statistical analysis

All experiments were performed at least twice. The data shown are means ± standard deviations. Statistical analyses were performed with student t-tests (two-tailed) using Microsoft Excel. Differences were considered to be statistically significant when the p value was less than 0.05.

Conflict of interest

The authors state no conflict of interest.

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641

642

643

644 **Figure legends**

645 **Figure 1. Effects of cutaneous LTA exposure on mild cutaneous inflammation.**

646 (a) Mice were sensitized with 80 µl 0.5% FITC onto the shaved abdomen at day 0
647 and1, and on day 7 mice were challenged by applying FITC onto the ears. Shortly
648 prior to challenge, LTA or PBS (control) was applied intracutaneously. (b) 8 hours
649 after FITC challenge cytokine mRNA expression was compared between LTA-
650 exposed and PBS-exposed skin by quantitative RT-PCR (normalized to a
651 housekeeping gene HPRT, (mean +/- SD of triplicates???). (c) Ear skin draining
652 lymph nodes were isolated at several different time points after challenge. Cells were
653 either stimulated *ex vivo* by FITC or anti-CD3/anti-CD28 Abs for 3 days and their
654 proliferation was analyzed as counts per minute (cpm) of ^{3}H-thymidine incorporation
655 (mean +/- SD of triplicates), (**: $p<0.01$, ***: $p<0.001$).

656

657 **Figure 2. LTA exposure suppressed murine and human T cell proliferation and**
658 **cytokine production.**

659 (a, b) Proliferation of murine $CD4^+$ T cells isolated from untreated mice (a) or human
660 $CD4^+$ T cells isolated from PBMCs (b) was analyzed (mean +/- SD of triplicates) after
661 stimulation under several conditions: (a) anti-CD3/anti-CD28 Abs and different
662 concentrations of Pam2Cys or LTA; (b) anti-CD3/anti-CD28 Abs, PMA/Iono, Con A,
663 SEB and different concentrations LTA. (c) T cell cytokines was analyzed 72 hours
664 following activation and exposure to LTA. (d) Murine $CD4^+$ T cells were stimulated by
665 anti-CD3/anti-CD28 Abs and *S. aureus* LTA, *S. epidermidis* LTA, or TGF-β were
666 added and the cell proliferation was analyzed (as cpm) (mean +/- SD of triplicates) (*:
667 $p<0.05$, **: $p<0.01$, ***: $p<0.001$, NS: $p>0.05$).

Figure 3. Unequivocal viability of LTA exposed and control T cells and restored responsiveness upon re-stimulation in the absence of LTA.

(a) $CD4^+$ T cells exposed to LTA were stained with Annexin V and PI and analyzed by flow cytometry. LTA-exposed T cells showed Annexin V- and PI-staining comparable to controls (mean +/- SD of triplicates). (b) $CD4^+$ T cells were primarily activated by anti-CD3/anti-CD28 Abs and exposed to LTA. These cells were then washed and rested with 10 U/ml IL-2. Secondary activation was done with anti-CD3/anti-CD28 Abs alone and their proliferation was analyzed (as cpm) (NS: $p>0.05$).

Figure 4. LTA-mediated suppression of $CD4^+$ T cells independent of TLR2 and MyD88.

(a) $CD4^+$ cells were polyclonally activated for 96 hours. LTA or Pam2Cys was added on indicated days. Pam2Cys acted as costimulator solely during the phase of T cell-activation. In contrast, LTA exposure suppressed T cell proliferation for up to two days following T cell activation. (b) $CD4^+$ T cells from wild type, $TLR2^{-/-}$, and $MyD88^{-/-}$ mice were polyclonally activated and incubated with different concentrations of staphylococcal LTA or Pam2Cys for 4 days. Cell proliferation was analyzed (as cpm) (mean +/- SD of triplicates). The proliferation of the control group of all three strains was normalized as 100% (*: $p<0.05$, **: $p<0.01$).

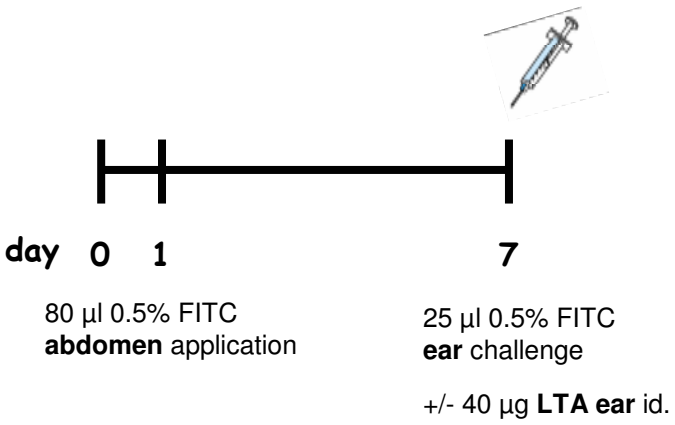
Figure 5. Consequences of LTA exposure for cell cycling of T cells.

691 $CD4^+$ T cells were stimulated *in vitro* by anti-CD3/anti-CD28 Abs and different
692 concentrations of Pam2Cys or staphylococcal LTA were added. Cell cycle analysis
693 was performed by DNA staining and the amount of DNA was determined by flow
694 cytometry. A representative FACS plot (a) and means +/- SD (*n*=3???) (b) is shown.

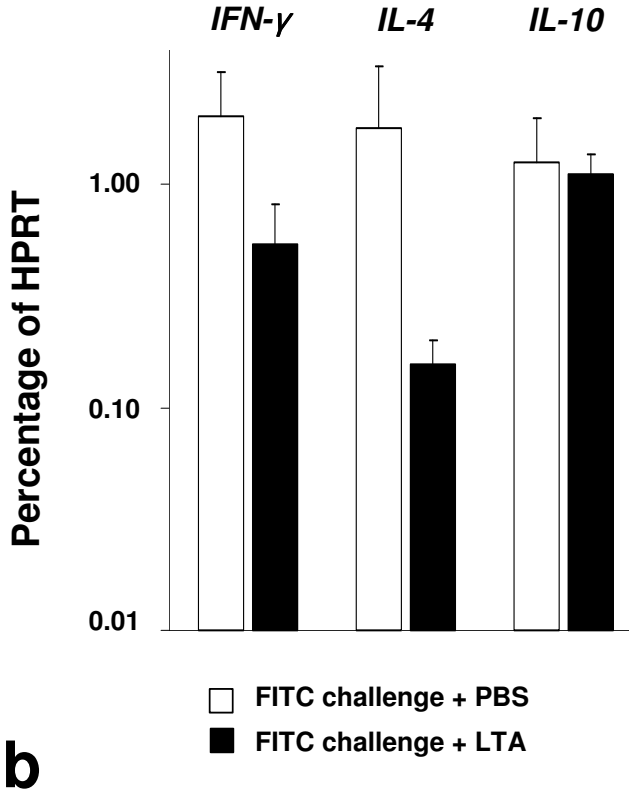
695

696 **Figure 6. LTA exposure suppressed contact hypersensitivity reactions *in vivo*.**

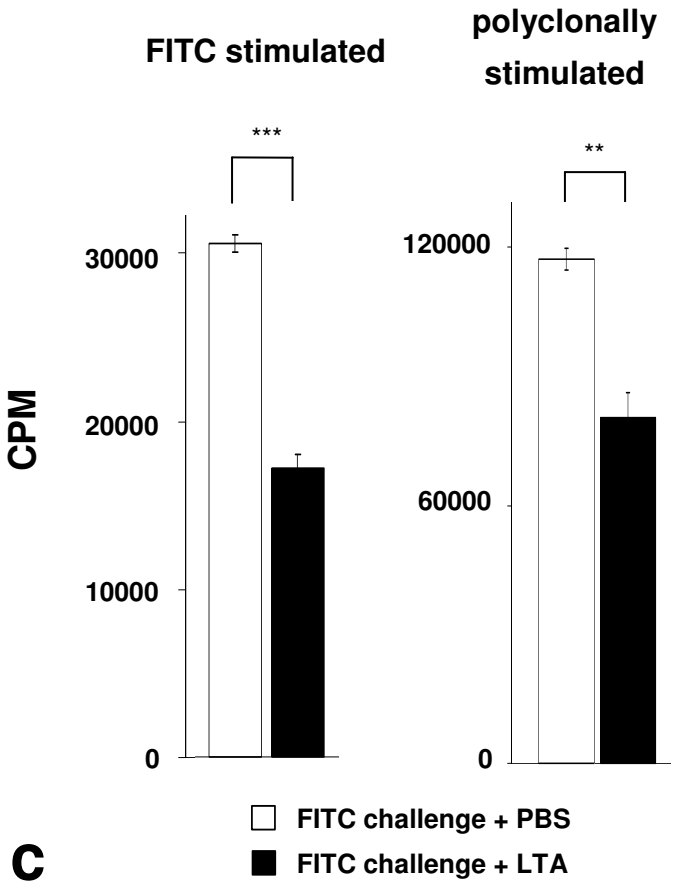
697 (a) Donor mice were sensitized with FITC 6 times and thereafter $CD3^+$ T cells were
698 isolated from axillary and inguinal LNs, and spleens. 7.5 x 10^6 T cells were then
699 adoptively transferred into recipient mice pre-treated as described for Figure 1a. The
700 next day, LTA or PBS was intracutaneously applied to the recipient mice, which were
701 then challenged by FITC or vehicle control. Ear swelling as the consequence of T cell
702 mediated dermatitis was monitored at 24 hours (mean +/- SD, *n*=5). (b) OVA-specific
703 Th2 cells were adoptively transferred together with OVA antigen into naïve recipient
704 wild type and $TLR2^{-/-}$ BL/6 mice with or without LTA (mean +/- SD, *n*=5). (*: $p<0.05$,
705 **: $p<0.01$, ***: $p<0.001$).


day 0 1 7
80 µl 0.5% FITC
abdomen application
25 µl 0.5% FITC
ear challenge
+/- 40 µg **LTA ear** id.

a


IFN-γ
IL-4
IL-10
Percentage of HPRT
1.00
0.10
0.01
FITC challenge + PBS
FITC challenge + LTA

b


FITC stimulated
polyclonally stimulated
***
**
CPM
30000
20000
10000
0
120000
60000
0
FITC challenge + PBS
FITC challenge + LTA

c

Figure 1

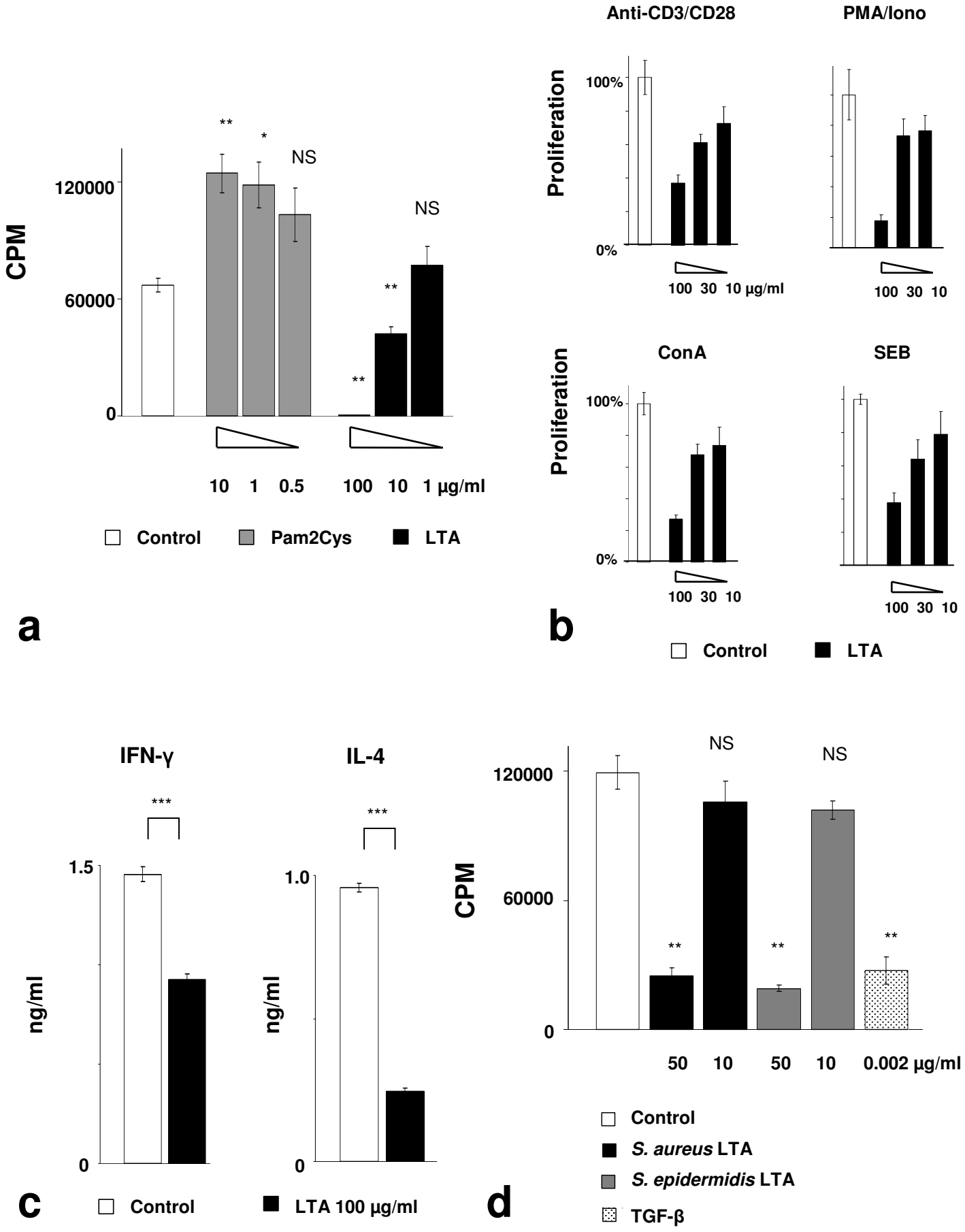
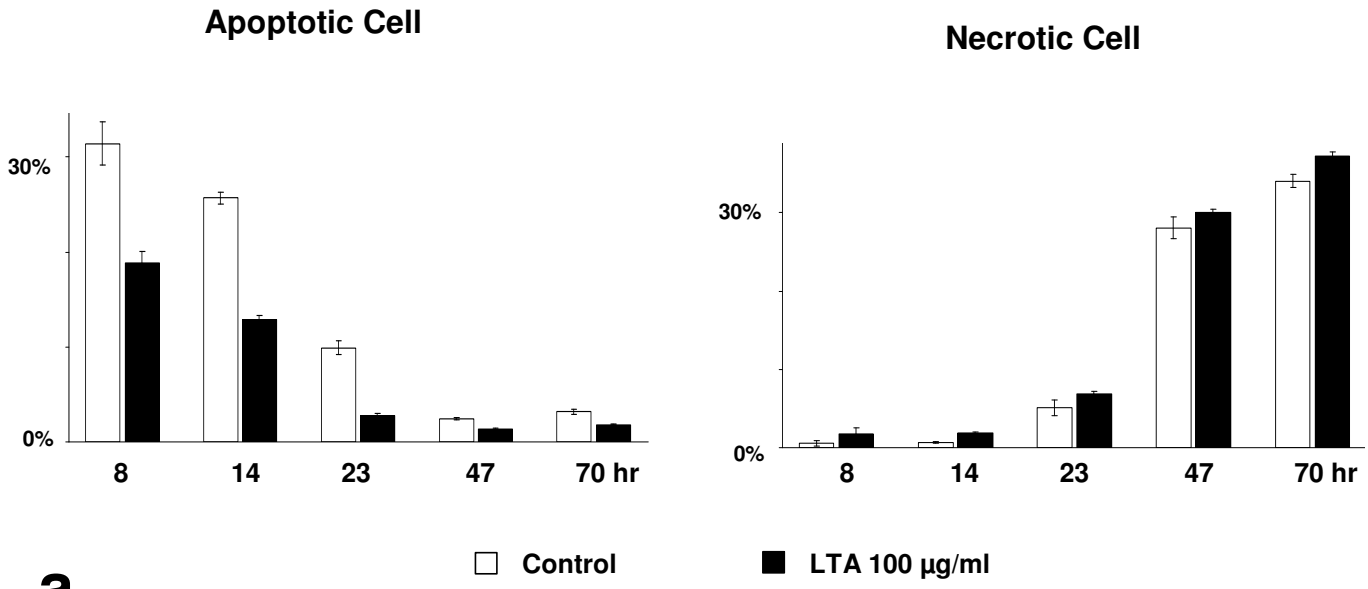
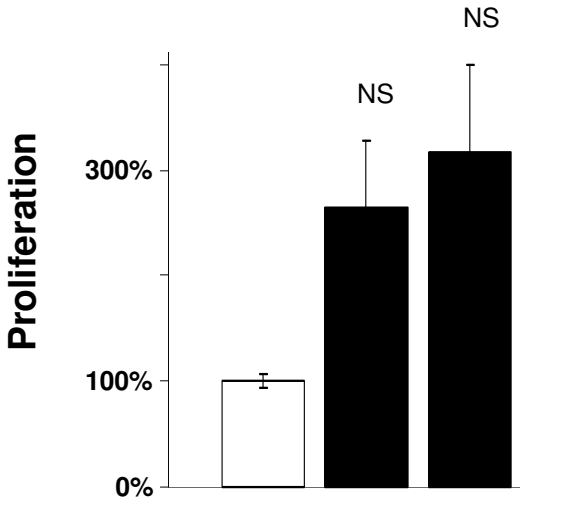

a
CPM
120000
60000
0
**
*
NS
NS
**
**
10 1 0.5
100 10 1 µg/ml
Control
Pam2Cys
LTA
b
Anti-CD3/CD28
PMA/Iono
Proliferation
100%
0%
100 30 10 µg/ml
100 30 10
ConA
SEB
100 30 10
100 30 10
Control
LTA
c
IFN-γ
IL-4
***
***
1.5
1.0
ng/ml
0
Control
LTA 100 µg/ml
d
CPM
120000
60000
0
NS
NS
**
**
**
50 10 50 10 0.002 µg/ml
Control
S. aureus LTA
S. epidermidis LTA
TGF-β

Figure 2



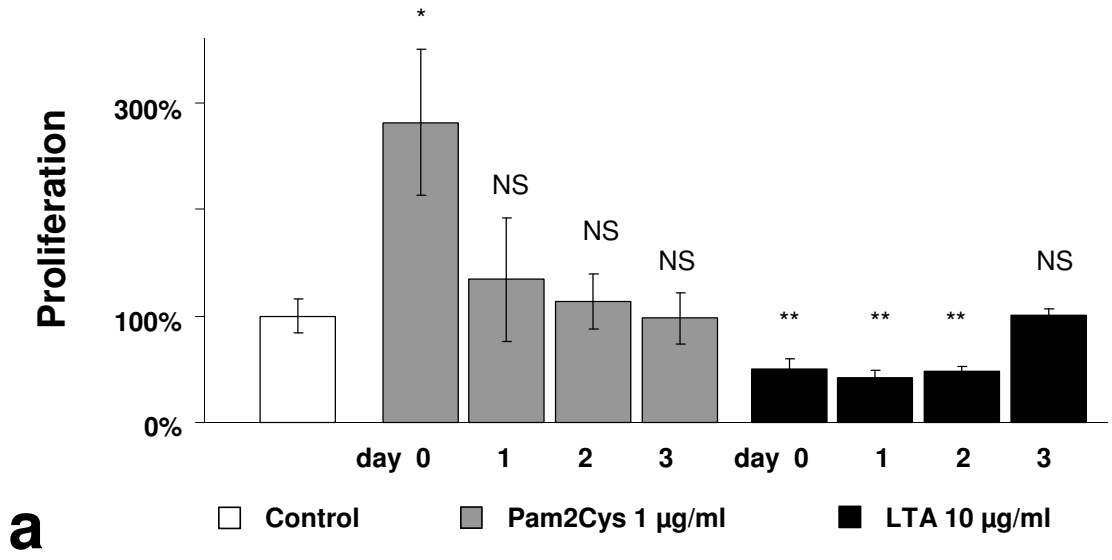
a



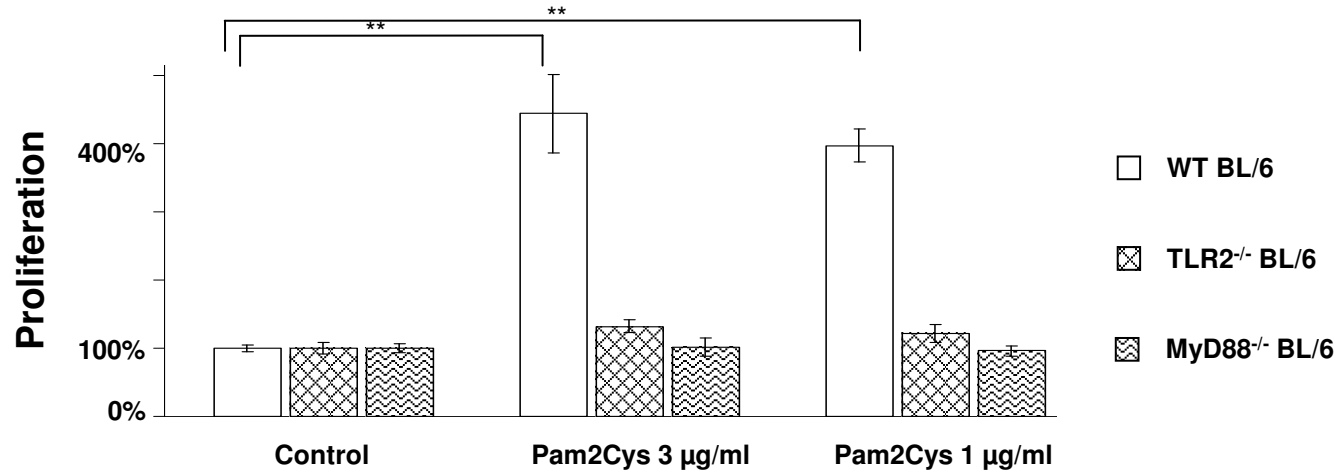
Primary Stimulation	anti-CD3/ anti-CD28	+	+	+
	LTA	0	100	50 µg/ml
Secondary Stimulation	anti-CD3/ anti-CD28	+	+	+
	LTA	0	0	0 µg/ml

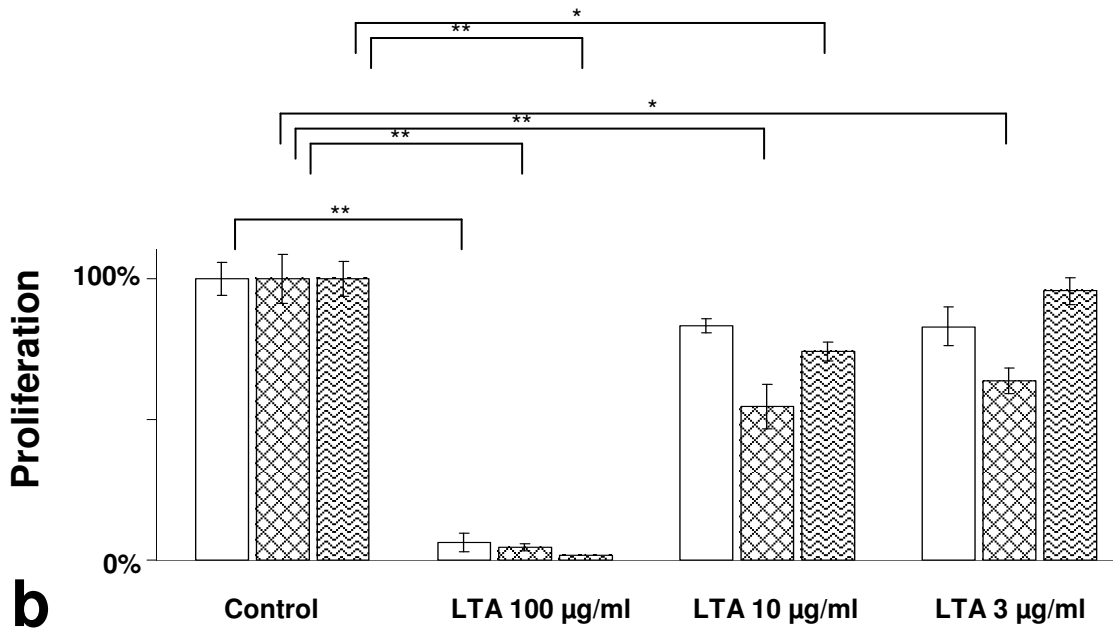
b

Figure 3


Proliferation
300%
100%
0%
*
NS
NS
NS
NS
**
**
**
day 0
1
2
3
day 0
1
2
3
Control
Pam2Cys 1 µg/ml
LTA 10 µg/ml

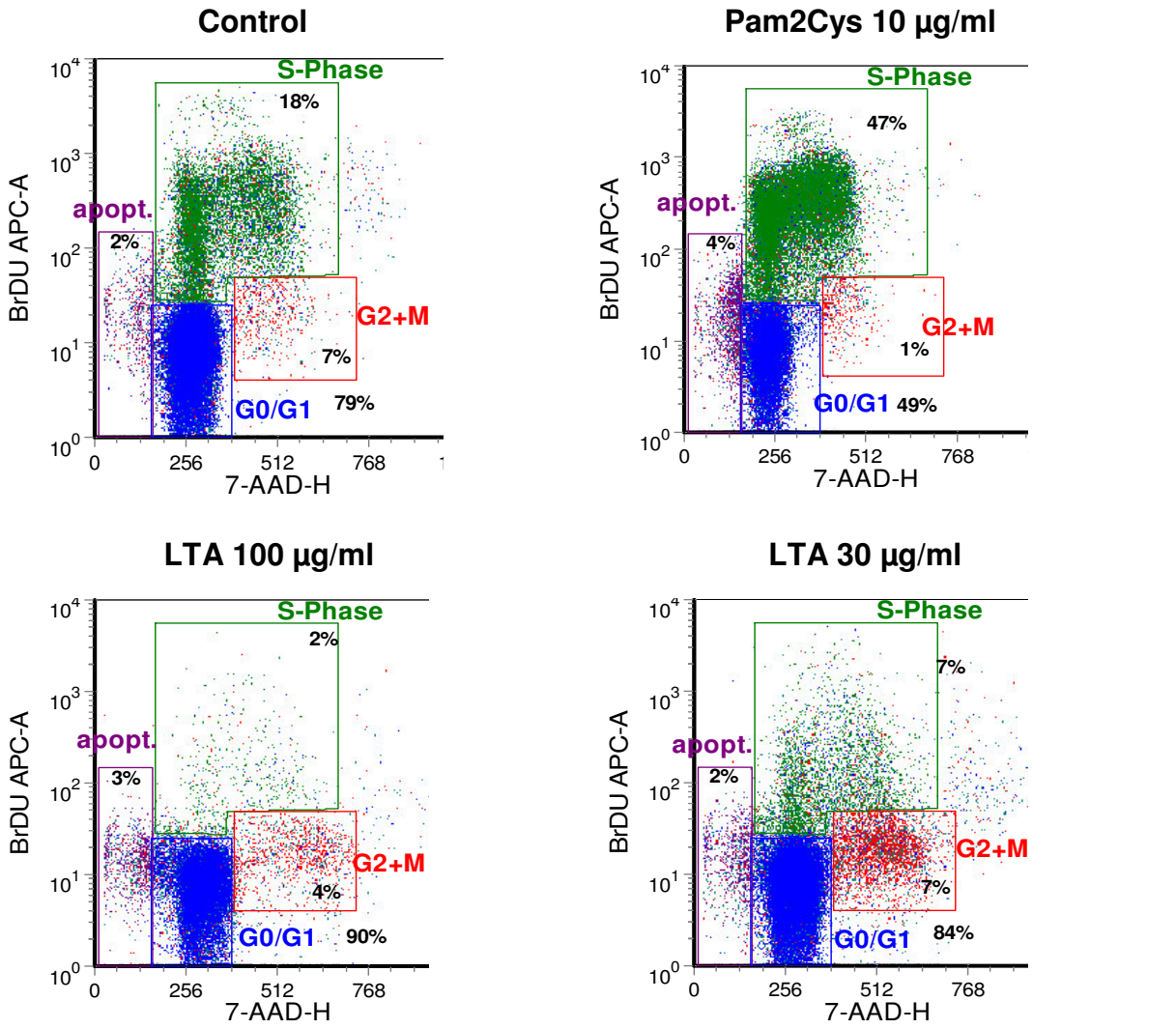
a


Proliferation
400%
100%
0%
**
**
Control
Pam2Cys 3 µg/ml
Pam2Cys 1 µg/ml
WT BL/6
TLR2-/- BL/6
MyD88-/- BL/6

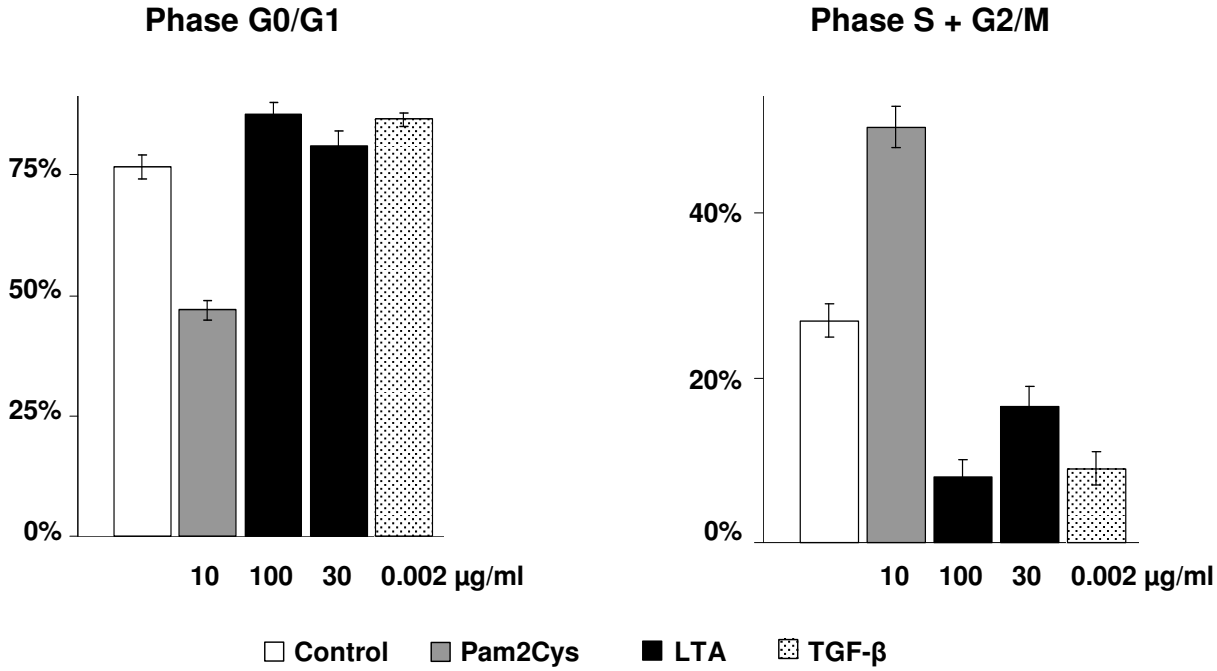

Proliferation
100%
0%
*
**
*
**
**
**
Control
LTA 100 µg/ml
LTA 10 µg/ml
LTA 3 µg/ml

b

Figure 4

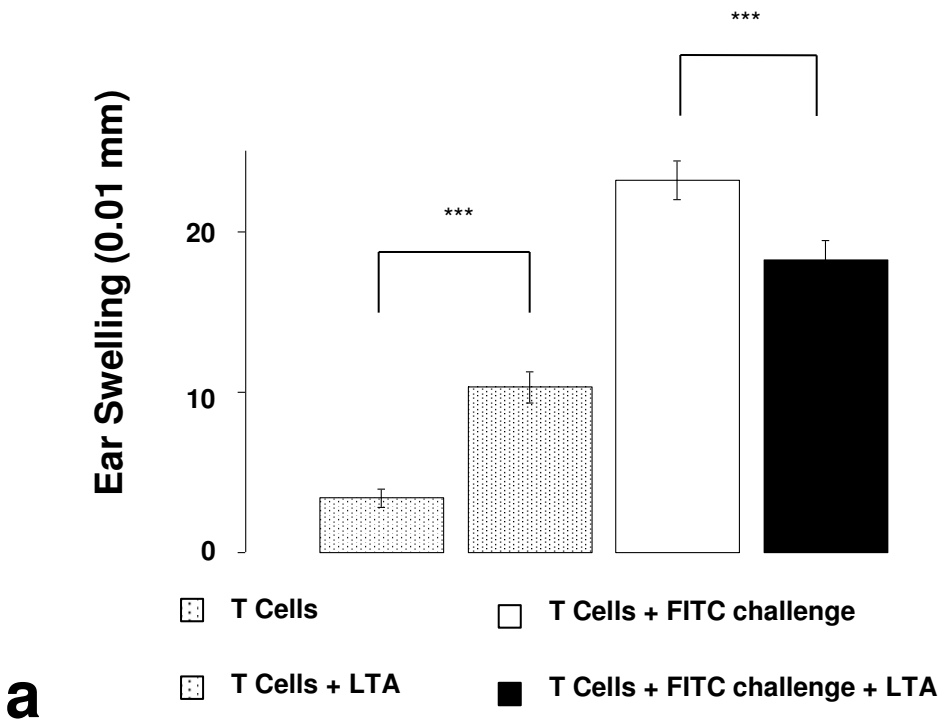

Control
S-Phase
18%
apopt.
2%
G2+M
7%
G0/G1
79%
BrDU APC-A
7-AAD-H
Pam2Cys 10 µg/ml
S-Phase
47%
apopt.
4%
G2+M
1%
G0/G1
49%
LTA 100 µg/ml
S-Phase
2%
apopt.
3%
G2+M
4%
G0/G1
90%
LTA 30 µg/ml
S-Phase
7%
apopt.
2%
G2+M
7%
G0/G1
84%

a

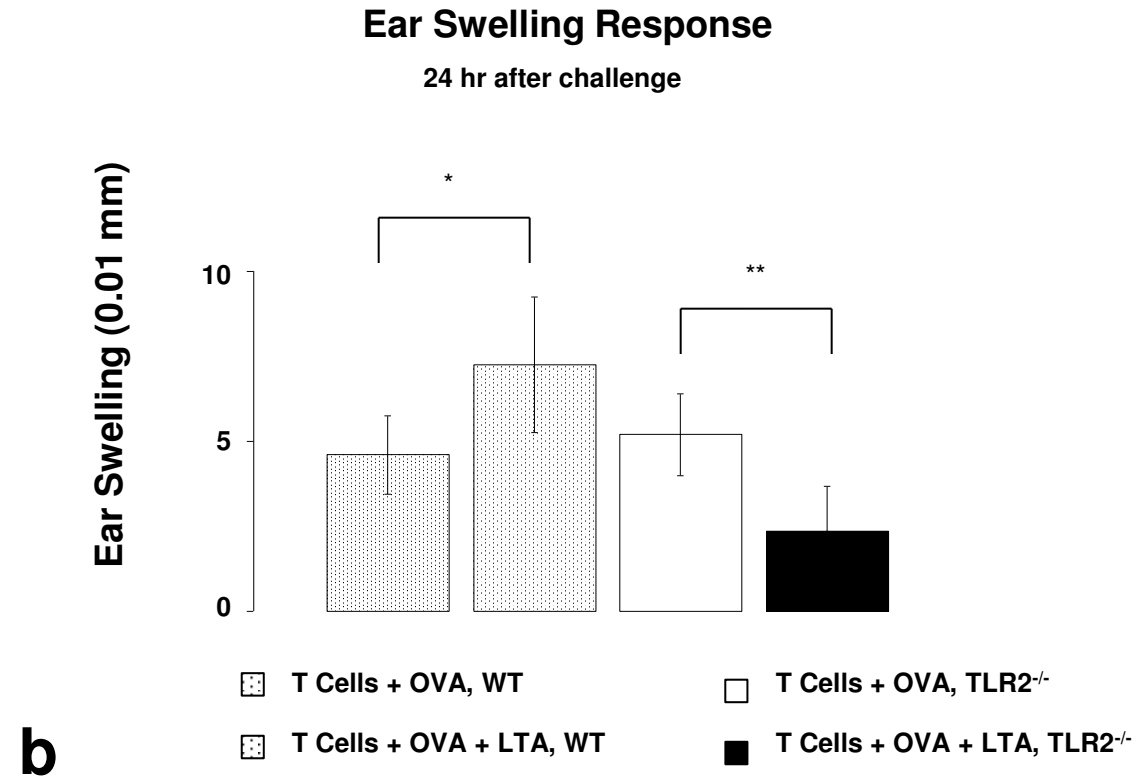

Phase G0/G1
75%
50%
25%
0%
10
100
30
0.002 µg/ml
Phase S + G2/M
40%
20%
0%
10
100
30
0.002 µg/ml
Control
Pam2Cys
LTA
TGF-β

b

Figure 5


Ear Swelling Response
24 hr after challenge
Ear Swelling (0.01 mm)
0
10
20
***
***
T Cells
T Cells + FITC challenge
T Cells + LTA
T Cells + FITC challenge + LTA

a


Ear Swelling Response
24 hr after challenge
Ear Swelling (0.01 mm)
0
5
10
*
**
T Cells + OVA, WT
T Cells + OVA, TLR2-/-
T Cells + OVA + LTA, WT
T Cells + OVA + LTA, TLR2-/-

b

Figure 6