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Comprehensive Analysis of CD4⁺ T Cells in the Decision between Tolerance and Immunity In Vivo Reveals a Pivotal Role for ICOS

Timo Lischke, Anika Hegemann, Stephanie Gurka, Dana Vu Van, Yvonne Burmeister, Kong-Peng Lam, Olivia Kershaw, Hans-Joachim Mollenkopf, Hans Werner Mages, Andreas Hultoff, and Richard A. Kroczek

We have established a comprehensive in vivo mouse model for the CD4⁺ T cell response to an “innocuous” versus “dangerous” exogenous Ag and developed an in vivo test for tolerance. In this model, specific gene-expression signatures, distinctive upregulation of early T cell-communication molecules, and differential expansion of effector T cells (Teff) and regulatory T cells (Treg) were identified as central correlates of T cell tolerance and T cell immunity. Different from essentially all other T cell-activation molecules, ICOS was found to be induced in the immunity response and not by T cells activated under tolerogenic conditions. If expressed, ICOS did not act as a general T cell costimulator but selectively caused a massive expansion of effector CD4⁺ T cells, leaving the regulatory CD4⁺ T cell compartment largely undisturbed. Thus, ICOS strongly contributed to the dramatic change in Ag.

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T.L. and A. Hegemann designed and performed experiments, analyzed and interpreted the data, and contributed to the writing of the manuscript; S.G., D.V.V., Y.B., H.W.M., and A. Hutloff provided specialized expertise and/or assisted with some experiments; K.-P.L. provided mice; O.K. performed the histology; H.-J.M. assisted in the gene-expression experiments; and R.A.K. conceived the project, designed experiments, interpreted the data, and wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ATCC, American Type Culture Collection; Ct, cycle threshold; DIG, digoxigenin; ICOS-L, ICOS ligand; i.n., intranasally; KO, knockout; LN, lymph node; Teff, effector T cell; Treg, regulatory T cell; WT, wild-type.

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It is well understood that Ag, when recognized in the context of pathogen-specific structural elements like LPS, dsRNA, or dsDNA, induces CD4⁺ T cells to massively proliferate and to express a variety of inflammatory cytokines as part of the immune defense. In contrast, the effect of innocuous exogenous Ags on the CD4⁺ T cell compartment is much less well defined mechanistically, and the same is true for the general concept of “peripheral tolerance” to harmless environmental Ags entering the body (1, 2).

In the past, numerous approaches have been undertaken in vitro to better understand the decision between tolerance and immunity (3), but these experimental systems were strongly reductionistic and, therefore, only partially reflected the process in vivo. It is clear that the decision between tolerance and immunity results from a reciprocal communication between CD4⁺ T cells and dendritic cells presenting an Ag in the peripheral immune system. However, the molecular switches driving CD4⁺ T cells into “defense” versus “tolerance” modes in response to exogenous Ags remain ill defined.

The molecular study of immunity versus tolerance requires robust in vivo models, allowing a systematic dissection of the involved molecular pathways. The goal of the present work was to establish such a model of peripheral immunity and tolerance to an exogenous Ag, to comprehensively describe the respective immune-response patterns, and to identify key elements in the dialogue of CD4⁺ T cells with dendritic cells mediating these responses. We decided to use a system of CD4⁺ T cell activation in vivo based on the application of the Ag OVA in two forms: endotoxin free, representing an innocuous exogenous Ag, or in combination with LPS, as a model for a pathogenic Ag. OVA was administered as a whole protein (and not as peptide), to ensure processing and presentation by professional APCs, in sufficient amounts to avoid partial T cell activation and transiently to mimic an influx of an environmental Ag. The T cell response was mea-
served with adoptively transferred, OVA-specific naive DO11.10 CD4+ T cells, a powerful system pioneered by Jenkins and colleagues (4). We also determined the humoral response to obtain a comprehensive view of adaptive immunity. Using this system, we were able to define distinct gene-expression signatures, specific T cell-activation patterns, and differential expansion of effector T cells (Teff) and regulatory T cells (Treg) as new correlates of tolerance and immunity in vivo. Furthermore, ICOS was identified as a central determinant in the decision between immunity and tolerance to an exogenous Ag.

ICOS, a T cell-specific molecule that is upregulated following cell activation, has early on been identified as essential for a successful humoral immune response (5). Further work elucidated the critical role for ICOS in the development of follicular Th cells and, thus, for effective T cell help for B cells (6, 7). However, the overall function of ICOS for all other Teff remained less clear (5). ICOS was assumed to be a potent costimulator of T cell activation, amplifying the induction of early cell surface molecules and the proliferation of T cells (8, 9). Furthermore, many in vivo experimental data indicated an important role for ICOS in the differentiation of naive T cells to effector cells secreting inflammatory cytokines (5).

In our model of immunity and tolerance, ICOS emerged as one of the very few T cell-communication molecules that are selectively upregulated by an inflammatory, but not by an innocuous, Ag. Using a gene-deletion mouse model, we demonstrate that ICOS has no role in the early activation of T cells and does not drive the differentiation of CD4+ T cells to cytokine-secreting effectors in a general way. Instead, ICOS plays a specific role in the early massive expansion of “innamatory” CD4+ Teff, leaving the Treg compartment largely unaffected. Thus, ICOS strongly contributes to the dominance of T cells over Treg observed in an inflammatory response. Through this newly identified role for the Treg/Treg balance, together with its established function in the B cell immune system, ICOS emerges as a central mediator of adaptive immunity. Given its exceptionally specific induction under inflammatory conditions, ICOS can be regarded as a pivotal effector molecule in the early decision between tolerance and immunity to an exogenous Ag.

Materials and Methods

Mice

OVA-TCR–transgenic DO11.10 mice were from The Jackson Laboratory (Bar Harbor, ME), and BALB/cAnNcrl mice and C57BL/6 mice were from Charles River (Sulzfeld, Germany). ICOS knockout (KO) mice (10) were backcrossed 10 times onto C57BL/6, ICOS ligand (ICOS-L) knockout mice (11) were backcrossed 10 times onto BALB/cAnNcrl (C. B7R-P-1 KO) and onto C57BL/6 (B6.B7R-P-1 KO), respectively. OVA-TCR–transgenic OT-II mice (12) were backcrossed 10 times onto C57BL/6 mice and additionally crossed with B6.PL mice (The Jackson Laboratory); these mice were further crossed to ICOS KO mice. All mice were bred under specific pathogen-free conditions in the animal facility of the Federal Institute for Risk Assessment (Berlin, Germany). Experiments were performed according to state guidelines and were approved by local authorities.

Adoptive-transfer system and immunization

Splenocytes were obtained by mashing spleens through cell sieves into PBS, followed by erythrocyte lysis with ACK lysing buffer (Invitrogen, Carlsbad, CA). Naive OVA-TCR–transgenic CD4+ T cells (4 × 10^6, unless otherwise indicated), enriched from spleens of DO11.10 mice by depletion of CD8+ cells with CD8 beads and subsequent positive sorting with CD62L beads (Miltenyi Biotec, Bergisch Gladbach, Germany), were transferred i.v. into syngeneic BALB/c or C.B7R-P-1 KO mice. Transferred DO11.10 CD4+ T cells typically contained ~5% Foxp3+ Treg and <1% Foxp3+ CD62L−CD44hi “effector” T cells. For in vivo proliferation analysis, cells were labeled prior to adoptive transfer with CFSE (Invitrogen), according to standard methods. Recipient mice were immunized i.v. with 2 mg endotoxin-free OVA 24 h later; some mice received at the same time 30 μLPS from Escherichia coli O55:B5 (Sigma-Aldrich, St. Louis, MO). Endotoxins were removed from OVA (Sigma-Aldrich) using Detoxi-Gel (Pierce, Rockford, IL) or EndoTrap red (Hyglos, Regensburg, Germany), resulting in <0.5 U endotoxin/mg protein, as determined by the Limulus amebocyte lysate assay (Charles River, Wilmington, NC). Detoxified OVA injected i.v. did not upregulate CD86 or PD-L1 on splenic dendritic cells, as measured 5 h later (A. Hegemann, unpublished observations).

In vivo blockade of ICOS–ICOS-L interactions

Mice were injected i.p. with 150 μg anti–ICOS-L Ab (MIL-5733) every other day, starting on the day of immunization. MIL-5733 is a nondepleting, blocking mAb (13). The control group was injected with 150 μg rat control Ab of the same isotype (IgG2a).

Flow cytometric analysis of cells

Cells were counted using an hemocytometer or the PCA cell analyzer with the ViaCount Assay (Guava Technologies, Hayward, CA). For surface staining, cells were first incubated with 100 μg/ml 2.4G2 (FcγRIII/BII, American Type Culture Collection [ATCC], Manassas, VA) and 50 μg/ml purified rat Ig (Nordic, Täliburg, The Netherlands) to minimize specific Ab binding. Staining was performed on ice with mAb conjugated to FITC, PE, PE-Cy7, Alexa Fluor, Pacific Blue, digoxigenin (DIG), or biotin, according to standard methods. 11-26c (specific for IgG1), ID3 (CD19), 53-6,72 (CD8α), GK1.5 (CD4), GL7 (CD7), IM7.8.1 (CD16/CD32), MR1-1 (Clec16A), MEL-14 (CD206), N418 (CD11c), UC10-4FI0 (CTLA-4) were from ATCC; 2E2 (CD25) (14), mBT-1622 (BTLA) (15), KJ1-26 (OVA-TCR), KT3 (CD3) (16), MIC-280 (ICOS) (17), MIC-2043 (ICOS) (18), OX-86 (OXR0), RA3-6822 (B220) or RB6-8C5 (Ly-6G/C), 2G8 (CDxR5), and ES0-2440 (Siglec-F) were from BD Biosciences (San Jose, CA); 17BS (4-1BB), 30-F11 (CD45), 53-6,7 (CD8α), CNX46-3, (CD160), H1F123 (CD86), J43 (PD-1), RMP1-30 (PD-1), and RA3-6822 (B220) were from eBioscience (San Diego, CA); 6D5 (CD19), 1K225S (RANKL), RA3-6822 (B220) from BioLegend (San Diego, CA); and PNA was from Vector Laboratories (Burlingame, CA). Commercial Abs were coupled to the above-described fluorophores or to allophycocyanin-Cy7, allophycocyanin-eFluor 780, and allophycocyanin-A750. Alexa Fluor 647-conjugated polyclonal sheep anti-DIG (Roche, Mannheim, Germany) and PE-Cy7-conjugated streptavidin (eBioscience) were used as secondary reagents. For staining of intracellular Ags (CD40L, CTLA-4) or cytokines, cells were labeled with 1.34 μM Pacific Orange prior to fixation (to exclude dead cells) and fixed with 2% formaldehyde. Intracellular staining was then performed in 0.5% saponin (Sigma-Aldrich) at room temperature, containing 100 μg/ml 2.4G2 and 50 μg/ml purified rat Ig. Staining for Foxp3 was performed with allophtococyanin-conjugated mAb FJK-16s, according to the manufacturer’s instructions (eBioscience). Cells were analyzed on a LSR II flow cytometer after gating out DAP7+ dead cells. Cell sorting was performed using a FACSaria II (both from BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Cytokine detection

Secretion of cytokines was measured directly ex vivo for 45 min at 37°C (without restimulation) using the appropriate Cytokine Secretion Assay (Miltenyi Biotec). The capacity of flow-sorted splenic KJ1-26+ CD4+ T cells or T cells obtained from lungs to generate cytokines was measured after stimulation with 0.02 μg/ml PMA and 1 μg/ml ionomycin in complete medium for 4.5 h, with 5 μg/ml brefeldin A (all from Sigma-Aldrich) present for the last 3.5 h. The following mAb were used for cytokine staining: 11B11 (IL-4; ATCC), AN18.17.24 (IFN-γ) (19), JES5-5H4 (IL-2), JES5-2A5 (IL-10), 7C11 (IL-17A), BioLegend, mBT-1622 (BTLA) (15), KJ1-26 (OV A-TCR), KT3 (CD3) (16), 18F10 (IL-17F; eBioscience). Stained cells were analyzed by flow cytometry; analysis gates were set on CD4+ T cells after exclusion of B cells and cells that had died before fixation. Further analysis was described above.

Immunohistochemistry

Spleens of BALB/c recipient mice were snap-frozen in NaCl solution (0.9% [w/v], NaCl) and were processed, rinsed, and frozen in TissueTek Cryostat sections (8 μm) were fixed in acetone. Spleen sections were stained with KJ1-26mAb, 11-26cFITC (IgD), and GL7mAb using a VECTASTAIN ABC-AP Kit (Vector Labs, Burlingame, CA); anti-DIG mAb coupled to alkaline phosphatase (Roche) and anti-FITC mAb coupled to HRP (Roche) were used as secondary reagents.
Substrate reactions were performed using Alkaline Phosphatase Substrate Kits 1 and 3 and InnoFECT DAB (both from Vector Labs). Lung sections were stained with KJ1-26 mAb, followed by anti-DIG-HRP, a 3-aminoo-9-ethylcarbazole substrate reaction, and counterstained with hematoxylin (Merck Chemicals, Darmstadt, Germany). Digitalized images were captured using an Axioscam HRc CCD camera on an Axioskop 40 microscope (both from Zeiss, Jena, Germany).

ELISA for OVA-specific Abs

Flat-bottom MaxiSorp 96-well immunoplates (Nunc, Roskilde, Denmark) were coated with 100 µg/mL OVA in PBS overnight at 4°C, blocked with 1% BSA in PBS for 1 h at room temperature, and incubated with serum dilutions in PBS + 1% BSA for 1 h at 37°C. OVA-specific Ig were detected with goat anti-mouse Ig (IgM+IgG+IgA)-, IgG1-, IgG2a-, IgG2b-, or IgG3-specific Abs coupled to HRP (Southern Biotech Abs, Birmingham, U.K.) and 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) as a substrate.

TagMan Low Density Array

Mice were immunized s.c. with 100 µg OVA, with or without 10 µg LPS. KJ1-26+ CD4+ T cells were isolated from popliteal lymph nodes (LN) 24 h later and flow sorted to purity (>98% live). RNA was extracted (High Pure RNA Isolation Kit; Roche) and reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Custom-made TaqMan Low Density Arrays were loaded with cDNA samples (~2.6 ng cDNA/target gene), according to the manufacturer’s instructions, and run on a 7900HT Fast Real-Time PCR System; raw data were analyzed using SDS 2.2.2 software (all from Applied Biosystems). Cycle threshold (Ct) values of target genes were normalized using the multilocus algorithm (20); Ct values > 35 were set to 35.

Airway Ag challenge and preparation of cells from lungs

Mice were anesthetized and challenged intranasally (i.n.) with 50 µg OVA + 0.25 µg LPS in 50 µl PBS. For analysis of lung-infiltrating cells, lungs were perfused with 10 ml PBS through the right heart ventricle, removed, cut into small pieces, and dissociated using gentleMACS (Miltenyi Biotec), followed by digestion with Collagenase D (500 µg/ml) and DNase I (20 µg/ml; both from Roche, Grenzach-Wyhlen, Germany) for 25 min at 37°C in RPMI 1640 containing 0.5% BSA (Biochrom, Berlin, Germany). After addition of EDTA (10 mM) for 5 min, the tissue was further dissociated with gentleMACS and filtered through a 70-µm nylon sieve (BD Falcon, Bedford, MA) into PBS. After erythrocyte lysis with ACK buffer, cells were resuspended and counted before flow cytometric analysis.

Statistical analysis

All statistical analyses were performed with Prism software (GraphPad Software, San Diego, CA). Differences between groups of mice were analyzed by the Mann–Whitney U test; a p value < 0.05 was considered significant.

Results

Adaptive transfer of T cells and application of Ag and adjuvant

To examine the proliferation pattern of T cells under immunogenic versus tolerogenic conditions in vivo, naive OVA-TCR-transgenic DO11.10 CD4+ T cells were transferred i.v. into BALB/c mice, which were immunized i.v. with 2 mg OVA or with 2 mg OVA + 30 µg LPS 24 h later. Previous extensive comparisons, in which we injected increasing amounts (1, 10, 100, and 1000 µg) of endotoxin-free OVA, with or without LPS s.c., or 2 mg i.v., with or without LPS, showed that local T cell responses in the draining LN are qualitatively identical to T cell responses in the spleen after systemic application of Ag (T. Lischke and A. Hegemann, unpublished observations); therefore, we used the systemic approach throughout the study with few exceptions. Injection of Ag by the i.v. route ensured that the CD4+ T cell response was synchronous within the lymphoid system and was not protracted by a continuous influx of naive T cells into a site of local Ag delivery. T cell reactions to 2 mg OVA with 30 µg LPS i.v. were qualitatively identical to 200 µg OVA with 30 µg LPS i.v., but they were quantitatively more pronounced, whereas 20 µg OVA with LPS i.v. elicited a minimal response. Without LPS, 2 mg OVA elicited again a qualitatively similar, but quantitatively higher T cell response compared to 200 µg OVA. We therefore decided to use in each instance 2 mg OVA i.v. to exclude limiting amounts of Ag. Injection of LPS alone resulted in no obvious T cell activation.

Expansion of CD4+ T cells under immunogenic versus tolerogenic conditions

In the presence of LPS, Ag-specific CD4+ T cells (detected with the clonotypic mAb KJ1-26) proliferated strongly, leading to an 18-fold increase in cell numbers at the observed peak of expansion around day 2.5 (Fig. 1A). With OVA alone, KJ1-26+ T cells expanded only ~3-fold (Fig. 1A). Under both experimental conditions, T cell numbers in the spleen decreased thereafter; on day 7, the numbers had decreased to less than the starting level with OVA but remained 3.5-fold higher with OVA+LPS (Fig. 1A). An analysis of the proliferation characteristics demonstrated that with LPS, almost all T cells had divided four to six times by day 2.5, whereas with OVA alone, the majority of T cells remained in generations one through three (Fig. 1B). Essentially the same results were observed on day 4, which excluded a delayed response to OVA (T. Lischke, unpublished observations). Together, these results indicated a substantial activation of T cells after triggering by OVA alone, which was clearly lower and much less sustained compared with the intense response to OVA+LPS.

Differentialization of CD4+ T cells into cytokine-secreting effector cells

To assess the differentialization of CD4+ T cells into effector cells, we determined the release of IL-2 using a cytokine-capture assay without in vitro restimulation. No IL-2 production was seen with adoptively transferred control T cells; OVA induced IL-2 secretion in ~14% of cells; and OVA+LPS induced IL-2 secretion in ~30% of T cells, with a peak at 12–18 h (Fig. 1C, 1D). Using the same assay system, release of IFN-γ, IL-4, or IL-10 could not be detected (T. Lischke, unpublished observations). We also determined the differentiation status of KJ1-26+ T cells at a later time point (day 5) by restimulating the cells with PMA+Ca2+ ionophore. Interestingly, the percentage of T cells producing IL-2 was similar, irrespective of previous exposure to LPS, whereas significant percentages of T cells generating IFN-γ, IL-17A, and IL-10 were seen only after previous exposure to OVA+LPS (Fig. 1E). On a single-cell basis, production of IFN-γ was strongly associated with generation of IL-2, cells producing IL-17A and IFN-γ were distinct populations, and IL-10 was produced either alone or in combination with IFN-γ (Fig. 1F). Production of IL-4, IL-5, IL-13, and IL-17F was not observed. Collectively, these experiments demonstrated that, under inflammatory conditions (OVA+LPS), T cells secrete substantially more IL-2 at early time points in vivo and selectively acquire the capacity to release IFN-γ, IL-10, and IL-17A compared with exposure to OVA alone.

Lack of follicular Th cell differentiation, germinal center development, and plasma cell generation under tolerogenic conditions

To determine the capacity of T cells to support the humoral immune response under tolerogenic versus immunogenic conditions, we analyzed the frequency of KJ1-26+ follicular Th cells, defined by the expression of both CXCR5 and PD-1 (21). After immunization with OVA, <1% of KJ1-26+ CD4+ T cells expressed both markers in the spleen on day 7, whereas with OVA+LPS, ~5% of T cells were positive (Fig. 2A). Germinal center B cells (GL7+ PNA+) showed a significant increase above the PBS control with OVA+LPS but not with OVA (Fig. 2B). At the same time, ~1% of B cells expressed the plasma cell marker CD138 with OVA+LPS.
whereas no significant increase in plasma cell frequency was observed after immunization with OVA (Fig. 2C). As a functional measure of successful T–B cooperation, we determined the levels of OVA-specific serum Ig on day 34. With OVA alone, some specific IgG1, but virtually no IgG2a, IgG2b, or IgG3, was detected (Fig. 2D). In stark contrast, OVA+LPS immunization induced high levels of all OVA-specific switched Ig isotypes, resulting in high total OVA-specific Ig levels (Fig. 2D). The immunohistological data were fully congruent with flow cytometry and functional results. The very few germinal centers (identified by staining for GL7) in splenic B cell zones of PBS-control animals were very small and devoid of KJ1-26+ T cells when analyzed on day 7 (Fig. 2E) and day 13 (T. Lischke, unpublished observations); virtually identical findings were obtained with OVA-immunized animals (Fig. 2E). In contrast, after immunization with OVA+LPS, the frequent germinal centers were large, contained numerous immigrated KJ1-26+ T cells (Fig. 2E), and were further enhanced in size on day 13 (T. Lischke, unpublished observations). Together, the obtained results demonstrated an effective CD4+ T cell helper activity for all isotypes under inflammatory conditions but only some IgG1 production without visible germinal centers under tolerizing conditions.

**Differential expansion of regulatory and effector CD4+ T cells**

Additional parameters were analyzed to further assess quantitative and qualitative differences in the response to OVA versus OVA+LPS. In a first step, proliferation of Foxp3+ Treg and all other (Foxp3−) T cells was compared using the CSFE dilution assay. In control recipients injected with PBS, the starting level of Treg was ∼8%, and no proliferation was seen (Fig. 3A). Remarkably, after challenge with OVA alone, the proportion of Foxp3+ T cells increased to 18% on day 5 (Fig. 3A), indicating a stronger expansion of Treg in the context of an overall modest 2.5-fold increase in KJ1-26+ T cells (Fig. 3B). With OVA+LPS, KJ1-26+ T cells had expanded 17-fold on day 5 (Fig. 3B), whereas the relative proportion of Foxp3+ T cells had decreased to 2.4% (Fig. 3A). On day 14, following the acute expansion and contraction phases of the T cell immune response, the total number of splenic KJ1-26+ T cells was still clearly higher with OVA+LPS, but it decreased to less than the starting level with OVA alone (Fig. 3C, Supplemental Table IA). By that time, the composition of the KJ1-26+ T cell populations had changed dramatically when analyzed for Treg (Foxp3+), Teff (Foxp3−) and either CD62Lhi CD44hi, CD62Llo CD44hi, or CD62lo CD44lo, Fig. 3D). Injection of OVA increased the proportion of Treg to 29% on day 14, but it did not affect the
proportion of Teff, which remained \( \sim \) 16% (Fig. 3D, right panel, Supplemental Table IA). In stark contrast, application of OV A+LPS strongly increased the proportion of Teff to 62% and decreased the proportion of Treg to \( \sim \) 3% (Fig. 3D, right panel, Supplemental Table IA). Thus, OV A alone induced a modest overall CD4+ T cell proliferation but within this process triggered a preferential expansion of Treg, reversing the initial Teff/Treg ratio of 1.3:1 to a dominance of Treg on day 14 (Teff/Treg ratio of 0.6:1). In contrast, addition of LPS to OV A effected a massive expansion of Teff, resulting in an overwhelming dominance of this subset 14 d later, with a Teff/Treg ratio of 21:1.

**CD4+ T cell response to Ag rechallenge in the lung**

In the next experiment, we tested the biological impact of the observed major changes in the size and composition of T cell subpopulations by Ag re-exposure on days 14 and 15. Intranasal application of OVA alone elicited a much lower influx of KJ1-26+ T cells on day 18 in the lungs of animals pre-exposed to OVA compared with unimmunized (i.e., PBS injected on day 0) animals undergoing a primary T cell response in the lungs (T. Lischke, unpublished observations). This observation indicated a state of tolerance in animals pre-exposed to OVA. In a more rigorous test,
we rechallenged all experimental groups with OVA+LPS i.n. Additon of LPS led to a mild general inflammation of the lung tissues in all animals, allowing an increased influx of Ag-specific T cells. Interestingly, in animals pre-exposed to OVA (1˚ OVA) alone, again fewer KJ1-26+ T cells were found in the lungs 4–10 d after application of OVA+LPS i.n. (Fig. 4A, days 18–24) compared with unimmunized animals (1˚ PBS; Fig. 4A). In contrast, in the lungs of animals preimmunized with OVA+LPS (1˚ OVA+LPS), greater numbers of Ag-specific T cells were present (Fig. 4A). Histological findings obtained from the lung tissues were fully congruent with these results. In recipients pre-exposed to OVA, few scattered KJ1-26+ T cells were found, whereas in animals exposed to Ag for the first time, and even more so in animals pre-exposed to OVA+LPS, KJ1-26+ T cells were identified in lymphoid infiltrates around lung vessels (Fig. 4B). The infiltrating OVA-specific T cell populations differed substantially in their composition, as determined by flow cytometry. In “naïve” animals injected with PBS only, 4 d after exposure to OVA+LPS i.n., ∼21% of KJ1-26+ cells were Foxp3+, and 72% were Teff (Fig. 4C). In animals preimmunized with OVA, more Foxp3+ T cells (50%) were present than Teff (46%). In stark contrast, in animals preimmunized with OVA+LPS, the Ag-reactive T cell pool was primarily composed of Teff (82%), with clearly fewer Foxp3+ cells (14%) present (Fig. 4C).

To assess the effector capacity of these infiltrating OVA-specific T cells, cells from the lungs were isolated on day 18 and restimulated in vitro with PMA+Ca2+ ionophore. Compared with animals exhibiting a primary lung response, OVA preimmunization decreased the number of T cells capable of generating IL-2 (Fig. 4D). In stark contrast, OVA+LPS pre-exposure increased the number of T cells generating IL-2, IFN-γ, IL-17A, and IL-10 (Fig. 4D). Collectively, these data demonstrated that pre-exposure to OVA decreased the number of T cells infiltrating the lungs and substantially changed the balance of Teff/Treg from 3.4:1 (naïve lung response) to 0.9:1. In contrast, preimmunization with OVA+LPS led to a greater influx of cells (Teff/Treg ratio of 5.8:1), which, at the same time, exhibited a high inflammatory cytokine potential. This experiment demonstrated that the changes in T cell subset composition observed in the spleen after primary exposure to OVA versus OVA+LPS (Fig. 3) translate into “immune” and “tolerant” reactions in a peripheral organ upon local Ag rechallenge, even if the second challenge was performed in the presence of LPS.

**Differential activation of CD4+ T cells after primary challenge by OVA versus OVA+LPS**

We used gene-expression analysis to understand the molecular events instructing Ag-specific T cells to differentially expand their Teff and Treg populations and to acquire different cytokine-secretion patterns. Animals were adoptively transferred with DO11.10 T cells and challenged with OVA versus OVA+LPS s.c. Twenty-four hours later, KJ1-26+ T cells were sorted to purity by OVA versus OVA+LPS and activated using LPS. The resulting CD4+ T cells were stimulated in vitro with OVA versus OVA+LPS (Fig. 3) translate into “immune” and “tolerant” reactions in a peripheral organ upon local Ag rechallenge, even if the second challenge was performed in the presence of LPS.

![FIGURE 4. T cell response on Ag rechallenge in the lung. Naive DO11.10 CD4+ T cells were transferred into BALB/c recipients, which were injected with PBS, OVA, or OVA+LPS under standard conditions. On days 14 and 15, all mice were rechallenged with OVA+LPS i.n. (A) Total number of KJ1-26+ T cells in the lungs 2–10 d after rechallenge. (B) Cryostat sections of lung tissues, 4 d after rechallenge (day 18), were stained with mAb KJ1-26 and counterstained with hematoxylin. (C) Proportions of Treg and Teff KJ1-26+ cells in the lungs on day 4 after Ag rechallenge (day 18). (D) Number of KJ1-26+ T cells in the lungs on day 4 after rechallenge capable of producing IL-2, IFN-γ, IL-17A, or IL-10 after in vitro restimulation with PMA+Ca2+ ionophore for 4.5 h in the presence of brefeldin A. Shown is one representative experiment of three. *p < 0.05, **p < 0.01.](http://www.jimmunol.org/)

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Role of ICOS in the activation, expansion, and differentiation of Ag-reactive CD4+ T cells

Because of its almost selective and, at the same time, strong induction by OVA+LPS, ICOS was chosen for further study. To assess the contribution of ICOS to the activation, expansion, and differentiation of Ag-reactive T cells and their subsets, DO11.10 T cells were transferred into wild-type (WT) BALB/c animals or into BALB/c recipients lacking the ligand for ICOS (ICOS-L KO). After challenging both animal groups with OVA versus OVA+LPS i.v. under standard conditions, the de novo expression of early cell-activation molecules was determined on splenic KJ1-26+ T cells. Interestingly, lack of ICOS costimulation essentially did not influence induction of 4-1BB, ICOS itself, OX40, CTLA-4, CD160, or CD69 under immunizing or tolerizing conditions over a period of 48 h (Fig. 6A); the same results were obtained in a different system in which OT-II T cells were transferred into C57BL/6 ICOS-L KO mice (Y. Burmeister, unpublished observations). The absence of ICOS did not affect the ability of KJ1-26+ T cells to secrete IL-2 and IFN-γ on day 5 upon restimulation with PMA+Ca2+ ionophore, but it clearly diminished their capacity to secrete IL-17A, nearly to the level seen with OVA alone (Fig. 6B).

A major functional impact of ICOS became apparent when we assessed the population size and subset composition of the responding CD4+ T cells. When analyzed on days 1–5, KJ1-26+ T cells transferred into ICOS-L KO recipients (and thus lacking ICOS costimulation) expanded in response to OVA to the same extent as did WT recipients (Fig. 6C and A. Hegemann, unpublished observations). However, in response to OVA+LPS, T cells without ICOS cosignaling expanded less than half compared with T cells with preserved ICOS signaling (Fig. 6C). After the contraction phase, on day 14, lack of ICOS signaling had no influence on the total number of KJ1-26+ T cells exposed to OVA, and their Teff/Treg ratio was 0.4:1, typical for this time point and mode of stimulation (Fig. 6D, Supplemental Table IB). In contrast, lack of ICOS costimulation diminished the residual T cell population on day 14 after OVA+LPS administration and strongly decreased the dominance of T eff, by changing the Teff/Treg ratio from 25:1 to 4.8:1 (Fig. 6D, Supplemental Table IB). In addition, lack of ICOS cosignaling fully abolished the development of follicular Th cells, germinal center B cells, and switched B cells and substantially lowered the level of plasma cells (T. Lischke and A. Hegemann, unpublished observations). We used additional experimental systems to verify the influence of ICOS on the size of the Teff and Treg populations. After standard transfer of KJ1-26+ T cells into BALB/c WT recipients and immunization, the interaction of ICOS with ICOS-L was blocked by injecting the nondepleting anti–ICOS-L mAb MIL-5733 over a period of up to 14 d. The reduction in Teff only and, thus, the shift in balance between Teff and Treg were also observed under these conditions (Supplemental Fig. 2A–C). Furthermore, the same phenomenon was seen in analogous experimental systems after transfer of OT-II ICOS KO T cells into C57BL/6 mice (Supplemental Fig. 2D) or OT-II WT T cells into C57BL/6 ICOS-L recipients (Supplemental Fig. 2E).

Together, these experiments revealed that ICOS, although substantially expressed on Treg (18), barely contributes to the tolerance reaction of KJ1-26+ T cells to OVA. In clear contrast, ICOS strongly boosts T cell immunity following OVA+LPS injection at three levels: by amplifying the expansion of the inflammatory T cell population (with its ICOS-independent capacity to secrete IL-2 and IFN-γ) at the height of the immune response, by enabling this T cell population to secrete the key inflammatory cytokine IL-17A, and by promoting the dominance of Teff over Treg at the later steady state.
Role of ICOS in the T cell response to Ag rechallenge in the lung

To determine the effector capacity of KJ1-26+ T cells transferred into WT BALB/c or ICOS-L KO recipients after priming with OVA versus OVA+LPS, all animal groups were rechallenged with OVA+LPS i.n. on days 14 and 15, as described previously. In ICOS-L KO recipients, KJ1-26+ T cells in the lungs were reduced on day 18 in all experimental groups but most prominently in animals pre-exposed to OVA+LPS (Fig. 7A). As a result, the absence of ICOS cosignaling strongly reduced the number of infiltrating KJ1-26+ T cells capable of generating IL-2, IFN-γ, and IL-17A, when removed on day 5 and restimulated in vitro for 4.5 h with PMA+Ca2+ ionophore in the presence of brefeldin A, fixed, and analyzed by intracellular flow cytometry. One representative experiment of two is shown. (C) Total number of KJ1-26+ cells in the spleen on day 5 after immunization. Shown is one representative experiment of four (each experiment was performed with at least two mice in the PBS WT group and three to five mice in all other groups). (D) Total number (left panel) and proportions (right panel) of Teff and Treg KJ1-26+ cells in the spleen on day 14 (mean ± SEM). Shown is one representative experiment of three (each experiment was performed with two or three mice in the PBS WT group and three to five mice in all other groups). *p < 0.05, **p < 0.01.

Discussion

In our model of tolerance and immunity, we used endotoxin-free OVA as an innocuous exogenous Ag and the combination of OVA+LPS as a model system for a pathogenic Ag.

Injection of endotoxin-free OVA induced a specific gene-expression pattern in the naive KJ1-26+ CD4+ T cell population. Among the early communication molecules, 4-1BB, RANKL, and CD160 were exclusively induced de novo by OVA and were not upregulated by OVA+LPS. With pure OVA, KJ1-26+ T cells secreted substantial amounts of IL-2 and proliferated to a modest degree. Interestingly, within the proliferating cell population, KJ1-26+ T cells expressing Foxp3 expanded preferentially relative to the "conventional" KJ1-26+ T cells, thus reversing the slight dominance of Teff over Treg observed at naive steady state to a slight dominance of Treg over Teff. The observed expansion of T cells was temporary and was followed by a decrease in the Ag-reactive T cell pool to less than the starting level 2 wk later. The CD4+ T cells failed to generate inflammatory cytokines, such as IFN-γ and IL-17A, and were not able to mount an inflammatory response on Ag rechallenge in the lung, established as a test for tolerance. At the same time, there were no signs of a significant humoral response to OVA. Although some of the described reaction patterns, such as reduced T cell expansion and the lack of a humoral response were described previously (22), we were able to advance the understanding of peripheral tolerance to a pure protein in several ways. Our data indicate that CD4+ T cells do not respond to an innocuous Ag by “anergy” or with a dampened, but otherwise similar, program as to a pathogen. Instead, CD4+ T cells react actively to a pure foreign protein with a complex “tolerance”
FIGURE 7. T cell response in ICOS-L KO mice on Ag rechallenge in the lung. Naive DO11.10 CD4+ T cells (2.2 × 10^6) were transferred into BALB/c or ICOS-L KO recipients, which were injected with PBS, OVA, or OVA+LPS under standard conditions. On days 14 and 15, all mice were rechallenged with OVA+LPS i.n. (A) Total number of KJ1-26+ T cells in the lungs 4 d after rechallenge (day 18). (B) Total number of KJ1-26+ T cells in the lungs on day 18 capable of producing IL-2, IFN-γ, IL-17A, or IL-10 after restimulation with PMA+Ca2+ ionophore for 4.5 h in vitro in the presence of brefeldin A. Shown is one representative experiment of three. *p < 0.05, **p < 0.01.

Several conclusions can be drawn from our observations. Most importantly, ICOS emerges as a key element in the decision between tolerance and immunity to foreign Ag in vivo. Until now, ICOS has primarily been viewed as an important molecule in T cell immunity. In contrast, our current in vivo analysis system, which we established by rechallenge, allowed us to recognize that the surface molecules ICOS, CTLA-4, and OX40 are almost exclusively induced under inflammatory conditions and, therefore, can be regarded as “key switches” for the “immune” reaction. Importantly, this finding was not specific for LPS, because essentially identical T cell-activation patterns were obtained with polyinosinic-polycytidylic acid, Pam3Cys, or CpG (A. Hegemann, unpublished observations). Our second major finding was that conventional CD4+ T cells expand in response to an inflammatory Ag to a much greater degree than do CD4+ Treg, which results in a dramatic dominance of Teff over Treg at the height of the immune response (days 3–6). This massive imbalance between Teff and Treg is also a part of short-term memory, when measured on day 14 through Ag rechallenge in a peripheral organ (lung). Thus, with regard to both the tolerance and immune reactions, the ratio of Teff/Treg is a key element in the response to a foreign Ag.

After establishing an extensive in vivo analysis system, we decided to examine potential key switches between tolerance and immunity. Intrigued by its strong and almost specific expression in immunity, we studied ICOS in detail. Contrary to expectations, the absence of ICOS costimulation had no effect on the early activation of CD4+ T cells, measured through upregulation of a greater number of cell surface molecules, and it did not influence their newly acquired capacity to generate IFN-γ. Only the generation of IL-17A+ T cells, which did not exhibit the phenotype of follicular Th cells (data not shown), was significantly reduced as a result of the absence of ICOS costimulation. This finding seems to support an ICOS-dependent mechanism for IL-17 differentiation, as reported by Park et al. (23), and it runs contrary to the observations of Bauquet et al. (24) that ICOS is dispensable for this process. Most strikingly, the lack of ICOS signaling strongly decreased the number of T cells around day 5, as well as their survival later in the response. More extensive analysis revealed that the absence of ICOS signaling primarily affected the expansion of Teff, thus diminishing their dominance over Treg in the inflammatory response. The absence of ICOS essentially shut down the B cell response, revealing that the known adjuvant effect of LPS on B cells in this system (22) is mediated by ICOS. Altogether, without the contribution of ICOS, the CD4+ T cell response under inflammatory conditions became more similar to a tolerance reaction.

Treg express high levels of ICOS (18, 25). It remains unclear why the absence of ICOS signaling did not substantially affect the expansion of Treg in our experimental system, either with OVA or with OVA+LPS. Until now, the involvement of ICOS in Treg differentiation and function has usually been examined in complex in vivo models (26–28) that cannot provide a clear answer, because ICOS is expressed on both Teff and Treg. Only the availability of animal models using a selective ablation of ICOS in Treg will provide conclusive data about the contribution of ICOS to Treg physiology.

Several conclusions can be drawn from our observations. Most importantly, ICOS emerges as a key element in the decision between tolerance and immunity to foreign Ag in vivo. Until now, ICOS has primarily been viewed as an important molecule in T cell help for B cells (5). This perspective originated from early observations of high ICOS expression on T cells in germinal centers (8), the dysfunction of the humoral immune system in mice deficient for ICOS (29–32), and the lack of mature B cell immunity in ICOS-deficient patients (33). Later studies corroborated the central role for ICOS in B cell immunity by demonstrating the importance of ICOS in the development of follicular Th cells (6, 7). However, the function of ICOS in T cell immunity was less well characterized. In the original studies performed in vitro, ICOS acted as a broad amplifier of T cell activation, upregulating cell surface molecules, cytokine synthesis, and proliferation, similarly to CD28 but without the capacity to induce IL-
levels of CD4+ T effector memory and Treg and demonstrated that recently identified a significant role for ICOS in the steady-state significantly contributes to the dominance of Teff over Treg in the severely compromising the tolerance reaction to innocuous Ags. The T cells may be beneficial for autoimmune patients, without se-

References

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Disclosures


