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Cutaneous Innate Immune Sensing of Toll-like Receptor 2-6 Ligands Suppresses T Cell Immunity by Inducing Myeloid-Derived Suppressor Cells

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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 and 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-binding lipopeptides, 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., 2012).

Staphylococcus aureus (*S. aureus*) is one of the most potent skin pathogens and is found to colonize skin of about 30%–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 coreceptor 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., 2012).

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 counterregulation.

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 available online). 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 (Figures 1B and 1C). 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 Δ*lgt 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 to WT *S. aureus*, but not in mice exposed to lipoprotein-deficient Δ*lgt 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 day 3 after FITC challenge, MDSCs were also slightly increased in draining lymph nodes due to cutaneous WT *S. aureus* infection, corresponding to 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 (LCs, defined as $CD11c^{lo}CD205^{hi}$) and $CD11c^+$MHC-II^+ cells were also unchanged, and dermal dendritic cells (dDCs, defined as $CD11c^{hi}CD205^{lo}$) were slightly increased (Figure S1E). These data indicate that MDSCs function independently 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, because 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.

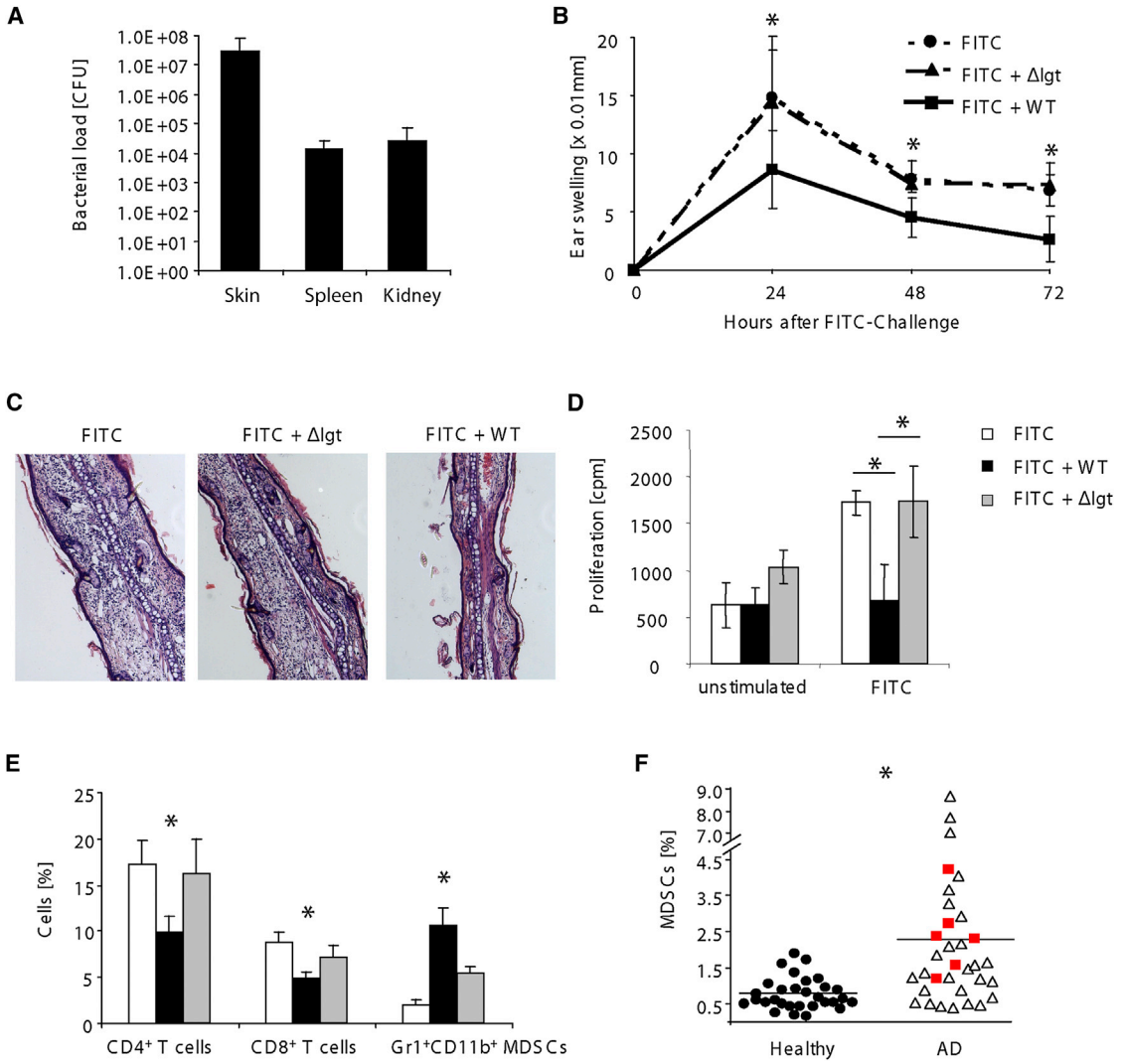

Figure 1. Cutaneous *Staphylococcus aureus* Induces Immune Suppression in Mice and Humans

(A–E) FITC-sensitized WT mice were treated following the protocol in Figure S1A (with living WT or lipoprotein mutant (Δ*lgt*) *S. aureus*). Bacterial load as colony-forming units (cfu) (mean ± SD, n = 5) (A), ear swelling (mean ± SD, n = 5) (B), histology (H&E staining) (C), proliferation of skin-draining lymph node (LN) cells stimulated ex vivo with FITC (detected as counts per minute [cpm] of ^{3}H-thymidine incorporation) (mean ± SD of triplicates) (D), and the percentage of cell populations in the spleen (mean ± SD, n = 5) (E) were investigated. *p < 0.05.

(F) PBMCs from atopic dermatitis (AD) patients (n = 33) and healthy volunteers (n = 30) were analyzed for MDSCs, defined as CD11b^{+}CD33^{+}HLA-DR^{-}CD14^{-} cells. The dots represent individual values, and the horizontal bar is the group mean. Red squares represent MDSCs of patients with severe AD and eczema herpeticum. *p < 0.05 (Mann-Whitney test). Data are representative of at least two independent experiments. See also Figure S1.

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 (Figures 2A and 2B), 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 (Figures 2E–2H). In contrast to Pam2Cys, the TLR2-TLR1 ligand Pam3Cys failed to suppress FITC-specific dermatitis and T cell proliferation (Figures 2E–2G). 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

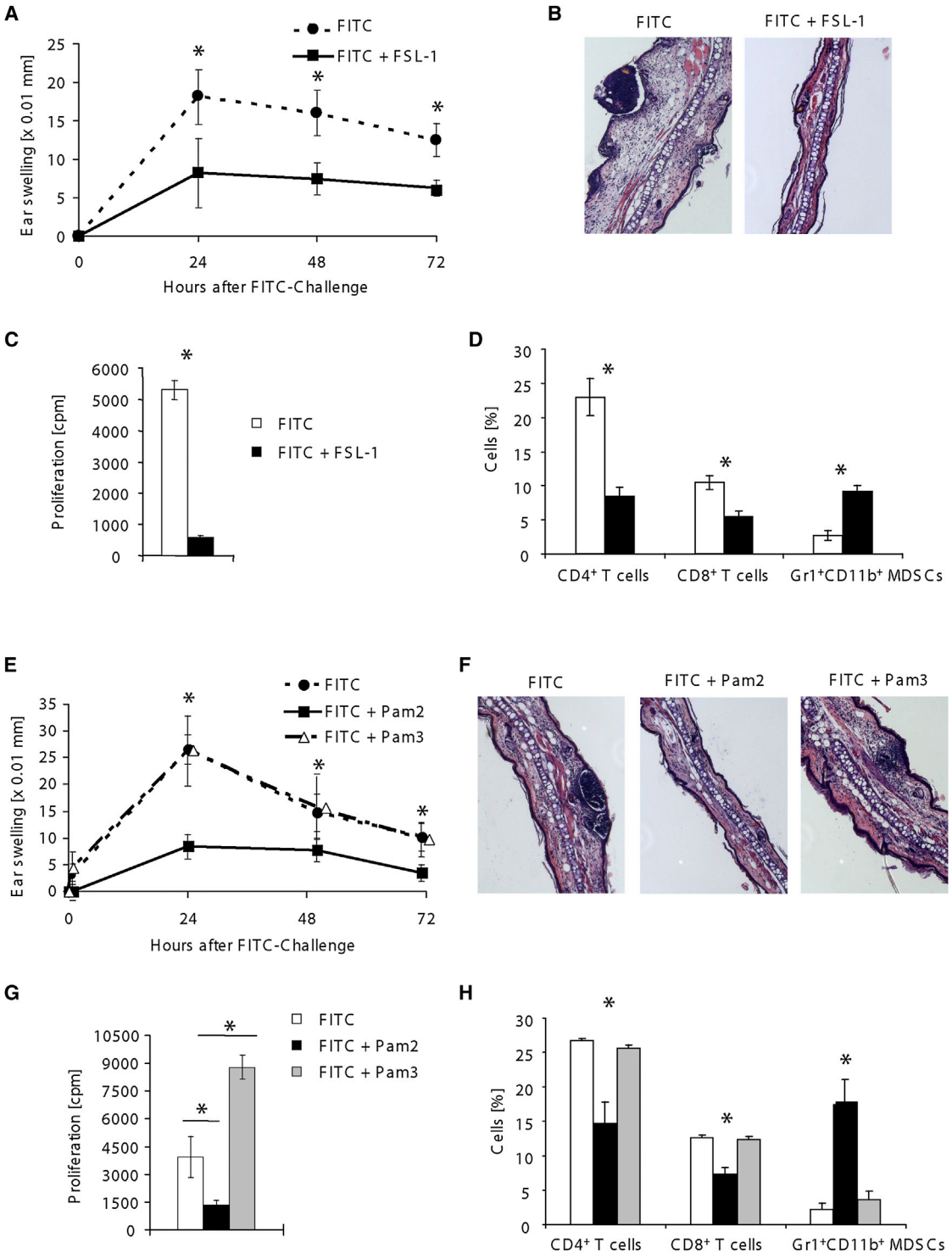

Figure 2. Cutaneous Exposure to TLR2-6 but Not TLR2-1 Ligands Ameliorates T Cell-Mediated Recall Responses of the Skin

WT mice were treated following the protocol shown in Figure S1A. Mice were cutaneously exposed to FSL-1 in (A)–(D) and Pam2Cys or Pam3Cys in (E)–(H). Ear swelling response (mean ± SD, n = 5) (A and E), histology (H&E staining) (B and F), proliferation of skin-draining LN cells stimulated ex vivo with FITC (mean ± SD of triplicates) (C and G), and the percentage of cell populations in the spleen (mean ± SD, n = 5) (D and H) are shown. Data are representative of at least two independent experiments. Experiments shown in (A) were performed with FSL-1 from two different providers. *$p < 0.05$.

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 hr 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 (dDCs, LCs) 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 (Figures S1J–S1L), 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 (Figures 3B and 3C). 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 seven of eight 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 downregulated in AD patients (Figure 3E), which is known to be one of the major features of MDSC-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 in (1) the bone marrow (BM), its primary source (Figure 4A, left), and (2) 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 BM 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 hr 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-CD28 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 downregulation 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 (Biedermann et al., 2002).

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 and 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 $Gr\text{-}1^{hi}Ly\text{-}6G^+CD11b^+$ ($Ly6G^+$) MDSCs from Pam2Cys-exposed mice and cocultured them with naive splenocytes (responder cells) activated with anti-CD3-CD28 antibodies (Abs) at different ratios. Following coculture 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 Th1-, Th2-, and Th17-polarized cells (Figure S2D). MDSCs' immunosuppressive activity is reported to be a result of the activation of inducible NOS

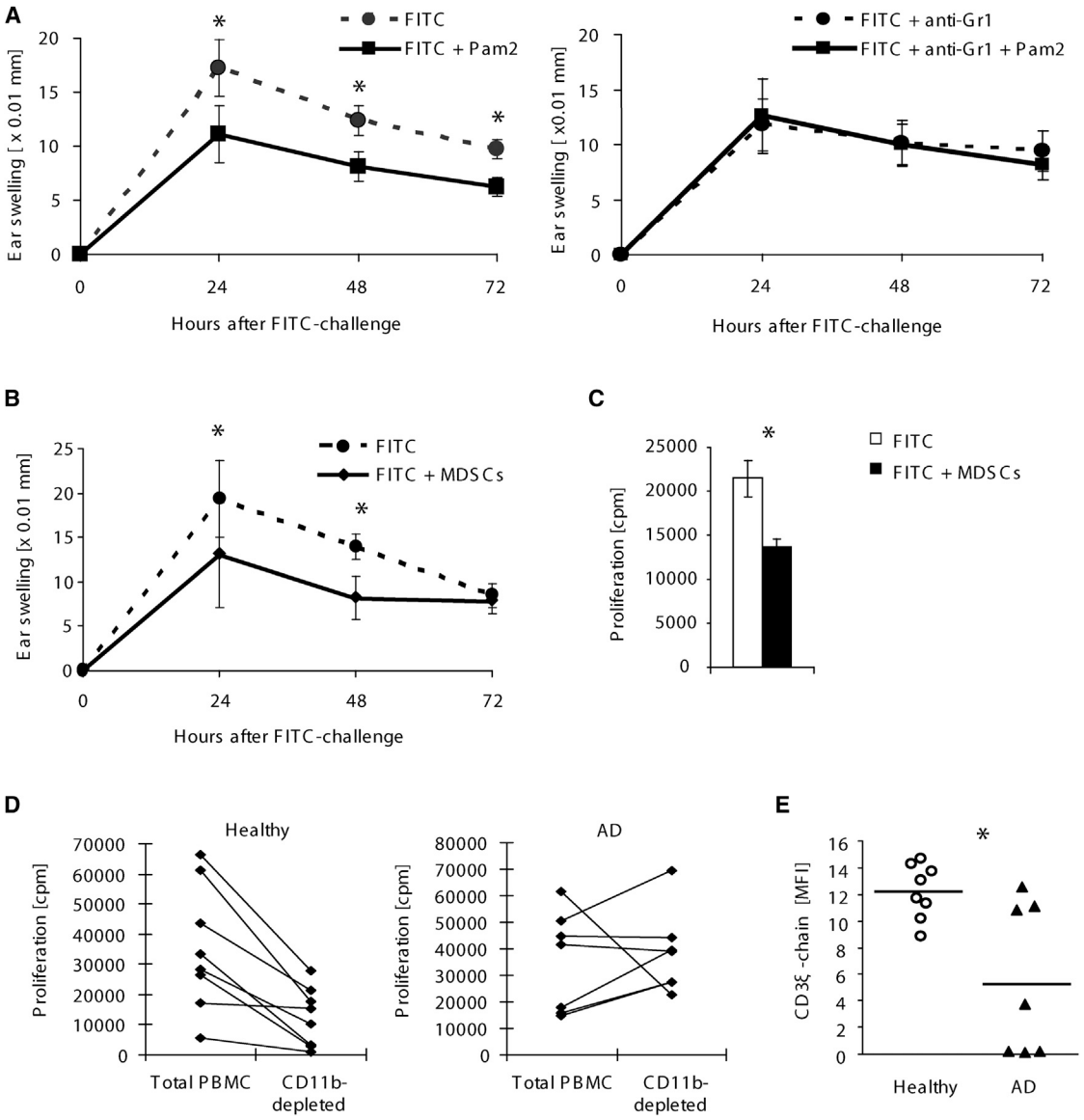

Figure 3. Myeloid-Derived Suppressor Cells Are Responsible for Skin-Infection-Induced Immune Suppression

(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 and 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 naive 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, and remaining PBMCs were 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, and the horizontal bar is the group's mean. *$p < 0.05$ (Mann-Whitney test). See also Figure S2.

(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 coculture 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

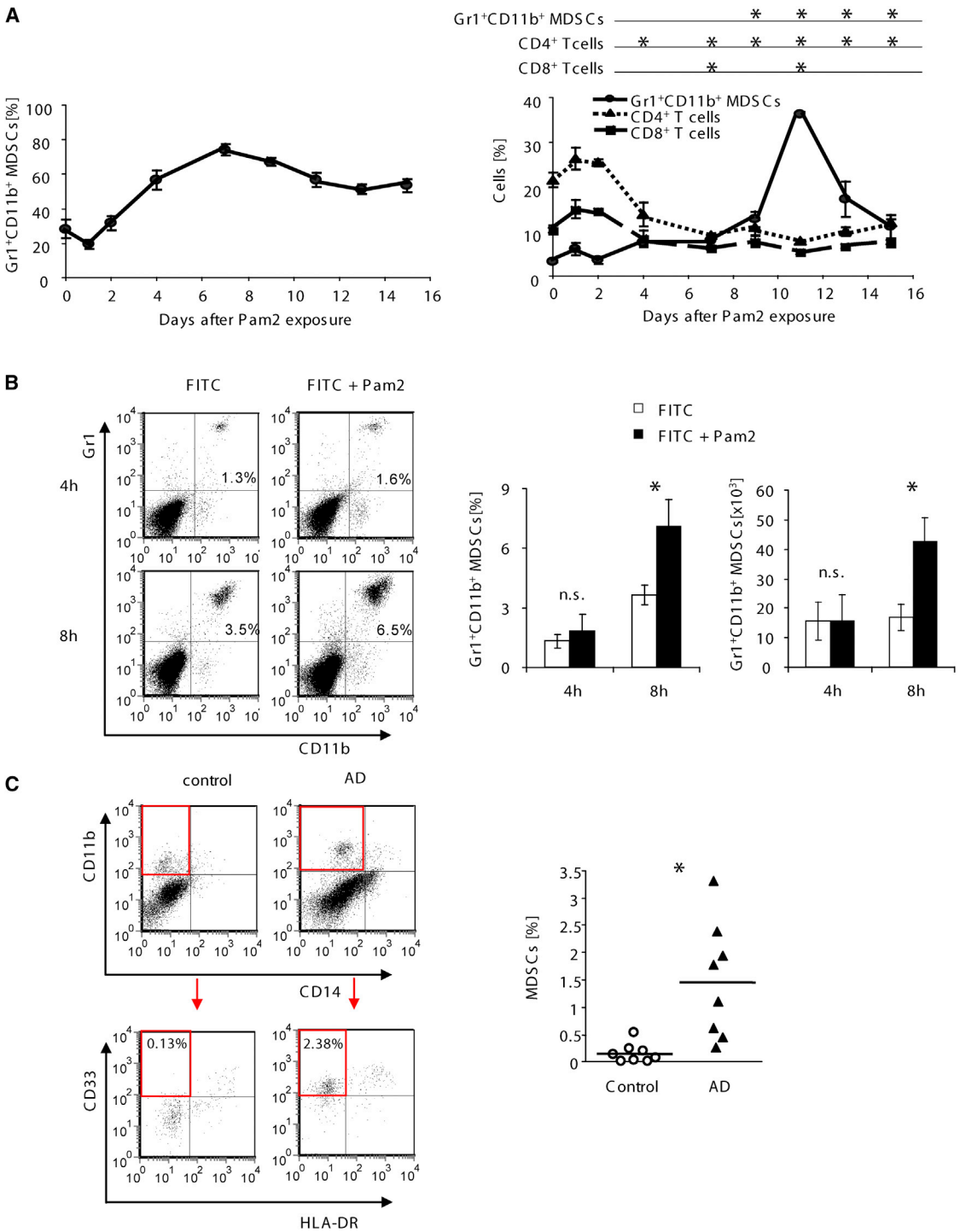

Figure 4. Skin Infection-Induced MDSCs Accumulate in the Skin in Mice and Humans

(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 BM (left) and spleen (right) (mean ± SD, n = 3). Asterisks show significant differences compared with t = 0 determined by one-way ANOVA followed by Dunnett's post test. *$p < 0.05$. Data are representative of two independent experiments.

(B) Cells from ear skin, isolated 4 hr or 8 hr 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) are 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.

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 might 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 BM cells (Figure S3B). The percentage of MDSCs was analyzed following the protocol shown in Figure S1A. WT mice, reconstituted with WT BM cells (WT + WT-BM) and WT mice, reconstituted with *Tlr2*$^{-/-}$ BM cells (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 *Cxcl2* was most dominantly induced by Pam2Cys and Pam3Cys (Figure 7B). Upregulation of *Il6* 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 *Il6* 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 bloodstream (Chalmin et al., 2010). Indeed, IL-6 concentrations in mouse sera strongly increased 1 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 (Figures S4C and S4D), 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 significant and unequivocal increase of Gr1$^+$CD11b$^+$ cells in both conditions (Figure S4E), and the adoptive transfer of MDSCs into *Il6*$^{-/-}$ mice showed a suppression of immune responses, comparable to what is observed in WT mice (Figure S4F). To investigate whether IL-6 plays a role for MDSC development, we analyzed MDSCs generation in vitro. BM-derived MDSCs (see Supplemental Experimental Procedures) were treated with IL-6 during development, and their suppressive function was investigated in a suppression assay with responder cells. As shown in Figure 7H, the exposure of MDSCs to IL-6 during generation enhanced their suppressive function. These data indicate that IL-6 supports induction and development of suppressive MDSCs, but not their migration to the skin.

Taken together, these data suggest a scenario in which Pam2Cys 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, 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

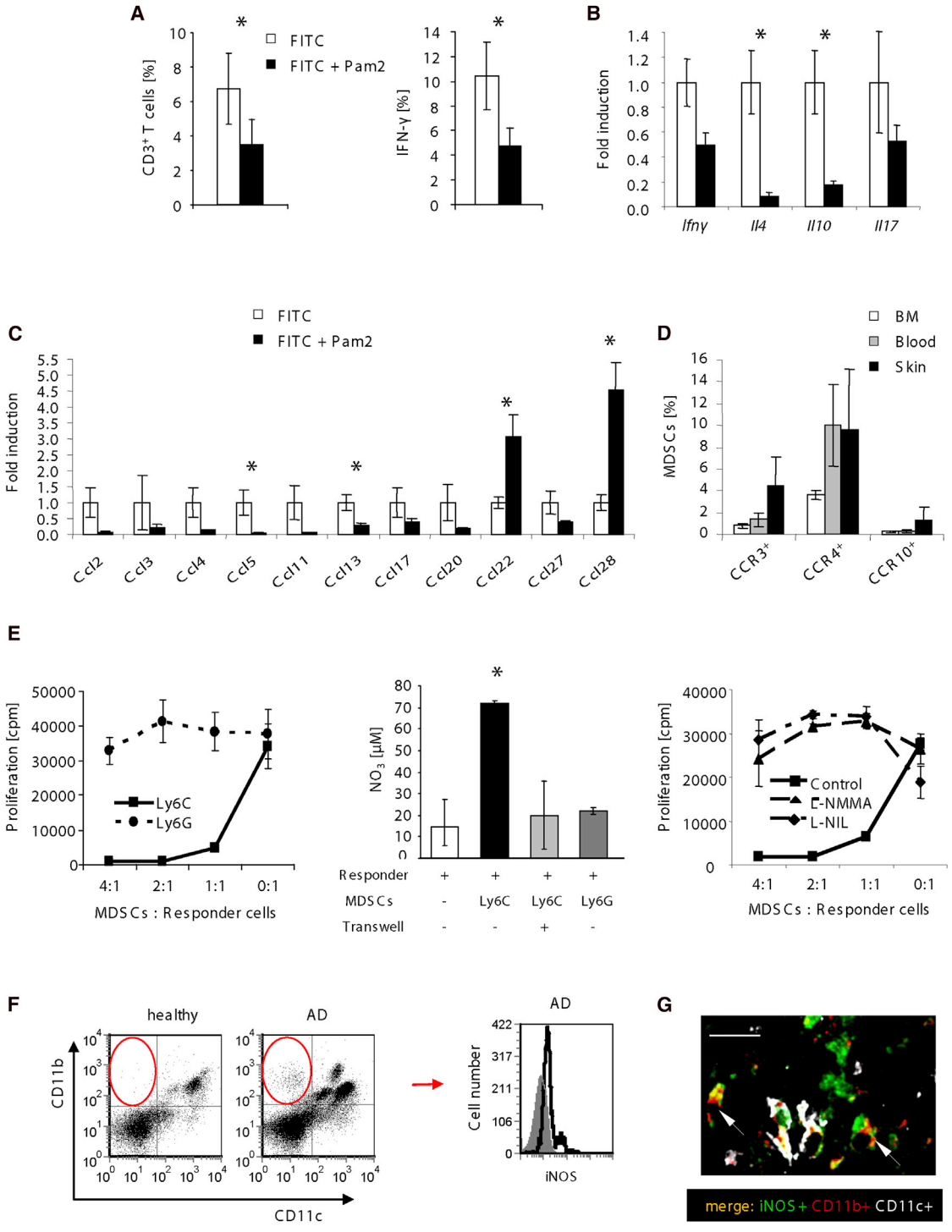

Figure 5. Skin Infection-Induced MDSCs Suppress T Cell Activation through Mechanisms Requiring NO Production

(A) WT mice were treated following the protocol in Figure S1A. 24 hr (A and B) or 8 hr (C and D) after FITC challenge ear tissue cells were analyzed. (A) Flow cytometry for $CD3^+$ cells (left) and IFN-γ production (right). A cumulative result (means ± SD, n = 5) is shown.

(B and C) Quantitative RT-PCR analysis for cytokines (B) or chemokines (C) (normalized to housekeeping genes *Actb-Gapdh*) 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 BM, 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 cocultured 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), and iNOS inhibitors L-NMMA and

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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 might differ in their potential to activate different TLR2 heterodimers and to regulate immune responses as well. Consequently, acylation properties might 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 postexponential 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 postexponential growth phase of *S. aureus* can be assumed. Consequently, lipoproteins from *S. aureus* on the skin are more diacylated. On the basis of our data and also own recently published data (Kaesler et al., 2014), we hypothesize that diacylation of lipoproteins induces acute inflammation followed by immune suppression as a consequence. Further, one can also assume that pathogenic and nonpathogenic skin microflora might have different acylation properties and therefore different compositions of TLR2 ligands and thus overall differ in regard to their immune consequences.

Previous data obtained 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 us to propose a model of 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 have further 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 might serve not only as danger signal for the initiation of effective immune responses but also might be able to counterregulate inflammation and potently control and suppress immune responses.

EXPERIMENTAL PROCEDURES

Animals

Specific-pathogen-free, WT BALB/c mice were purchased from Charles River. *Tlr2*$^{-/-}$ mice (C57BL/6) were from C. Kirschning (Institute of Medical Microbiology, University Duisburg-Essen) and were backcrossed to BALB/c for ten generations. *Il6*$^{-/-}$-BALB/c mice were from Dr. M. Kopf (Swiss Federal Institute of Technology). 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 3 × 10^8 WT or *lgt* mutant *S. aureus* Newman in 30 μl PBS or PBS control were added to filter paper discs placed onto the prepared skin and covered by Finn Chambers on Scanpor (Smart Practice). 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) on the shaved abdomen on days −8 and −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 day 7, 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) 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 0.3 μM CpG 1668 (0.2 μM,

L-NIL were added to the coculture (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 seven 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.

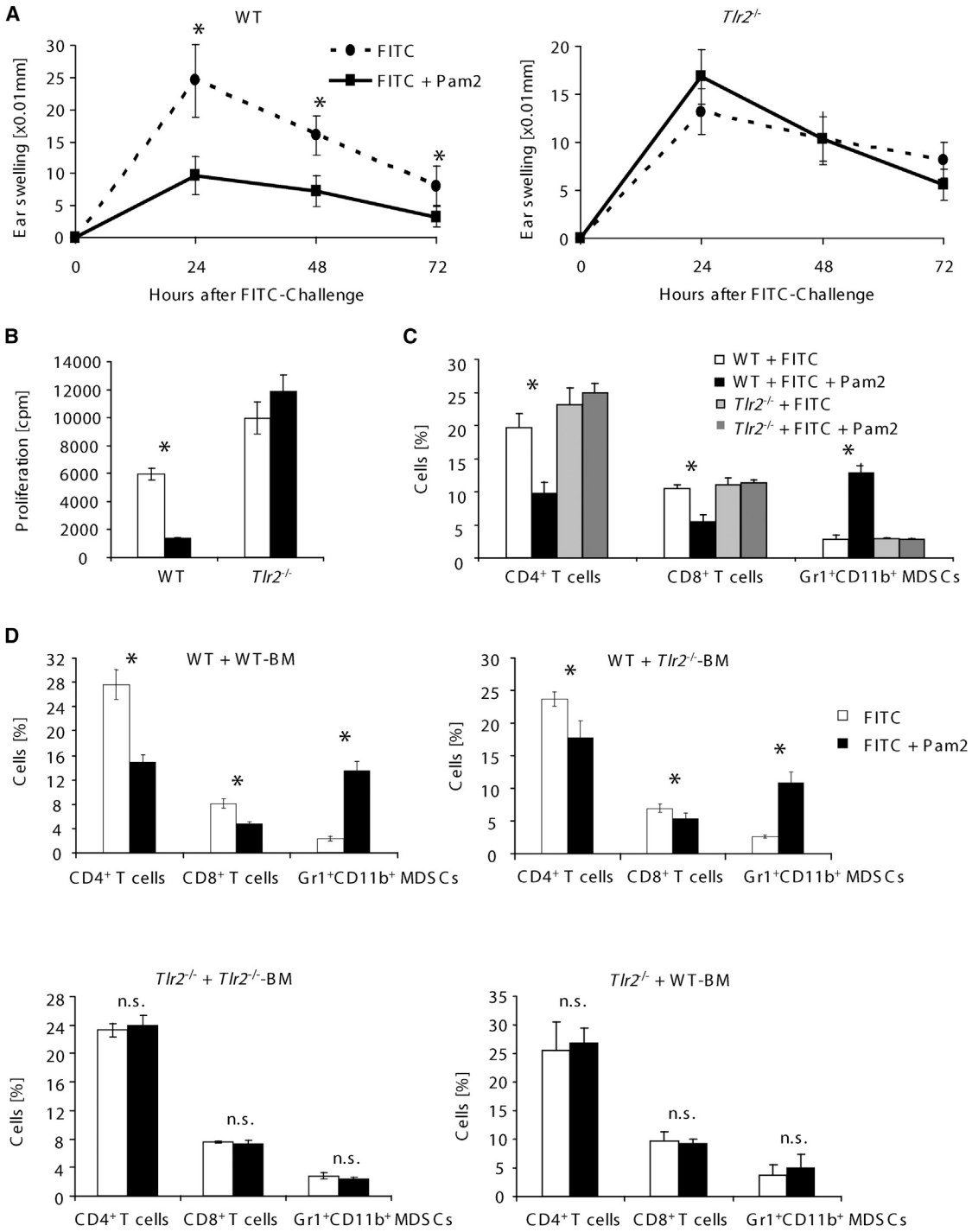

Figure 6. Pam2Cys-Induced Immune Suppression Is Dependent on TLR2

(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 or *Tlr2*$^{-/-}$ mice were irradiated and reconstituted with WT or *Tlr2*$^{-/-}$ BM cells (see Figure S3A). Seven 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.

Eurofins Genomics), 1 μg/mouse LPS (from Salmonella minnesota R595, Alexis Biochemicals), cyclophoshamide (2 mg/mouse, Sigma-Aldrich), 20 μg/mouse rmIL-6 (20 μg/mouse) or 50 μg/mouse anti-IL-6 (BioLegend).

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

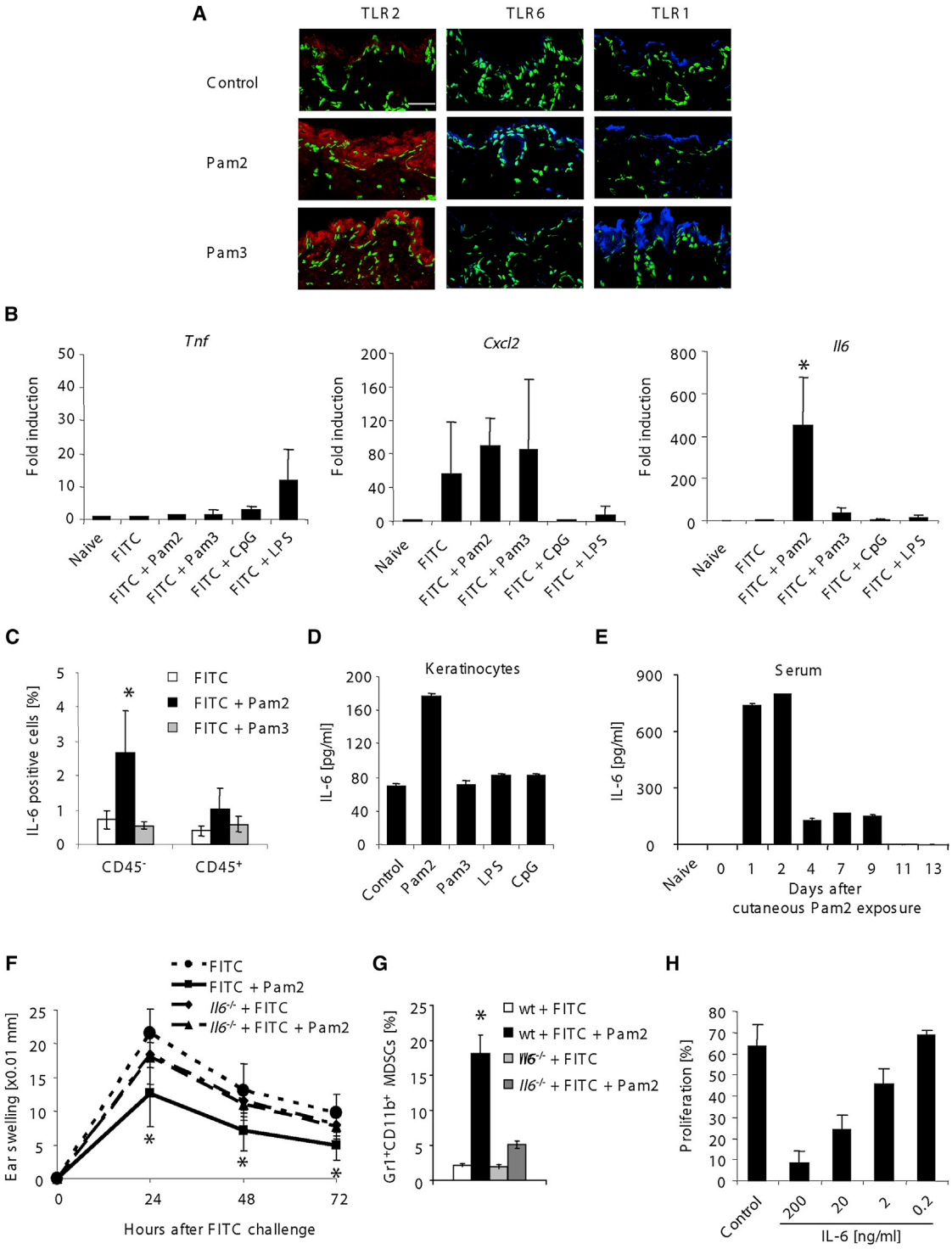

Figure 7. IL-6 Is Required for Induction of Gr1$^+$CD11b$^+$ Cells and Pam2Cys-Induced Immune Suppression

(A–C) WT mice were treated following a protocol similar to that shown in Figure S1A. 24 hr after cutaneous exposure to TLR ligands or PBS (control), immunofluorescence 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*, *Cxcl2*, and *Il6* mRNA by quantitative RT-PCR analysis (normalized to housekeeping gene *Actb*). Expression in the skin of untreated mice (naive) was set as 1 (mean ± SD, n = 5). (C) Skin cells were isolated and analyzed for IL-6 production by intracellular flow cytometry, and a cumulative analysis (mean ± SD, n = 5) is shown.

(D) Primary human keratinocytes were isolated and treated with TLR ligands for 24 hr, and the production of IL-6 was measured by ELISA (mean ± SD of triplicates).

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subjects (project number 344/2011BO2, 345/2011BO2, 396/2011BO2, 040/2013BO2, 180/2013BO2). PBMCs were obtained from heparinized blood by centrifugation (800 g for 30 min) using Ficoll-Histopaque (Biochrom). 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 on the next day BM cells (10^6 cells per recipient) were intravenously injected into recipient mice. To confirm the chimerism of mice, we conducted genotyping of BM cells by PCR for the WT and the mutated *Tlr2* gene (Figure S3B).

Depletion of $CD11b^+$ Cells

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

Statistical Analysis

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

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.009.

AUTHOR CONTRIBUTIONS

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 Δ*lgt S. aureus*; S.K., M.S., H.-G.R., and M.R. contributed to project development by fruitful discussions; K.-M.C., W.E.K., D.D., and F.G. participated in the manuscript preparation.

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(E) WT mice were treated following a protocol similar to that shown in Figure S1A, and IL-6 concentrations in the sera were analyzed by ELISA (mean ± SD of triplicates).
(F and G) WT and $Il6^{-/-}$ mice were treated following the protocol shown in Figure S1A and ear swelling (mean ± SD, n = 5) (F) and the percentage (mean ± SD, n = 5) of $Gr1^+CD11b^+$ cells (G) were analyzed.
(H) BM-derived MDSCs were treated with IL-6 (in indicated concentrations) during generation and their suppressive activity was measured in a coculture with activated spleen cells (responder cells) in ratio 1:4. Proliferation of responder cells without MDSCs was set as 100%. Data are representative of two independent experiments. *$p < 0.05$. See also Figure S4.

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