Propionibacterium acnes Promotes Th17 and Th17/Th1 Responses in Acne Patients

Magdalena Kistowska[1], Barbara Meier[1], Tatiana Proust[1], Laurence Feldmeyer[2], Antonio Cozzio[1], Thomas Kuendig[1], Emmanuel Contassot[1,3] and Lars E. French[1,3]

Propionibacterium acnes is a Gram-positive commensal bacterium thought to be involved in the pathogenesis of acne vulgaris. Although the ability of *P. acnes* in the initiation of pro-inflammatory responses is well documented, little is known about adaptive immune responses to this bacterium. The observation that infiltrating immune cells consist mainly of $CD4^+$ T cells in the perifollicular space of early acne lesions suggests that helper T cells may be involved in immune responses caused by the intra-follicular colonization of *P. acnes*. A recent report showing that *P. acnes* can induce IL-17 production by T cells suggests that acne might be a T helper type 17 (Th17)-mediated disease. In line with this, we show in this work that, in addition to IL-17A, both Th1 and Th17 effector cytokines, transcription factors, and chemokine receptors are strongly upregulated in acne lesions. Furthermore, we found that, in addition to Th17, *P. acnes* can promote mixed Th17/Th1 responses by inducing the concomitant secretion of IL-17A and IFN-γ from specific $CD4^+$ T cells *in vitro*. Finally, we show that both *P. acnes*-specific Th17 and Th17/Th1 cells can be found in the peripheral blood of patients suffering from acne and, at lower frequencies, in healthy individuals. We therefore identified *P. acnes*-responding Th17/Th1 cells as, to our knowledge, a previously unreported $CD4^+$ subpopulation involved in inflammatory acne.

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INTRODUCTION

Propionibacterium acnes (*P. acnes*) is a Gram-positive normal skin commensal bacterium present in all individuals. The intra-follicular colonization and proliferation of *P. acnes* has been associated with the pathogenesis of acne, the most common human inflammatory skin disorder (Leeming *et al.*, 1988; Leyden *et al.*, 1998). It has been proposed that *P. acnes* contributes to the development of inflammatory lesions by releasing chemotactic substances and attracting polymorphonuclear leukocytes to the site of inflammation. Infiltrating cells are locally activated and release pro-inflammatory cytokines including IL-6, tumor necrosis factor-α, IL-12, IL-8, and IL-1β (Kim *et al.*, 2002; Kalis *et al.*, 2005; Nagy *et al.*, 2005; Kistowska *et al.*, 2013). Surprisingly, in early acne lesions, infiltrating cells consist mainly of $CD4^+$ T cells (Norris and Cunliffe, 1988; Layton *et al.*, 1998). These memory/effector $CD4^+$ T cells are detected in close proximity to macrophages and are also present in uninvolved follicles from acne patients but not in non-acne controls (Jeremy *et al.*, 2003). In addition, *P. acnes* has been shown to induce T-cell proliferation (Jappe *et al.*, 2002), and reactive *P. acnes*-specific $CD4^+$ T cells have been isolated from early inflamed acne lesions (Mouser *et al.*, 2003). These T cells have the capacity to secrete IFN-γ, but not IL-4, upon stimulation with *P. acnes* and have therefore revealed to be Th1 cells.

$CD4^+$ T helper (Th) cells have a key role in the regulation of adaptive immune responses by secreting cytokines and chemokines that activate and/or recruit effector cells. Because of the discovery of an IL-17-producing T-cell subset, known as Th17, numerous studies have revealed IL-17 as a pro-inflammatory cytokine involved in the pathogenesis of autoimmune disorders as well as in response to certain pathogens at both the barrier site and at a systemic level (O'Connor *et al.*, 2010; Miossec and Kolls, 2012). Th17 cells have a role in the protection against extracellular bacteria and fungi (Zhu and Paul, 2008; Zhu *et al.*, 2010), particularly those colonizing the respiratory and gastrointestinal tracts and the skin (Peck and Mellins, 2010). The protective effects of IL-17-producing cells have been demonstrated in patients with hyper-immunoglobulin E syndrome, who suffer from recurrent infections with *Candida albicans* (*C. albicans*) and *Staphylococcus aureus* (*S. aureus*) due to the impairment in Th17 development (Milner *et al.*, 2008; Puel *et al.*, 2011). In addition to their protective effect, Th17 have also been reported to be pathogenic. Indeed, Th17 cells are associated with many autoimmune and inflammatory disorders including Crohn's disease, colitis, psoriasis, multiple sclerosis, rheumatoid arthritis, and chronic graft-versus-host disease (Wilke *et al.*,

[1]*Department of Dermatology, University Hospital, Zürich, Switzerland and* [2]*Department of Dermatology and Venereology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*

[3]*These authors contributed equally to this work.*

Correspondence: Lars E. French, Department of Dermatology, University Hospital, Gloriastrasse 31, Zürich 8006, Switzerland.
E-mail: lars.french@usz.ch

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2011; McGeachy, 2013). Moreover, Th17 cells are suspected to have a role in cutaneous inflammatory disorders, including psoriasis, allergic contact dermatitis, or atopic dermatitis (Asarch *et al.*, 2008). In a recent report, Agak *et al.* (2013) showed that the secretion of IL-17 by naïve $CD4^+$ T cells can be induced by *P. acnes*, thus suggesting that both Th1 and Th17 responses may be involved in responses to this bacterium.

In this work, we show that, in addition to IL-17A, both Th1 and Th17 effector cytokines, transcription factors, and chemokine receptors are strongly upregulated in acne lesions. We also observed that, besides Th17, *P. acnes* can promote mixed Th17/Th1 responses by inducing the concomitant secretion of IL-17A and IFN-γ from specific $CD4^+$ T cells *in vitro*. Finally, we show that both *P. acnes*-reactive Th17 and Th17/Th1 cells can be found in the peripheral blood of patients suffering from acne and, at lower frequencies, in healthy individuals. Our data suggest that, in addition to Th17, Th17/Th1 T cells may have a role in acne pathogenesis.

RESULTS

T cells of Th1 and Th17 phenotypes are found in acne lesions

We first assessed the expression of Th effector cytokines in human acne biopsies. We detected significantly increased levels of *IFN-γ* and, in line with the recent report from Agak *et al.* (2013), *IL-17A* mRNA in human acne samples when compared with normal skin. IL-21, an essential cytokine for the differentiation and homeostasis of Th17 cells (Korn *et al.*, 2007; Weaver *et al.*, 2007; Wei *et al.*, 2007), was also found to be highly expressed in acne biopsies (Figure 1a). In accordance with previous studies (Mouser *et al.*, 2003; Agak *et al.*, 2013), the levels of *IL-4* mRNA were not higher in acne lesions compared with those in normal skin (Figure 1a). *IL-22* mRNA levels were also not increased in acne lesions in comparison with normal skin (Figure 1a). Noteworthy, we observed a correlation between the mRNA levels of IL-17A and IFN-γ in acne biopsies (Figure 1b). The immunohistochemical analysis of acne biopsies not only confirmed a strong expression of IL-17A but also revealed strong IFN-γ expression in the same areas of the immune cell infiltrates (Figure 1c).

Differentiation of Th subsets is tightly regulated by master transcription factors, signaling transducers, and activators of transcription (signal transducer and activator of transcriptions (STATs)). Th1 differentiation depends on STAT4 and T-bet (TBX-21), whereas Th2 cells require STAT5a and GATA3, and RAR-related orphan receptor gamma (ROR-γt) (RORC) and STAT3 are critical factors for Th17 subset differentiation (for review (Zhu *et al.*, 2010)). In acne biopsies, increased levels of Th1 and Th17 transcription factor mRNA were observed, whereas transcription factors required for Th2 cells remained unchanged when compared with normal skin (Figure 1d).

Analysis of chemokine receptors in skin biopsies revealed elevated *CXCR3*, *CCR4,* and *CCR6* mRNA levels in acne lesions when compared with normal skin (Figure 1e). These receptors are preferentially expressed in Th1 (CXCR3) or Th17 (CCR4 and CCR6) subsets (Bromley *et al.*, 2008; Brodie *et al.*, 2013). In contrast, the expression of the Th2 chemokine receptor *CRTh2* in acne lesions was not different from the one of normal skin. Our detailed analysis of $CD4^+$ T-cell subset markers further supports the presence of cells of Th1 and Th17 in inflammatory lesions of patients suffering from acne.

P. acnes triggers Th17 and mixed Th17/Th1 responses *in vitro*

To further characterize Th responses to *P. acnes*, peripheral blood mononuclear cells (PBMCs) from healthy donors were exposed to live *P. acnes*, at different multiplicities of infection (MOI). After 24 hours, we observed a strong secretion of Th1 and Th17 polarizing cytokines: namely IL-12 (Th1) and IL-1β, IL-6, and IL-23 (Th17) (Supplementary Figure S1a online). Moreover, we could confirm that, after 6 days of incubation, a robust release of Th1 and Th17 effector cytokines, namely IFN-γ and IL-17A, respectively, could be detected, whereas *P. acnes* failed to induce the secretion of IL-4 and IL-22 from PBMCs (Supplementary Figure S1b online). However, using intracellular stainings and flow-cytometric analysis, we observed that IL-17A was secreted by two distinct cell populations upon *P. acnes* exposure. In accordance with the recent report from Agak *et al.* (2013), we also found a first population secreting IL-17A alone. Interestingly, we identified a second cell population secreting IL-17A and IFN-γ concomitantly (Figure 2a). In line with ELISA data, we did not detect cells producing IL-4 or IL-22 upon stimulation with *P. acnes*. The induction of *P. acnes*-reactive $IL\text{-}17A^+$ $IFN\text{-}\gamma^-$ and $IL\text{-}17A^+$ $IFN\text{-}\gamma^+$ cells was significant in 100% of the tested healthy donors (Figure 2b). In contrast, induction of $IL\text{-}17A^-$ $IFN\text{-}\gamma^+$ cells was observed in only 40% of the tested donors (Figure 2b). Nearly 100% of the *P. acnes*-induced $IL\text{-}17A^+$ cells were $CD4^+$ (Figure 2c). Taken together, these data show that *P. acnes* not only induces Th17 ($IL\text{-}17A^+$ $IFN\text{-}\gamma^-$) but also Th17/Th1 ($IL\text{-}17A^+$ $IFN\text{-}\gamma^+$) responses.

P. acnes-exposed monocytes activate $CD4^+$ T cells in an major histocompatibility complex II-dependent manner

These findings prompted us to further characterize the $CD4^+$ T-cell response to *P. acnes.* Therefore we isolated $CD4^+$ T cells from PBMCs from healthy donors and stimulated them with live bacteria in the presence of autologous monocytes. After 6 days, we assessed the production of IL-17A and IFN-γ by intracellular staining. Flow-cytometric analysis showed that both $IL\text{-}17A^+$ $IFN\text{-}\gamma^-$ and $IL\text{-}17A^+$ $IFN\text{-}\gamma^+$ $CD4^+$ cells are strongly induced in response to *P. acnes* (Figure 3a and b). In about 50% of tested donors also $IL\text{-}17A^-$ $IFN\text{-}\gamma^+$ T cells were increased upon *P. acnes* exposure (Figure 3b). It has been previously shown that human leukocyte antigen-DR (HLA-DR) is upregulated in acne lesions and that HLA-DR-expressing cells are present in close association with $CD4^+$ T cells surrounding the lesions (Layton *et al.*, 1998). We addressed the role of major histocompatibility complex (MHC) class II in the induction of T-cell response upon *P. acnes* exposure by blocking its function with a neutralizing antibody. Treatment with an anti-HLA-DR antibody, but not with an irrelevant control antibody, led to a significant decrease in IL-17A single producers as well as IL-17A and IFN-γ double producers in all analyzed donors (Figure 3c and d). Anti-HLA-DR also led to a decrease in the number of T cells producing

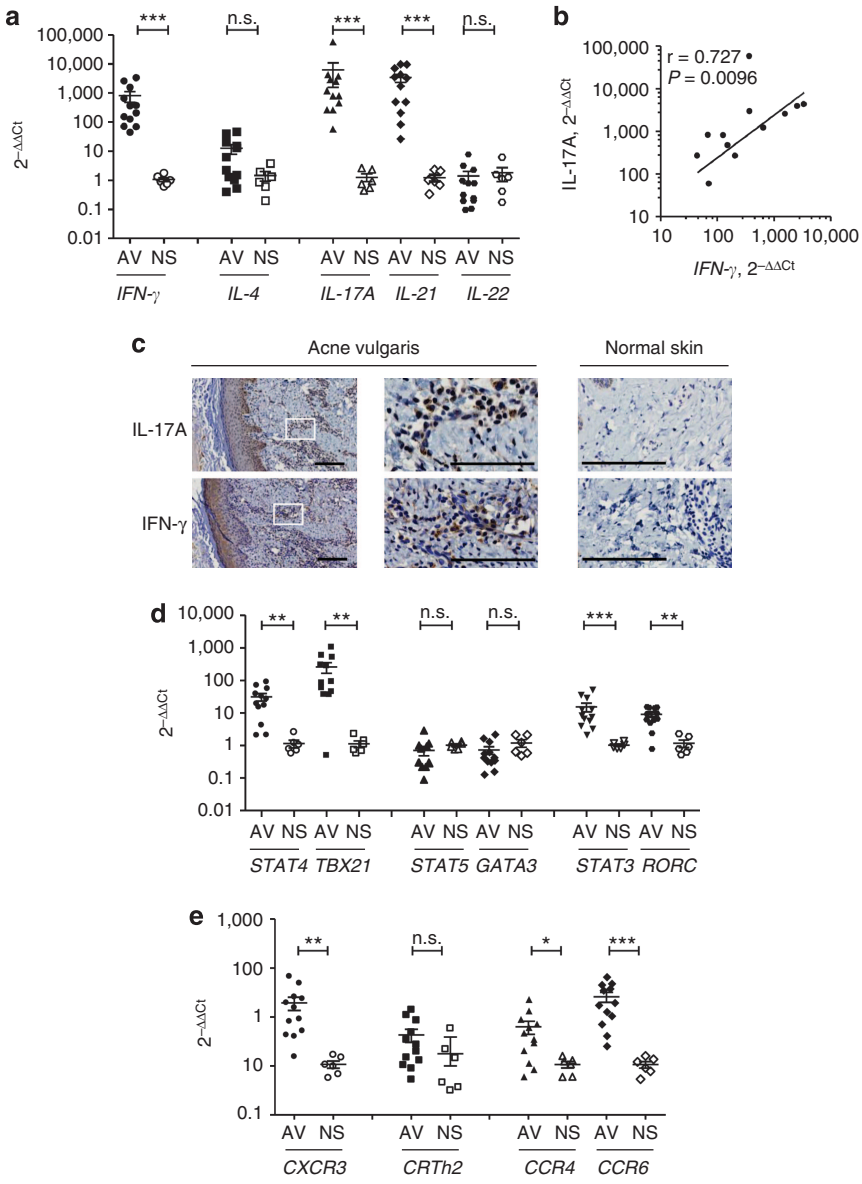

Figure 1. Cells with T helper type 1 (Th1) and Th17 profile are found in acne lesions. (**a**) Th cell effector cytokine gene expression in acne lesions (AV, $n=12$) and normal skin (NS, $n=6$) measured by quantitative reverse transcriptase in real time (qPCR). (**b**) Scatter plot showing the correlation (Spearman's rank correlation) between IL-17A and IFN-γ mRNA expression in human acne biopsies. (**c**) Immunohistochemical analysis of IL-17 and IFN-γ in an acne lesion compared with healthy skin (scale bar = 500 μm). (**d**) Transcription factors and (**e**) chemokine receptor gene expression in acne lesions (AV, $n=12$) and normal skin (NS, $n=6$) measured by qPCR. The relative mRNA abundance of indicated genes against ribosomal protein L27 (RPL27) mRNA levels was analyzed and normalized with the average of normal skin. *$P\leqslant0.01$, **$P\leqslant0.001$, and ***$P\leqslant0.0001$ by the Mann–Whitney two-tailed test for acne lesions compared with normal skin. Presented are mean and SEM.

only IFN-γ in about 80% of the donors, as shown in Figure 3d. However, for unknown reasons, the extent of this reduction was not as strong as the one obtained in IL-17A single producer or IL-17A and IFN-γ double-producer reduction and was not statistically significant. Moreover, the blockade of MHC II resulted in a strong reduction of T-cell proliferation upon *P. acnes* exposure (Figure 3e). These data demonstrate that proliferation and cytokine secretion by $CD4^+$ T cells upon *P. acnes* exposure are dependent on MHC II.

IL-1β and IL-12/IL-23 modulate the T-cell response to *P. acnes*

The IL-1 family cytokines and STAT activators are able to influence effector cytokine production from differentiated Th subsets. IL-12 activates STAT4 and, together with IL-18, induces IFN-γ production from Th1 cells (Guo *et al.*, 2009). Secretion of IL-17A from Th17 cells can be promoted by IL-1 and STAT3 activators, including IL-6, IL-21, and IL-23 (Chung *et al.*, 2009; Guo *et al.*, 2009). The observation that IL-1β, IL-12, and IL-23 are secreted by PBMCs upon *P. acnes*

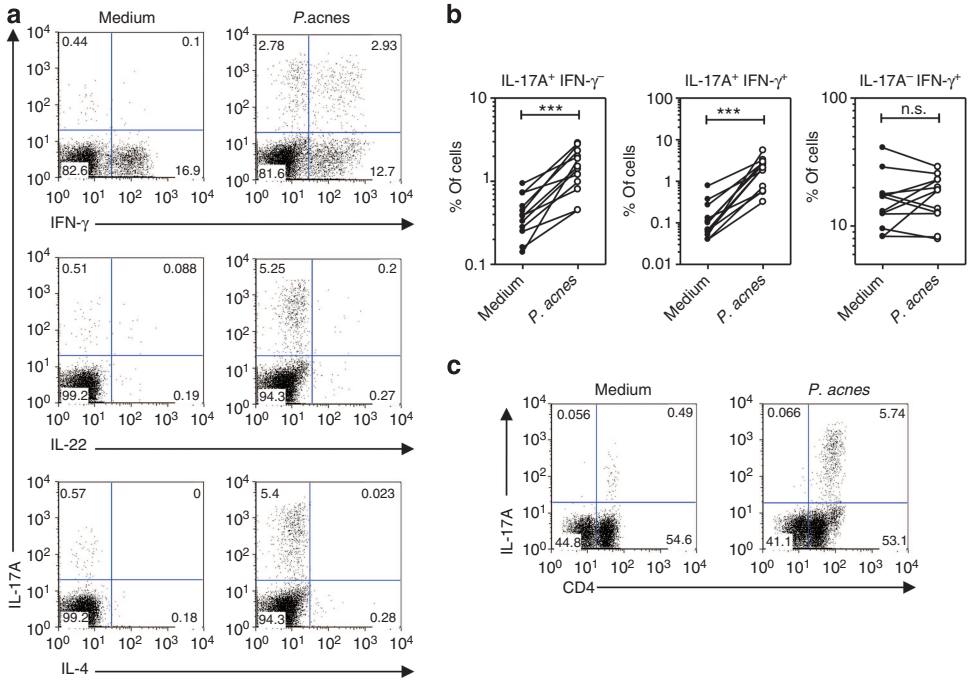

Figure 2. *P. acnes* induces production of IL-17A and IFN-γ by CD4$^+$ T cells. Peripheral blood mononuclear cells (PBMCs) were exposed to *P. acnes* (multiplicity of infection (MOI) = 3) or incubated with medium. On day 6, cells were stimulated for 4.5 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin, fixed, permeabilized, and stained with antibodies against CD4, IL-17A, and IFN-γ or IL-22 or IL-4. (**a**) Representative flow-cytometric analysis. (**b**) Percentages of IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$, and IL-17A$^-$IFN-γ$^+$ detected in PBMCs from healthy donors untreated (●) or stimulated with *P. acnes* (○) ($n = 12$). Each symbol represents a unique donor. ***$P \leqslant 0.0001$ and ****$P \leqslant 0.00001$ by two-tailed paired Student's *t*-test for *P. acnes*-exposed PBMCs compared with medium-exposed PBMCs. (**c**) Representative flow-cytometric analysis of CD4 expression among IL-17A$^+$ PBMCs stimulated with *P. acnes*.

exposure (Supplementary Figure S1a online) prompted us to investigate whether these cytokines may have a role in shaping T-cell responses. Sorted CD4$^+$ T cells were stimulated with live *P. acnes* in the presence of various neutralizing mAb. The inhibition of IL-1β resulted in a reduction in IL-17A$^+$ IFN-γ$^-$ and IL-17A$^+$ IFN-γ$^+$ T cells, whereas the neutralization of the IL-12/23-p40 common subunit alone reduced the response of IL-17A$^+$ IFN-γ$^+$ cells but did not affect IL-17A$^+$ IFN-γ$^-$ T cells unless used together with anti-IL-1β (Figure 4). The effect of anti-IL-12/23 blocking mAb on IL-17A$^+$ IFN-γ$^+$ T cells was further enhanced when used in combination with anti-IL-1β mAb. In contrast, tumor necrosis factor-α inhibition did not significantly affect the T-cell response to *P. acnes*. These results suggest that the stimulation of *P. acnes*-induced Th17 and Th17/Th1 T cells can be efficiently prevented by blocking IL-12/23-p40 and IL-1β, whereas the responses of IL-17A$^-$ IFN-γ$^+$ cells are less susceptible to the blockade of these cytokines.

Acne patients exhibit stronger Th17 and Th17/Th1 responses to *P. acnes*

The presence of *P. acnes*-reactive T cells in the blood of healthy individuals raised the question whether these cells could be detected at higher frequencies in acne patients. To address this question and assess the specificity of responding CD4$^+$ T cells, the ability of *P. acnes* to induce Th17 and Th17/Th1 responses was compared with *Staphylococcus aureus* (*S. aureus*). As *S. aureus* could not be used in its live form, cells were stimulated with bacterial lysates. *P. acnes* lysates induced comparable number of IL-17A$^+$IFN-γ$^-$ as well as IL-17A$^+$IFN-γ$^+$ cells as live bacteria (Supplementary Figure S2 online). Then, we compared the response to *P. acnes* and *S. aureus* in healthy donors and in acne patients. In healthy donors, both *P. acnes* and *S. aureus* induced, at a similar extent, a discrete increase of IL-17A$^+$ IFN-γ$^-$ as well as IL-17A$^+$IFN-γ$^+$ CD4$^+$ cells (Figure 5). In contrast, *P. acnes* was able to induce a dramatic increase in both IL-17A$^+$IFN-γ$^-$ and IL-17A$^+$IFN-γ$^+$ CD4$^+$ cells, whereas the induction of both CD4$^+$ subsets remained discrete and comparable with that of healthy donors upon exposure to *S. aureus* lysates (Figure 5). Neither *P. acnes* nor *S. aureus* was able to induce IL-17A$^-$IFN-γ$^+$ cells in both healthy individuals' and acne patients' cells. Altogether, these data show that acne patients have increased frequencies of circulating *P. acnes*-specific Th17 and Th17/Th1 cells.

DISCUSSION

In the present study, we have investigated the adaptive immune response to the skin commensal bacterium *P. acnes*. Previous studies have shown that *P. acnes*-specific Th1 are cells present in early inflamed acne lesions (Mouser *et al.*, 2003). In addition to IFN-γ, we have found significantly increased levels of IL-17A but not IL-4 or IL-22 in acne

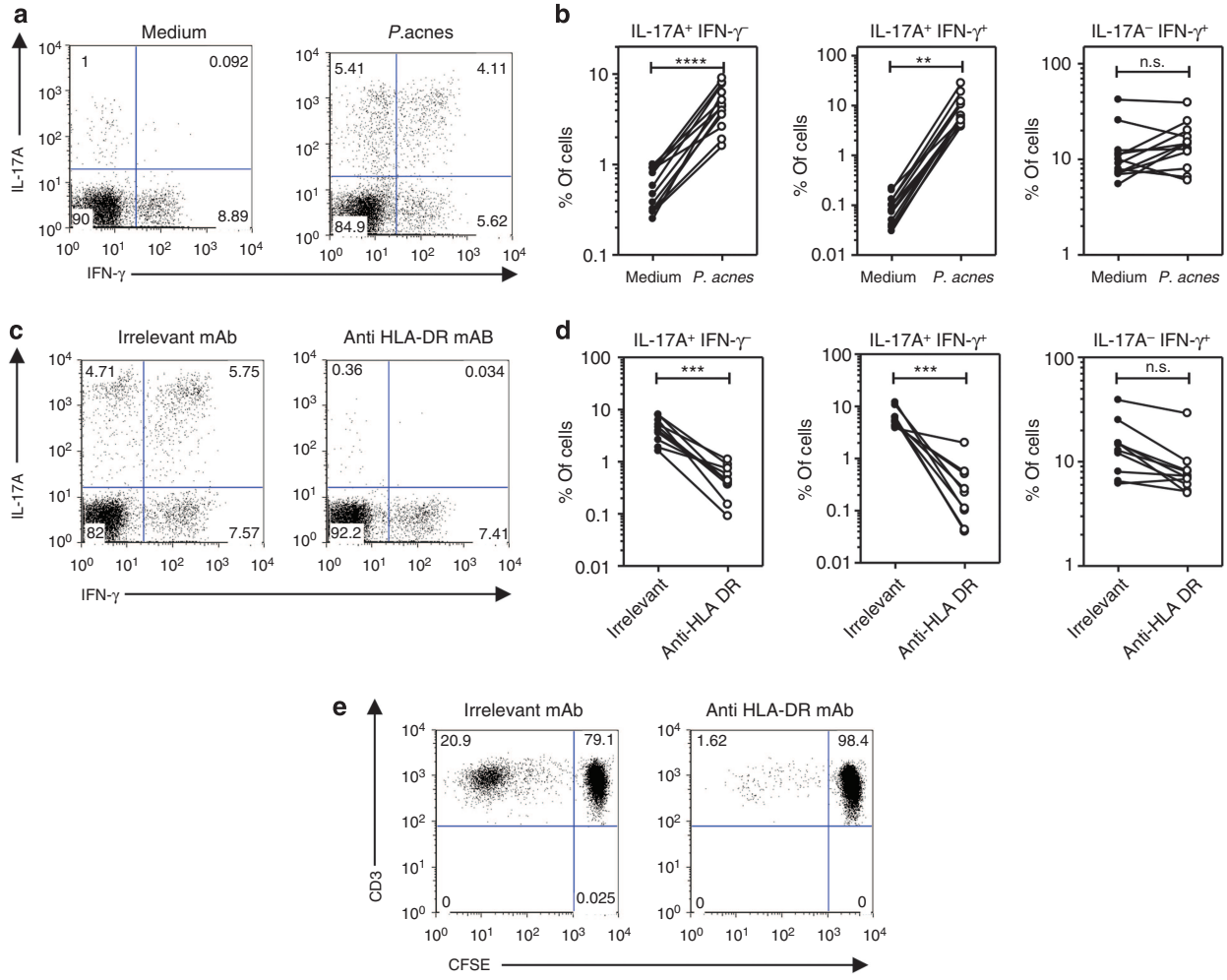

Figure 3. Major histocompatibility complex II (MHC II)-dependent response of CD4$^+$ T cells to *P. acnes* infection. (**a**, **b**) Freshly isolated CD4$^+$ T cells from healthy donors were left untreated or exposed to *P. acnes* in the presence of autologous monocytes. On day 6, cells were stimulated with P/I and stained with antibodies against CD4, IL-17A, and IFN-γ. (**a**) Representative flow-cytometric analysis. (**b**) Percentages of IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$, and IL-17A$^-$IFN-γ$^+$ within CD4$^+$ T cells untreated (●) or stimulated with *P. acnes* (○) ($n=12$). (**c**, **d**) IL-17A and INF-γ production by CD4$^+$ T cells stimulated with *P. acnes* in the presence of irrelevant or anti-HLA-DR-neutralizing mAb determined by intracellular staining following P/I stimulation on day 6. (**c**) Representative flow-cytometric analysis. (**d**) Percentages of IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$, and IL-17A$^-$IFN-γ$^+$ within CD4$^+$ T cells stimulated with *P. acnes* in the presence of irrelevant mAb (●) or anti-HLA-DR mAb (○) ($n=10$). (**e**) Carboxyfluorescein succinimidyl ester (CFSE) profile of CD4$^+$ T cells stimulated with autologous monocytes exposed to *P. acnes* in the presence of irrelevant mAb or anti-HLA-DR mAb. Data are representative of at least three different experiments. (**b**, **d**) Each symbol represents an individual donor. ***$P\leqslant0.0001$ and ****$P\leqslant0.00001$ by two-tailed paired Student's *t*-test. HLA-DR, human leukocyte antigen-DR; P/I, phorbol 12-myristate 13-acetate (PMA)/ionomycin.

biopsies. *In vitro,* we demonstrated that *P. acnes* has the ability to induce the secretion of IL-17A and IFN-γ, therefore confirming the recent observation by Agak *et al.* (2013) that *P. acnes* induces Th17 differentiation. However, in contrast to the data reported in the same study, we show that IL-17A is produced by two distinct subsets of T cells producing either IL-17A alone or in conjunction with IFN-γ. We also observed that Th1 cells could be induced in only 40% of the tested donors, whereas Th17 and Th17/Th1 cells could be induced by *P. acnes* in 100% of them. This is also in apparent contradiction with the recent study by Agak *et al.* (2013), who concluded that both Th17 and Th1 cells could be induced in 100% of the tested PBMC donors. In fact, we show here that, in addition to Th17 and Th1 cells, a third subset secreting both IL-17A and IFN-γ may be involved in the inflammatory response to *P. acnes*. Interestingly, our data suggest that the use of anti-IL-1β and anti-IL-12/IL-23 may represent an efficient strategy for the targeting of Th17/Th1 cells. While anti-IL-1β had a discrete but significant effect on the induction of IL-17A$^+$/IFN-γ$^-$ cells, an anti-IL-12/IL-23-p40 antibody alone did not impact the response of these cells, suggesting that, in this setting, the induction of Th17 cells by *P. acnes* does not require IL-23, as previously reported (Agak *et al.*, 2013). In contrast, anti-IL-1β and anti-IL-12/IL-23-p40 had pronounced additive inhibitory effects on cells producing both IL-17 and IFN-γ, suggesting that IL-1β and IL-12 or/and IL-23 are key cytokines in the differentiation of these cells. We also found elevated levels of IL-21 mRNA in acne lesions, whereas, surprisingly, IL-22 mRNA levels were similar to those found in healthy skin.

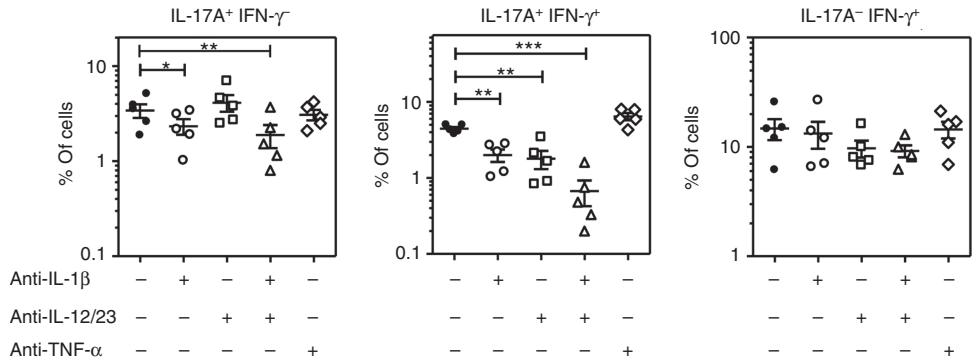

Figure 4. T helper type 17 (Th17) and Th17/Th1 responses are prevented by blocking differentiation cytokines. Freshly isolated CD4$^+$ T cells from healthy donors ($n=5$) were left untreated or exposed to *P. acnes* in the presence of autologous monocytes. On day 6, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin and stained with antibodies to CD4, IL-17A, and IFN-γ. Percentages of IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$, and IL-17A$^-$IFN-γ$^+$ within CD4$^+$ T cells stimulated with *P. acnes* in the absence or presence of the indicated blocking antibodies. $^{**}P\leqslant0.001$ and $^{***}P\leqslant0.0001$ by two-tailed paired Student's *t*-test. Results are presented as mean and SEM.

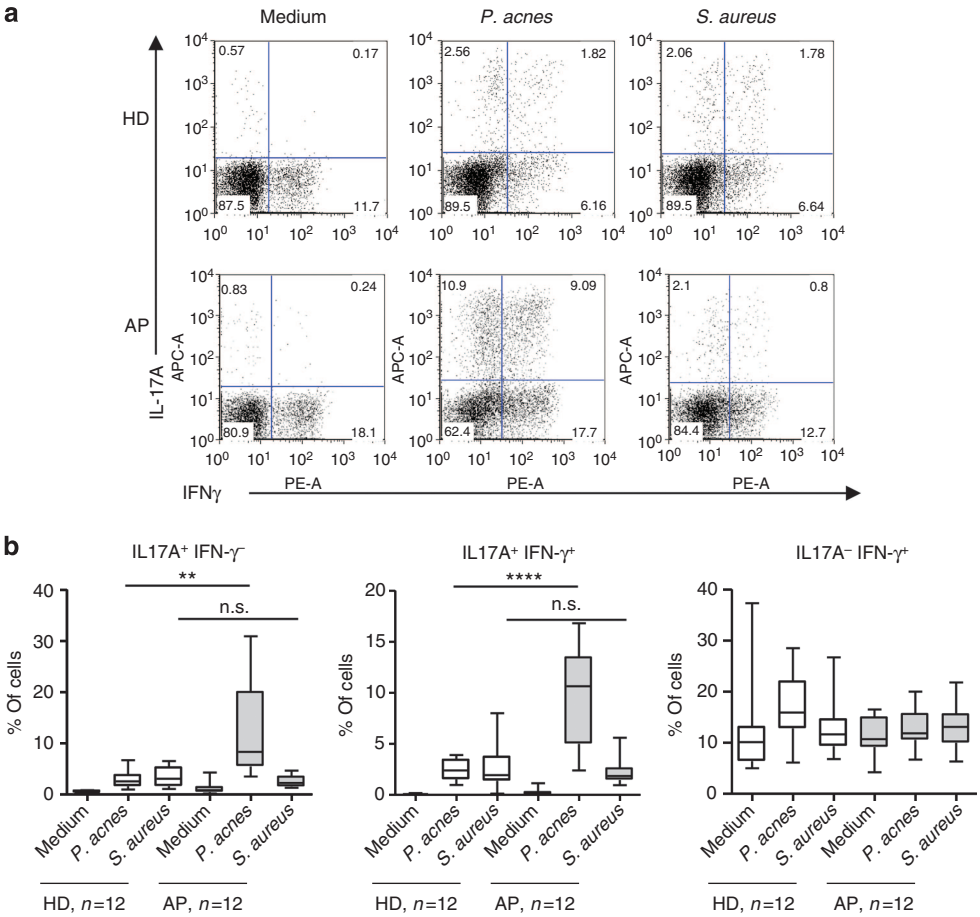

Figure 5. Acne patients have enhanced T-cell responses to *P. acnes*. Freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors (HD, $n=12$) and acne patients (AP, $n=12$) were exposed to bacterial lysates from *P. acnes* or *S. aureus* or incubated with medium. On day 6, cells, after 4.5 hours stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, were subjected to intracellular staining with antibodies against CD4, IL-17A, and IFN-γ. (**a**) Representative flow-cytometric analysis. (**b**) Percentages IL-17A$^+$IFN-γ$^-$, IL-17A$^+$IFN-γ$^+$, and IL-17A$^-$IFN-γ$^+$ detected among CD4$^+$ cells from healthy donors and acne patients untreated or stimulated with *P. acnes* or *S. aureus*. $^{**}P\leqslant0.001$ and $^{****}P\leqslant0.00001$ by two-tailed unpaired Student's *t*-test for HD-PBMCs compared with AP-PBMCs exposed to indicated bacteria.

Th17 effector cytokines, IL-17 and IL-22, have the capacity to stimulate the production of various antimicrobial peptides (Peck and Mellins, 2010). Moreover, IL-17 induces the production of neutrophil-recruiting chemokines such as IL-8 (CXCL8) in epithelial cells (Laan *et al.*, 1999; Ye *et al.*, 2001; Annunziato *et al.*, 2012). It has been previously shown that neutrophils infiltrate late-stage acne lesions (Norris and Cunliffe, 1988) and that the expression levels of IL-8 in acne biopsies are elevated (Trivedi *et al.*, 2006). In addition to its ability to induce pro-inflammatory cytokine secretion in the perifollicular area, *P. acnes* is thought to produce low-molecular weight chemotactic factors, resulting in the accumulation of neutrophils at the site of acne comedones. Our observation that IL-17A is found in acne lesions and that *P. acnes* has the ability to induce IL-17-secreting $CD4^+$ T cells supports an additional T-cell–dependent role of this bacterium in the recruitment of neutrophils at the site of acne lesions.

The coproduction of IFN-γ and IL-17 has been previously observed in memory T cells residing in the gut of patients with Crohn's disease (Annunziato *et al.*, 2007) and in T cells responding to the mycobacterial antigen purified protein derivative (Acosta-Rodriguez *et al.*, 2007). Moreover, it has been shown that *Candida albicans*-specific T cells have the capacity to produce IL-17A in combination with IFN-γ and co-express ROR-γt and T-bet (Zielinski *et al.*, 2012). These cells with a mixed Th17/Th1cytokine profile most likely derive from Th17 subsets exhibiting a certain plasticity and can acquire functional characteristics of Th1 cells (Muranski and Restifo, 2013).

Alhough Th17 cells have been extensively studied, the precise role of Th17/Th1 cells in host defense and diseases remains to be investigated. The development of these mixed T-cell subset depends on the cytokine microenvironment (Zielinski *et al.*, 2012) but also might be induced by chronic stimulation of Th17 cells (Dileepan *et al.*, 2011).

In the present work, we also demonstrate that recall responses could be elicited by *P. acnes* and that such responses are mainly mediated by Th17 and Th17/Th1 cells. Interestingly, the Th17- and Th17/Th1-mediated recall responses could be observed in all the healthy donors tested ($n = 12$), suggesting that such a pool of memory cells is present in a majority of healthy individuals. The increased responses of IL-17A- and IL-17A/IFN-γ-producing $CD4^+$ cells found in individuals with an active disease revealed higher frequencies of circulating *P. acnes*-specific T cells in acne patients. In contrast, responses to *S. aureus* were not enhanced in PBMCs from patients with acne when compared with healthy individuals.

Altogether, our data suggest that $CD4^+$ T cells harboring a Th17 and Th17/Th1 cytokine profile may be involved in the inflammatory response associated with acne. The targeting of effector cytokines—namely, IL-17 and IFN-γ—or differentiation cytokines—namely, IL-1β and IL-23—may represent a new therapeutic approach for the treatment of acne. However, a better knowledge of the precise contribution of these T-cell subsets in the pathogenesis of this common skin disease remains to be further investigated.

MATERIALS AND METHODS

Human samples

All human skin biopsies and peripheral blood samples were collected with informed written consent upon approval from Local Ethical Committees and were conducted according to the Declaration of Helsinki Principles. All acne patients included in this study had a moderate form of acne vulgaris. All of them were 19–25-year-old men who were not treated at the time of biopsy.

Cell sorting

PBMCs were isolated from buffy coats (obtained from healthy donors, Blood Donation Center, Schlieren, Switzerland) and from peripheral blood from acne patients using a density gradient (Ficoll-Paque, Pharmacia, Glattbrugg, Switzerland). $CD4^+$ T cells were sorted from PBMCs by negative selection using magnetic beads ($CD4^+$ T Cell Isolation Kit II, MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) with a purity of 97% (determined by staining with CD3 and CD4 antibodies and flow-cytometric analysis). Monocytes were sorted with anti-CD14-labeled magnetic beads according to the manufacturer's instructions (MACS, Miltenyi Biotech). The purity was over 96% as determined by CD14 staining and flow-cytometric analysis.

Bacteria

P. acnes (DSM 1897, DMSZ, Braunschweig, Germany) was cultured under anaerobic conditions as previously described (Kistowska *et al.*, 2013). Bacteria were harvested by centrifugation at 5,000 r.p.m. for 10 minutes, washed, and suspended in phosphate-buffered saline (PBS) or medium for experiments. To prepare bacterial lysates, bacterial pellets were resuspended in PBS and heat inactivated for 1 hour at 65 °C and then subjected to five freeze–thaw cycles followed by two rounds of sonication (5 minutes each). Protein concentration was measured using the bicinchoninic acid assay (BCA Protein Assay Reagent, Pierce, Rockford, IL) according to the manufacturer's instructions.

T-cell–stimulation assays

Cells were cultured in RPMI 1640 medium (Invitrogen, Basel, Switzerland), supplemented with 5% human serum (Blood Donation Center, Schlieren, Switzerland), 1% Antibiotic-Antimycotic (Invitrogen, Life Technologies, Zug, Switzerland), 1 mM sodium pyruvate (Invitrogen), and 2 mM GlutaMAX solution (Invitrogen). PBMCs from healthy donors and patients were exposed to live *P. acnes* at the indicated MOI or to bacterial lysates (2.5 μg ml^{-1}) for 6 days. Sorted $CD4^+$ T cells were cocultured for 6 days with autologous monocytes (ratio 2:1) pre-exposed to live *P. acnes* at the indicated MOI. In some experiments, T-cell activation was performed in the presence of the following neutralizing antibodies (all at 10 μg ml^{-1}): anti-human leukocyte antigen HLA-DR (L243, kindly provided by Prof G. De Libero, Basel, Switzerland), irrelevant mouse IgG2a (MG2a-53, Biolegend, San Diego, CA), anti-IL-1β (Canakinumab, Novartis, Basel, Switzerland), anti-IL-12/IL-23 (Ustekinumab, Janssen Biotech, Zug, Switzerland), and tumor necrosis factor-α inhibitor (5 μg ml^{-1} Etanercept, Pfizer, Zürich, Switzerland).

Cytokine analysis

Cell culture supernatants were collected at indicated time points, and the following cytokines were measured by ELISA: IL-1β IL-6, IL-17A, IFN-γ IL-22 (R&D Systems, Minneapolis, MN), IL-4, IL-12p70 (Biolegend), and IL-23 (eBioscience, San Diego, CA). All ELISAs were

performed according to the manufacturer's instructions. For intracellular cytokine stainings, cells were stimulated at indicated time points for 4.5 hours with phorbol-12-myristate-13-acetate (50 ng ml^{-1}, Sigma-Aldrich, Buchs, Switzerland) and ionomycin (500 ng ml^{-1}, Sigma-Aldrich) in the presence of brefeldin A (5 μg ml^{-1}, Sigma-Aldrich). Cells were fixed with paraformaldehyde (2%, Sigma-Aldrich), permeabilized with saponin (0.1%, Sigma-Aldrich), and stained with anti-IL-17A (BL168), anti-IFN-γ (B27), anti-IL-4 (MP4-25D2), and anti-IL-22 (BG/IL-22) (all from Biolegend) and were analyzed by flow cytometry (FacsCanto A, Becton-Dickinson, Basel, Switzerland). Flow-cytometric data were analyzed by FlowJo (Tree Star, Ashland, OR).

Gene expression analysis

Total RNA was isolated from human skin samples obtained from healthy donors ($n=6$) or from acne patients ($n=12$) using the RNeasy Fibrous Tissue Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. RNA was converted into cDNA by standard reverse transcription with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Life Technologies, Carlsbad, CA). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies). The following primers were used:

RPL27: forward 5′-ATCGCCAAGAGATCAAAGATAA-3′, reverse 5′-TCTGAAGACATCCTTATTGACG-3′

IFN-γ: forward 5′-TCGGTAACTGACTTGAATGTCCA-3′, reverse 5′-TCGCTTCCCTGTTTTAGCTGC-3′

IL-4: forward 5′-CCGTAACAGACATCTTTGCTGCG-3′, reverse 5′-GAGTGTCCTTCTCATGGTGGCT-3′

IL-17A: forward 5′-CAATCCCACGAAATCCAGGATG-3′, reverse 5′-GGTGGAGATTCCAAGGTGAGG-3′

IL-21: forward 5′-CCAAGGTCAAGATCGCCACATG-3′, reverse 5′-TGGAGCTGGCAGAAATTCAGGG-3′

IL-22: forward 5′-GCTTGACAAGTCCAACTTCCA-3′, reverse 5′-GCTCACTCATACTGACTCCGTG-3′

STAT3: forward 5′-CTTTGAGACCGAGGTGTATCACC-3′, reverse 5′-GGTCAGCATGTTGTACCACAGG-3′

STAT4: forward 5′-CAGTGAAAGCCATCTCGGAGGA-3′, reverse 5′-TGTAGTCTCGCAGGATGTCAGC-3′

STAT5a: forward 5′-GTTCAGTGTTGGCAGCAATGAGC-3′, reverse 5′-AGCACAGTAGCCGTGGCATTGT-3′

RORC: forward 5′-GAGGAAGTGACTGGCTACCAGA-3′, reverse 5′-GCACAATCTGGTCATTCTGGCAG-3′

TBX-21: forward 5′-ATTGCCGTGACTGCCTACCAGA-3′, reverse 5′-GGAATTGACAGTTGGGTCCAGG-3′

GATA3: forward 5′-ACCACAACCACACTCTGGAGGA-3′, reverse 5′-TCGGTTTCTGGTCTGGATGCCT-3′

CXCR3: forward 5′-ACGAGAGTGACTCGTGCTGTAC-3′, reverse 5′-GCAGAAAGAGGAGGCTGTAGAG-3′

CRTh2: forward 5′-TGGAGTCATCCTCTTCGTGGTG-3′, reverse 5′-AGTAGGTGAAGAAGGGCAGGGA-3′

CCR4: forward 5′-CTCTGGCTTTTGTTCACTGCTGC-3′, reverse 5′-AGCCCACAGTATTGGCAGAGCA-3′

CCR6: forward 5′-CTGAACCCTGTGCTCTACGCTT-3′, reverse 5′-CACAGGAGAAGCCTGAGGACTT-3′

The real-time PCR included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 1 minute, and 72 °C for 1 minute, and one cycle of 95 °C for 1 minute, 55 °C for 30 seconds, and 95 °C for 30 seconds.

Immunohistochemistry

Human acne skin samples ($n=12$) and normal skin samples ($n=3$) from distinct individuals were cross-sectioned, and immunohistochemistry stainings were performed. After deparaffinization by heat and ascending alcohol series, the slides were stored in Retrieval Solution (DAKO, Agilent Technologies, Santa Clara, CA) and heated under pressure in a pressure cooker for 25 minutes for antigen demasking. Unspecific binding sites were blocked by incubation in 5% (wt/v) BSA and 5% (v/v) normal goat serum (DAKO) in PBS for 1 hour. Antibodies against IFN-γ (Abcam, Cambridge, UK) and Interleukin-17 (Abcam) were diluted in antibody diluent (DAKO S0809), and an appropriate isotype control antibody was substituted for every primary antibody on a separate section. Slides were washed with PBS, and secondary antibody goat anti-rabbit (Southern Biotech, Birmingham, AL) was added and incubated for 1 hour at room temperature. Slides were again washed with PBS and mounted with an Avidin–Biotin complex (Vectastain, Vector Laboratories, Burlingame, CA). After 45 minutes, incubation slides were washed with PBS and mounted with (3,3′-diaminobenzidine) HRP substrate (Vectastain) to produce a brown reaction product. After the washing steps, a counterstain with hematoxylin was performed. The sections were mounted in mounting medium (DAKO) and imaged using Aperio ScanScope (Leica, Nussloch, Germany).

Statistics

The statistical analysis for quantitative reverse transcriptase in real time on human samples was performed using the Mann–Whitney two-tailed test. Data obtained from *in vitro* experiments were subjected to two-tailed paired or unpaired Student's *t*-test as indicated. Differences were considered significant when $*P \leqslant 0.05$, $**P \leqslant 0.001$, $***P \leqslant 0.0001$, and $****P \leqslant 0.00001$. All statistical analyses were performed using GraphPad Prism software (La Jolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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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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