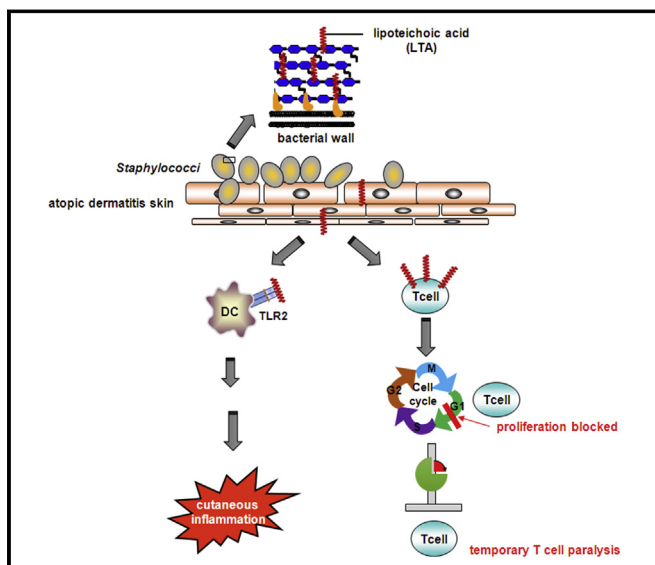


***Staphylococcus aureus*–derived lipoteichoic acid induces temporary T-cell paralysis independent of Toll-like receptor 2**

Susanne Kaesler, PhD,^{a,b,*} Yuliya Skabytska, PhD,^{a,*} Ko-Ming Chen, MD, DSc,^{a,f,*} Wolfgang E. Kempf, MSc,^{a,b} Thomas Volz, MD,^{a,b} Martin Köberle, PhD,^{a,b} Florian Wölbing, MD,^{a,b} Ulrike Hein, MD,^a Thomas Hartung, MD, PhD,^{c,d} Carsten Kirschning, PhD,^e Martin Röcken, MD,^a and Tilo Biedermann, MD^{a,b} *Tübingen, Munich, Konstanz, Essen, and Düsseldorf, Germany, and Baltimore, Md*

GRAPHICAL ABSTRACT



Background: The interplay between microbes and surface organs, such as the skin, shapes a complex immune system with several checks and balances. The first-line defense is mediated by innate immune pathways leading to inflammation. In the second phase specific T cells invade the infected organ, amplifying inflammation and defense. Consecutively, termination of inflammation is crucial to avoid chronic inflammation triggered by microbes, such as in patients with atopic dermatitis.

Objective: We aimed to elucidate how the *Staphylococcus aureus*–derived cell-wall component lipoteichoic acid (LTA) governs the second phase of immune responses when high concentrations of LTA access T cells directly through disrupted skin.

Methods: We analyzed the direct exposure of T cells to LTA *in vitro*. For *in vivo* analyses, we used fluorescein isothiocyanate contact hypersensitivity and ovalbumin-induced dermatitis as models for T_H2-mediated cutaneous inflammation.

Results: We observed that LTA potently suppressed T-lymphocyte activation in a Toll-like receptor 2–independent manner. LTA-exposed T cells did not proliferate and did not produce cytokines. Importantly, these T cells remained completely viable and were responsive to consecutive activation signals on subsequent removal of LTA. Thus LTA exposure 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.

Conclusion: We identified a new mechanism through which bacterial compounds directly but temporarily modulate adaptive immune responses. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Lipoteichoic acid, T cell, temporary unresponsiveness, *Staphylococcus aureus*, atopic dermatitis, cell cycle

Surface organs, such as the skin, are developed under the pressure of constant exposure to microbes and microbial products. As a consequence, a highly complex and regulated immune

From ^athe Department of Dermatology, Eberhard Karls University, Liebermeisterstr. Tübingen; ^bthe Department of Dermatology and Allergy, Technische Universität München, Munich; ^cthe Center for Alternatives to Animal Testing Europe, University of Konstanz; ^dJohns Hopkins University, Bloomberg School of Public Health, Baltimore; ^ethe Institute of Medical Microbiology, University of Duisburg-Essen, Essen; and ^fDerma Labor Düsseldorf, Düsseldorf.

*These authors contributed equally to this work.

Supported by contract research “Allergologie 2” of the Baden-Württemberg Stiftung (P-LS-AL2/4) and by grants of the Deutsche Forschungsgemeinschaft (DFG; B1696/10-1, B1696/5-1, B1696/5-2, SFB 685 A06, C01, SFB 824 B10) and Wilhelm Sander-Stiftung (2012.056.1).

Disclosure of potential conflict of interest: S. Kaesler has received grants from Landesstiftung Baden-Württemberg and Deutsche Forschungsgemeinschaft. K.-M. Chen has received a grant from Baden-Württemberg Stiftung. T. Volz has received payment for lectures from La Roche Posay. M. Köberle has received grants from Deutsche Forschungsgemeinschaft (B1696/5-1, B1696/5-2). U. Hein has received a grant from Landesstiftung Baden-Württemberg. M. Röcken has received grants from Deutsche Forschungsgemeinschaft (SFB TRR 156/1 TP B 06, RO 764/15-1 AOBJ);

has consultant arrangements with Almirall Hermal, Biogen Idec, and Regeneron; is employed by Government Baden-Württemberg; has a pending patent (DE 10 2012 024 749.4); and has received travel support from Deutsche Dermatologische Gesellschaft e. V. and European Academy of Dermatology and Venereology. T. Biedermann has received grants from Deutsche Forschungsgemeinschaft (B1696/10-1, B1696/5-1, B1696/5-2, SFB 685, SFB 824), Helmholtz, Novartis, and Phadia; has received payment for development of educational presentations from Phadia; and has consultant arrangements with Alk-Abelló, Astellas, Bencard, Biogen, Janssen, Leo, Meda, MSD, Novartis, Phadia, and Thermo Fisher. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 9, 2015; revised November 9, 2015; accepted for publication November 20, 2015.

Corresponding author: Tilo Biedermann, MD, Technische Universität München, Department of Dermatology and Allergy, Biedersteinerstr 29, 80802 Munich. E-mail: Tilo.Biedermann@tum.de.

0091-6749/\$36.00

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2015.11.043>

Abbreviations used

AD:	Atopic dermatitis
CFU:	Colony-forming units
CHS:	Contact hypersensitivity
dLN:	Draining lymph node
FITC:	Fluorescein isothiocyanate
LN:	Lymph node
LTA:	Lipoteichoic acid
MyD88:	Myeloid differentiation primary response gene-88
OVA:	Ovalbumin
PAMP:	Pathogen-associated molecular pattern
TCR:	T-cell receptor
TLR:	Toll-like receptor
WT:	Wild-type

system evolved to conserve the integrity of both the surface organ and the whole organism.

Cutaneous infections effectively induce adaptive immune responses, even allowing the first vaccination strategy to successfully eradicate vaccinia virus.¹ On the other hand, chronic inflammatory diseases of the gut, such as Crohn disease, or of the skin, such as atopic dermatitis (AD), are believed to be triggered by microbes²⁻⁵ and mediated by T cells, which migrate into the gut or skin to initiate and maintain inflammation.⁶⁻⁹ Initiation and maintenance of T cell-mediated inflammation is regulated differently because clinically unaffected skin in patients with AD already contains a sparse perivascular T-cell infiltrate in the absence of detectable signs of dermatitis,^{10,11} and compared with normal nonatopic skin, clinically unaffected skin in patients with AD harbor an increased number of T_H2 cells,¹² which are known to dominate early visible AD lesions.¹²

Staphylococcus aureus most commonly elicits skin, wound, and systemic infections and is a major pathogen in both community-acquired and nosocomial infections.^{13,14} Even minor infections lead to specific elicitation of *S aureus*-specific adaptive immune responses in most subjects, who also had long-lived immune memory.¹⁵ *S aureus* and *S aureus*-derived substances trigger skin inflammation. More than 90% of patients with AD have *S aureus* colonization or infection, and effective treatment protocols for these patients include antiseptics.¹⁶ The presence of microbes on the skin might elicit or increase an immune response, and constituents of *S aureus* function as proinflammatory adjuvants. Pathogen-associated molecular patterns (PAMPs) that activate resident skin cells^{3,17,18} have been identified for *S aureus*. Lipoteichoic acid (LTA), lipoproteins, and peptidoglycan are part of the bacterial cell wall of *S aureus* and bind Toll-like receptor (TLR) 2, which is expressed by several immune cells present in the skin.¹⁹⁻²³ *S aureus* 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*.²⁴ Moreover, Travers et al²⁴ showed that similar LTA levels are found in many infected AD lesions, and they demonstrated a correlation between the amount of LTA in AD lesions and AD aggravation. This study indicated that LTA plays a role in exacerbating AD. Indeed, we recently showed in a mouse model of AD that TLR2 ligands potently aggravate inflammation, leading to chronic dermatitis.³ Using the TLR2 ligands LTA or lipoprotein Pam2 in adoptive transfer experiments, we have found that activation of TLR2 in combination with IL-4 enhances cutaneous inflammation and contributes to its chronification. These findings

corroborate other models of AD, in which the development of skin lesions depends on conventional housing conditions for the animals. Animals do not have dermatitis lesions when kept in specific pathogen-free conditions, indicating a role for microbes and PAMPs in patients with AD.²⁵ On the other hand, we showed that cutaneous microbes were able to ameliorate AD inflammation by inducing regulatory T cells and IL-10.²⁶ Moreover, our group discovered recently that activation of TLR2/6 in the skin leads to immune suppression mediated by myeloid-derived suppressor cells.²⁷ Thus different mechanisms to terminate and limit inflammation have evolved to avoid tissue damage or as immune escape mechanisms for microorganisms. Given the role of cutaneous microbiota for tolerance induction, elucidation of these mechanisms is pivotal for understanding the regulation of the microbial-cutaneous immune interface. LTA is a surface-associated macroamphiphilic adhesion molecule of Gram-positive bacteria. It is released during bacterial cell lysis and during bacterial cell wall turnover in the course of normal cell growth and maintenance.²⁸ The physiochemical properties of LTA are postulated to be similar to those of LPS in Gram-negative bacteria.¹³ Notably, 10 µg/mL LTA or greater from *S aureus* stimulates the production of multiple proinflammatory cytokines and chemokines in different leukocytes.²⁹⁻³¹ Thus LTA activates the innate immune system, but its direct effects on the adaptive immune system are unclear. Our purpose was to study the effect of the Gram-positive cell-wall component LTA on T-lymphocyte function.

We show that exposure to LTA during initial contacts with fluorescein isothiocyanate (FITC) significantly suppressed lesional T-cell cytokine expression. The proliferation of T cells from the draining lymph nodes (dLNs) of LTA-exposed skin was also significantly reduced. Moreover, LTA also significantly suppressed T-cell proliferation *in vitro*, which was independent of TLR2 signaling. LTA-exposed T cells were viable, and LTA exposure did not induce T-cell apoptosis. FITC-induced contact hypersensitivity (CHS) and ovalbumin (OVA)-induced dermatitis were investigated as models for T_H2-mediated cutaneous inflammation in patients with AD to investigate whether T-cell suppression by LTA is operative *in vivo*. LTA potently suppressed both FITC-induced CHS and OVA-induced dermatitis independent of TLR2.

In sum, T cell-mediated immune responses are susceptible to LTA-induced immune suppression. These newly discovered consequences of LTA exposure might be functional after effective immune defense of *S aureus* to terminate inflammation. Likewise, this pathway represents another means of immune escape by *S aureus* or a mechanism through which Gram-positive bacteria avoid initiation of adaptive immune responses. Importantly, this LTA-induced T-cell paralysis is temporary in nature, avoiding prolonged periods of immune suppression.

METHODS

Animals

Pathogen-free, 6- to 12-week-old wild-type (WT) BALB/c and C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). *Myd88*^{-/-} BL/6 mice were provided by Akira's group (Osaka University, Osaka, Japan), and *Thr2*^{-/-} mice were from C. Kirschning (University Duisburg-Essen). All mice were kept under specific pathogen-free conditions in accordance with the guidelines of the Federation of European Laboratory Science Association in the animal facility of Eberhard Karls University. Age-matched female mice were used. All animal experiments were in compliance with both European Union and German laws and approved by the local authorities (HT1/10).

FITC-induced antigen-specific CHS

BALB/c mice were sensitized by means of administration of 0.5% FITC (Sigma-Aldrich, Taufkirchen, Germany) solution (dissolved in 1:1 acetone/dibutyl phthalate) on the shaved abdomen on days 0 and 1. After 6 days, mice were challenged by means of application of 0.5% FITC solution on both sides of the ears. Ear thickness was measured with a micrometer (Kroeplin, Schlüchtern, Germany), and data were expressed as change in ear thickness before treatment. For *ex vivo* analysis, dLN cells were isolated and stimulated with FITC (10 μ g/mL) or anti-CD3/anti-CD28.

Real-time PCR analysis

Total RNA was isolated with a Macherey-Nagel isolation kit (Düren, Germany) and reverse transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). Primers were as follows: *Ifn γ* forward 5'-CTCTGAGACAATGAACGCTAC-3' and reverse 5'-TCTTCCACATC TATGCCACTT-3'; *Il4* forward 5'-GACGGCACAGAGCTATTGATG-3' and reverse 5'-ACCTTGAAGCCCTACAGACG-3'; and *Il10* forward 5'-CAA CATACTGCTAACCGACTC-3' and reverse 5'-CATTTCATGGCCTTGTA GAC-3'. Real-time PCR assay was carried out with an iCycler (Bio-Rad). Values were normalized to hypoxanthine phosphoribosyltransferase (*Hprt*).

CD4⁺ T-cell isolation and stimulation for *in vitro* experiments

Murine CD4⁺ T cells were purified with a CD4⁺ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Human PBMCs, including CD4⁺ T cells, were obtained from heparinized blood samples from healthy volunteers by means of density gradient centrifugation (800g for 30 minutes). T cells were stimulated with 4 μ g/mL (murine) or 0.5 μ g/mL (human) anti-CD3 and 2 μ g/mL (murine) or 0.5 μ g/mL (human) anti-CD28 (BioLegend, San Diego, Calif). For some assays, phorbol 12-myristate 13-acetate/ionomycin (1 ng/mL; 500 ng/mL), concanavalin A (2 μ g/mL), *Staphylococcus enterotoxin B* (4 μ g/mL), or TGF- β (2 ng/mL) was added.

Proliferation assays

For proliferation assays, cells were seeded in 96-well plates and stimulated. After 3 days, 0.25 μ Ci of tritiated thymidine (GE Healthcare, Freiburg, Germany) was added to each well, and cells were harvested 12 to 18 hours later. Incorporated tritium was measured with a MicroBeta counter (PerkinElmer, Wiesbaden, Germany).

Western blot analysis

Protein extracts from ear dLNs were separated on 18% Tris-HCl gradient gel along with 10 ng of LTA as a positive control and transferred to polyvinylidene difluoride membrane. The blot was probed with mAbs against LTA (QED Bioscience, San Diego, Calif) and β -actin (Merck Millipore, Schwalbach, Germany) followed by IRDye-conjugated secondary antibodies (Li-Cor Biotechnology, Bad Homburg, Germany) and scanned with an Odyssey Infrared Imaging System (Li-Cor Biotechnology).

Apoptosis detection

Apoptosis and necrosis detection was performed with an Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, Calif).

Cell-cycle analysis

Cell-cycle analyses were performed with APC BrdU Flow Kits (BD Biosciences).

Adoptive transfer

FITC-induced CHS model. Naive mice serving as donors were sensitized with FITC on days -15, -14, -8, -7, -1, and 0. On day 6, CD3⁺

cells were isolated from secondary lymphoid organs with a Pan T Cell Isolation Kit (Miltenyi Biotec) and transferred into recipient mice that were previously sensitized with FITC. The next day, recipients were injected with LTA or PBS and challenged with FITC or vehicle control (see Fig E1, A, in this article's Online Repository at www.jacionline.org).

OVA-induced dermatitis model. OVA-specific T cells were harvested from OVA-sensitized mice and expanded *in vitro* under T_H2-promoting conditions. For adoptive transfer, 1×10^6 OVA-specific T_H2 cells and 5 μ g of OVA protein with or without 10 μ g of LTA were intracutaneously injected into the ear skin of WT and *Tlr2*^{-/-} BL/6 recipient mice (see Fig E1, B). Ear thickness was measured with a micrometer and expressed as the change in ear thickness after treatment.

Statistical analysis

All figures represent one of at least 2 independently performed experiments. Unless otherwise stated, quantitative results are expressed as means $\pm$ SDs, and differences were compared by using the unpaired Student *t* test with the Dunnett *post hoc* test for correction of multiple testing. Analysis was performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). *P* values of less than .05 were considered statistically significant and marked as follows. Additional information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

LTA exposure during cutaneous inflammation suppresses T-cell cytokines and T-cell proliferation

Chronic inflammatory diseases of the skin, such as AD, are believed to be triggered by microbes. We first examined whether TLR2 ligands can transform nonlesional skin with a detectable but sparse T-cell infiltrate, as found in patients with AD,^{10,11} into overt dermatitis. CHS to the hapten FITC after up to 6 repetitive sensitizations with FITC is associated with FITC-specific IgE and T_H2 cells (see Fig E2 in this article's Online Repository at www.jacionline.org). We now established a model in which the weak hapten FITC was applied only twice to the shaved abdomens of mice before an FITC challenge was applied to the ear skin. In this model no obvious ear swelling was detectable (see Fig E3 in this article's Online Repository at www.jacionline.org). However, T-cell recruitment to the skin can be identified because T-cell cytokine levels were significantly upregulated 4 to 8 hours after challenge (data not shown). Moreover, FITC-specific T cells were readily detectable in these mice. Thus this model of mild T-cell inflammation is ideal for identifying microbial constituents that trigger and/or amplify cutaneous inflammation. Surprisingly, exposure to up to 40 μ g of LTA per ear did not significantly amplify ear swelling in mildly FITC-sensitized animals. Therefore we investigated the dynamics of T-cell cytokine expression in animals exposed to either LTA or PBS (Fig 1, A). At 8 hours after challenge, cutaneous *Il4* mRNA expression was suppressed by greater than 10-fold after LTA exposure. *Ifn γ* mRNA levels were reduced by a factor of 3, and *Il10* expression was unchanged (Fig 1, B). Western blot analysis confirmed LTA in skin dLNs at 8 hours after application (Fig 1, C). dLNs were prepared after exposure to FITC with or without LTA to identify the functional consequences of LTA exposure *in vivo*. *Ex vivo* stimulation of T cells with anti-CD3/CD28 antibodies, as well as FITC, demonstrated significantly reduced T-cell proliferation (Fig 1, D), indicating consequences of LTA exposure on both FITC-specific T cells and T-cell bystanders. This suppressive effect was not systemic because proliferation of splenic T cells was not affected by local LTA application (data not shown).

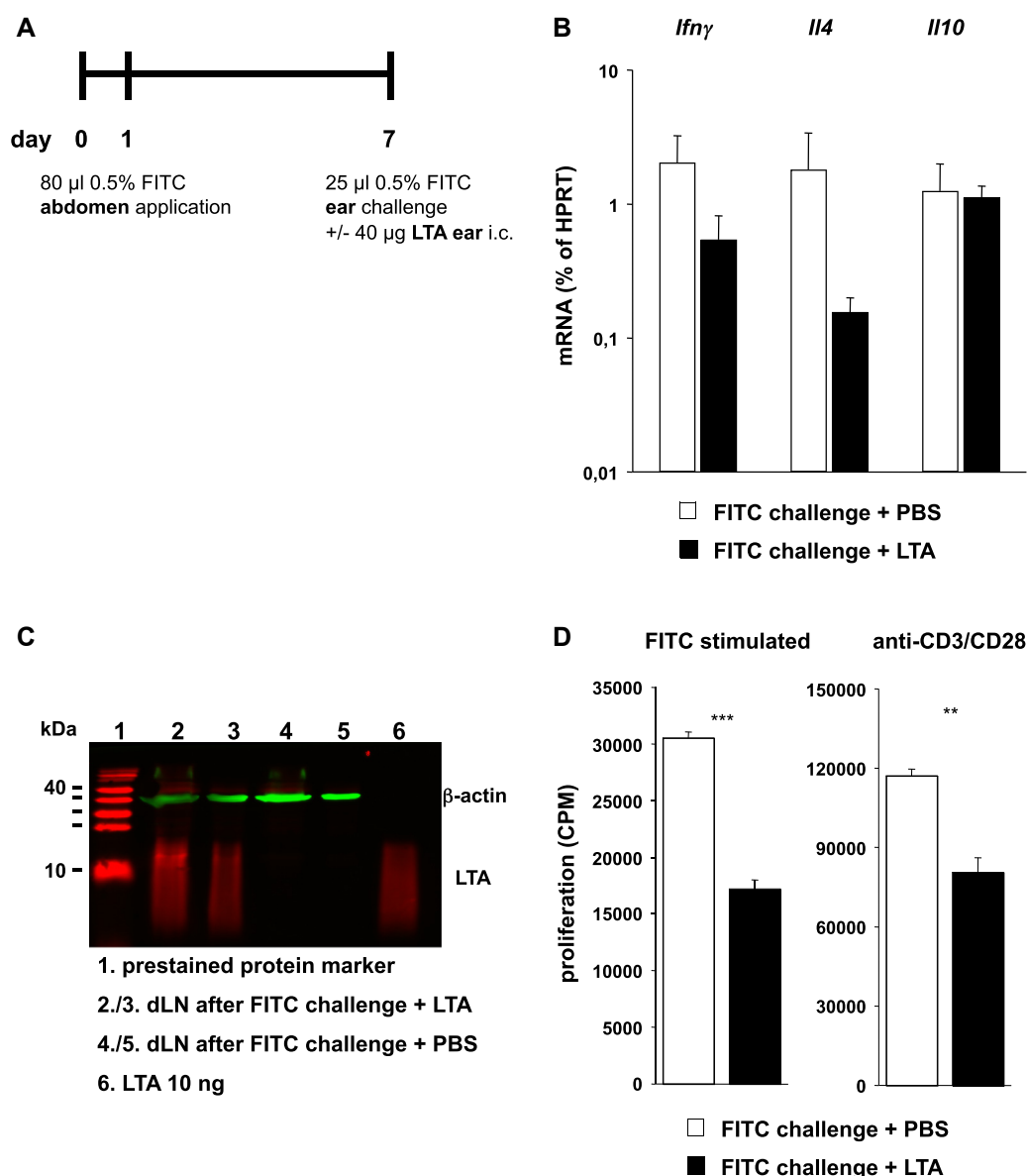


FIG 1. Cutaneous LTA suppresses cutaneous inflammation. **A**, Mice were sensitized with FITC (days 0 and 1) and challenged (day 7). LTA was applied intracutaneously (*i.c.*). **B**, mRNA expression was analyzed by using quantitative RT-PCR (normalized to *Hprt*, mean $\pm$ SD, $n = 4$ for both groups). **C**, Western blot analysis of ear dLNs isolated 8 hours after challenge with or without LTA. The blot was stained for LTA (red) and β -actin (green). **D**, Ear skin dLN cells were stimulated *ex vivo* with FITC or anti-CD3/CD28 antibodies, and their proliferation was determined based on tritiated thymidine uptake (mean $\pm$ SD). ** $P < .01$ and *** $P < .001$.

LTA directly suppresses CD4⁺ T-cell proliferation and cytokine production *in vitro*

CD4⁺ T cells were isolated from untreated BALB/c mice and activated by anti-CD3/anti-CD28 antibodies *in vitro* to determine whether LTA mediated immune suppression by directly targeting T cells. These T cells were then treated with the TLR2 ligands Pam2Cys, staphylococcal LTA, or control medium. Pam2Cys was a potent T-cell costimulator, acting in a dose-dependent manner and reaching a maximum at 10 μ g/mL, as previously published (Fig 2, A).³² In contrast, at the same concentration of 10 μ g/mL, LTA significantly suppressed T-cell proliferation (Fig 2, A). Moreover, increasing LTA concentrations further enhanced T-cell suppression, almost completely abrogating T-cell

proliferation at 100 μ g/mL (Fig 2, A). Because these experiments were performed with murine T cells, we next investigated human T cells. LTA most potentially inhibited human T-cell activation that was elicited by T-cell receptor (TCR) activation, such as with anti-CD3/CD28 antibodies and superantigen *Staphylococcus enterotoxin B* (Fig 2, B). This result confirmed the functional relevance of LTA in patients with AD because staphylococcal superantigens have been implicated in exacerbating this disease.³³ Interestingly, LTA also blocked human T-cell activation induced by mitogens, although with less potency (Fig 2, B). Importantly, LTA not only affected T-cell proliferation but also inhibited production of T-cell cytokines, such as IFN- γ and IL-4, in murine (Fig 2, C) and human (data not shown) T cells. This suppression

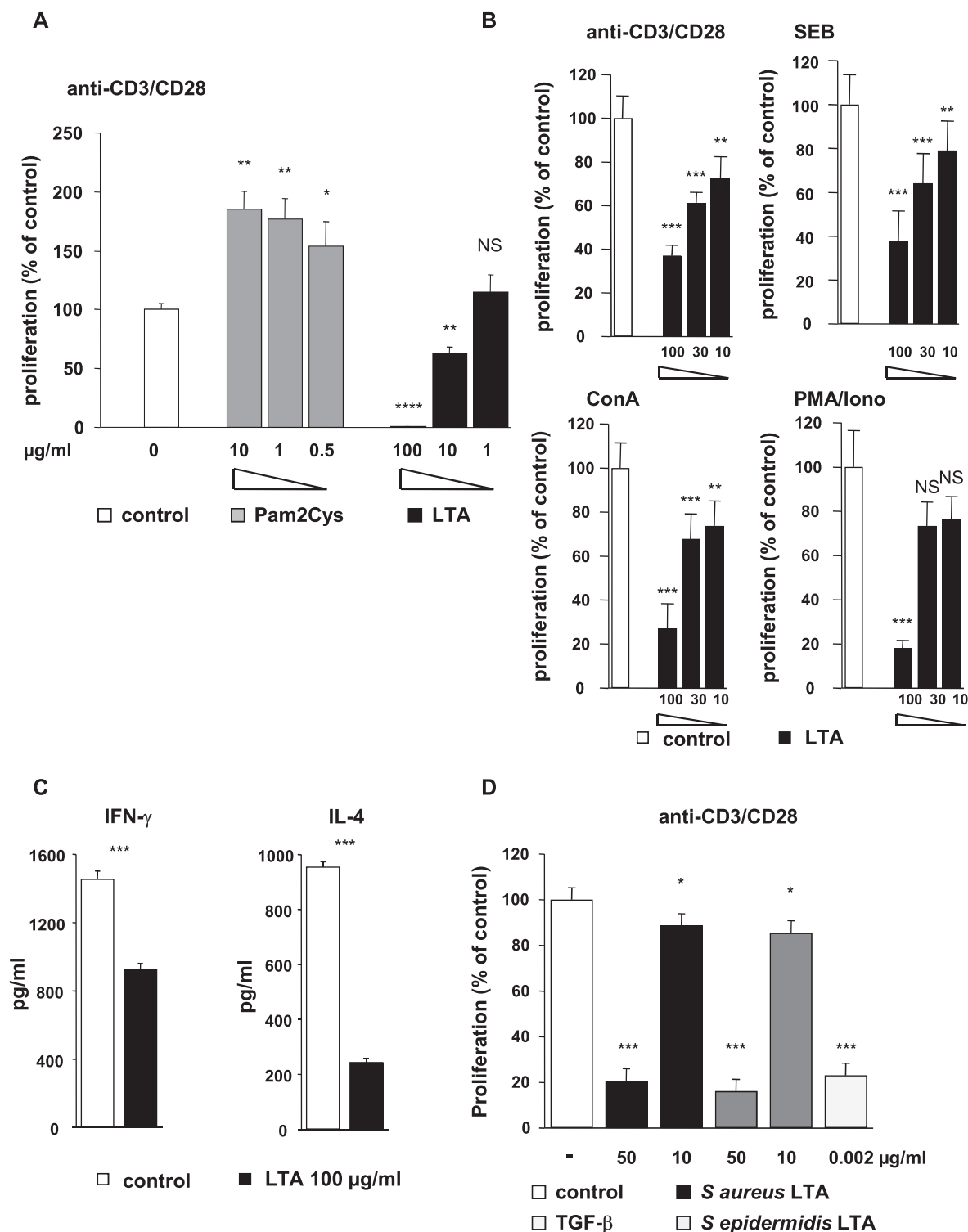


FIG 2. LTA exposure suppresses murine and human T-cell proliferation and cytokine production. **A** and **B**, Proliferation of murine T cells (Fig 2, **A**) or human (Fig 2, **B**) PBMCs was analyzed (mean $\pm$ SD) after stimulation under the following conditions: Fig 2, **A**, anti-CD3/CD28 antibodies, Pam2Cys, or LTA; Fig 2, **B**, anti-CD3/CD28 antibodies, phorbol 12-myristate 13-acetate/ionomycin (*PMA/Iono*), concanavalin A (*ConA*), *Staphylococcus enterotoxin B* (*SEB*), or LTA. **C**, T-cell cytokines were analyzed after activation and exposure to LTA. **D**, Murine CD4⁺ T cells were stimulated by anti-CD3/CD28 antibodies; *S aureus* LTA, *S epidermidis* LTA, or TGF- β was added; and proliferation was analyzed by using tritiated thymidine uptake relative to the control. Controls were set as 100%. NS, Not significant. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

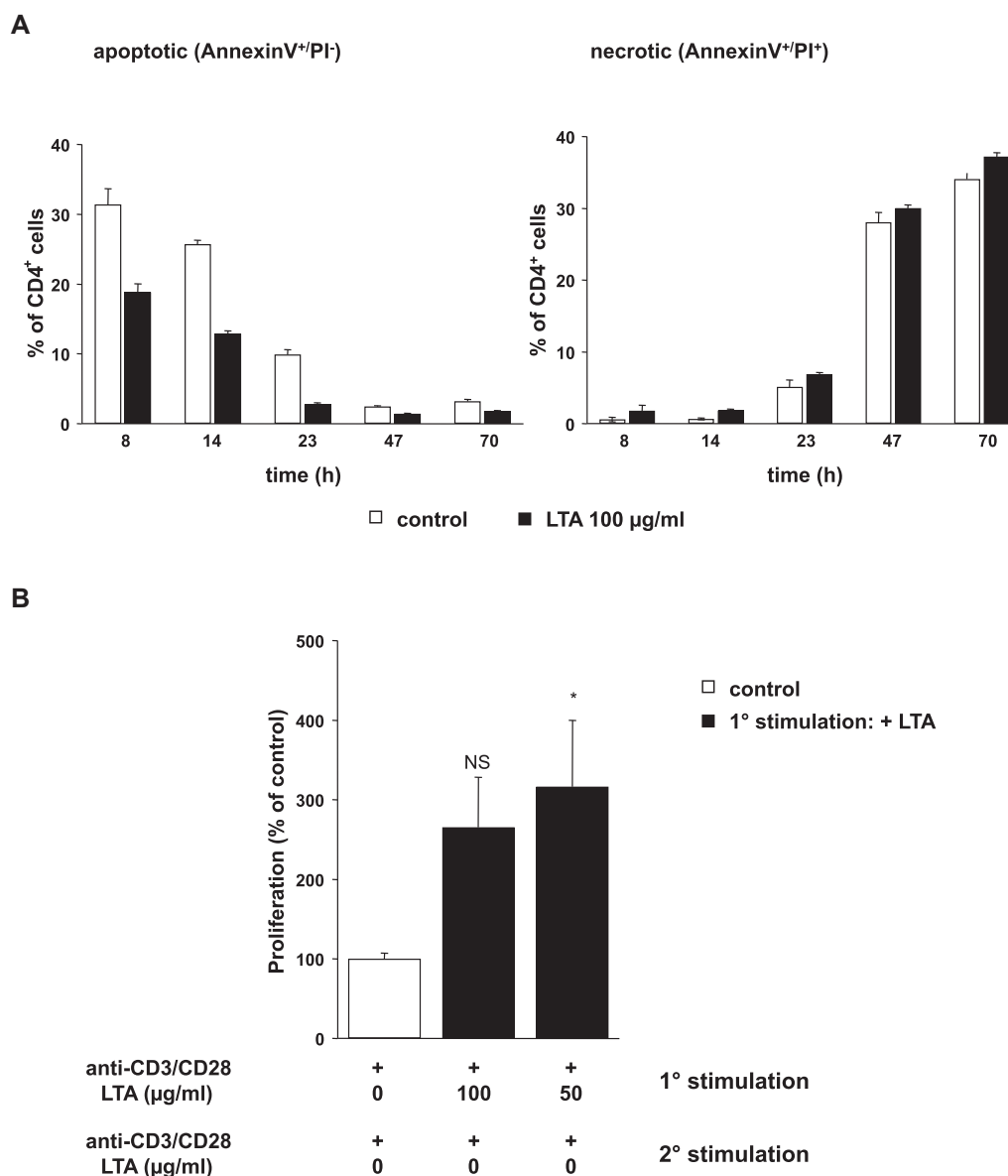


FIG 3. Unequivocal viability of LTA-exposed and control T cells and restored responsiveness on restimulation in the absence of LTA. **A**, Anti-CD3/CD28-activated CD4⁺ T cells exposed to LTA were analyzed for Annexin V and propidium iodide (PI; mean $\pm$ SD). **B**, CD4⁺ T cells, which were primarily activated by anti-CD3/CD28 antibodies and exposed to LTA, were washed and rested with 10 U/mL IL-2 before secondary activation with anti-CD3/CD28 antibodies alone. Proliferation was analyzed by using tritiated thymidine uptake relative to the control without LTA exposure (mean $\pm$ SD). NS, Not significant. * $P < .05$.

was not restricted to a specific T_H subtype but was found for the proliferation and corresponding cytokine production of T_H1, T_H2, and T_H17 cells (see Fig E4 in this article's Online Repository at www.jacionline.org).

These data indicate that *S aureus*-derived LTA potently blocks T-cell activation, thereby representing one mechanism of immune evasion. However, *Staphylococcus epidermidis*, another member of the skin microbiota that is tolerated by the host in most circumstances, also contains large quantities of LTA. For comparison, LTA was isolated from both pathogenic *S aureus* and nonpathogenic *S epidermidis* and analyzed for T-cell suppression in comparison with TGF- β , a known suppressor of T-cell proliferation.³⁴ LTA from both *S aureus* and *S epidermidis*

potently suppressed T-cell activation, indicating a general effect of this class of PAMP (Fig 2, D).

Paralysis but not apoptosis or necrosis in LTA-exposed T_H cells

One possible explanation for the LTA-mediated reduction of T_H cell cytokine production or proliferation could be apoptosis of T cells after LTA exposure. T_H cells were stained with Annexin V and propidium iodide after activation and exposure to LTA to investigate this possibility. Surprisingly, LTA-exposed T_H cells exhibited even fewer apoptotic cells (Fig 3, A): these T_H cells seemed to be completely viable after LTA exposure. CD4⁺

T cells were activated *in vitro* by anti-CD3/CD28 and exposed to LTA for 4 days to further demonstrate T_H cell viability. Afterward, T_H cells were cultured with low concentrations of IL-2 until resting and then stimulated in the absence of bacterial constituents. In contrast to the primary stimulation, T_H cells previously exposed to LTA readily responded to anti-CD3/CD28 treatment. These data demonstrated that T_H cells completely recovered from LTA exposure (Fig 3, B). Thus T_H cells exposed to LTA were viable and remained fully responsive to subsequent stimulation. This result indicated that LTA-mediated suppression is only temporary, reflecting a transient T_H cell paralysis.

LTA-induced suppression of T_H cell activation is independent of TLR2 and myeloid differentiation primary response gene-88 (MyD88)

Next, we analyzed whether LTA functions only during incoming TCR signals, as is expected for TLR2 ligands acting as T-cell costimulators. T_H cells were activated by anti-CD3/CD28, and LTA or Pam2Cys was added up to 3 days after activation. As expected, the costimulatory role of Pam2Cys was functional only in parallel to TCR activation (Fig 4, A). In sharp contrast, LTA-induced paralysis was functional up to 48 hours after T_H cell activation, indicating a mechanism independent of TCR signaling (Fig 4, A). Consequently, we assumed that LTA is functional through structures different from TLRs. To this end, WT, *Tlr2*^{-/-}, and *Myd88*^{-/-} CD4⁺ T cells were analyzed. LTA suppressed the activation of T_H cells of all 3 genotypes, whereas costimulation by Pam2Cys was dependent on TLR2 and MyD88 (Fig 4, B). Direct interaction with LTA independent of TLR2 was confirmed by using immunofluorescence of *Tlr2*^{-/-} CD4⁺ T cells (see Fig E5 in this article's Online Repository at www.jacionline.org).

LTA induces cell-cycle arrest in activated T cells

TGF- β inhibits T-cell proliferation³⁴ through mechanisms that directly target cell-cycle regulators.³⁵⁻³⁷ The consequences of LTA exposure on cell-cycle regulation were investigated to better understand the underlying mechanisms of LTA-mediated T_H cell suppression. In a dose-dependent manner the addition of LTA resulted in significantly fewer CD4⁺ T cells in the S and G2/M phases (Fig 5, A). However, Pam2Cys-stimulated T cells were prone to divide, corresponding to Pam2Cys-induced proliferation (Fig 1, A). As previously noted,³⁴ most T_H cells remained in the G1 phase after TGF- β treatment (Fig 5, B). Thus these data reinforced the suppressive effect of LTA exposure on T_H cells and provided an explanation as to why this suppressive effect (1) is also functional when adding LTA at later time points, (2) is functional in the absence of apoptosis and necrosis, and (3) is transient, allowing normal activation of T cells after a period of resting.

LTA suppresses 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. We adoptively transferred CD3⁺ T cells from FITC-sensitized mice into mice treated as indicated in Fig 1, A, to increase the precursor frequency of FITC-specific T cells. The next day, recipients were challenged

with FITC or vehicle control and exposed to either LTA or PBS (see Fig E1, A). Interestingly, with vehicle-only challenge in the absence of antigen, LTA elicited an ear swelling response, confirming the previously reported direct proinflammatory effects of LTA.³ Similar to the inhibition of T-cell activation *in vitro*, LTA significantly reduced cutaneous inflammation in the presence of antigen and T-cell activation (Fig 6, A). These data indicate that LTA functions as a TLR2 ligand on skin-resident cells by activating the innate immune system and leading to inflammation. In contrast, T cell-dependent CHS is significantly suppressed by a TLR2-independent mechanism. However, proinflammatory and immunosuppressive consequences of LTA exposure might compensate in part for each other in this model. 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.³ In this model we adoptively transferred OVA-specific T_H2 cells and antigen to the ear skin of previously untreated WT and *Tlr2*^{-/-} mice and monitored the T cell-driven ear swelling response in the presence or absence of LTA (see Fig E1, B). Interestingly, LTA profoundly suppressed OVA-specific dermatitis in recipient mice deficient for TLR2, which confirmed our hypothesis (Fig 6, B). This reflects the suppressive effect of LTA on T cells, which is otherwise covered by the proinflammatory, TLR2-dependent LTA effect on non-T cells in WT mice. 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 on non-T cells and produces inflammation, whereas the later and T cell-mediated immune response is susceptible to immune suppression, allowing termination of inflammation. Importantly, the latter effect is transient in nature, avoiding prolonged periods of immunosuppression.

DISCUSSION

Apparently, host cells encounter high concentrations of LTA at sites of bacterial infection. For example, the 50% lethal dose of live *S aureus* in an intraperitoneal infection model in mice is 10⁹ colony-forming units (CFU),³⁸ and this amount of *S aureus* contains approximately 200 μ g of LTA. With a cavity fluid of less than 0.1 mL, this corresponds to 2000 μ g/mL LTA in the peritoneal cavity. Although LPS triggers severe inflammatory responses at low concentrations (in the nanogram per milliliter range^{39,40}), relatively large amounts of LTA (approximately 1-10 μ g/mL) are required to elicit cellular responses *in vitro*.¹³ However, LTA is a major component of Gram-positive bacterial cell walls, whereas Gram-negative bacterial cell walls harbor much less LPS. The active concentrations of LTA (10 μ g or 10⁷ to 10⁸ CFU) and LPS (20 ng or 10⁷ CFU) are comparable with respect to bacterial cell equivalents.³¹

LTA from *S aureus* is a potent stimulant of neutrophil recruitment through triggering the production of cytokines and chemokines in macrophages and monocytes.^{30,31} After induction of innate immune signals, activation of adaptive immunity is functional.⁴¹ LTA has been reported as a proinflammatory effector for the host immune system and especially for innate immunity. Accordingly, LTA is considered the LPS of Gram-positive bacteria.^{13,42} However, as a result of long-lasting coexistence with human subjects, *S aureus* is a well-adapted pathogen that has developed several evasion mechanisms.⁴³ This organism secretes proteins that inhibit complement activation, block neutrophil chemotaxis, lyse neutrophils, or neutralize antimicrobial defensin

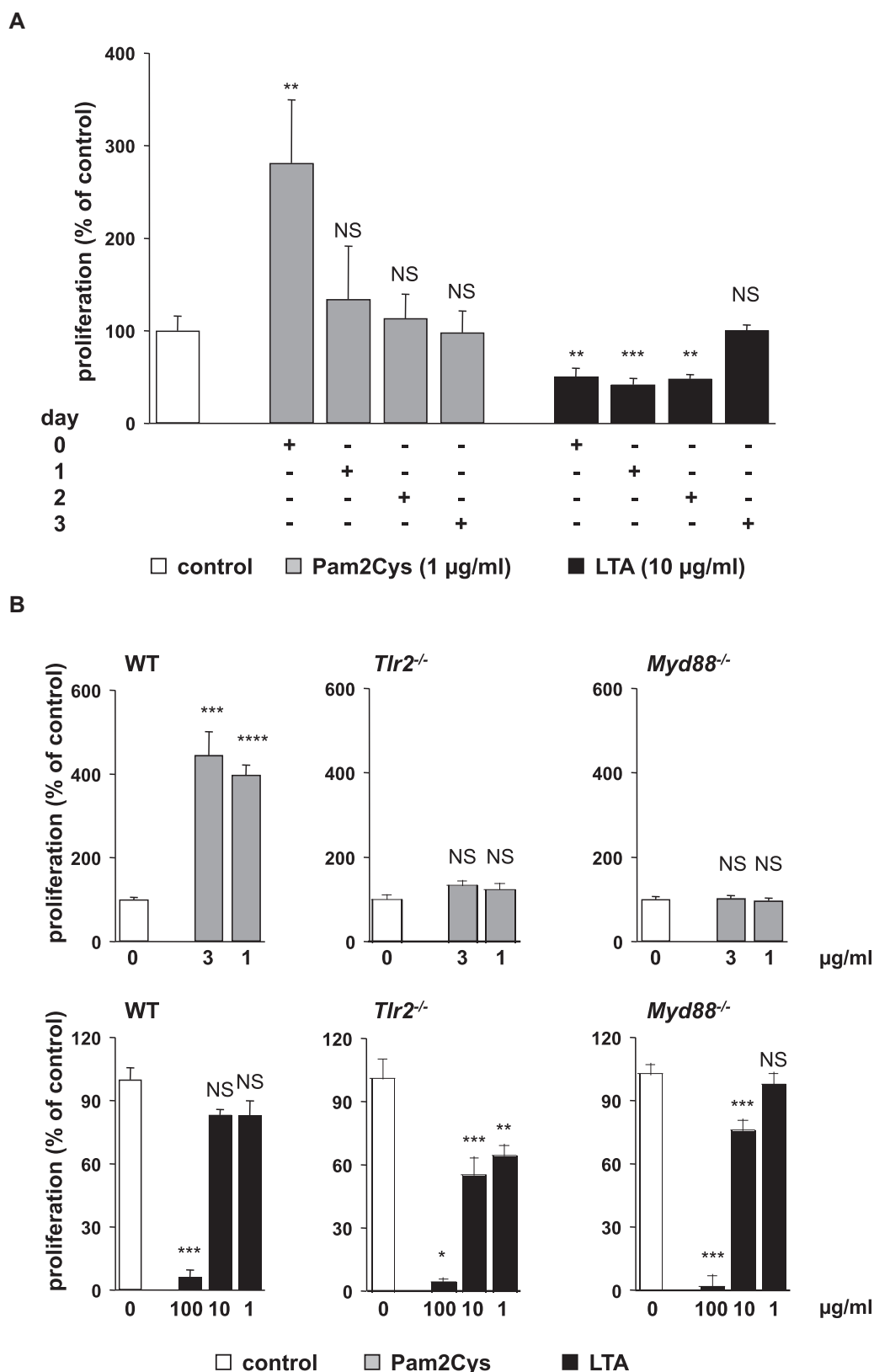


FIG 4. LTA-mediated suppression of CD4⁺ T cells is independent of TLR2 and MyD88. **A**, CD4⁺ cells were activated with anti-CD3/CD28 and LTA or Pam2Cys was added once at different time points, as indicated. **B**, CD4⁺ T cells from WT, *Tlr2*^{-/-}, and *Myd88*^{-/-} mice were activated with anti-CD3/CD28 and incubated with LTA or Pam2Cys. Cell proliferation was analyzed by using tritiated thymidine uptake relative to control groups, which were set as 100% (mean $\pm$ SD). NS, Not significant. * P < .05, ** P < .01, *** P < .001, and **** P < .0001.

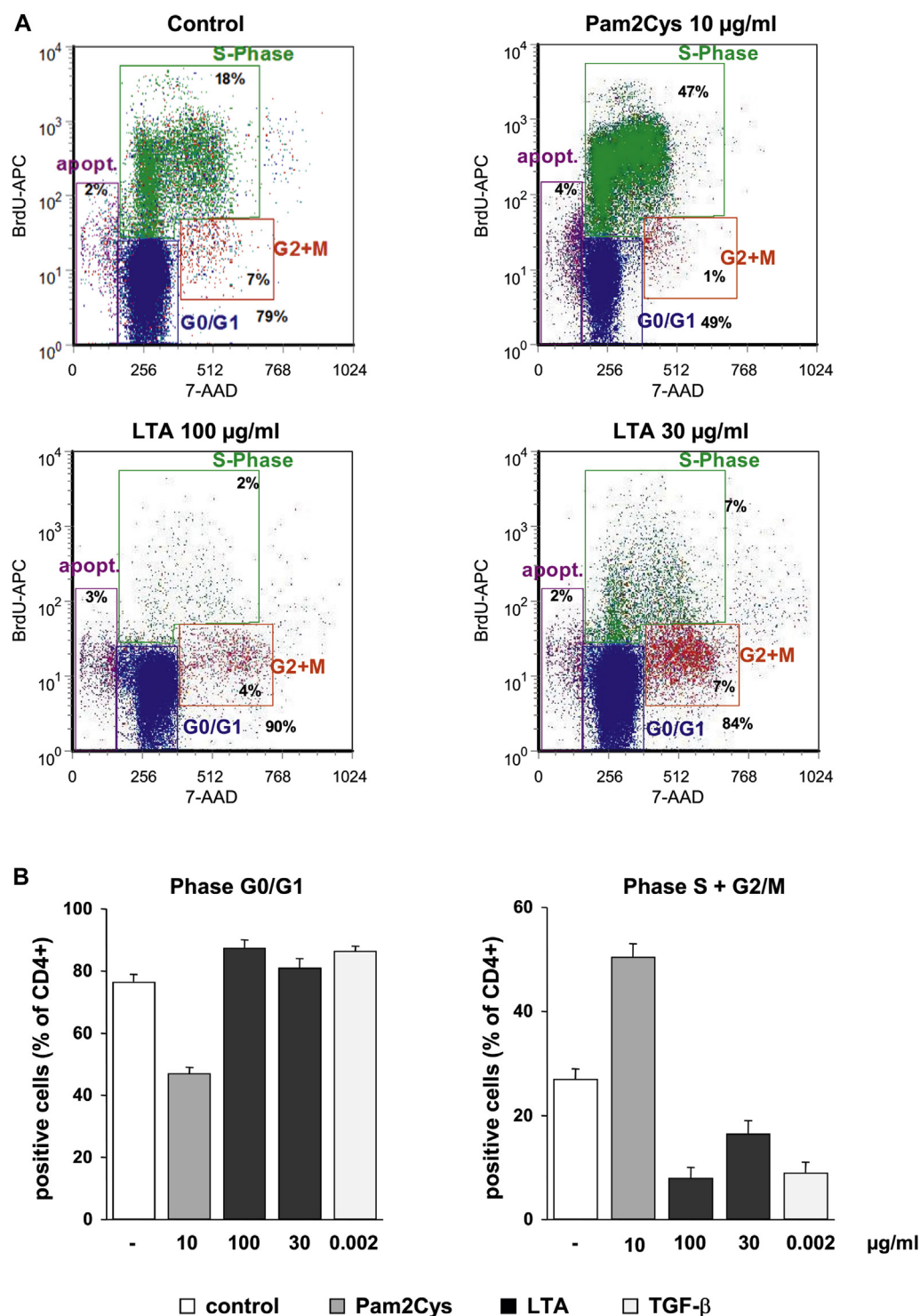


FIG 5. LTA induces cell-cycle arrest in T cells. CD4⁺ T cells were stimulated *in vitro* by anti-CD3/CD28 antibodies, and different concentrations of Pam2Cys or staphylococcal LTA were added. Cell-cycle analysis was performed by means of DNA staining, and the amount of DNA was determined by using fluorescence-activated cell sorting. Representative dot plots (**A**) and means of a similar experiment $\pm$ SD ($n = 5$; **B**) are shown. 7-AAD, 7-Aminoactinomycin D; BrdU, 5-bromo-2'-deoxyuridine.

peptides, and its cell surface is modified to reduce the effectiveness of immune responses.⁴⁴ *S aureus* survives in phagosomes and expresses polysaccharides and proteins that inhibit opsonization by antibody and complement; its cell wall is resistant to

lysozyme. Moreover, *S aureus* expresses several types of superantigens, which disturb the normal humoral immune response and result in anergy, apoptosis, and immunosuppression.⁴⁵⁻⁴⁷ Bacterial components from *S aureus* also inhibit fibroblast proliferation

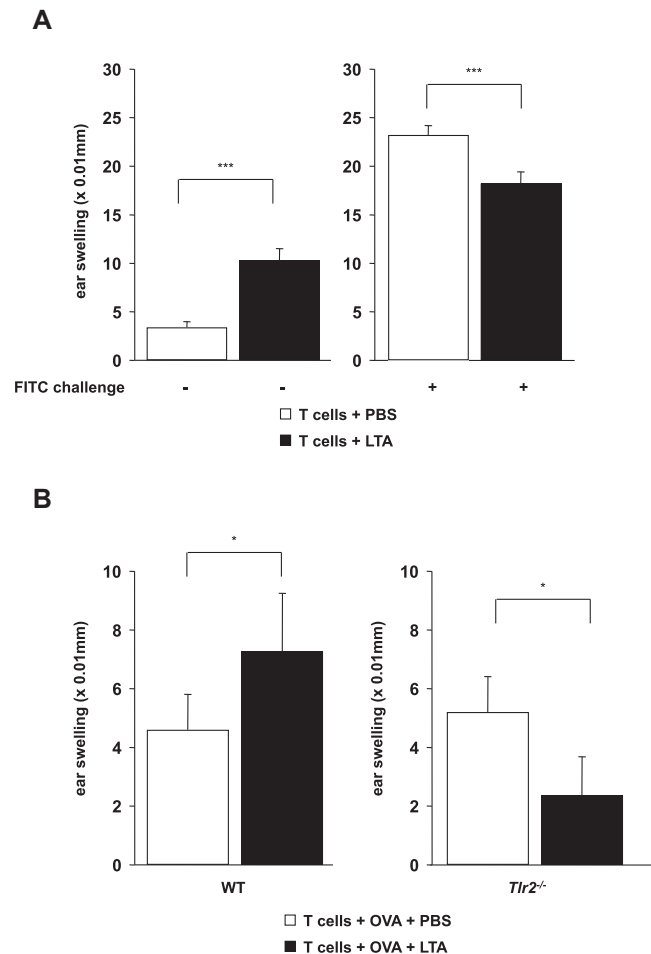


FIG 6. LTA exposure suppresses CHS reactions *in vivo*. Ear swelling measurement of mice was done 24 hours after treatment. **A**, CD3⁺ T cells of FITC-sensitized donor mice were isolated and adoptively transferred into FITC-pretreated recipient mice, as described in Fig 1, A. The next day, LTA or PBS was applied intracutaneously to the recipient mice, which were then challenged with FITC or PBS. **B**, OVA-specific T_H2 cells were adoptively transferred together with OVA antigen and LTA or PBS into naive WT or Tlr2^{-/-} recipient mice. Ear swelling was monitored in Fig 6, A and B (mean $\pm$ SD, n = 5). **P* < .05 and ****P* < .001.

in vitro,⁴⁸ and LTA isolated from *S aureus* inhibits platelet function and platelet-monocyte aggregation.⁴⁹ In addition to *S aureus*, other bacteria of the physiologic skin microbiome, such as *S epidermidis*, contain LTA. Furthermore, *S epidermidis* is a frequent commensal of the human skin. Recently, it was shown that a unique LTA produced by *S epidermidis* inhibits uncontrolled skin inflammation during skin injury.⁵⁰ After skin injury, the host RNA from damaged cells activates TLR3 in keratinocytes, leading to the release of inflammatory cytokines and subsequent inflammation. Staphylococcal LTA inhibits both inflammatory cytokine release from keratinocytes and inflammation triggered by injury through a TLR2-dependent mechanism.⁵¹ However, how proinflammatory macroamphiphilic molecules directly affect adaptive immunity is yet unknown. Functional TLR2 is expressed on T cells,^{21,52} and *Bacteroides fragilis* signals through TLR2 on forkhead box protein 3–positive regulatory T cells to promote immunologic tolerance.⁵³ Therefore it is reasonable to postulate that LTA exerts its modulatory effect directly on adaptive immunity.

Surface organs, such as the skin or gut, are constantly colonized with bacteria called microbiota, which do not induce detectable inflammation.^{54,55} The mechanisms that prevent or inhibit inflammation in response to these nonpathogenic bacteria are not completely understood. Interestingly, TLRs and dendritic cells are critical for the induction of active tolerance to nonpathogenic bacteria,²⁶ and some microbiota elicit immune signals that inhibit inflammation and lead to tolerance through induction of regulatory T cells.^{56,57} Here we demonstrated that the *S aureus*-derived cell-wall component LTA, a known PAMP and TLR2 ligand, potentially suppressed T-lymphocyte activation. This suppression was independent of TLR signaling and effected transient cell-cycle arrest. Immunofluorescent staining indicated that LTA binds to the membrane, possibly to a yet unknown receptor (see Fig E5). Because suppression was not limited to *S aureus* LTA but was also visible for *S epidermidis*, we assume that the shift to *S aureus* monoculture accompanied by the decrease in bacterial diversity,⁵⁸ and subsequent abundant LTA in the skin could be a reason for the disadvantages for AD through *S aureus* LTA. Thus we identified a novel mechanism through which bacterial compounds temporarily directly modulate the adaptive immune system. This new mechanism of T-cell paralysis might (1) terminate inflammation in Gram-positive infections, (2) enable pathogenic bacteria immune evasion, and/or (3) allow for colonization with bacteria from the microbiota in the absence of detectable inflammation. Thus T-cell paralysis represents another level of regulation in the complex interplay between microbes and the host and indicates a possible new class of bacterial compounds to be further explored, possibly also for therapeutic purposes.

We thank Cornelia Grimm (FACS Core Facility of the Interdisciplinary Center for Clinical Research of the University Hospital of Tübingen) for expert technical assistance. We thank Shizuo Akira for providing Myd88^{-/-} mice.

Key messages

- LTA suppresses lesional T-cell cytokines in a mouse model of cutaneous inflammation.
- LTA suppresses T-cell proliferation *in vitro* independent of TLR2 signaling.
- LTA suppresses both FITC-induced CHS and OVA-induced dermatitis independent of TLR2.

REFERENCES

1. Tian T, Liu L, Freyschmidt EJ, Murphy GF, Kupper TS, Fuhlbrigge RC. Overexpression of IL-1 α in skin differentially modulates the immune response to scarification with vaccinia virus. *J Invest Dermatol* 2009;129:70-8.
2. Bieber T. Atopic dermatitis. *N Engl J Med* 2008;358:1483-94.
3. Kaesler S, Volz T, Skabytska Y, Koberle M, Hein U, Chen KM, et al. Toll-like receptor 2 ligands promote chronic atopic dermatitis through IL-4-mediated suppression of IL-10. *J Allergy Clin Immunol* 2014;134:92-9.
4. Lipinski S, Rosenstiel P. Debug your bugs—how NLRs shape intestinal host-microbe interactions. *Front Immunol* 2013;4:479.
5. Kuo IH, Yoshida T, De Benedetto A, Beck LA. The cutaneous innate immune response in patients with atopic dermatitis. *J Allergy Clin Immunol* 2013;131:266-78.
6. Akdis CA, Akdis M, Bieber T, Bindslev-Jensen C, Boguniewicz M, Eigenmann P, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergy and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *J Allergy Clin Immunol* 2006;118:152-69.

7. Boguniewicz M, Eichenfield LF, Hultsch T. Current management of atopic dermatitis and interruption of the atopic march. *J Allergy Clin Immunol* 2003; 112(suppl):S140-50.
8. Kapp A, Papp K, Bingham A, Fölster-Holst R, Ortonne JP, Potter PC, et al. Long-term management of atopic dermatitis in infants with topical pimecrolimus, a nonsteroid anti-inflammatory drug. *J Allergy Clin Immunol* 2002;110:277-84.
9. Mosli MH, Rivera-Nieves J, Feagan BG. T-cell trafficking and anti-adhesion strategies in inflammatory bowel disease: current and future prospects. *Drugs* 2014; 74:297-311.
10. Leung DY, Bhan AK, Schneeberger EE, Geha RS. Characterization of the mononuclear cell infiltrate in atopic dermatitis using monoclonal antibodies. *J Allergy Clin Immunol* 1983;71:47-56.
11. Mihm MC Jr, Soter NA, Dvorak HF, Austen KF. The structure of normal skin and the morphology of atopic eczema. *J Invest Dermatol* 1976;67:305-12.
12. Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994;94:870-6.
13. Fournier B, Philpott DJ. Recognition of *Staphylococcus aureus* by the innate immune system. *Clin Microbiol Rev* 2005;18:521-40.
14. Myles IA, Datta SK. *Staphylococcus aureus*: an introduction. *Semin Immunopathol* 2012;34:181-4.
15. Broker BM, Holtfreter S, Bekeredjian-Ding I. Immune control of *Staphylococcus aureus*—regulation and counter-regulation of the adaptive immune response. *Int J Med Microbiol* 2014;304:204-14.
16. Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003;361:151-60.
17. Nakamura Y, Oscherwitz J, Cease KB, Chan SM, Munoz-Planillo R, Hasegawa M, et al. *Staphylococcus delta-toxin* induces allergic skin disease by activating mast cells. *Nature* 2013;503:397-401.
18. Volz T, Kaesler S, Biedermann T. Innate immune sensing 2.0—from linear activation pathways to fine tuned and regulated innate immune networks. *Exp Dermatol* 2012;21:61-9.
19. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783-801.
20. Biedermann T. Dissecting the role of infections in atopic dermatitis. *Acta Derm Venereol* 2006;86:99-109.
21. Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci U S A* 2004;101:3029-34.
22. Thoma-Uszynski S, Kiertscher SM, Ochoa MT, Bouis DA, Norgard MV, Miyake K, et al. Activation of toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. *J Immunol* 2000;165:3804-10.
23. Volz T, Nega M, Buschmann J, Kaesler S, Guenova E, Peschel A, et al. Natural *Staphylococcus aureus*-derived peptidoglycan fragments activate NOD2 and act as potent costimulators of the innate immune system exclusively in the presence of TLR signals. *FASEB J* 2010;24:4089-102.
24. Travers JB, Kozman A, Mousdicas N, Saha C, Landis M, Al-Hassani M, et al. Infected atopic dermatitis lesions contain pharmacologic amounts of lipoteichoic acid. *J Allergy Clin Immunol* 2010;125:146-52, e1-2.
25. Jin H, He R, Oyoshi M, Geha RS. Animal models of atopic dermatitis. *J Invest Dermatol* 2009;129:31-40.
26. Volz T, Skabytska Y, Guenova E, Chen KM, Frick JS, Kirschning CJ, et al. Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells. *J Invest Dermatol* 2014; 134:96-104.
27. Skabytska Y, Wölbing F, Günther C, Köberle M, Kaesler S, Chen KM, et al. Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. *Immunity* 2014;41:762-75.
28. Ginsburg I. Role of lipoteichoic acid in infection and inflammation. *Lancet Infect Dis* 2002;2:171-9.
29. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E, Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infect Immun* 1996;64:1906-12.
30. Standiford TJ, Arenberg DA, Danforth JM, Kunkel SL, VanOtteren GM, Strieter RM. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. *Infect Immun* 1994;62:119-25.
31. von Aulock S, Morath S, Hareng L, Knapp S, van Kessel KP, van Strijp JA, et al. Lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus for neutrophil recruitment. *Immunobiology* 2003;208:413-22.
32. Okusawa T, Fujita M, Nakamura J, Into T, Yasuda M, Yoshimura A, et al. Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6. *Infect Immun* 2004;72:1657-65.
33. Cardona ID, Cho SH, Leung DY. Role of bacterial superantigens in atopic dermatitis: implications for future therapeutic strategies. *Am J Clin Dermatol* 2006;7:273-9.
34. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 1986;163: 1037-50.
35. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci U S A* 1995;92:5545-9.
36. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994;371:257-61.
37. Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, et al. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 1994;8:9-22.
38. Dziarski R, Platt KA, Gelius E, Steiner H, Gupta D. Defect in neutrophil killing and increased susceptibility to infection with nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice. *Blood* 2003; 102:689-97.
39. Hoetzenecker W, Echtenacher B, Guenova E, Hoetzenecker K, Wölbing F, Bruck J, et al. ROS-induced ATF3 causes susceptibility to secondary infections during sepsis-associated immunosuppression. *Nat Med* 2011;18:128-34.
40. Kusunoki T, Hailman E, Juan TS, Lichenstein HS, Wright SD. Molecules from *Staphylococcus aureus* that bind CD14 and stimulate innate immune responses. *J Exp Med* 1995;182:1673-82.
41. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature* 2007;449:819-26.
42. Morath S, Stadelmaier A, Geyer A, Schmidt RR, Hartung T. Synthetic lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus of cytokine release. *J Exp Med* 2002;195:1635-40.
43. Massey RC, Horsburgh MJ, Lina G, Hook M, Recker M. The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? *Nat Rev Microbiol* 2006;4:953-8.
44. Lowy FD. How *Staphylococcus aureus* adapts to its host. *N Engl J Med* 2011;364: 1987-90.
45. Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol* 2005;3:948-58.
46. Kaesler S, Sobiesiak M, Kneilling S, Volz T, Kempf WE, Lang PA, et al. Effective T-cell recall responses require the taurine transporter Taut. *Eur J Immunol* 2012;42:831-41.
47. Rooijackers SH, Ruyken M, van Roon J, van Kessel KP, van Strijp JA, van Wamel WJ. Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus*. *Cell Microbiol* 2006;8:1282-93.
48. Edds EM, Bergamini TM, Brittan KR. Bacterial components inhibit fibroblast proliferation in vitro. *ASAIO J* 2000;46:33-7.
49. Sheu JR, Lee CR, Lin CH, Hsiao G, Ko WC, Chen YC, et al. Mechanisms involved in the antiplatelet activity of *Staphylococcus aureus* lipoteichoic acid in human platelets. *Thromb Haemost* 2000;83:777-84.
50. Lai Y, Di Nardo A, Nakatsuji T, Leichtle A, Yang Y, Cogen AL, et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nat Med* 2009;15:1377-82.
51. Lai Y, Cogen AL, Radek KA, Park HJ, Macleod DT, Leichtle A, et al. Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. *J Invest Dermatol* 2010; 130:2211-21.
52. Reynolds JM, Pappu BP, Peng J, Martinez GJ, Zhang Y, Chung Y, et al. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity* 2010;32: 692-702.
53. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 2011;332:974-7.
54. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, et al. Topographical and temporal diversity of the human skin microbiome. *Science* 2009; 324:1190-2.
55. Kong HH, Segre JA. Skin microbiome: looking back to move forward. *J Invest Dermatol* 2012;132:933-9.
56. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* 2010;107:12204-9.
57. Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 2011;34:794-806.
58. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res* 2012;22:850-9.

METHODS

T-cell polarization

CD4⁺ T cells were purified from naive WT BALB/c mice and stimulated with anti-CD3 (4 µg/mL) and anti-CD28 (2 µg/mL) in culture medium. Polarization to different T_H subtypes was achieved by further addition of 0.2 µmol/L CpG (Eurofins, Ebersberg, Germany) and 1 µg/mL anti-IL-4 (clone 11B11, purified from hybridoma supernatant) for T_H1, 50 ng/mL IL-4 (PromoCell, Heidelberg, Germany) and 5 µg/mL anti-IFN-γ (BioLegend) for T_H2, and 10 ng/mL IL-6 (BioLegend), 2 ng/mL TGF-β (R&D Systems, Abington, United Kingdom), 5 µg/mL anti-IL-4, and 5 µg/mL anti-IFN-γ for T_H17.

Immunofluorescence

Purified CD4⁺ T cells from naive *Tlr2*^{-/-} mice were cultured for 24 hours with or without LTA, washed 3 times with culture medium, transferred to poly-L-lysine-coated slides, and dried overnight. For immunofluorescence, cells were fixed by using methanol/acetone, incubated with α-LTA antibody (QED Bioscience) overnight, and stained with goat-anti-mouse DyLight 488 (Abcam, Cambridge, United Kingdom) and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen Life Technologies GmbH, Darmstadt, Germany).

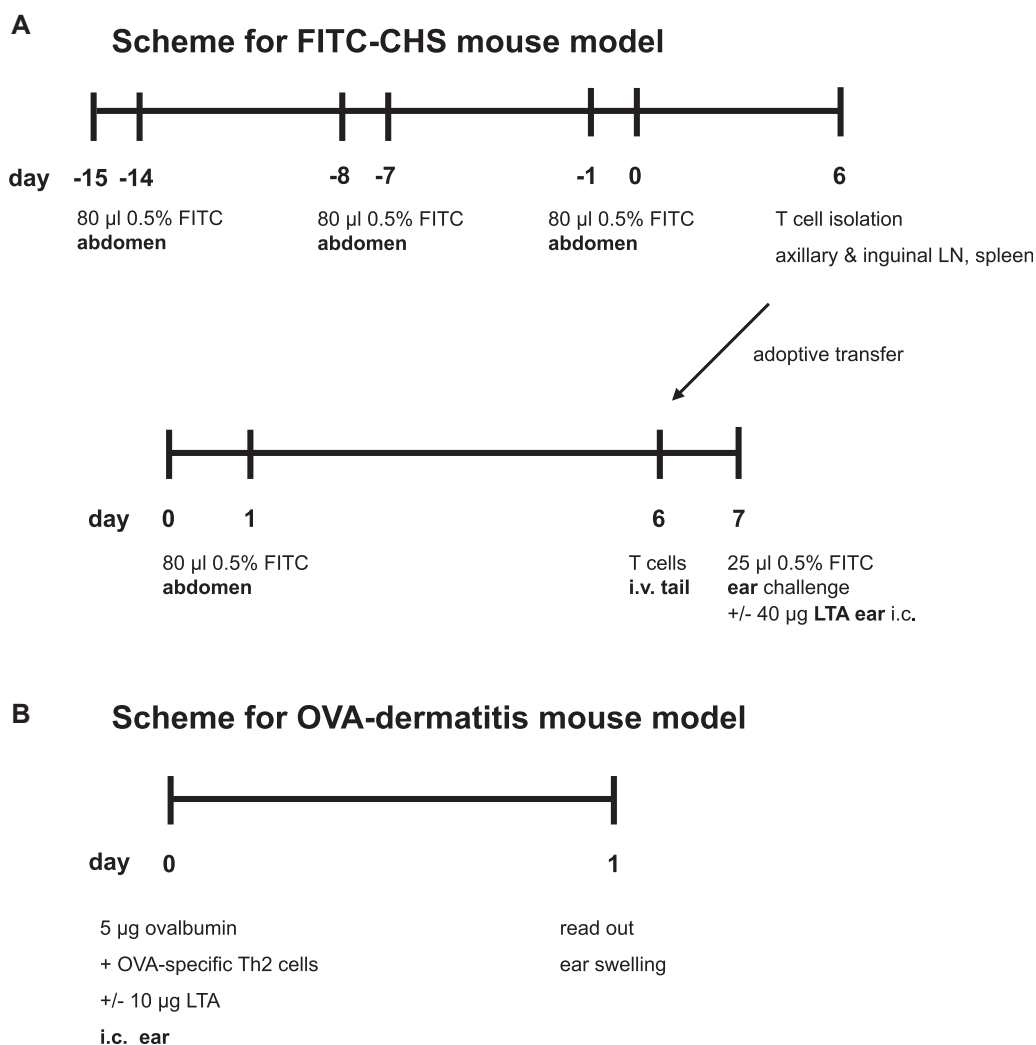


FIG E1. Scheme for experimental procedures shown in Fig 6. **A**, After 6 episodes of FITC sensitization, T cells were isolated from donor mice and adoptively transferred to mice that had been sensitized only twice. The next day, recipient mice were challenged, and ear swelling was determined 24 hours later. **B**, OVA-specific T_H2 cells and their specific antigen, OVA, were injected with or without LTA into the ear skin of naive mice. After 24 hours, ear swelling was measured. *i.c.*, Intracutaneous.

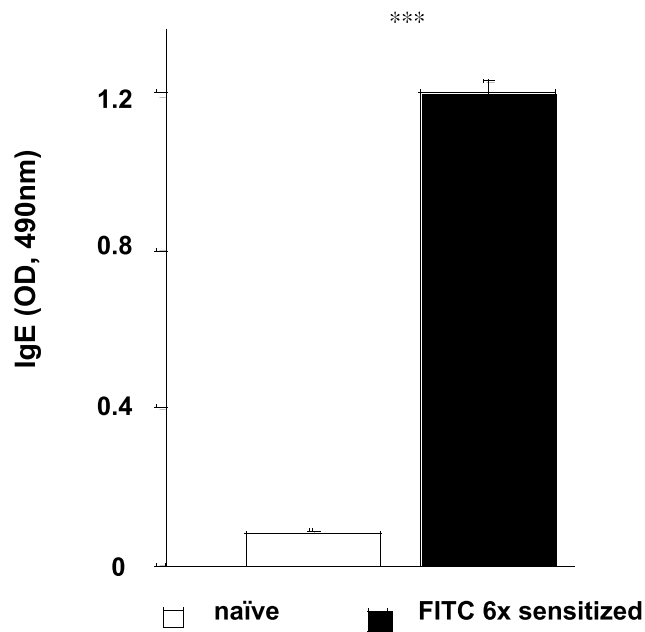
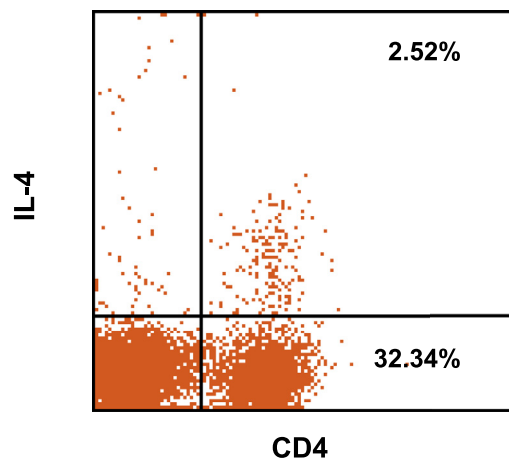
A**B**

FIG E2. FITC-induced CHS induces specific IgE and T_H2 cells. **A**, Mice were sensitized with FITC on days 0, 1, 7, 8, 14, and 15, and on day 21, mice were challenged with FITC. FITC-specific IgE levels were analyzed in sera ($n = 3$). **B**, dLNs were isolated after ear exposure to FITC, and IL-4 production was analyzed. *** $P < .001$.

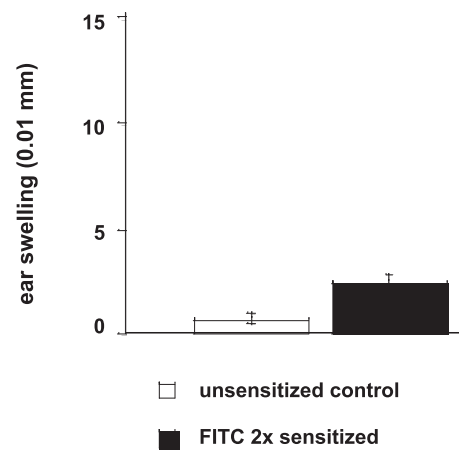


FIG E3. Minimal cutaneous inflammation is induced by FITC. Mice were sensitized with FITC on days 0 and 1, and on day 7, mice were challenged with FITC. Mice in the control group received vehicle sensitization and then FITC challenge. Ear swelling responses were recorded (mean $\pm$ SD, $n = 5$).

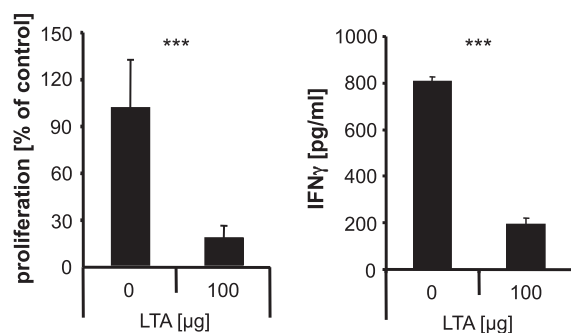
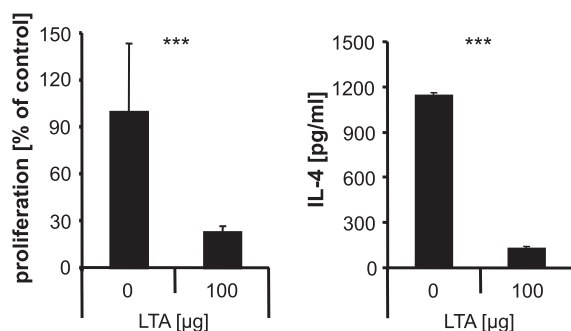
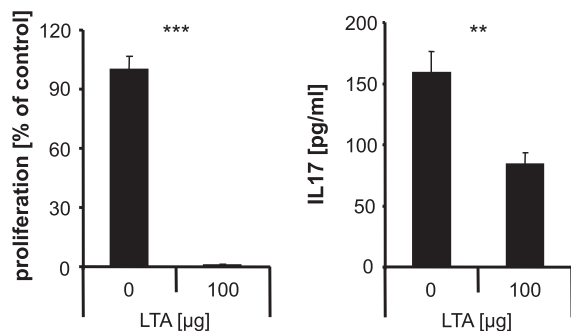
A Th1**B Th2****C Th17**

FIG E4. LTA-mediated suppression of CD4⁺ T cells is independent of T_H polarization. Purified CD4⁺ T cells were cultured under different T_H-polarizing conditions and restimulated with anti-CD3/CD28 antibodies with or without LTA. Proliferation was measured based on tritiated thymidine uptake. Cytokine production was determined by means of ELISA from supernatants of T_H1 (A), T_H2 (B), and T_H17 cells (C). ***P* < .01 and ****P* < .001.

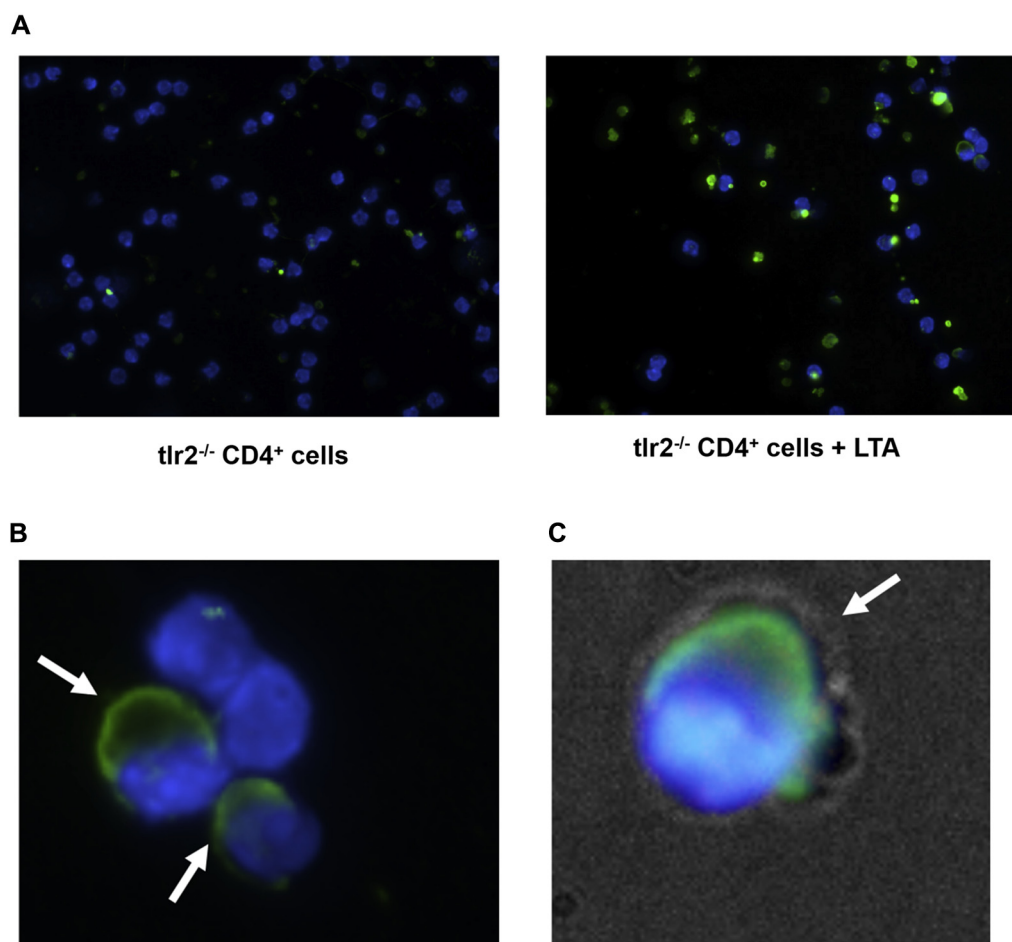


FIG E5. LTA directly interacts with CD4⁺ T cells in the absence of TLR2. **A**, Immunofluorescence of immobilized purified CD4⁺ T cells from TLR2-deficient mice after overnight culture with or without LTA. *Tlr2*^{-/-} CD4⁺ T cells were stained for DNA (*blue*) and LTA (*green*). **B** and **C**, Magnification at $\times 40$ (Fig E5, **B**) and magnification of $\times 100$ (Fig E5, **C**) of *Tlr2*^{-/-} CD4⁺ T cells cultured in the presence of LTA, depicting membrane localization of LTA (*white arrows*).