ICOS Mediates the Generation and Function of CD4+CD25+Foxp3+ Regulatory T Cells Conveying Respiratory Tolerance

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ICOS Mediates the Generation and Function of CD4+CD25+
Foxp3+ Regulatory T Cells Conveying Respiratory Tolerance

Mandy Busse,* Mathias Krech,*,† Almut Meyer-Bahlburg,* Christian Hennig,* and Gesine Hansen*

Costimulatory molecules like ICOS are crucial in mediating T cell differentiation and function after allergen contact and thereby strongly affect the immunologic decision between tolerance or allergy development. In this study, we show in two independent approaches that interruption of the ICOS signaling pathway by application of a blocking anti-ICOSL mAb in wild-type (WT) mice and in ICOS−/− mice inhibited respiratory tolerance development leading to eosinophilic airway inflammation, mucus hypersecretion, and Th2 cytokine production in response to OVA sensitization. Respiratory Ag application almost doubled the number of CD4+Foxp3+ regulatory T cells (Tregs) in the lung of WT mice with 77% of lung-derived Tregs expressing ICOS. In contrast, in ICOS−/− mice the number of CD4+CD25+Foxp3+ Tregs did not increase after respiratory Ag application, and ICOS−/− Tregs produced significantly lower amounts of IL-10 than those of WT Tregs. Most importantly, in contrast to WT Tregs, ICOS−/− Tregs did not convey protection when transferred to “asthmatic” recipients demonstrating a strongly impaired Treg function in the absence of ICOS signaling. Our findings demonstrate a crucial role of ICOS for the generation and suppressive function of Tregs conveying respiratory tolerance and support the importance of ICOS as a target for primary prevention strategies. The Journal of Immunology, 2012, 189: 000–000.
phenotype in WT animals, the phenotype in tolerized ICOS−/− mice was comparable to that of allergic WT mice. Accordingly, development of tolerance and thereby the protection from an allergic phenotype was inhibited in WT animals by application of a blocking anti-ICOSL (B7RP-1) mAb before tolerization. Moreover, the number of CD4+CD25+Foxp3+ Tregs in the lung of WT mice increased 1.8-fold after respiratory tolerance induction with 77% of lung-derived Tregs expressing ICOS. In contrast, the application of respiratory Ag in ICOS−/− mice did not significantly increase the number of CD4+CD25+Foxp3+ Tregs. An adoptive cell transfer experiment showed that in contrast to WT Tregs, ICOS−/− Tregs did not transfer suppression of an allergic phenotype in “allergic” recipients in vivo, whereas transfer of WT Tregs conferred protection.

Our findings demonstrate a crucial role of ICOS signaling for the development of respiratory tolerance by mediating the development and function of CD4+CD25+Foxp3+ Tregs.

Materials and Methods

Animals

Female B6.129P2-Icostm1Mak/J (ICOS−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), C57BL/6J WT mice (6–8 wk of age) were obtained from Charles River Laboratories (Sulzfeld, Germany), and BALB/cByJ WT mice were obtained from Janvier (St. Berthevin, France) and bred and maintained in the animal facilities at the Martin-Luther University (Halle/Saale, Germany) and at Hannover Medical School. All animal experiments were performed according to institutional and state guidelines. The Committee on Animal Welfare approved animal protocols used in this study.

Induction of respiratory tolerance and immunization

Anesthetized mice were tolerized intranasally (i.n.) with 500 μg OVA in normal saline twice according to the protocols in Figs. 1 and 6A (“tolerance protocol”). Mice were subsequently sensitized i.p. with OVA (20 μg, grade V; Sigma-Aldrich) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (alum; Fischer Scientific grade V; Sigma-Aldrich) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (alum; Fischer Scientific International) followed by a six-time OVA challenge i.n. (20 μg OVA in 40 μl normal saline) (“asthma protocol”). Some mice were only referred to the asthma protocol (“OVA”) or received alum without OVA i.p. and 0.9% NaCl i.n. instead of OVA (“Alum”). Other mice were first referred to the tolerance protocol and afterward to the asthma protocol (“TOL”).

In vivo Ab treatments

BALB/cByJ WT mice were injected i.p. with 200 μg anti-ICOSL mAb (clone HK5.3) or alternatively with isotype control rat IgG2b (both from eBioscience, San Diego, CA) 1 d before first tolerization (see Fig. 1). Subsequently, mice received OVA in alum normal saline twice according to the protocols in Figs. 1 and 6A (“tolerance protocol”). Some mice were only referred to the asthma protocol (“OVA”) or received alum without OVA i.p. and 0.9% NaCl i.n. instead of OVA (“Alum”). Other mice were first referred to the tolerance protocol and afterward to the asthma protocol (“TOL”).

Analysis of bronchoalveolar lavage fluid

Two days after the last challenge, mice were sacrificed by CO2 asphyxiation; blood was taken, the left lung was tied off for histology, and bronchoalveolar lavage (BAL) was performed as described previously (33–35).

Lung histology was assessed as described previously (5), and cytokine production was analyzed as described previously (5, 35).

Stimulation of Tregs with CD3/CD28 Dynabeads

CD4+CD25+Foxp3+ Tregs from tolerized ICOS−/− mice or C57BL/6J WT mice were cultured in the presence of Dynabeads Mouse CD3/CD28 T Cell Expander (1:100; Invitrogen, Oslo, Norway) for 4 d.

Measurement of cell proliferation

The number of viable cells was measured using a cell titer glow assay (Promega, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, 100 μl cell suspension and 100 μl freshly prepared glow reagent were mixed and measured using a GloMax Multi-Detection System (Promega).

OVA-specific IgE assay

OVA-specific IgE serum levels were measured by ELISA according to a standard protocol as described previously (5, 36).

Flow cytometry and Abs

Single-cell suspensions from spleen and lung were incubated with fluorescently labeled Abs for 20 min at 4°C in staining buffer (PBS with 0.5% BSA). Abs used in this study included reagents specific for CD4 (RM4-5) and CD8 (53-6,7) from BD Pharmingen (San Diego, CA) and ICOS (C5984A) and Foxp3 (FJK-16s) from eBioscience (San Diego, CA). Intracellular staining of Foxp3 was done using the eBioscience reagents according to the manufacturer’s instructions. Briefly, cells were surface stained and subsequently fixed for 45 min in fixation buffer. Cells were washed twice with perm buffer and incubated with Ab to Foxp3 for 30 min at 4°C. Finally, data were collected on a FACScanto flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). The data were analyzed using a bpsomnial transformation function for complete data visualization.

Adaptive transfer of Tregs

For adoptive transfer of CD4+CD25+ T cells, ICOS−/− mice or C57BL/6J WT mice were tolerized i.n. with 500 μg OVA in normal saline on days −6 and −3. On day 0, spleens were harvested, and CD4+CD25+ T cells were selected by MACS (Regulatory T Cell Isolation Kit; Miltenyi Biotec). Purity of isolated CD4+CD25+ cells was >90% with >85% coexpressing Foxp3. CD4+CD25+ T cells (2 × 106) were adoptively transferred i.v. into OVA-sensitized C57BL/6J WT recipient mice. Recipient animals were subsequently sensitized for a second time and challenged four times with 20 μg OVA i.n. (see protocol in Fig. 6A).

Statistical analysis

The Student t test was used to determine the statistical significance of differences between groups, and *p < 0.05, **p < 0.005, and ***p < 0.001 were considered as significant.

Results

Blockade of ICOSL in WT animals impairs the development of respiratory tolerance

In WT mice, mucosal application of OVA before immunization completely protects the animals from the development of an allergic phenotype. To analyze the role of ICOS for the development of respiratory tolerance, we first applied a blocking anti-ICOSL or control mAb to naive BALB/c mice 24 h before tolerization ([4]; see protocol in Fig. 1). Subsequently, mice received OVA in alum systemically and were challenged with OVA i.n. Application of anti-ICOSL mAb compared with control mAb inhibited tolerance development and resulted in high total cell numbers and eosinophil numbers in the BAL fluid (Fig. 2A). While tolerized mice that had received anti-ICOSL mAb showed dense eosinophilic peribronchial cell infiltrates and mucus hypersecretion in HE- and periodic acid/Schiff (PAS)-stained lung sections, which was comparable to allergic mice, a strong reduced degree of inflammation and mucus impact was detected in mice that received control mAb (Fig. 2B). The level of the Th2 cytokines IL-5 and IL-13 in cell culture supernatants of OVA-restimulated bronchial lymph node (BLN) cells was significantly reduced in tolerized WT mice that had received control mAb, whereas it was comparable to allergic mice when anti-ICOSL mAb was applied (Fig. 2C). Moreover, proliferation of BLN cells in response to OVA restimulation was comparable to allergic mice when anti-ICOSL mAb was applied but not when control mAb was applied (Fig. 2D). These data emphasize a crucial role of ICOS/B7RP-1 signaling in the development of mucosal tolerance and the protection from allergy and asthma.

ICOS-deficient mice are severely impaired in developing respiratory tolerance

To analyze further the role of ICOS for the development of respiratory tolerance, we next analyzed ICOS−/− mice in comparison with WT mice in our respiratory tolerance model (37).
As shown in Fig. 1, ICOS−/− and WT mice were first tolerized to OVA by intranasal Ag application. Subsequently, they were immunized with the same Ag by systemic and intranasal OVA application and finally referred to our murine asthma model. The inflammatory response after OVA immunization was stronger in ICOS−/− mice compared with WT mice as seen by higher cell numbers in the BAL fluid (Fig. 3A) as well as higher IL-5 and IL-13 levels in BLN cell culture supernatants (Fig. 3C).

Although respiratory tolerance induction before immunization completely inhibited the inflammatory phenotype of WT mice, this was not the case in ICOS−/− mice where the inflammatory response was reduced but still as high as in allergic WT mice. In detail, the total cell number and number of eosinophils in the BAL fluid of tolerized WT mice was comparable to naive control animals. In contrast, in ICOS−/− mice it was as high as in allergic controls although reduced in comparison with their allergic phenotype (Fig. 3A). Similarly, H&E- and PAS-stained lung sections of tolerized WT and ICOS−/− mice revealed an almost complete inhibition of eosinophilic inflammation and mucus hypersecretion in WT but not in ICOS−/− mice (Fig. 3B). In tolerized ICOS−/− mice, the inflammatory response and mucus hypersecretion was comparable to allergic WT mice.

Moreover, the level of the Th2 cytokines IL-5 and IL-13 in OVA-restimulated cell culture supernatants (Fig. 3C) was reduced in tolerized ICOS−/− mice compared with allergic ICOS−/− mice. However, the cytokine production in tolerized ICOS−/− mice was still as high as in allergic WT mice and significantly higher compared with tolerized WT mice. Furthermore, ex vivo proliferation of OVA-restimulated BLN cells was significantly reduced in tolerized WT mice but not in tolerized ICOS−/− mice (Fig. 3D).

Tolerization of WT mice resulted in a significant reduction of OVA-specific serum IgE levels in response to OVA immunization. In accordance with previous reports (38), ICOS−/− mice did not produce OVA-specific IgE in response to OVA immunization demonstrating the crucial role of ICOS/ICOSL signaling for the differentiation of B cells and IgE-producing plasma cells (data not shown).

In summary, our data demonstrate that mucosal application of Ag completely inhibits the allergic phenotype in WT mice whereas ICOS−/− mice remain allergic, although the allergic phenotype is attenuated. Our finding again supports an important role of ICOS in the development of respiratory tolerance.

![FIGURE 1. Protocols. BALB/cByJ WT mice, ICOS−/− mice, and C57BL/6J WT mice received OVA in alum i.p. on days 0 and 14 and were challenged with OVA in NaCl 0.9% i.n. on days 14–16 and 21–23 (“allergy protocol”; OVA). For induction of respiratory tolerance, mice received OVA in NaCl 0.9% i.n. on days –6 and –3 (“tolerance protocol”; TOL) before being referred to the allergy protocol, started on day 0. Anti-ICOSL mAb or control mAb was applied i.p. to a group of BALB/cByJ WT mice 1 d before tolerization on day –7.](http://www.jimmunol.org/)

![FIGURE 2. Blockade of ICOS signaling by anti-ICOSL mAb impairs respiratory tolerance. Mucosal application of OVA before immunization (see Fig. 1) completely inhibited the development of an allergic phenotype. Application of anti-ICOSL mAb but not control mAb before tolerization resulted in significantly increased total cell numbers and eosinophil numbers in the BAL fluid (A) and dense cellular infiltrates and mucus in H&E- and PAS-stained lung sections (original magnification ×200) (**OVA versus TOL, ***TOL + aICOSL versus TOL + Isotype, n.s., OVA versus TOL + aICOSL) (B), increased production of the Th2 cytokines IL-5 and IL-13 ([C]; **OVA versus TOL, ***TOL versus TOL + aICOSL, **TOL + aICOSL versus TOL + Isotype), and enhanced proliferation of OVA-restimulated BLN cells ([D]; OVA versus TOL, *TOL versus TOL + aICOSL, *TOL + aICOSL versus TOL + Isotype) in response to OVA immunization. Data are expressed as means ± SEM (n = 12 animals per group, *p < 0.05, **p < 0.005, ***p < 0.001).](http://www.jimmunol.org/)
ICOS is upregulated on Tregs upon induction of respiratory tolerance

To investigate the role of ICOS for respiratory tolerance induction on the cellular level, we determined ICOS expression on CD4+ Foxp3+ Tregs in lung and spleen after mucosal Ag application. For this analysis, WT mice were tolerized by i.n. OVA application on days 26 and 23 and sacrificed on day 0 for analysis. The percentage of CD4+ICOS+Foxp3+ Tregs in nonmanipulated naive WT mice was significantly higher in the lung compared with the spleen (representative samples for staining of ICOS on CD4+ Foxp3+ Tregs in lung and spleen are shown in Supplemental Fig. 1): the frequency of CD4+ICOS+Foxp3+ Tregs detected in the lung was on average 46% compared with 22% in the spleen, suggesting an important role of ICOS signaling especially in the lung. Upon tolerance induction, ICOS+ Tregs increased significantly in both lung and spleen to 77 and 33% on average, respectively (Fig. 4). However, the absolute numbers of total and as well ICOS+ Tregs did not change in the spleen of WT mice upon tolerization (Supplemental Fig. 2).

ICOS deficiency impairs the generation and IL-10 production of CD4+Foxp3+ Tregs in the lung

Next, we analyzed the role of ICOS signaling for the generation of CD4+Foxp3+ Tregs in lung and spleen. ICOS−/− and WT mice were tolerized with OVA i.n. on days −6 and −3 and sacrificed on day 0. ICOS−/− mice had significantly lower numbers of CD4+Foxp3+ Tregs as well in the lung as in the spleen compared with WT mice. Induction of tolerance resulted in a significant increase in CD4+Foxp3+ Tregs in the lung as detected by flow cytometry. One representative example is shown in Fig. 5A (left side). In the lung, the percentage of CD4+Foxp3+ Tregs in WT mice increased in mean from 9.2 to 16.5% (Fig. 5A, right side). In the spleen of WT mice, the absolute numbers of CD4+Foxp3+ Tregs increased upon induction of tolerance from mean 1.1 × 10^6 cells to 1.5 × 10^6 cells (Fig. 5B). In contrast, no significant increase in CD4+Foxp3+ Tregs in ICOS−/− mice was observed in spleen or lung after mucosal tolerization (Fig. 5C).
Moreover, the ratio of Foxp3^+CD4^+ cell to Foxp3^+CD4^+ Treg as well as the percentage of Foxp3^+CD4^+ cells changed only in the lung of WT mice, whereas it was unaffected in ICOS^−/− mice (Supplemental Fig. 3). These data demonstrate the important role of ICOS signaling for the generation of CD4^+Foxp3^+ Tregs. Tregs use both direct and indirect ways to suppress T cells. Beside close cell/cell contact, Tregs function via secretion of cytokines such as IL-10. As it was shown that ICOS^+ Tregs strongly produce IL-10 (39), we analyzed next the capacity of WT and ICOS^−/− Tregs to produce IL-10. CD4^+CD25^+ Tregs isolated from the lung of ICOS^−/− mice produced significantly less IL-10 compared with Tregs isolated from WT mice upon stimulation by CD3/CD28 cross-linking (Fig. 5C). This finding supports the importance of ICOS for generation of functional Tregs.

WT Tregs but not ICOS^−/− Tregs suppress the allergic phenotype when transferred into allergic recipients

Finally, we examined the suppressive function of ICOS^−/− Tregs compared with WT Tregs purified from tolerized ICOS^−/− and WT mice in vivo. For this purpose, we isolated CD4^+CD25^+ T cells from tolerized WT or ICOS^−/− mice using MACS and determined via intracellular FACS staining that >85% of purified CD4^+CD25^+ cells coexpressed the Treg transcription marker Foxp3. These CD4^+CD25^+ cells were adoptively transferred into OVA-sensitized WT recipients (Fig. 6A). Our data clearly show that CD4^+CD25^+ WT Tregs protected the host efficiently from the development of an allergic phenotype. The total cell number in the BAL fluid (Fig. 6B), Th2-cytokine production in cell cultures from bronchoalveolar lymph node cells (Fig. 6C), and OVA-specific IgE serum levels (Fig. 6D) were significantly reduced in OVA-sensitized WT recipients that received CD4^+CD25^+ Tregs from WT mice. In contrast, CD4^+CD25^+ Tregs isolated from ICOS^−/− mice did not transfer protection to OVA-immunized WT hosts. The recipients showed a severe allergic phenotype comparable to the phenotype of OVA-sensitized control mice. These data suggest that ICOS plays a key role not only for the generation but also for the suppressive function of CD4^+CD25^+ Tregs in vivo.

Discussion

In this study, we demonstrate a crucial role of ICOS for the generation and suppressive function of Tregs conveying respiratory tolerance and protection from the development of an allergic phenotype.

5A, 5B). Moreover, the ratio of Foxp3^−CD4^+ cell to Foxp3^+CD4^+ Treg as well as the percentage of Foxp3^+CD4^+ cells changed only in the lung of WT mice, whereas it was unaffected in ICOS^−/− mice (Supplemental Fig. 3). These data demonstrate the important role of ICOS signaling for the generation of CD4^+Foxp3^+ Tregs.

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Discussion

In this study, we demonstrate a crucial role of ICOS for the generation and suppressive function of Tregs conveying respiratory tolerance and protection from the development of an allergic phenotype.
The absence of ICOS/ICOSL interaction, either in ICOS−/− mice or in WT mice, where ICOS signaling is blocked via neutralizing ICOSL mAb, resulted in failure of tolerance development and thereby missing protection from severe pulmonary inflammation, Th2 cytokine production, and high serum IgE levels (for WT mice) in response to OVA immunization. Investigating the mechanisms contributing to this incompetence in tolerance development, we found that CD4+CD25+Foxp3+ Tregs were quantitatively and qualitatively compromised in the absence of normal ICOS signaling in spleen and lung demonstrating that ICOS co-stimulation is critical for the development of Tregs conveying protection. Tregs purified from tolerized ICOS−/− mice were not able to transfer suppression into asthmatic WT recipients in vivo. These data demonstrate the important role of ICOS not only for the development but also for the function of Tregs.

Notably, the number of ICOS−/−Foxp3+ Tregs was twice as high in the lung as in the spleen of naive WT mice (46 versus 22%) already indirectly supporting an important role of ICOS signaling in the lung. Upon induction of respiratory tolerance in WT mice, we measured an impressive increase in the number of ICOS+/CD4+Foxp3+ Tregs especially in the lung, with a high frequency of cells coexpressing ICOS (77%). In ICOS−/− mice, CD4+CD25+Foxp3+ T cells were quantitatively and qualitatively compromised in spleen and lung after intranasal tolerization. This observation is in line with recent findings by Burmeister et al. (8), who describe that signaling via ICOS substantially contributes to the survival and expansion of Foxp3+ Tregs and does not only influence the pool size of effector and memory T cells. Moreover, recent reports support the importance of ICOS for homeostasis of Tregs (40, 41) indicating that the missing protection of ICOS−/− Tregs upon transfer into presensitized recipients might not only be a result of their strong reduced suppressive capacity but could also partially be due to their decreased life span.

Although CD28 has major impact upon the generation of Tregs in the thymus (42), in the human thymus ICOS+ and ICOS− Foxp3+ Tregs were detected indicating a role for ICOS in the generation of a specific thymic Treg population (43). However, the finding that thymic Foxp3+ T cells in ICOS−/− mice are not impaired (36) shows that ICOS influences the induction of Tregs mainly in the periphery as found in our model. Our observation that the absence of ICOS in respiratory tolerance is associated with a defect in the generation of Foxp3+ Tregs has been similarly observed in a model of atherosclerosis and infection (44, 45). Moreover, the impaired generation of Foxp3+ Tregs has also been shown in ICOS-deficient patients (15). In contrast, transgenic mice expressing elevated levels of ICOS had increased numbers of Foxp3+ Tregs (46), clearly indicating a correlation between expression of ICOS and the number of Tregs. Moreover, ICOS+ Tregs were described to elicit superior suppressive activity (15, 39, 45).

An important role of ICOS/ICOSL interaction in mucosal tolerance has been shown previously in a study indicating that pulmonary DCs in the BLNs of mice exposed to respiratory allergen induced the costimulation of Tregs via the ICOS/ICOSL pathway (32). In this study, CD4+ T cells were derived from DO11.10 OVA-specific TCR-transgenic mice and generated through IL-10–secreting DCs in vitro. Their main characteristics were production of IL-10 and smaller amounts of IL-4, and accordingly, they were referred to as Tregs. In our study, we support this finding and complement it by using Foxp3 as the now most established marker for Tregs and by working with this specifically defined population in vivo in WT mice. We directly show in vivo that CD4+CD25+ Foxp3+ T cells are upregulated in both spleen and lung after intranasal tolerization and that this effect is significantly inhibited.
when ICOS signaling is blocked. Moreover, we further support and complement these findings by adding a second, independent model where we show the importance of ICOS signaling for the generation and suppressive function of CD4+CD25+Foxp3+ Treggs and thereby for the respiratory tolerance development in ICOS−/− mice demonstrating several important parameters in this model.

The finding that ICOS−/− mice show enhanced susceptibility to EAE further stresses a potential role for ICOS in limiting of autoimmune pathology (16) and is in line with our finding that ICOS−/− mice are not able to develop respiratory tolerance protecting from allergy development. The important role of ICOS for the development and maintenance of tolerance in models of autoimmune diseases like allergic encephalomyelitis (EAE) and autoimmune type 1 diabetes was also shown by blockade of ICOS signaling using anti-ICOSL mAbs, which interrupted the balance between regulatory and effector T cells and worsened disease (19, 31).

In addition to our findings in the respiratory tolerance model, we showed by immunological and functional analysis of ICOS−/− mice that the Th2 response was pronounced and complete except for IgE production leading to the phenotype of allergic inflammation in ICOS−/− mice including eosinophilic airway inflammation, mucus hypersecretion, and Th2 cytokine production. The failure to cell-switch to IgE is in accordance with previous studies showing that ICOS+ T cells promote the infiltration of pulmonary B cells and the production of allergen-specific IgE production in the lung (38). Reports in ICOS−/− animals indicate that deficient class-switch recombination resulted from insufficient CD40L upregulation (13, 47).

However, our finding that ICOS−/− mice develop eosinophilic airway inflammation is in contrast to earlier studies that have shown that blockade of ICOS signaling also attenuates Th2-driven airway inflammation (18, 23, 48). Moreover also in B7R1−/− mice, an enhanced Th2 asthmatic phenotype was detected (49). We hypothesize that in our model, an appropriate route of Ag application in combination with a strong adjuvant (alum) compensates for ICOS deficiency in the case of Th2-mediated immune responses. In accordance with this hypothesis, an infection model compensates for ICOS deficiency in the case of Th2-mediated immune responses. In accordance with this hypothesis, an infection model permits development of mucosal tolerance.

In summary, by using two different and independent, comprehensive models, we show an important role of ICOS for the induction and maintenance of respiratory tolerance by controlling the generation and function of Tregs mediating tolerance to allergens. Our data support an important role of ICOS as a target for modulation of immune responses as in allergic diseases or autoimmune diseases where tolerance to Ags is highly desirable.

Disclosures

The authors have no financial conflicts of interest.

References


Suppl. figure 1: Staining of ICOS on CD4+Foxp3+ Treg cells in lung and spleen

Shown are representative samples for stainings of ICOS on CD4+Foxp3+ Treg cells in lung and spleen of WT mice before (naïve) or 6 days after tolerization (TOL) were analyzed by flow cytometry.

Suppl. figure 2: Absolute Treg numbers in the spleen of naïve and OVA-tolerized WT mice

The absolute numbers of total Treg cells as well as ICOS+ Treg cells in the spleen of WT mice before (naïve) or 6 days after tolerization (TOL) were analyzed by flow cytometry.

Suppl. figure 3: CD4+Foxp3- T cells in lung and spleen of naïve and OVA-tolerized WT and ICOS-/- mice

The relative ratios of Foxp3-CD4+ cells to Foxp3+CD4+ Treg (A) and the percentages of Foxp3-CD4+ cells in the lung and the absolute numbers of Foxp3-CD4+ T cells in spleen (B) of WT and ICOS-/- mice before (naïve) or 6 days after tolerization (TOL) were analyzed by flow cytometry.
suppl. figure 1

ICOS

Foxp3

naive

TOL

lung

spleen

56% 93%

44% 7%

22% 37%

78% 63%
Suppl figure 2
Suppl. figure 3

A

lung

spleen

B

Foxp3-CD4+/Foxp3+CD4+

Treg

Foxp3-CD4+ cells (% of lymphocytes)

Foxp3-CD4+ cells (x 10^6 cells)

WT naive
WT TOL
ICOS naive
ICOS TOL

WT naive
WT TOL
ICOS naive
ICOS TOL

Suppl. figure 3