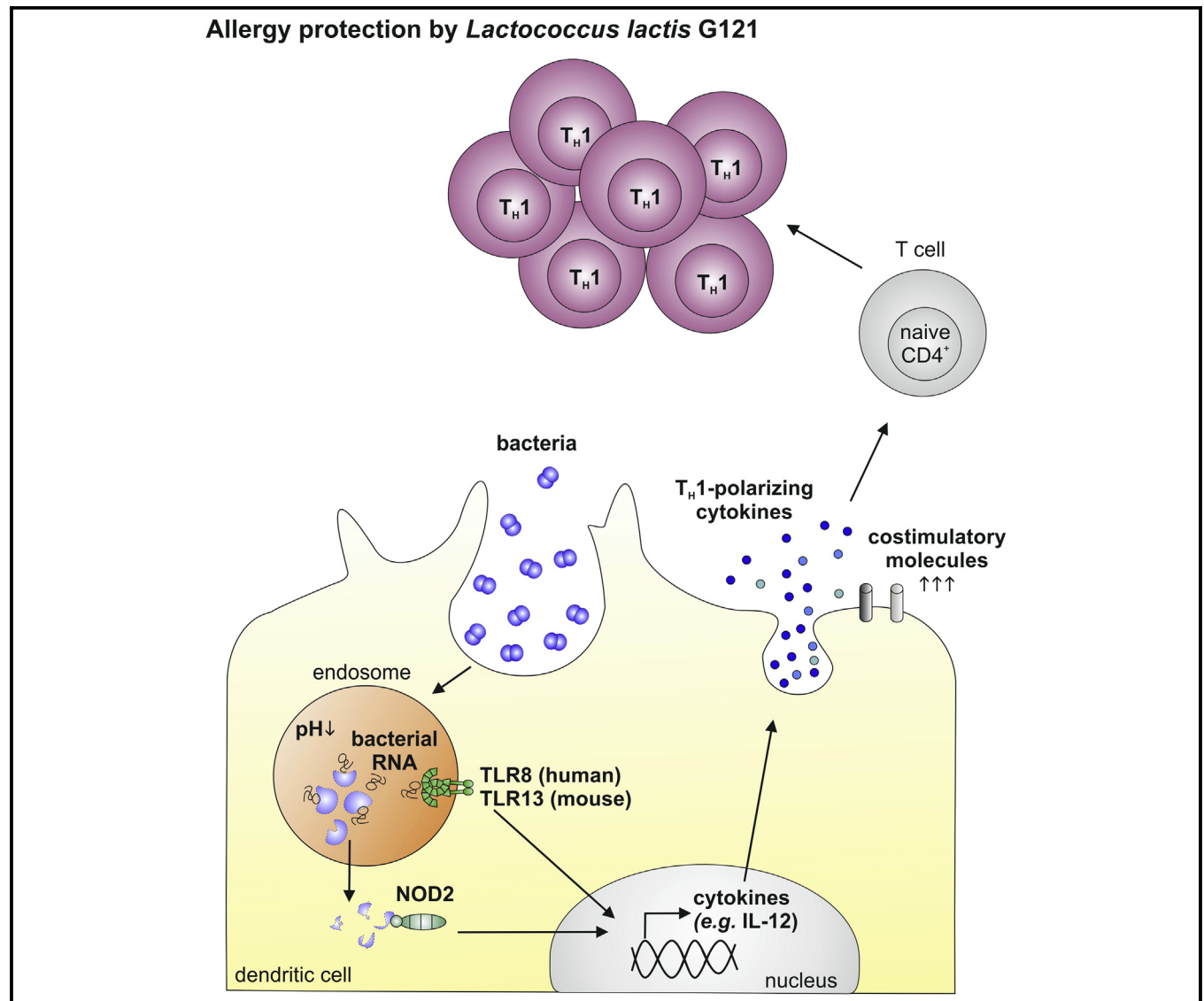


Endosomal recognition of *Lactococcus lactis* G121 and its RNA by dendritic cells is key to its allergy-protective effects

Karina Stein, PhD,^a Stephanie Brand, PhD,^b André Jenckel, PhD,^a Anna Sigmund, PhD,^c Zhijian James Chen, PhD,^d Carsten J. Kirschning, PhD,^c Marion Kauth, PhD,^b and Holger Heine, PhD^a *Borstel, Gelsenkirchen, and Essen, Germany, and Dallas, Tex*

GRAPHICAL ABSTRACT



From ^athe Division of Innate Immunity, Research Center Borstel, Airway Research Center North, German Center for Lung Research (DZL), Germany; ^bProtectimmun GmbH, Gelsenkirchen; ^cthe Institute of Medical Microbiology, University of Duisburg-Essen, Essen; and ^dthe Department of Molecular Biology, Howard Hughes Medical Institute, UT Southwestern Medical School, Dallas.

Supported by the German Research Foundation, DFG, SFB/Transregio 22, project A02. Disclosure of potential conflict of interest: K. Stein receives grant support from DFG and BMBF. S. Brand is an employee of Protectimmun GmbH. A. Jenckel receives grant support from BMBF. M. Kauth receives grant support from EU-FP7 grant and KBBE-

211911. H. Heine receives grant support from DFG, BMBF, and Fa. Pohl-Boskamp. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 23, 2015; revised June 2, 2016; accepted for publication June 13, 2016.

Corresponding author: Holger Heine, PhD, Research Center Borstel, Division of Innate Immunity, Parkallee 22, 23845 Borstel, Germany. E-mail: hheine@fz-borstel.de. 0091-6749/\$36.00

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2016.06.018>

Background: Bacterial cowshed isolates are allergy protective in mice; however, the underlying mechanisms are largely unknown. We examined the ability of *Lactococcus lactis* G121 to prevent allergic inflammatory reactions.

Objective: We sought to identify the ligands and pattern recognition receptors through which *L lactis* G121 confers allergy protection.

Methods: *L lactis* G121-induced cytokine release and surface expression of costimulatory molecules by untreated or inhibitor-treated (bafilomycin and cytochalasin D) human monocyte-derived dendritic cells (moDCs), bone marrow-derived mouse dendritic cells (BMDCs), and moDC/naive CD4⁺ T-cell cocultures were analyzed by using ELISA and flow cytometry. The pathology of ovalbumin-induced acute allergic airway inflammation after adoptive transfer of BMDCs was examined by means of microscopy.

Results: *L lactis* G121-treated murine BMDCs and human moDCs released T_H1-polarizing cytokines and induced T_H1 T cells. Inhibiting phagocytosis and endosomal acidification in BMDCs or moDCs impaired the release of T_H1-polarizing cytokines, costimulatory molecule expression, and T-cell activation on *L lactis* G121 challenge. *In vivo* allergy protection mediated by *L lactis* G121 was dependent on endosomal acidification in dendritic cells (DCs). Toll-like receptor (*Tlr*) 13^{-/-} BMDCs showed a weak response to *L lactis* G121 and were unresponsive to its RNA. The T_H1-polarizing activity of *L lactis* G121-treated human DCs was blocked by TLR8-specific inhibitors, mediated by *L lactis* G121 RNA, and synergistically enhanced by activation of nucleotide-binding oligomerization domain-containing protein (NOD) 2.

Conclusion: Bacterial RNA is the main driver of *L lactis* G121-mediated protection against experimentally induced allergy and requires both bacterial uptake by DCs and endosomal acidification. In mice *L lactis* G121 RNA signals through TLR13; however, the most likely intracellular receptor in human subjects is TLR8. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Allergy protection, dendritic cells, endosomal acidification, *Lactococcus lactis* G121, pattern recognition receptor, Toll-like receptor 13, Toll-like receptor 8, bacterial RNA

Growing up in a traditional farming environment reduces the risk of having allergic diseases, such as asthma, later in life.^{1,2} Specifically, exposure to the immunostimulatory products of environmental microorganisms found on farms protects against asthma.^{3,4} Dendritic cells (DCs) transmit signals received from environmental pathogens to the adaptive immune system, thereby activating the immune responses that cause asthma.⁵⁻⁸ However, DCs can also prevent asthma,^{9,10} for example, through therapeutic induction of a T_H2-counterbalancing T_H1-mediated immune response or a suppressive regulatory T cell response.¹¹ Polarization of T cells is driven by the cytokine milieu created by DCs. The key players in DC polarization are IL-12 (a T_H1-polarizing cytokine)¹² and the immune-suppressive cytokine IL-10.¹³

Previously, we showed that intranasal inoculation of mice with the bacterial cowshed isolates *Acinetobacter lwoffii* F78, *Lactococcus lactis* G121, and *Staphylococcus sciuri* W620 prevented the development of asthma symptoms.¹⁴⁻¹⁶ The gram-positive

Abbreviations used

Baf A1:	Bafilomycin A1
BMDC:	Bone marrow-derived dendritic cell
cfu:	Colony-forming units
DC:	Dendritic cell
Foxp3:	Forkhead box protein 3
MDP:	Muramyl dipeptide
moDC:	Monocyte-derived dendritic cell
NOD2:	Nucleotide-binding oligomerization domain-containing protein 2
OVA:	Ovalbumin
PAMP:	Pathogen-associated molecular pattern
PFA:	Paraformaldehyde
PRR:	Pattern recognition receptor
ROR:	Retinoic acid-related orphan receptor
T-bet:	T-box transcription factor
TLR:	Toll-like receptor
WT:	Wild-type

bacterium *L lactis* G121 and the gram-negative bacterium *A lwoffii* F78 strongly stimulate human DCs to initiate an allergy-protective T_H1-polarizing program that results in robust production of IL-12.¹⁴ We identified *A lwoffii* F78-derived LPS as the main driver of IL-12 production, which occurs through activation of Toll-like receptor (TLR) 4.¹⁷ However, the major allergy-protective pathogen-associated molecular pattern (PAMP) of *L lactis* G121 remains elusive. Here we show that *L lactis* G121-mediated activation of monocyte-derived dendritic cells (moDCs) is dependent on bacterial uptake and endosomal acidification. Also, adoptive transfer of *L lactis* G121-challenged bone marrow-derived mouse dendritic cells (BMDCs) protected mice from ovalbumin (OVA)-induced allergic inflammation through the establishment of a T_H1-polarizing cytokine milieu. This was reversed by inhibiting endosomal acidification in *L lactis* G121-stimulated BMDCs. Moreover, we also identified TLR13, which is expressed by most vertebrates (but not primates)¹⁸⁻²⁰ and the receptor for a specific bacterial 23S rRNA segment, as the main host sensor responsible for the allergy-protective effects of *L lactis* G121 in mice. Finally, we provide clear evidence that this response is induced in human subjects by bacterial RNA as well and mediated through TLR8.

METHODS

Bacteria

L lactis G121 was grown for 5 hours at 37°C in Tryptic Soy Broth liquid medium containing 0.3% yeast extract. The harvested bacteria were treated with PBS containing 1000 U/mL penicillin and 1 mg/mL streptomycin (PAA Laboratories, Pasching, Austria) for 30 minutes at 37°C. The *L lactis* G121 used for animal experiments was grown in Tryptic Soy Broth medium for 12 hours at 30°C. Bacteria were then lyophilized and resuspended in PBS before inoculation into animals.

Ethics statement

The study was approved by the Institutional Ethics Committee at the University of Lübeck (Lübeck, Germany; Az. 08-150) and conducted in accordance with the Declaration of Helsinki. All donors provided written informed consent.

Generation and stimulation of human moDCs

PBMCs were isolated from healthy donors by using Ficoll (PAA Laboratories), as previously described.²¹ Monocytes were isolated by means of counterflow elutriation centrifugation,²² cultured at a density of 10^6 cells/mL in RPMI 1640 containing stable glutamine (PAA Laboratories), and supplemented with 10% heat-inactivated FCS (Biochrom AG, Berlin, Germany), 100 U/mL penicillin (PAA Laboratories), 100 μ g/mL streptomycin (PAA Laboratories), 500 U/mL GM-CSF, and 500 U/mL IL-4 (both from ImmunoTools, Friesoythe, Germany). Half of the medium was exchanged (including cytokines) every other day. At day 7, cells were harvested and recultured in complete RPMI 1640 (density, 10^6 cells/mL).

MoDCs were preincubated with either 10 nmol/L bafilomycin A1 (Baf A1; Merck Millipore, Darmstadt, Germany), 1 μ mol/L cytochalasin D (AppliChem, Darmstadt, Germany), or different amounts of IRS 957 (Eurofin MWG Operon, Munich, Germany) for 1 hour before stimulation for 20 hours with 100 ng/mL LPS derived from *Salmonella enterica* sv. Friedenau (a kind gift from Professor Helmut Brade, Research Center Borstel, Borstel), 5 μ g/mL CL097, 1 μ mol/L R848 (both from InvivoGen, San Diego, Calif), or 10^7 or 10^6 cfu/mL *L. lactis* G121.

For RNA transfection, moDCs were recultured in RPMI 1640 containing 10% heat-inactivated FCS (cell density, 0.75×10^6 cells/mL). Different amounts of RNA were pretreated or not with RNase A (Thermo Scientific, Waltham, Mass) for 30 minutes and delivered to cells by using N-(1-[2,3-Dioleoyloxy]propyl)-N,N,N-trimethylammonium methyl-sulfate (DOTAP; Roth, Karlsruhe, Germany). Concomitant with RNA transfection, moDCs were stimulated (or not) with 500 ng/mL of the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) ligand muramyl dipeptide (MDP; a kind gift from Professor Shoichi Kusumoto, Osaka, Japan) for 20 hours.

Isolation of human and bacterial RNA

L. lactis G121 cells were lysed by using a FastPrep-24 instrument and Lysing Matrix B (both MP Biomedicals, Santa Ana, Calif), and total RNA was isolated with Trizol (Thermo Fisher Scientific). Total RNA was extracted from human Calu-3 cells (HTB-55; ATCC, Washington, DC) by using a NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany). After purification, RNA was treated with DNase I to remove contaminating DNA (Roche, Mannheim, Germany).

Isolation of autologous naive CD4⁺ T cells, coculture with moDCs, and bacterial challenge

Autologous lymphocytes were obtained by means of counterflow elutriation and naive CD4⁺ T cells purified by using negative MACS selection with the Naïve CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; >90% purity). LPS- or *L. lactis* G121-treated moDCs were washed and cultured with naive CD4⁺ T cells at a ratio of 1:10. After 5 days, cocultures were restimulated for 5 hours with 50 ng/mL phorbol 12-myristate 13-acetate (Merck Millipore) and 2 μ g/mL ionomycin (Thermo Fisher Scientific).

Animals

Female BALB/c mice, C57BL/6 wild-type (WT) mice (6–8 weeks old; Charles River, Sulzfeld, Germany), and C57BL/6 *Tlr13*^{−/−20} mice were housed under pathogen-free conditions. Water and an OVA-free diet were supplied *ad libitum*. All animal experiments were performed in accordance with German and international guidelines and approved by local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany).

Generation and stimulation of BMDCs

For the transfer model, bone marrow was isolated from WT BALB/c mice, and BMDCs were generated by means of culture with 20 ng/mL recombinant murine GM-CSF (ImmunoTools, Friesoythe, Germany) for

6 days, with one exchange of medium (RPMI 1640, 5% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin) after 3 days. Cells were then preincubated with 10 nmol/L Baf A1 for 1 hour and then stimulated with 10^8 cfu/mL *L. lactis* G121 for 24 hours. On day 9, cells were pulsed for 24 hours with 100 μ g/mL endotoxin-free OVA (Hyglos, Bernried, Germany) and 1 ng/mL LPS derived from *S. enterica* sv. Friedenau. For *in vitro* experiments, bone marrow from C57BL/6 WT or *Tlr13*^{−/−} mice was cultured for 5 days with 20 ng/mL recombinant murine GM-CSF (ImmunoTools), followed by an additional 3 days with 10 ng/mL recombinant murine GM-CSF. Subsequently, cells were stimulated for 24 hours with *L. lactis* G121 and LPS or transfected with 100 pmol Sa19 (InvivoGen) or 0.25 and 0.5 μ g of *L. lactis* G121 RNA per 200 μ L by using DOTAP.

Sensitization of mice with OVA-pulsed BMDCs and aerosol challenge

Mice were sensitized by means of intranasal administration of 10^6 OVA-pulsed BMDCs in 50 μ L of PBS. Sham-treated groups received PBS-pulsed BMDCs only. On days 13, 14, and 15, all animals were exposed to aerosolized OVA (Grade V; Sigma, Steinheim, Germany) for 20 minutes.

Bronchoalveolar lavage and differential cell counts

Two days after the final OVA challenge, lungs were lavaged with 1 mL of PBS through the trachea, and leukocytes in lavage fluid were counted. Cytospin preparations were prepared for differential cell count analysis and stained by using a fast-staining procedure (HAEME-Schnellfärbung; Labor+Technik Eberhard Lehmann, Berlin, Germany). Macrophages, lymphocytes, eosinophils, and neutrophils were identified according to standard morphologic criteria.

Lung histology

Lungs were fixed with 4% paraformaldehyde (PFA; injected through the trachea), removed, and stored in 4% PFA. Lung tissues were embedded in paraffin, and sections (3 μ m thick) were stained with hematoxylin and eosin or periodic acid–Schiff. Goblet cell hyperplasia was expressed as the number of goblet cells per millimeter of basement membrane.

Measurement of cytokine levels

Cytokine levels in supernatants from murine BMDCs were measured by using IL-10 (BD Biosciences, San Jose, Calif), TNF- α (Thermo Fisher Scientific), and IL-12p70 (R&D Systems, Minneapolis, Minn) ELISAs. Concentrations of human CXCL8, IL-6, IL-10, TNF- α , IFN- γ , IL-4, and IL-5 (Thermo Fisher Scientific), as well as IL-12p70 and IL-23 (R&D Systems), in the supernatants of moDCs and moDC/T-cell cocultures were measured by using commercial ELISA kits.

Flow cytometric analysis

Stimulated moDCs were harvested; stained with antibodies specific for the costimulatory molecules CD40, CD80, and CD86 (BD Biosciences) or with appropriate isotype controls; and fixed with 1.5% PFA. Flow cytometry was performed in an LSRII cytometer (BD Biosciences), and data were analyzed with FlowJo V 7.6.1 software (TreeStar, Ashland, Ore).

Statistical analysis

All numeric data are expressed as means + SEMs, unless stated otherwise. Human data were analyzed for significance by using 2-way ANOVA and either the Tukey or Bonferroni *post hoc* test. Mouse data were analyzed by using 1-way ANOVA and the Tukey *post hoc* test. *P* values of less than .05 were considered statistically significant. All calculations were performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

L lactis G121 induces moDCs to produce proinflammatory and T cell-polarizing cytokines

MoDCs were challenged with *L lactis* G121 preparations, and cytokine production was measured (Fig 1, A). Marked induction of proinflammatory mediators, such as CXCL8, IL-6, and TNF- α , as well as IL-23 and IL-10, was observed. In accordance with the findings of previous experiments,¹⁴ *L lactis* G121 also induced strong production of IL-12p70.

L lactis G121-stimulated moDCs induce T cells to produce IFN- γ and IL-10 on coculture

To investigate the capacity of *L lactis* G121-stimulated moDCs to polarize T cells, we cocultured them with autologous naive CD4⁺ T cells. *L lactis* G121-challenged moDCs stimulated T cells to produce IL-10 and large amounts of IFN- γ (Fig 1, B, and see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). Neither of these cytokines was detected in cultures of moDCs alone (data not shown). IL-4 and IL-5 release in cocultures was low. Furthermore, we observed predominant occurrence of T-box transcription factor (T-bet)⁺ and comparatively low numbers of GATA3⁺, forkhead box protein 3 (Foxp3)⁺, and retinoic acid-related orphan receptor (ROR) γ t⁺ T cells (see Fig E3 in this article's Online Repository at www.jacionline.org).

Phagocytosis of *L lactis* G121 by moDCs is required to induce cytokine production

Activation of T cells by bacterial antigens requires their presentation by antigen-presenting cells, such as DCs. Therefore we next examined whether uptake of *L lactis* G121 is pivotal for cytokine production by moDCs. The phagocytosis inhibitor cytochalasin D (which disrupts actin skeleton formation²³) inhibited *L lactis* G121 uptake by moDCs (Fig 2, A) and strongly impaired the release of IL-12p70, IL-23, and IL-10 by *L lactis* G121-challenged cells (Fig 2, B). No cytokine reduction occurred when cells were treated with LPS in the presence of cytochalasin D, with the exception of TNF- α , an effect that has been reported before.²⁴

Cytokine release and expression of T-cell costimulatory molecules by moDCs requires endosomal acidification

Activation of endosomal TLRs, such as TLR3, TLR7, TLR8, TLR9, and TLR13, depends on acidification of the endosomal compartments.²⁵ Therefore we next used Baf A1 to examine whether endosomal pattern recognition receptors (PRRs) mediate activation of moDCs by *L lactis* G121. Baf A1 blocks V-ATPases and disables endosomal acidification. LysoTracker staining confirmed that uptake of *L lactis* G121 by DCs led to acidification of bacteria containing endosomes, which was abrogated by Baf A1 treatment (Fig 3, A).

Next, we examined the effect of Baf A1 treatment on cytokine induction by moDCs. Cells treated with an endosome acidification-independent stimulus (LPS) were used as a negative control, whereas cells exposed to the TLR7/8 ligand CL097 (recognition of which is acidification dependent) were used as a positive control (Fig 3, B). Baf A1 treatment resulted in a marked

reduction in cytokine release on *L lactis* G121 challenge. These results clearly emphasized the requirement for endosomal acidification for *L lactis* G121-mediated induction of T cell-polarizing cytokines by moDCs.

DC-mediated activation of T cells not only requires T-cell receptor binding of antigen-loaded MHC molecules on the surfaces of antigen-presenting cells but also costimulation. Thus we examined the expression of costimulatory receptors on the surfaces of moDCs by using flow cytometry. We found that CD40, CD80, and CD86 expression was induced on moDCs after *L lactis* G121 challenge (Fig 4, A). This induction was significantly inhibited by Baf A1; however, expression was not inhibited when cells were exposed to LPS rather than *L lactis* G121.

DC endosomal acidification is required for IFN- γ and IL-10 production by T cells in coculture

Baf A1-treated and control moDCs were stimulated with *L lactis* G121, CL097, and LPS and then incubated with naive CD4⁺ T cells to investigate the effect of inhibitor treatment on the induction of IFN- γ and IL-10 by moDCs on coculture. As already shown in Fig 1, B, both *L lactis* G121- and LPS-activated moDCs induced IL-10 and IFN- γ production by T cells, as well as CL097 (Fig 4, B). However, pretreatment of moDCs with Baf A1 resulted in a strong reduction in levels of both cytokines only when moDCs were stimulated with *L lactis* G121 or CL097, but not with LPS, again emphasizing the importance of intracellular acidification for *L lactis* G121-mediated DC activation and T-cell polarization.

Endosomal acidification in DCs is essential for *L lactis* G121-mediated prevention of OVA-induced allergic airway inflammation

To examine whether endosomal processing of *L lactis* G121 is essential for allergy prevention *in vivo*, we induced allergic airway inflammation in naive mice by means of intranasal transfer of OVA-pulsed BMDCs. Before transfer, BMDCs were incubated (or not) with Baf A1 and then either treated with *L lactis* G121 or sham treated with PBS. Concentrations of IL-10 and IL-12p70 in the BMDCs supernatants were then measured. The results revealed that *L lactis* G121 induced strong production of both of these cytokines (Fig 5, A). In addition, the production of IL-10 and IL-12p70 was clearly reduced when BMDCs were pretreated with Baf A1; however, the inhibitor had no effect in the absence of the bacterium. These data corroborate the results obtained with human moDCs (Fig 3, B). Transfer of sham-treated OVA-pulsed BMDCs resulted in clear induction of allergic airway inflammation associated with an influx of eosinophils and lymphocytes into the lungs (Fig 5, B). Histologic analysis of airway and lung tissues revealed significant infiltration by eosinophils and mononuclear cells and increased mucus production by goblet cells (Fig 5, C). By contrast, transfer of *L lactis* G121-treated BMDCs inhibited the development of the allergic phenotype, as reflected by a significant reduction in the number of cells in the bronchoalveolar lavage fluid compared with those in the sham-treated OVA control group. This reduction was mainly due to a decrease in the number of eosinophils and lymphocytes (Fig 5, B, middle and right panels). Histologic analysis of lung tissue revealed an almost complete absence of

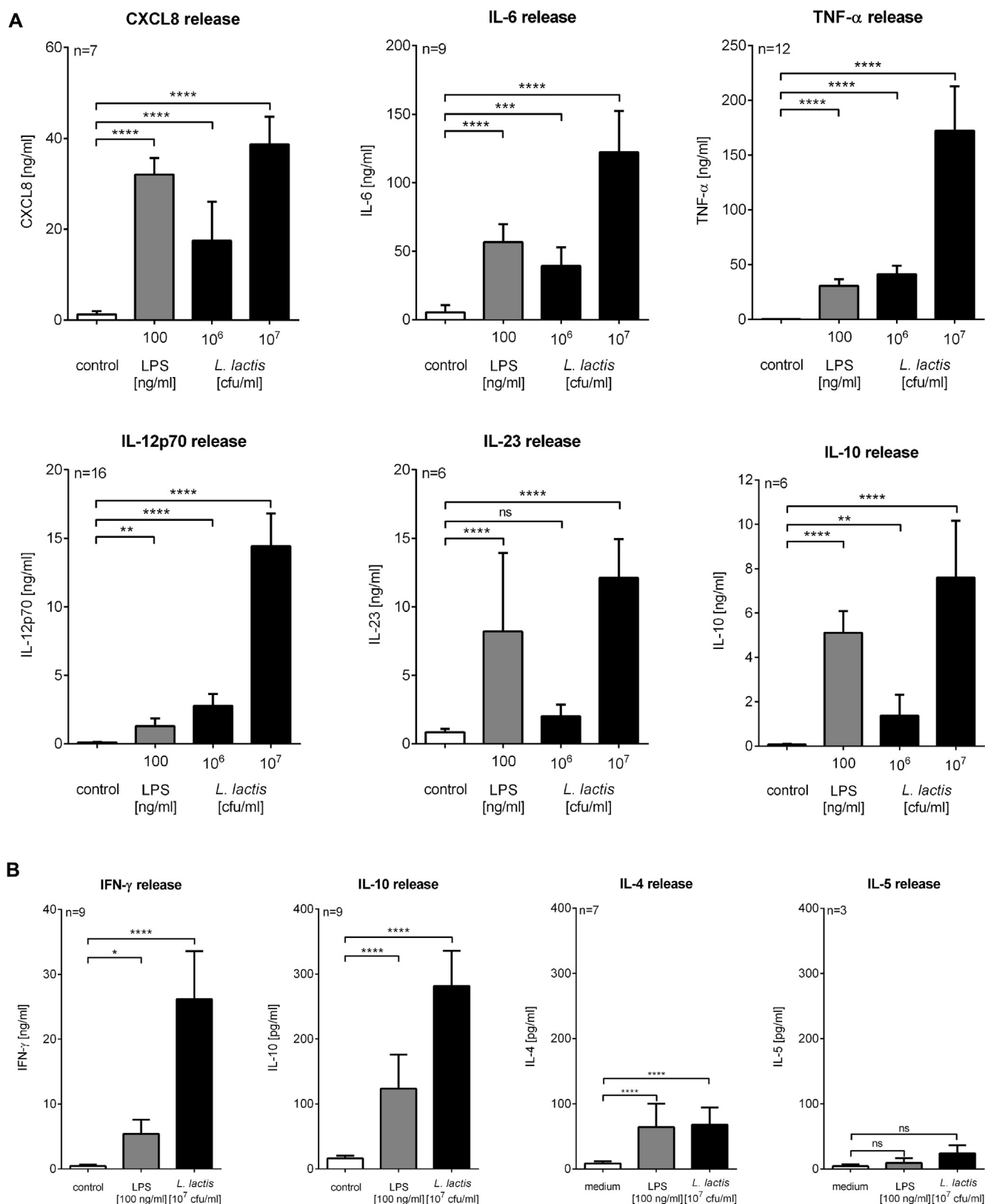


FIG 1. *L. lactis* G121-induced cytokine release by moDCs and in moDC/T-cell cocultures. **A**, Cytokine levels of moDCs (n $\geq$ 5). **B**, Cytokine release of moDC/T-cell cocultures. Statistical analysis was performed by using 2-way ANOVA and the Bonferroni *post hoc* test. **P* < .05, ***P* < .005, ****P* < .001, and *****P* < .0001. ns, Not significant.

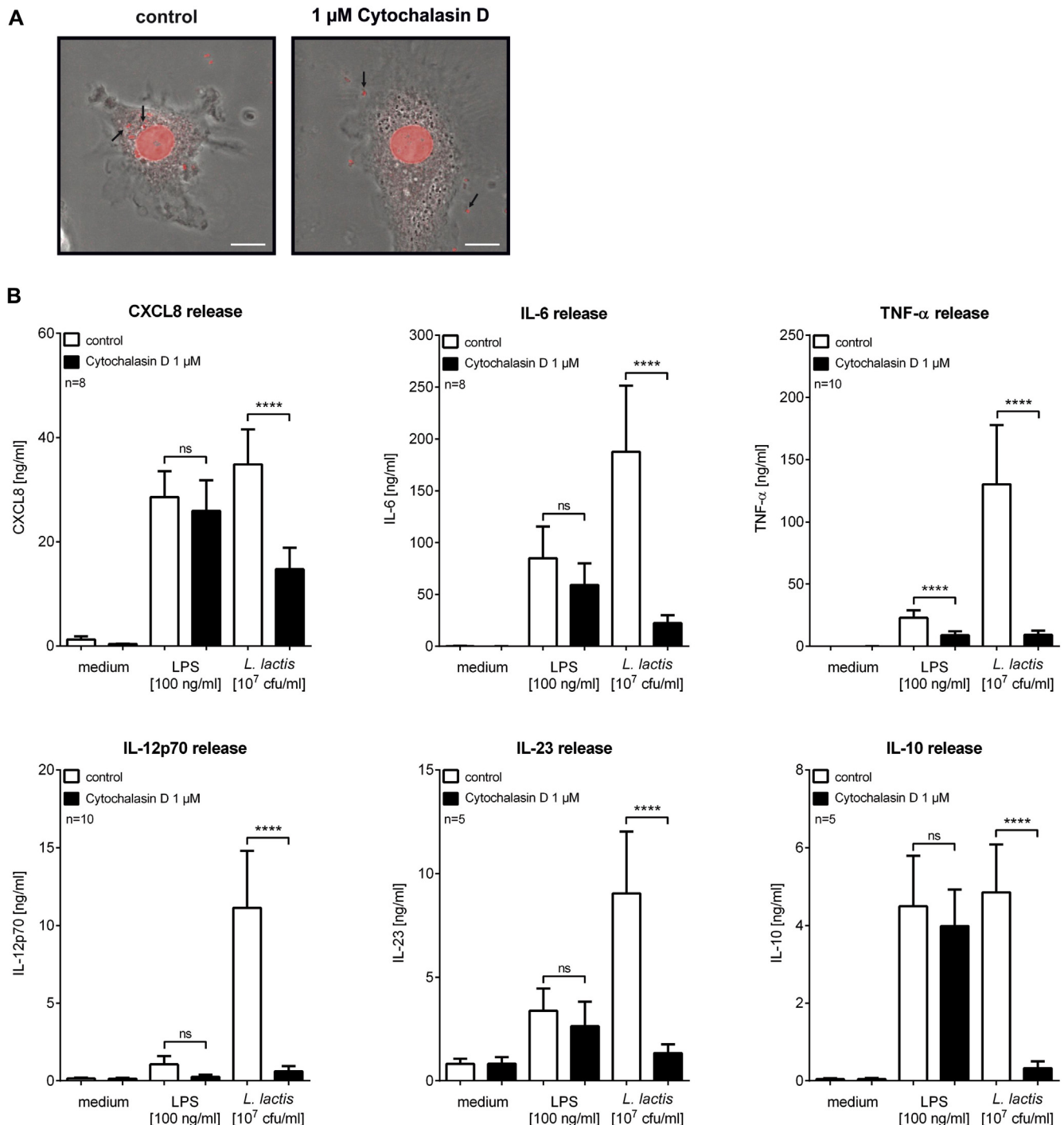


FIG 2. *L. lactis* G121-induced cytokine release by moDCs is dependent on phagocytosis. **A**, Confocal images of *L. lactis* G121-treated human moDCs preincubated with cytochalasin D (nucleus, red; white bars = 10 μ m). Arrows point to bacteria inside and outside the cells (n = 2). **B**, Cytokine release of cytochalasin D-pretreated and stimulated moDCs (n = 5). Statistical analysis was performed by using 2-way ANOVA and the Tukey *post hoc* test. *****P* < .0001. ns, Not significant.

peribronchial and perivascular airway inflammation and mucus production (Fig 5, C and D). However, pretreatment of BMDCs with both Baf A1 and *L. lactis* G121 before transfer almost completely abolished their allergy-preventive effect, as evidenced by a significant increase in the number of leukocytes invading the airway lumen (mainly lymphocytes and eosinophils; Fig 5, B,

middle and right panels). Histologic analysis also revealed marked accumulation of inflammatory cells and goblet cell hyperplasia, which were comparable with those in the sham-treated OVA control group (Fig 5, C and D). Importantly, the results for the Baf A1 control group were not different from those for the sham-treated OVA control group (Fig 5, B-D), ruling out the

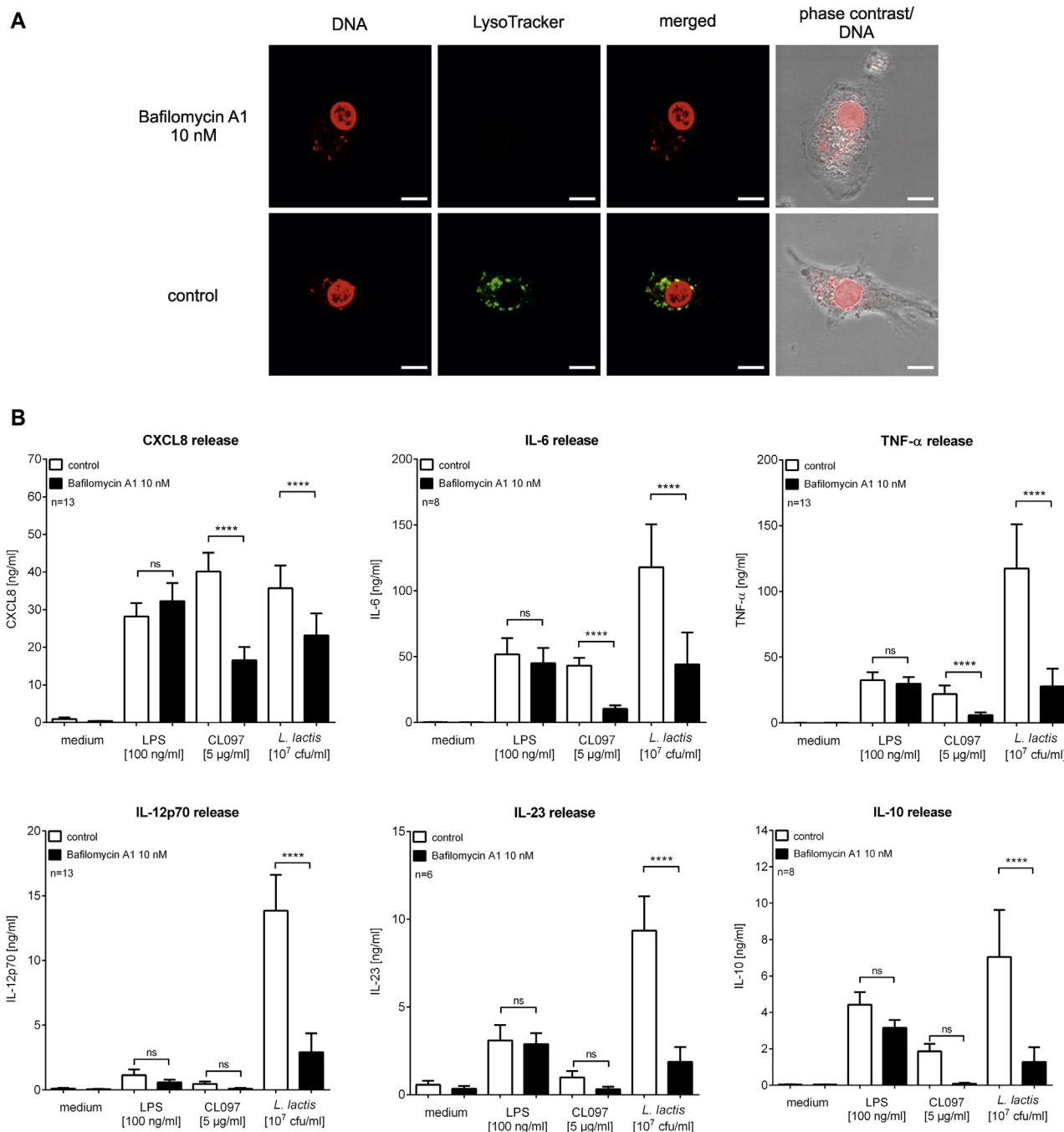


FIG 3. *L. lactis* G121-induced cytokine release by moDCs is dependent on endosomal acidification. **A**, Confocal images of *L. lactis* G121-stimulated human moDCs pretreated with Baf A1 (nucleus, red; acidic vesicles, green; white bars = 10 μ m; n = 3). **B**, Cytokine release of Baf A1-pretreated and stimulated moDCs (n $\geq$ 5). Statistical analysis was performed by using 2-way ANOVA and the Tukey *post hoc* test. **** $P < .0001$. ns, Not significant.

possibility that the immunomodulatory effects were mediated by the inhibitor.

Recognition of *L. lactis* G121 in mice and human subjects

Immune recognition of *L. lactis* G121 by murine cells was dependent on myeloid differentiation response gene 88 but not

on TLR2, TLR3, TLR4, TLR7, TLR8, or TLR9 (data not shown), suggesting that TLR13 (known to be activated by 23S bacterial rRNA),¹⁸ was the primary candidate molecule. Therefore we next generated BMDCs from WT and *Tlr13*^{-/-} mice, which we then challenged with different amounts of *L. lactis* G121 bacteria, as well as isolated total RNA. IL-12 release by BMDCs on *L. lactis* G121 challenge was largely dependent on TLR13 (Fig 6, A, left panel); IL-12 release from *Tlr13*^{-/-} BMDCs was not induced

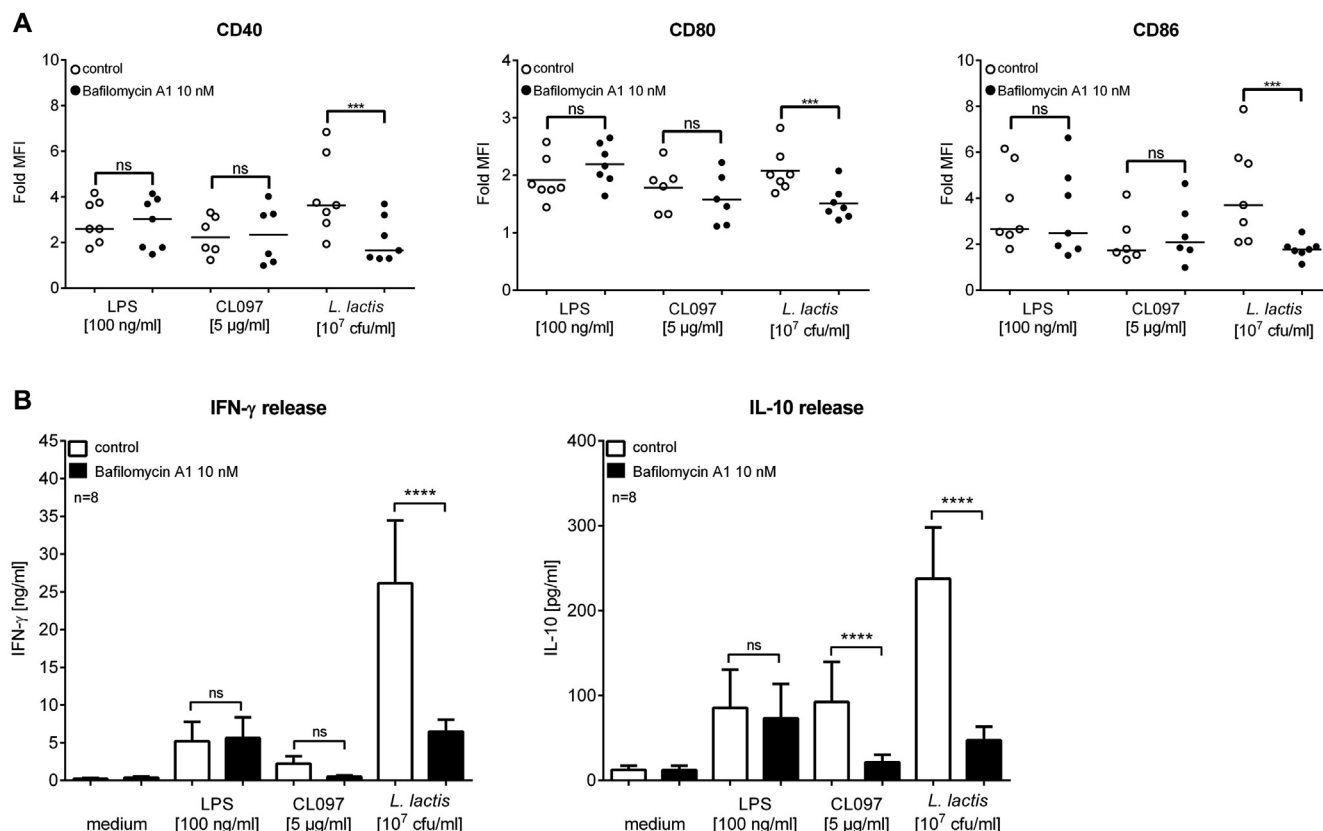


FIG 4. Baf A1 inhibits upregulation of costimulatory molecules on the surfaces of *L. lactis* G121-stimulated moDCs and reduces cytokine release in DC/T-cell cocultures. Human moDCs were stimulated in the presence of Baf A1. **A**, Surface expression of costimulatory molecules shown as fold increase of the mean fluorescence intensity (Fold MFI) with medians. **B**, Cytokine release by moDC/T-cell cocultures (n = 9). Statistical analysis was performed by using 2-way ANOVA and the Tukey *post hoc* test. ****P* < .001 and *****P* < .0001. ns, Not significant.

at a bacterial concentration of 10⁶ colony-forming units (cfu)/mL and was markedly reduced (although still detectable) on application of larger amounts (10⁷ cfu/mL) compared with WT control mice. The response of BMDCs toward *L. lactis* RNA and the 23S rRNA-derived oligoribonucleotide Sa19 was completely dependent on TLR13 expression (Fig 6, A). Because human subjects do not express TLR13,²⁶ we reasoned that another TLR might fulfill the same function. Recently, a number of reports identified human TLR8 as a receptor for bacterial RNA²⁷⁻³⁰; thus we first examined whether *L. lactis* G121 and TLR8 colocalized in moDCs on bacterial uptake (Fig 6, B). TLR8 was highly expressed in endosomal compartments, which were also positive for *L. lactis* G121 DNA and LysoTracker staining, indicating the presence of acidified endosomes. Although colocalization of *L. lactis* and TLR8 is a prerequisite for recognition and intracellular signaling, it does not provide definitive proof. Hence we next used the TLR8-specific immunoregulatory DNA sequence, IRS 957,³¹ to block TLR8 signaling. Treatment with IRS 957 led to a dose-dependent reduction in *L. lactis* G121- and R848-induced cytokine release but did not affect LPS-dependent signaling (Fig 6, C).

Finally, we wanted to verify that *L. lactis* G121 RNA acts as a cytokine-inducing PAMP in human DCs. Thus we isolated bacterial RNA and used it to stimulate moDCs in the presence of DOTAP to ensure endosomal delivery.^{32,33} In addition, we

used MDP (a NOD2 agonist) to investigate possible synergistic effects because we previously reported NOD2-dependent activity in *L. lactis* G121 preparations.¹⁴ *L. lactis* G121 RNA clearly activated moDCs to produce IL-12p70, TNF-α (Fig 6, D), and IL-6 (data not shown). This activity was completely abrogated by RNase treatment and synergistically enhanced on NOD2 activation; human RNA induced no activity under these conditions.

DISCUSSION

We previously showed that *L. lactis* G121 induces production of IL-12, a major T_H1-polarizing cytokine, by human DCs.¹⁴ The present study adds to these findings by showing that *L. lactis* G121-treated moDCs induced T_H1 polarization of naive T cells, as demonstrated by the predominant appearance of T-bet⁺CD4⁺ T cells and release of high doses of IFN-γ. Furthermore, the anti-inflammatory cytokine IL-10 was released by CD4⁺ T cells on coculture with DCs, and a low frequency of Foxp3⁺ cells was observed, which might serve to limit T_H1-derived inflammation.³⁴

We also showed that both phagocytosis of *L. lactis* G121 and endosomal acidification are essential for activation of moDCs and the subsequent induction of T_H1 cells. As previously shown for *Staphylococcus aureus*,³⁵ lack of cell activation after

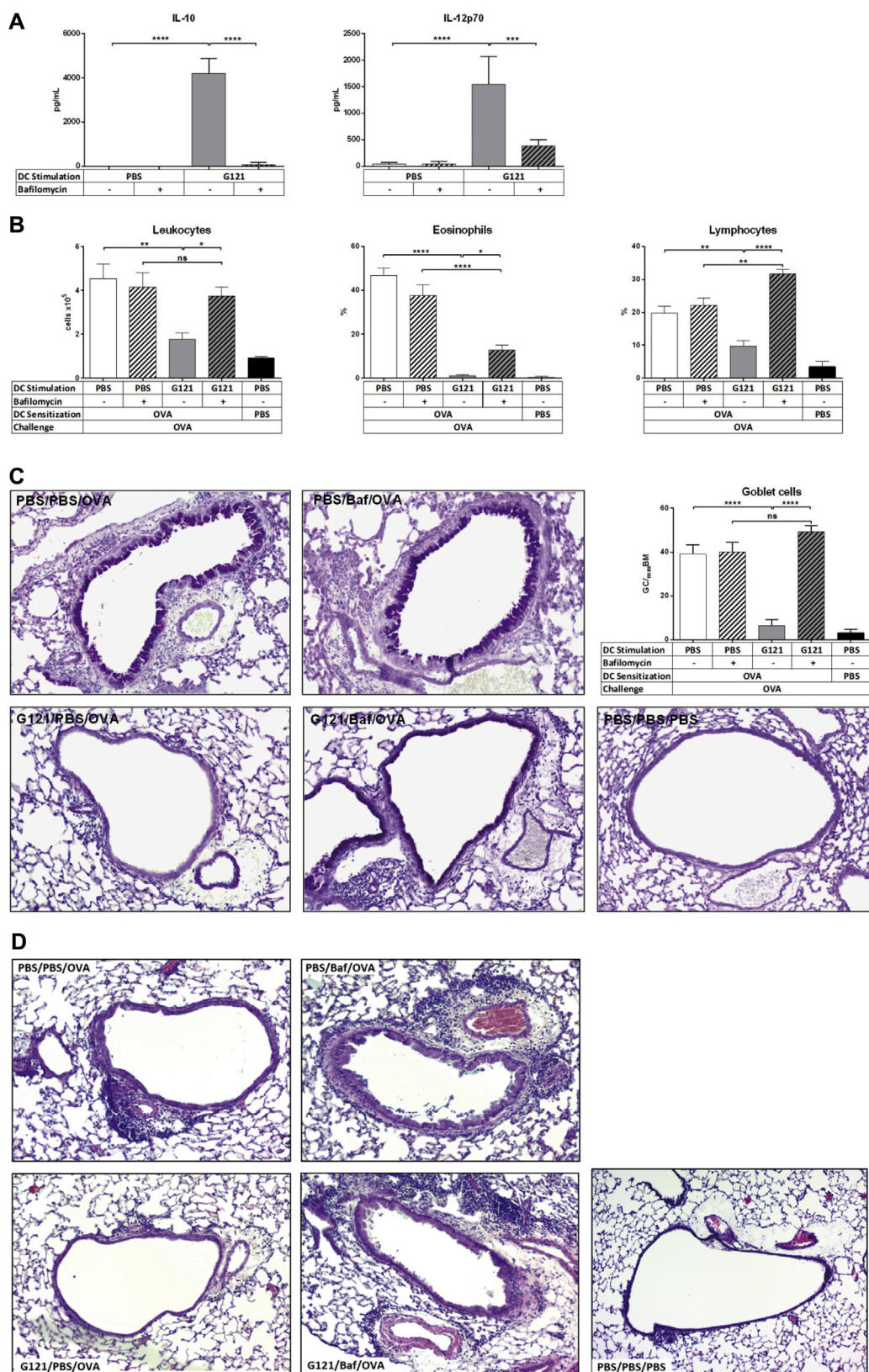


FIG 5. Effects of *L. lactis* G121- and/or Baf A1-treated BMDCs on OVA-induced allergic airway inflammation in a mouse model after adoptive transfer of BMDCs. **A**, Cytokine levels of BALB/c BMDCs preincubated with Baf A1. **B**, Leukocyte counts in bronchoalveolar lavage fluid of mice. **C**, Periodic acid-Schiff staining of mouse lung tissue and number of goblet cells (GC) per millimeter of basal membrane (BM). **D**, Hematoxylin and eosin staining of mouse lung tissue. Data are representative of 2 experiments involving 4 to 5 animals per group. Statistical analysis was performed by using 1-way ANOVA and the Tukey *post hoc* test. * $P < .05$, ** $P < .005$, *** $P < .001$, and **** $P < .0001$. ns, Not significant.

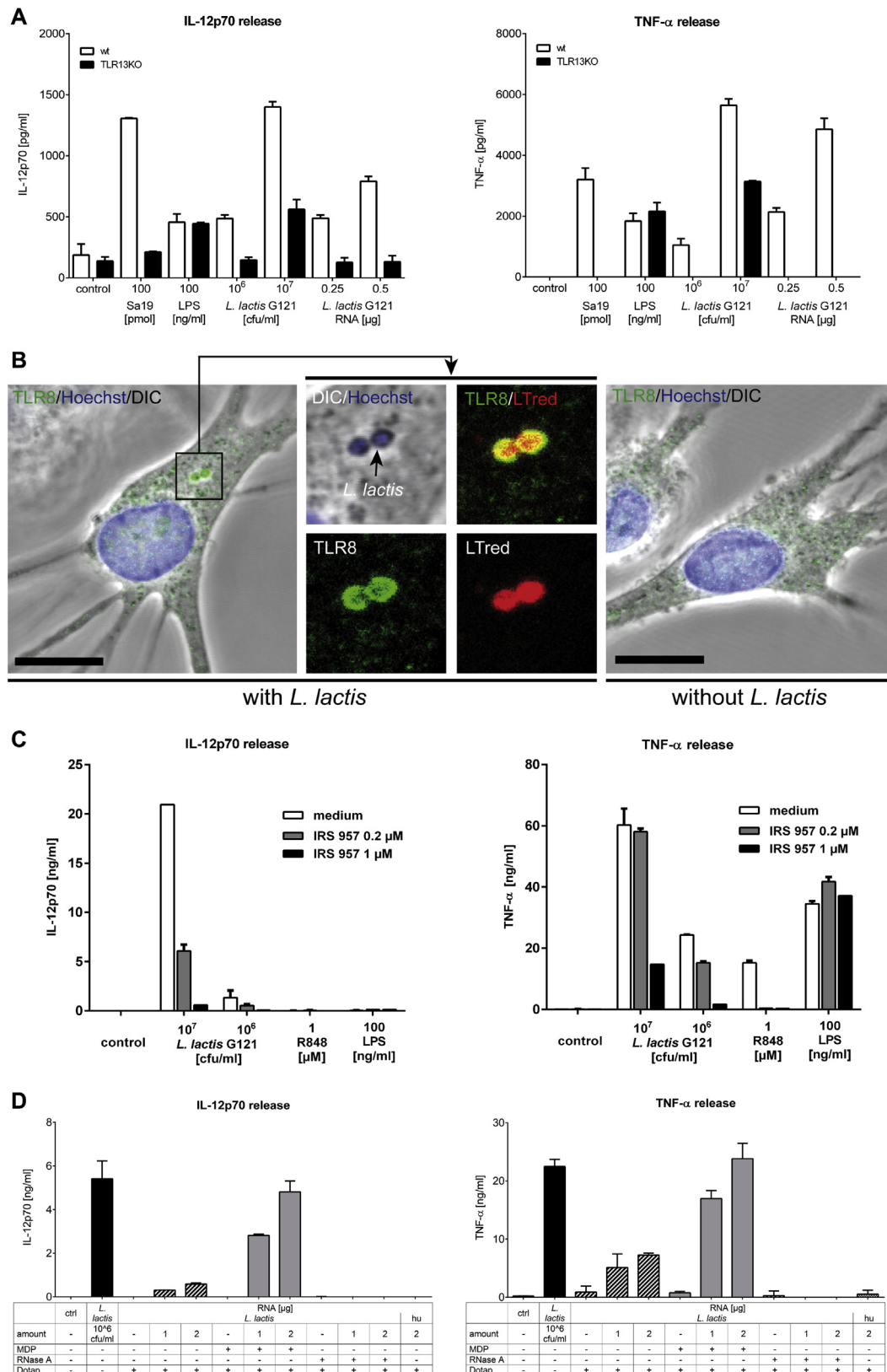


FIG 6. *L. lactis* G121 RNA is an activating PAMP in mice and human subjects acting through TLR13 and TLR8, respectively, and synergistically with MDP. **A**, Cytokine levels of C57BL/6 WT and *Tlr13*^{-/-} BMDCs. **B**, Confocal image of *L. lactis* G121-treated human moDCs stained for nucleus (blue), acidified compartments (red), and TLR8 (green; $n = 3$). **C**, Cytokine levels of moDCs preincubated with IRS 957. **D**, Cytokine levels of moDCs treated with whole bacteria, human RNA, *L. lactis* G121 RNA, and MDP, either with or without RNase treatment. All ELISA data are shown as representative data ($n \geq 3$; means $\pm$ SDs).

inhibition of uptake by moDCs is due to the prevention of PAMPs accessing intracellular PRRs. The data presented herein suggest that activation of moDCs by *L. lactis* G121 is mediated by both intracellular and acidification-dependent sensing mechanisms. Similar observations were made for *Borrelia burgdorferi*, in which TLR8 was implicated as a cellular sensor.³¹ In a previous study we implied that TLR2 and NOD2 act as *L. lactis* G121 sensors.¹⁴ However, TLR2 is activated only when *L. lactis* G121 is present in very high amounts ($>10^7$ cfu/mL, data not shown), suggesting that it plays a rather minor role in allergy-protective host cell activation by TLR2. Activation of NOD2 by its ligand, MDP, depends on vacuolar acidification, presumably because it enables leakage or active transport of MDP into the cytosol.³⁶ However, we observed no secretion of IL-12p70 after challenge of human moDCs with MDP alone; thus we concluded that at least 1 additional endosomal PRR is required for *L. lactis* G121-mediated moDC activation and T_H1 polarization.

The finding that endosomal acidification in *L. lactis* G121-stimulated moDCs was important for induction of a T_H1 immune response was underlined by coculture of moDCs with naive CD4⁺ T cells. Both IFN- γ and IL-10 production by T cells was reduced by Baf A1, indicating general impairment of the ability of *L. lactis* G121-challenged moDCs to activate T cells. On the one hand, this process is likely due to the strongly impaired secretion of T cell-polarizing cytokines (eg, IL-12 and IL-10) by Baf A1-treated and *L. lactis* G121-challenged moDCs because both of these cytokines play dominant roles in inducing T cells to produce IFN- γ and IL-10.^{37,38} On the other hand, it might be due to impaired expression of T-cell costimulatory molecules on the surfaces of DCs. Nonbinding of these receptors to their cognate ligands on T cells results in decreased T-cell activation.^{39,40}

We also examined an animal model of allergic airway inflammation generated by adoptive transfer of *in vitro*-derived and OVA-pulsed murine BMDCs into naive recipient mice. The results showed that the allergy-preventing effects mediated by *L. lactis* G121 were conveyed exclusively by DCs. When BMDCs were treated with *L. lactis* G121 before adoptive transfer, they protected mice from OVA-induced allergic airway inflammation. The capacity of DCs to convey protection against allergies has been reported previously.^{41,42} According to the *in vitro* results presented herein, *L. lactis* G121 might induce a T_H2-counterbalancing T_H1 immune response, thereby preventing an allergic reaction.

We also showed that Baf A1 inhibited cytokine release by BMDCs stimulated *in vitro* with *L. lactis* G121, a result comparable with that observed in moDCs. When *L. lactis* G121-conditioned BMDCs were transferred to recipient mice, they impaired the pathogenesis of asthmatic inflammation; however, this was not the case if the *L. lactis* G121-treated group was pretreated with Baf A1. Nonetheless, the number of eosinophils was not completely restored to levels observed in OVA-sensitized control mice, suggesting that a significant proportion of eosinophilic infiltrate occurs independently of endosome acidification. However, the allergy-preventive effect of *L. lactis* G121 was almost completely abrogated by inhibition of endosomal acidification in BMDCs.

Finally, we identified the major ligand and receptors through which *L. lactis* G121 conveys its allergy-protective effects: in mice induction of IL-12 and TNF- α largely depends on TLR13, the recently identified receptor for 23S rRNA.¹⁸ However, TLR13 is not expressed in human subjects, raising the question of

whether another nucleic acid-sensing TLR recognizes *L. lactis* G121 RNA. Human TLR8, which was first described as a receptor for viral single-strand RNA,^{43,44} is implicated in bacterial RNA recognition.^{28-30,45} Here we clearly show that *L. lactis* G121 and TLR8 colocalize in human DCs, that inhibiting TLR8 signaling strongly reduces cytokine production, and that RNA isolated from *L. lactis* G121 induces both IL-12 and TNF- α . Furthermore, we demonstrated that activation by *L. lactis* G121 RNA can be substantially increased by NOD2 activation, a synergistic response of TLR8 and NOD2 that has also been shown elsewhere.^{46,47} Therefore the apparent difference in the amount of IL-12 induced by whole bacteria versus RNA might be explained by the fact that the degree of activation after the uptake and processing of whole bacteria is much stronger because of synergistic activation processes.

In conclusion, the data presented herein demonstrate that the allergy-protective effects of *L. lactis* G121 are dependent on the T_H1-polarizing activity of DCs, which requires uptake of the bacterium and subsequent endosomal acidification, thereby enabling recognition of bacterial RNA through TLR13 in mice and TLR8 in human subjects.

We acknowledge the technical help of Ina Goroncy, Katrin Böhnstedt, Suhad Al-Badri, and Sarah Stellbogen. We thank the Fluorescence Cytometry Core Facility Borstel for providing support and access to flow cytometers and the confocal laser scanning microscope.

Key messages

- *L. lactis* G121 recognition by human and murine DCs is preceded by bacterial uptake and endosomal acidification, thereby protecting mice from allergy-induced inflammation.
- TLR13 and TLR8 (expressed by murine and human DCs, respectively) mediate *L. lactis* G121 RNA-induced cell activation.
- This activation is synergistically enhanced by NOD2-induced signaling.

REFERENCES

1. Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Bohm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 2000;30:187-93.
2. Douwes J, Travier N, Huang K, Cheng S, McKenzie J, Le Gros G, et al. Lifelong farm exposure may strongly reduce the risk of asthma in adults. *Allergy* 2007;62:1158-65.
3. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;347:869-77.
4. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C, et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med* 2011;364:701-9.
5. Bertorelli G, Bocchino V, Zhou X, Zanini A, Bernini MV, Damia R, et al. Dendritic cell number is related to IL-4 expression in the airways of atopic asthmatic subjects. *Allergy* 2000;55:449-54.
6. van Rijt LS, Jung S, Kleijman A, Vos N, Willart M, Duez C, et al. In vivo depletion of lung CD11c⁺ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005;201:981-91.
7. Bratke K, Lommatzsch M, Julius P, Kuepper M, Kleine HD, Luttmann W, et al. Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge. *Thorax* 2007;62:168-75.
8. Plantinga M, Guillems M, Vanheerswynghe M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells

- initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013;38:322-35.
9. Lambrecht BN, Hammad H. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat Rev Immunol* 2003;3:994-1003.
 10. Gill MA. The role of dendritic cells in asthma. *J Allergy Clin Immunol* 2012;129:889-901.
 11. Romagnani S. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* 2004;112:352-63.
 12. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995;182:1527-36.
 13. Muller G, Muller A, Tuting T, Steinbrink K, Saloga J, Szalma C, et al. Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells in vivo. *J Invest Dermatol* 2002;119:836-41.
 14. Debarry J, Garn H, Hanuszkiewicz A, Dickgreber N, Blumer N, von Mutius E, et al. *Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties. *J Allergy Clin Immunol* 2007;119:1514-21.
 15. Conrad ML, Ferstl R, Teich R, Brand S, Blumer N, Yildirim AO, et al. Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe *Acinetobacter lwoffii* F78. *J Exp Med* 2009;206:2869-77.
 16. Hagner S, Harb H, Zhao M, Stein K, Holst O, Ege MJ, et al. Farm-derived gram-positive bacterium *Staphylococcus sciuri* W620 prevents asthma phenotype in HDM- and OVA-exposed mice. *Allergy* 2013;68:322-9.
 17. Debarry J, Hanuszkiewicz A, Stein K, Holst O, Heine H. The allergy-protective properties of *Acinetobacter lwoffii* F78 are imparted by its lipopolysaccharide. *Allergy* 2010;65:690-7.
 18. Oldenburg M, Kruger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science* 2012;337:1111-5.
 19. Hidmark A, von Saint Paul A, Dalpke AH. Cutting edge: TLR13 is a receptor for bacterial RNA. *J Immunol* 2012;189:2717-21.
 20. Li XD, Chen ZJ. Sequence specific detection of bacterial 23S ribosomal RNA by TLR13. *Elife* 2012;1:e00102.
 21. Boyum A. Separation of white blood cells. *Nature* 1964;204:793-4.
 22. Turpin J, Hester JP, Hersh EM, Lopez-Berestein G. Centrifugal elutriation as a method for isolation of large numbers of functionally intact human peripheral blood monocytes. *J Clin Apher* 1986;3:111-8.
 23. Goddette DW, Frieden C. Actin polymerization. The mechanism of action of cytochalasin D. *J Biol Chem* 1986;261:15974-80.
 24. Shinji H, Akagawa KS, Yoshida T. Cytochalasin D inhibits lipopolysaccharide-induced tumor necrosis factor production in macrophages. *J Leukoc Biol* 1993;54:336-42.
 25. Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity* 2010;32:305-15.
 26. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, et al. The evolution of vertebrate Toll-like receptors. *Proc Natl Acad Sci U S A* 2005;102:9577-82.
 27. Nishibayashi R, Inoue R, Harada Y, Watanabe T, Makioka Y, Ushida K. RNA of *Enterococcus faecalis* strain EC-12 Is a major component inducing interleukin-12 production from human monocytic cells. *PLoS One* 2015;10:e0129806.
 28. Bergstrom B, Aune MH, Awuh JA, Kojen JF, Blix KJ, Ryan L, et al. TLR8 senses *Staphylococcus aureus* RNA in human primary monocytes and macrophages and induces IFN-beta production via a TAK1-IKKbeta-IRF5 signaling pathway. *J Immunol* 2015;195:1100-11.
 29. Tanji H, Ohto U, Shibata T, Taoka M, Yamauchi Y, Isobe T, et al. Toll-like receptor 8 senses degradation products of single-stranded RNA. *Nat Struct Mol Biol* 2015;22:109-15.
 30. Eigenbrod T, Pelka K, Latz E, Kreikemeyer B, Dalpke AH. TLR8 senses bacterial RNA in human monocytes and plays a nonredundant role for recognition of *Streptococcus pyogenes*. *J Immunol* 2015;195:1092-9.
 31. Cervantes JL, Dunham-Ems SM, La Vake CJ, Petzke MM, Sahay B, Sellati TJ, et al. Phagosomal signaling by *Borrelia burgdorferi* in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN-beta. *Proc Natl Acad Sci U S A* 2011;108:3683-8.
 32. Almofti MR, Harashima H, Shinohara Y, Almofti A, Baba Y, Kiwada H. Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization. *Arch Biochem Biophys* 2003;410:246-53.
 33. Yasuda K, Yu P, Kirschning CJ, Schlatter B, Schmitz F, Heit A, et al. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and independent pathways. *J Immunol* 2005;174:6129-36.
 34. Yang X, Gartner J, Zhu L, Wang S, Brunham RC. IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following *Chlamydia trachomatis* lung infection. *J Immunol* 1999;162:1010-7.
 35. Wolf AJ, Arruda A, Reyes CN, Kaplan AT, Shimada T, Shimada K, et al. Phagosomal degradation increases TLR access to bacterial ligands and enhances macrophage sensitivity to bacteria. *J Immunol* 2011;187:6002-10.
 36. Marina-Garcia N, Franchi L, Kim YG, Hu Y, Smith DE, Boons GJ, et al. Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation. *J Immunol* 2009;182:4321-7.
 37. Koch F, Stanzl U, Jennwein P, Janke K, Heufler C, Kampgen E, et al. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* 1996;184:741-6.
 38. Clausen BE, Girard-Madoux MJ. IL-10 control of dendritic cells in the skin. *Oncoimmunology* 2013;2:e23186.
 39. Karimi MH, Ebadi P, Pourfathollah AA, Moazzeni M, Soheili ZS, Samiee S. Comparison of three techniques for generation of tolerogenic dendritic cells: siRNA, oligonucleotide antisense, and antibody blocking. *Hybridoma (Larchmt)* 2010;29:473-80.
 40. Lim TS, Goh JK, Mortellaro A, Lim CT, Hammerling GJ, Ricciardi-Castagnoli P. CD80 and CD86 differentially regulate mechanical interactions of T-cells with antigen-presenting dendritic cells and B-cells. *PLoS One* 2012;7:e45185.
 41. Gorelik L, Kauth M, Gehlhar K, Bufer A, Holst O, Peters M. Modulation of dendritic cell function by cowshed dust extract. *Innate Immun* 2008;14:345-55.
 42. Takagi T, Taguchi O, Toda M, Ruiz DB, Bernabe PG, D'Alessandro-Gabazza CN, et al. Inhibition of allergic bronchial asthma by thrombomodulin is mediated by dendritic cells. *Am J Respir Crit Care Med* 2011;183:31-42.
 43. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol* 2002;3:499.
 44. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004;303:1526-9.
 45. Cervantes JL, La Vake CJ, Weinerman B, Luu S, O'Connell C, Verardi PH, et al. Human TLR8 is activated upon recognition of *Borrelia burgdorferi* RNA in the phagosome of human monocytes. *J Leukoc Biol* 2013;94:1231-41.
 46. Vissers M, Remijn T, Oosting M, de Jong DJ, Diavatopoulos DA, Hermans PW, et al. Respiratory syncytial virus infection augments NOD2 signaling in an IFN-beta-dependent manner in human primary cells. *Eur J Immunol* 2012;42:2727-35.
 47. Schwarz H, Posselt G, Wurm P, Ulbing M, Duschl A, Horejs-Hoeck J. TLR8 and NOD signaling synergistically induce the production of IL-1beta and IL-23 in monocyte-derived DCs and enhance the expression of the feedback inhibitor SOCS2. *Immunobiology* 2013;218:533-42.

METHODS

L. lactis G121–treated, LPS-treated, or untreated moDCs were washed and cultured with naive CD4⁺ T cells at a ratio of 1:10. After 5 days, cocultures were restimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Merck Millipore) and 2 µg/mL ionomycin (Thermo Fisher Scientific) for 5 hours, the last 4 hours in the presence of 1 µL/mL Brefeldin A (Thermo Fisher Scientific). Cocultured cells were treated with LIVE/DEAD Fixable Blue Dead Cell Stain (Thermo Fisher Scientific) and stained with CD4 (BioLegend, Aachen, Germany) and CD11c (Thermo Fisher Scientific) antibodies. Intracellular staining of cytokines and transcription factors was performed

with the Fixation and Permeabilization Kit or Foxp3/Transcription Factor Fixation/Permeabilization Kit, respectively (both from Thermo Fisher Scientific), according to the manufacturer's protocol. Antibodies used were IFN-γ, Foxp3, and RORγt (Thermo Fisher Scientific) and GATA3, T-bet, and IL-10 (BioLegend), as well as appropriate isotype controls. The gating strategy was as follows: (1) dead cell exclusion by means of discrimination of LIVE/DEAD Fixable Blue Dead Cell Stain–positive cells (except for [Fig E1](#)) and (2) doublet exclusion by plotting forward scatter A against forward scatter H. Flow cytometry was performed in an LSRII cytometer (BD Biosciences), and data were analyzed with FlowJo version 10.1 software (TreeStar).

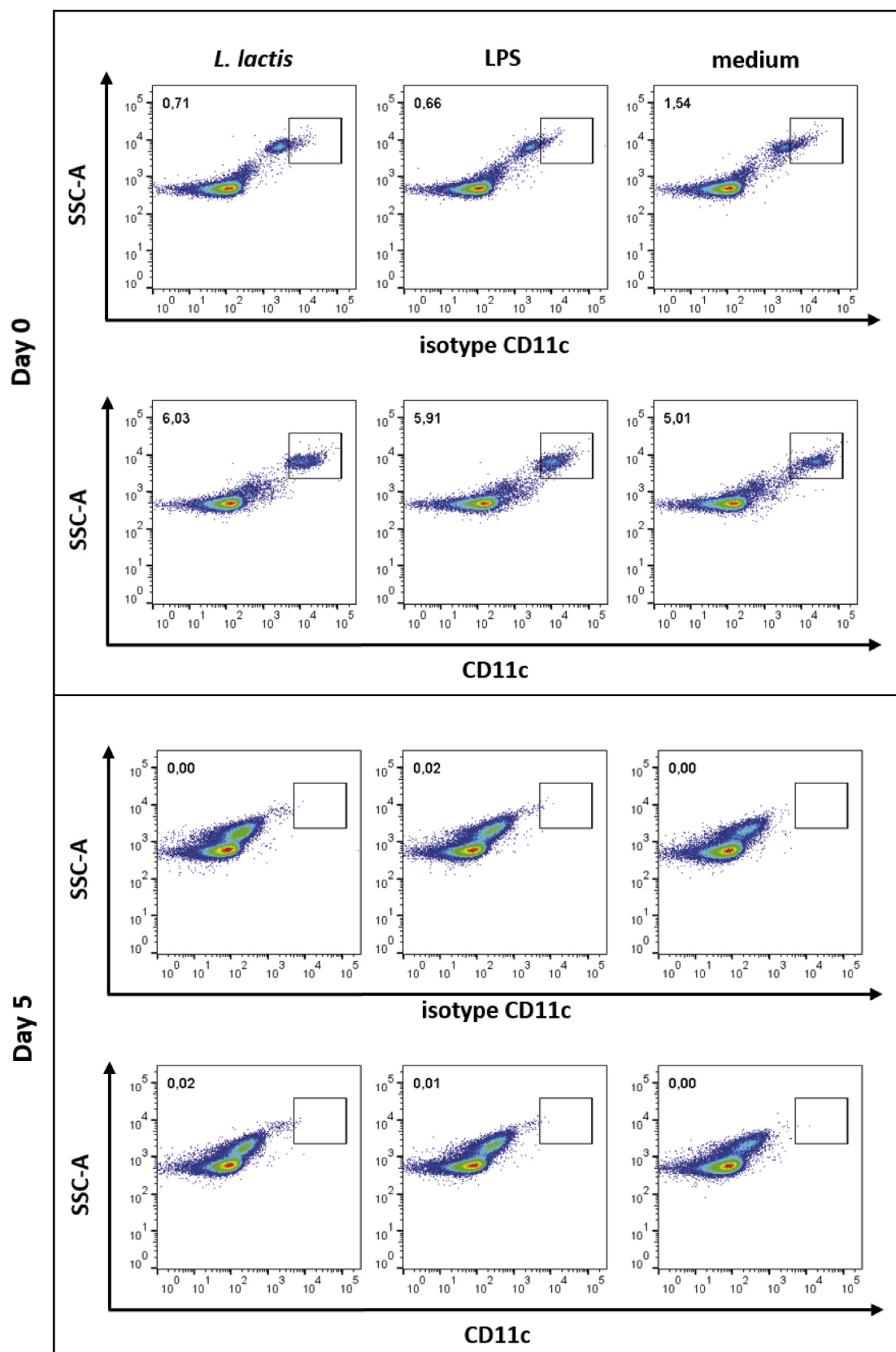


FIG E1. DC population is lost in the coculture after 5 days. Fluorescence-activated cell sorting staining of moDCs (CD11c⁺) in cocultures at days 0 and 5 is shown (representative data, n = 6). SSC, Side scatter.

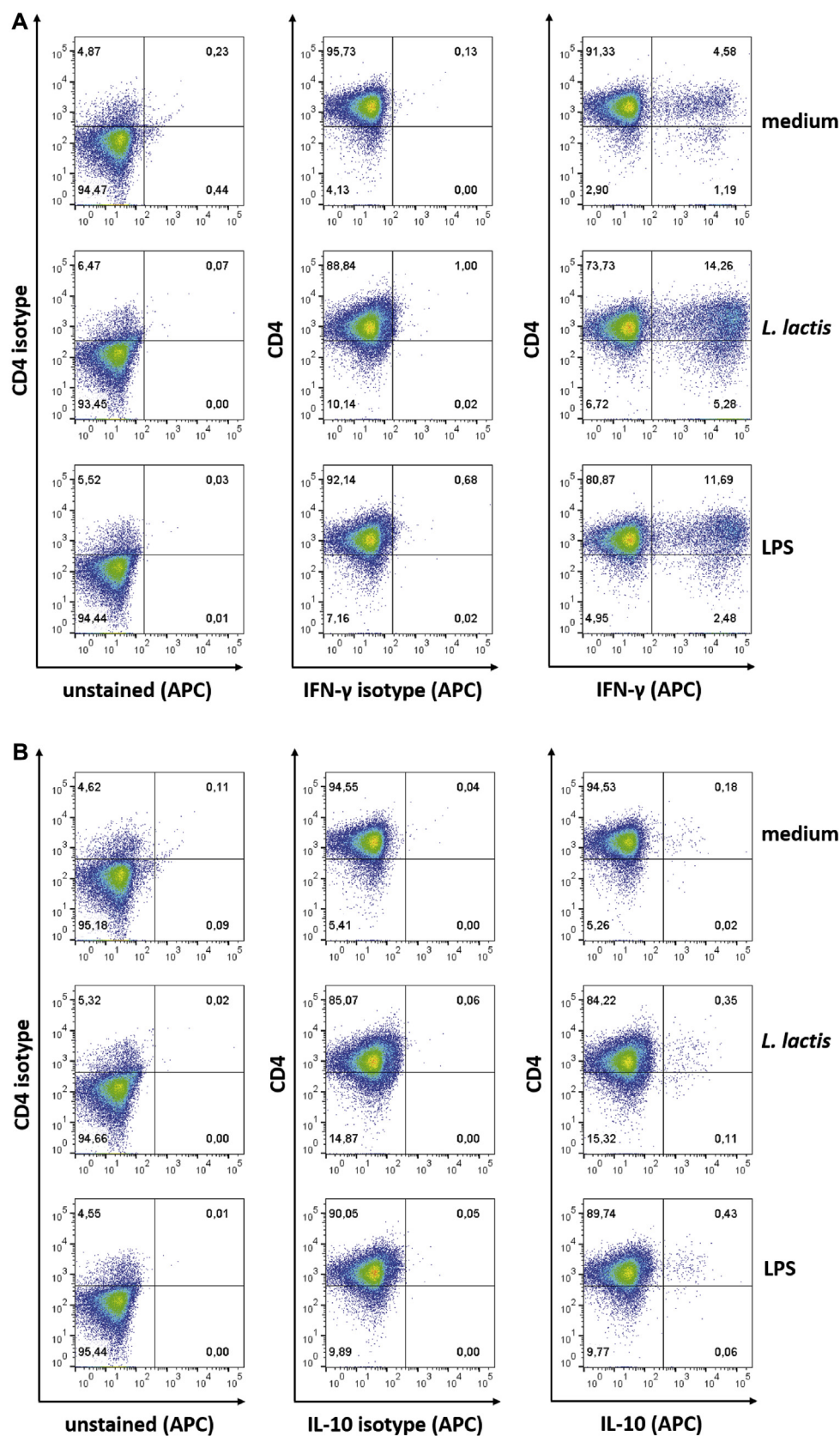


FIG E2. *L. lactis* G121-stimulated DCs induce IFN- γ ⁺ and IL-10⁺ T cells in coculture. Intracellular IFN- γ (A) and IL-10 (B) expression of CD4⁺ T cells cocultured for 5 days with stimulated or untreated moDCs is shown (representative data, n = 6).

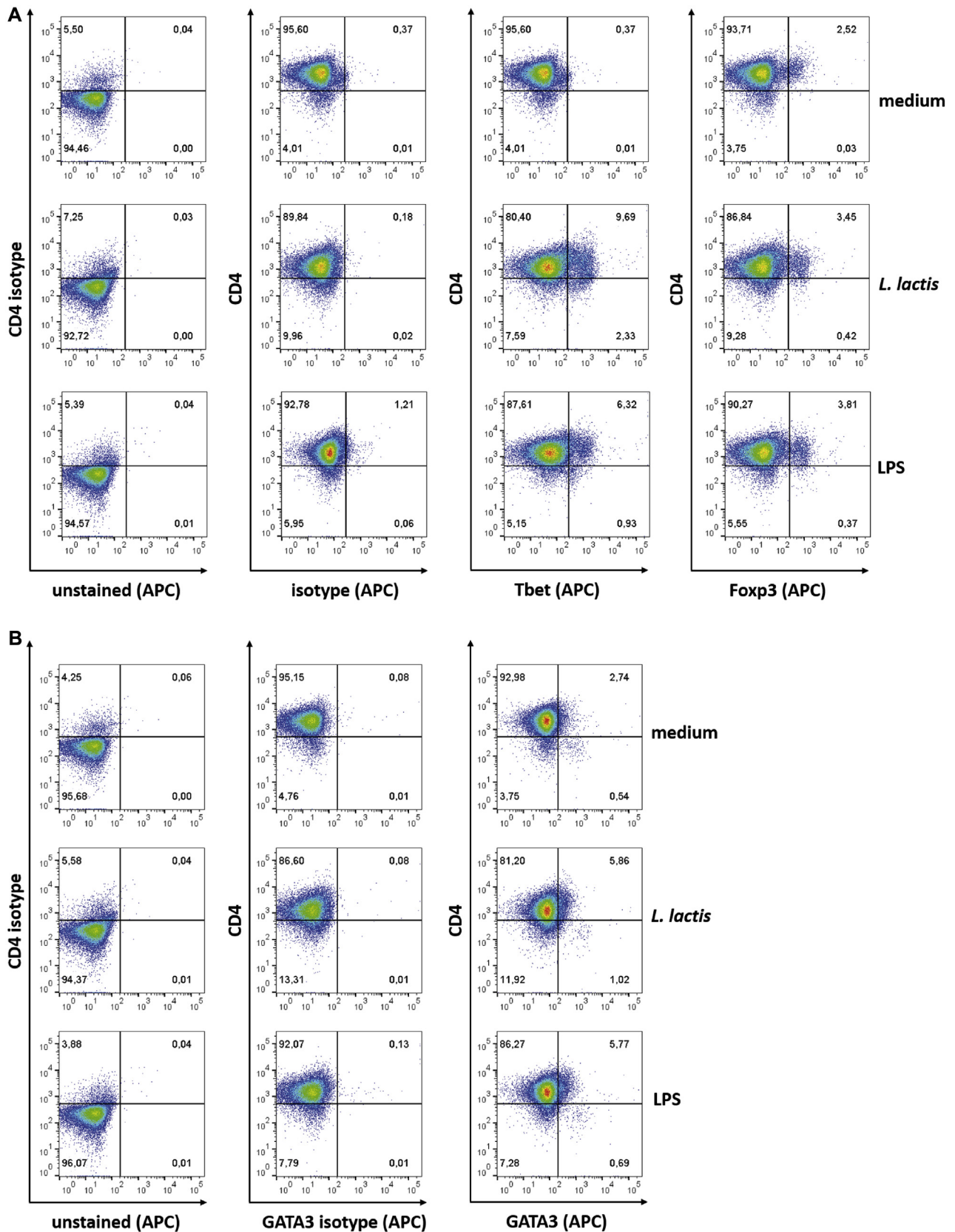


FIG E3. *L. lactis* G121-stimulated DCs induce predominantly T_H1 cells. Intracellular Foxp3 and Tbet (A), GATA3 (B), and ROR- γ t (C) expression of $CD4^+$ T cells cocultured for 5 days with stimulated or untreated moDCs is shown (representative data, $n = 6$).

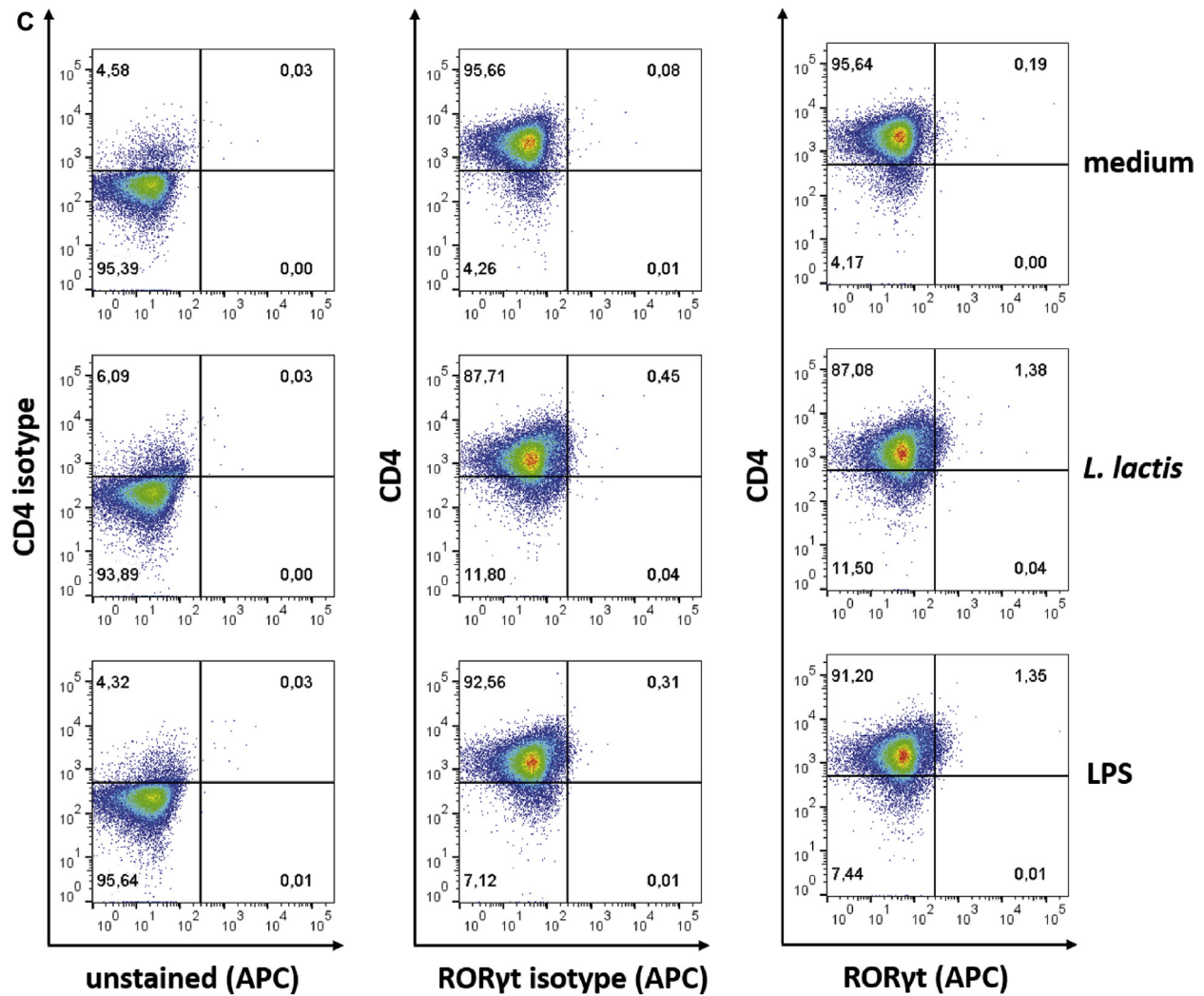


FIG E3. (Continued).