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*J Immunol* 2002; 169:5813-5817; doi: 10.4049/jimmunol.169.10.5813

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Inducible Costimulator Protein Controls the Protective T Cell Response Against *Listeria monocytogenes*¹

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The inducible costimulator protein (ICOS) was recently identified as a costimulatory molecule for T cells. Here we analyze the role of ICOS for the acquired immune response of mice against the intracellular bacterium *Listeria monocytogenes*. During oral *L. monocytogenes* infection, low levels of ICOS expression were detected by extracellular and intracellular Ab staining of Listeria-specific CD4⁺ and CD8⁺ T cells. Blocking of ICOS signaling with a soluble ICOS-Ig fusion protein markedly impaired the Listeria-specific T cell responses. Compared with control mice, the ICOS-Ig treated mice generated significantly reduced numbers of Listeria-specific CD8⁺ T cells in spleen and liver, as determined by tetramer and intracellular cytokine staining. In contrast, the specific CD8⁺ T cell response in the intestinal mucosa did not appear to be impaired by the ICOS-Ig treatment. Analysis of the CD4⁺ T cell response revealed that ICOS-Ig treatment also affected the specific CD4⁺ T cell response. When restimulated with listerial Ag in vitro, reduced numbers of CD4⁺ T cells from infected and ICOS-Ig-treated mice responded with IFN-γ production. The impaired acquired immune response in ICOS-Ig treated mice was accompanied by their increased susceptibility to *L. monocytogenes* infection. ICOS-Ig treatment drastically enhanced bacterial titers, and a large fraction of mice succumbed to the otherwise sublethal dose of infection. Thus, ICOS costimulation is crucial for protective immunity against the intracellular bacterium *L. monocytogenes*. The *Journal of Immunology*, 2002, 169: 5813–5817.

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Received for publication June 17, 2002. Accepted for publication September 12, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*¹M.K. was supported by the Graduiertenkolleg 276/2, and this work will be part of his Ph.D. thesis.

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³Abbreviations used in this paper: ICOS, inducible costimulator; EAE, experimental allergic encephalomyelitis; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O.

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Here we analyze the role of ICOS in the acquired immune response to the intracellular bacterium *Listeria monocytogenes*. *Listeria* Ags are presented via both the MHC class I and class II pathways. Consequently, *L. monocytogenes* infection induces a CD8\(^+\) T cell response and a Th1-polarized CD4\(^+\) T cell response (23). Both T cell populations are involved in the control of bacterial infection, and CD8\(^+\) T cells appear to be particularly important for protection against secondary infections (23).

Using ICOS-Ig fusion proteins, we demonstrate that blocking of ICOS signaling increases the susceptibility of mice against oral *L. monocytogenes* infection. ICOS-Ig-treated mice fail to mount a proper CD8\(^+\) T cell response and show diminished IFN-\(\gamma\) production by CD4\(^+\) and CD8\(^+\) T cells. Thus, ICOS cosignaling is crucial for the development of a protective immune response against an intracellular bacterial pathogen.

**Materials and Methods**

Abs and Ig fusion proteins

Anti-CD16/32 (clone 2.4G2), anti-IFN-\(\gamma\) mAb (clone R4-6A2, IgG1), anti-CD4 mAb (clone YTS191.1), anti-CD8a mAb (clone YTS169), and anti-CD62L mAb (clone Mel-14) were purified from hybridoma supernatants and conjugated according to standard protocols. FITC- and the PE-conjugated rat-IgG1 isotype control mAb (clone R3-34) and PE-conjugated anti-IL-4 mAb (clone 11B11, rat IgG1) were purchased from BD Pharmingen, San Diego, CA.

The construction and the purification of the mouse ICOS human IgG1 Fc fusion protein (ICOS-Ig) and the control IgG1 Fc protein (con-Ig) has been described previously (3).

To prepare the anti-ICOS mAb, 5-wk-old Lou rats (Harlan Sprague Dawley) received i.p. and s.c. injections at 4-wk intervals with 5 \(\times\) 10\(^5\) murine ICOS-CHO transfected cells in PBS. Purified murine ICOS-Ig was used for the final i.v. boost before fusion. Splenocytes from these animals were fused with Y3-Ag1.2.3 rat myeloma cells (CRL 1631; American Type Culture Collection, Manassas, VA). Ten to 14 days postfusion, conditioned media from hybridoma-containing wells were screened by ELISA against both ICOS-Ig and con-Ig. Hybridomas of interest were subcloned by limiting dilution and tested further by flow cytometric analysis.

**Bacteria and bacterial infection of mice**

*Listeria* strain EGD were grown in tryptic soy broth to late log phase and the bacteria were appropriately diluted in PBS. Bacteria were applied to BALB/c mice in a total volume of 200 \(\mu\)l PBS by gastric intubation. Three to 4 h before infection and from then on every other day, mice received 200 \(\mu\)g ICOS-Ig or con-Ig (in 200 \(\mu\)l PBS i.p.). Experiments were conducted according to German animal protection law. To determine the bacterial burdens, spleens were homogenized in PBS, serial dilutions of homogenates were plated on tryptic soy broth agar plates, and colonies were counted after 24-h incubation at 37°C.

**In vitro restimulation of spleen cells and flow cytometric determination of cytokines**

Spleen cells (3 \(\times\) 10\(^3\)/well) were stimulated for 5 h with 10 \(\times\) 10\(^6\) M of the peptide listeriolysin O\(_{\text{LMO}_{91-99}}\) (LL0\(_{91-99}\), GYDKDGNEYI, Jerini Bio Tools, Berlin, Germany) or heat-killed *Listeria* (5 \(\times\) 10\(^5\)/ml). During the final 4 h of culture, 10 \(\mu\)g/ml brefeldin A was added. Cells were stained extracellularly with Cy5-conjugated anti-IFN-\(\gamma\) mAb and anti-CD4 mAb or intracellularly with FITC-conjugated anti-IFN-\(\gamma\) mAb and PE-conjugated anti-IL-4 mAb or the corresponding FITC- and PE-conjugated isotype control mAbs as described previously (24). For detection of ICOS expression, the cells were stained extracellularly with PE-conjugated anti-CD4 mAb or anti-CD8a mAb and intracellularly with FITC-conjugated anti-IFN-\(\gamma\) mAb. ICOS staining was performed extracellularly or intracellularly with the Cy5-conjugated anti-ICOS mAb. For staining controls, anti-ICOS mAb was incubated for 15 min with a 30-fold molar excess of ICOS-Ig before adding it to the cell samples.

**Purification of cells and staining of cells with tetramers**

Intraepithelial lymphocytes and lamina propria lymphocytes from the small intestine and lymphocytes from the liver were isolated using a 40/60% Percoll gradient as previously described (25). Cells were stained with Cy5-conjugated anti-CD8a mAb, FITC-conjugated anti-CD62L mAb, and PE-conjugated MHC class I LLO\(_{91-99}\) tetramers (24) or with FITC-conjugated anti-CD8a mAb, Cy5-conjugated anti-ICOS mAb, and PE-conjugated tetramers. For staining controls, the anti-ICOS mAb was incubated for 15 min with a 30-fold molar excess of ICOS-Ig before adding it to the samples. Directly before analysis of cells, propidium iodide was added.

**Statistical analysis**

The statistical significance of the results was determined with the statistics program included in PRISM software (GraphPad, San Diego, CA). Bacterial titers were analyzed with the Mann-Whitney test, and frequencies and numbers of tetramer-positive or cytokine-expressing cells were determined by unpaired Student’s t test. A value of \(p < 0.05\) was considered a statistically significant difference.

**Results**

**Blocking of ICOS cosignaling increases susceptibility to *L. monocytogenes* infection**

Natural infection with *L. monocytogenes* originates from the consumption of contaminated food. Therefore, BALB/c mice were orally infected with 10\(^7\) *Listeria*, a dose that is well tolerated by BALB/c mice. ICOS signaling was blocked by repeated injections of a soluble ICOS-Ig fusion protein. This treatment had profound effects on the course of infection. By day 7, 15 of the 25 ICOS-Ig-treated mice (in five independent experiments) had succumbed to the oral *L. monocytogenes* infection, and several of the surviving mice were moribund. In contrast, none of the con-Ig-treated mice succumbed to infection (of 21 mice), and none of these mice showed overt signs of disease. On day 7, the surviving ICOS-Ig-treated mice had bacterial abscesses in the spleen and liver (data not shown), and their spleens contained high numbers of *Listeria*. Fig. 1 shows the result from an experiment in which four of five ICOS-Ig-treated mice survived the infection for 7 days. At this time point, the con-Ig-treated mice showed either low bacterial titers or had completely cleared *Listeria* from the spleen.

**ICOS-Ig treatment impairs the generation of Listeria-specific CD8\(^+\) T cells**

In BALB/c mice, the CD8\(^+\) T cell response to *Listeria* is concentrated on a few immunodominant epitopes. The most prominent of these listerial epitopes is derived from the secreted pore-forming protein, LLO (aa 91–99; LLO\(_{91-99}\)) (24). Therefore, we used LLO\(_{91-99}\)-loaded MHC class I tetramers to analyze the specific CD8\(^+\) T cell response to *L. monocytogenes* infection in ICOS-Ig-treated animals. Fig. 2 shows representative results for LLO\(_{91-99}\) tetramer and CD62L staining of CD8\(^+\) T cells isolated from different organs. CD62L is a surface molecule of CD8\(^+\) T cells that is down-regulated in spleen and liver following T cell activation.

![FIGURE 1. ICOS-Ig treatment increases the generation of Listeria-specific CD8\(^+\) T cells](http://www.jimmunol.org/)
The situation is different in the intestinal mucosa, where the vast majority of CD8+ T cells typically express low levels of CD62L. Consistent with published results (26, 27), we observed high frequencies and numbers of LLO91-specific CD8+ T cells in spleen, liver, and lamina propria and lower frequencies in intestinal epithelium of con-Ig-treated mice on day 7 of infection (Figs. 2 and 3). As expected, the vast majority of these cells were CD62Llow (Fig. 2). In contrast to the con-Ig treatment, the ICOS-Ig treatment markedly decreased the frequencies and numbers of LLO91-specific CD8+ T cells in spleens and livers of infected mice. This result was observed in all animals regardless of whether the mice were moribund (of the 10 ICOS-Ig-treated mice that survived infection, nine were individually analyzed in four independent experiments (note the different scales of the y-axes). * p < 0.05. NS, not significant (determined with Student’s t test); LPL, lamina propria lymphocytes; IEL, intraepithelial lymphocytes.

ICOS-Ig treatment markedly reduced the frequencies of CD8+ T cells that responded to LLO91 with IFN-γ production. To analyze the CD4+ T cell response to L. monocytogenes, spleen cells from infected mice were stimulated with heat-killed Listeria (Fig. 4). In con-Ig-treated mice, incubation with heat-killed Listeria increased frequencies of IFN-γ-producing CD4+ T cells. In ICOS-Ig-treated mice, the frequencies of IFN-γ+ CD4+ T cells were already elevated without restimulation. This was most likely due to the presence of high Listeria numbers in these mice. Restimulation with heat-killed Listeria did not further enhance the frequencies of IFN-γ+ CD4+ T cells, and the frequencies were lower than those in spleens from con-Ig-treated animals after Ag restimulation. Although we consistently observed higher IFN-γ production in the CD4+ T cells of con-Ig-treated mice compared with ICOS-Ig-treated mice, this difference never reached significance due to the high background levels of IFN-γ+ CD4+ T cells observed in the ICOS-Ig-treated mice. Frequencies of IFN-γ+ CD4+ T cells were reduced in spleens from ICOS-Ig-treated mice when the cells were polyclonally restimulated with anti-CD3 mAb and anti-CD28 mAb (data not shown). Independent from the stimuli, there was no significant induction of IL-4 production in CD4+ and CD8+ T cells from either con-Ig- or ICOS-Ig-treated animals.

ICOS expression on Listeria-specific CD4+ and CD8+ T cells
Several reports indicate that ICOS is not expressed on resting CD8+ T cells and is only slowly up-regulated after activation (3, 5, 20). It was therefore important to determine whether Listeria-specific CD8+ T cells express ICOS. Direct ex vivo costaining of
CD8⁺ T cells with LLO₉₁₋₉₉ tetramers and anti-ICOS mAb revealed that a significant population of these cells expressed ICOS on the surface on day 8 of infection (Fig. 5). Cells were also analyzed after in vitro culture for 5 h with the peptide LLO₉₁ in either ICOS-Ig or con-Ig on days 0, 2, 4, and 6. Three hours after the first injection, mice were infected orally with 10⁹ Listeria. On day 7, spleen cells were restimulated ex vivo for 5 h with LLO₉₁ or heat-killed Listeria (HKL) or were left untreated. Frequencies of IFN-γ and IL-4-producing cells were determined by flow cytometry. Intracellular staining with FITC- and PE-conjugated isotype control mAbs always resulted in <0.05% positive cells (not shown). The figures show representative results for CD4- or CD8-gated cells, and numbers represent the percentage calculated for CD4⁺ or CD8⁺ T cells. The x- and y-axes are logarithmic scales (log_{10}). Numbers below the figures give the mean percentage ± SD for IFN-γ⁺ cells from an experiment with three (con-Ig) or two (ICOS-Ig) individually analyzed mice per group and are representative of three independent experiments. The percentages of con-Ig- and ICOS-Ig-treated mice were compared with Student’s t test (*, p < 0.05; NS, not significant).

Spleen cells were incubated with heat-killed Listeria, and the expression of ICOS on IFN-γ⁺ CD4⁺ T cells was analyzed. ICOS staining revealed that after Ag stimulation, IFN-γ⁺ CD4⁺ T cells expressed low, but significant, levels of ICOS on their surface, and slightly higher levels were detected by intracellular staining.

**Discussion**

Our studies demonstrate a crucial role of ICOS signaling in the development of the protective CD8⁺ T cell response against oral L. monocytogenes infection. Blocking of ICOS signaling in vivo impaired the generation of Listeria-specific CD8⁺ T cells and Listeria-specific CD4⁺ Th1 cells, as determined by tetramer and intracellular cytokine staining. It has been suggested that ICOS signaling has only a limited influence on T cell priming, but affects the function of recently activated and effecter T cells (4, 13, 15). We did not address the T cell effector functions, but our experiments demonstrate that blocking of ICOS signaling affects priming and/or expansion of the Listeria-specific CD4⁺ and CD8⁺ T cell populations. The highly diminished T cell response in ICOS-Ig-treated mice also increased the susceptibility to oral L. monocytogenes infection.

A prerequisite for direct effects of ICOS on Listeria-specific T cells is its surface expression on these cells. A significant population of Listeria-specific CD8⁺ T cells expressed ICOS on the surface, although at a relatively low level. After the peptide incubation, there was no or only weak surface staining, but distinct intracellular ICOS staining was observed in the responding T cells. We assume that the intracellular enrichment of ICOS was due to the brefeldin A incubation, which hindered the transport of ICOS to the cell surface. However, we cannot exclude that ICOS expression after activation is a dynamic process, and that intracellular stores for the ICOS molecule exist, as described for the related
CTL-A-4 molecule (28). Overall, our results demonstrate that sub-populations of Listeria-specific CD8\(^+\) T cells and CD4\(^+\) Th1 cells either express ICOS or rapidly produce the molecule following Ag encounter.

ICOS-\(\alpha\) treatment reduced CD8\(^+\) T cell frequencies in spleens and livers, but not in intestinal mucosa, implying tissue-specific requirements for ICOS costimulation. This observation is different from the situation in CD28-deficient mice, where the CD8\(^+\) T cell response after oral infection was highly impaired in all organs analyzed (H.-W. Mitträcker, unpublished observations). For other cosignals, tissue-specific requirements have been reported (27). Following L. monocytogenes infection, the CD8\(^+\) T cell response is independent from CD4\(^+\) T cell help and the CD40/CD40L interaction in the spleen, but requires these signals in the intestinal mucosa (27). In our infection model the requirement for ICOS signaling showed an inverse pattern, with a high dependency in spleen and liver and a low or even absent influence in intestinal mucosa. It is possible that tissue-specific requirements are due to differential expression levels of the ICOS ligand, B7RP-1, in these tissues. However, it is also possible that the ICOS-\(\alpha\) protein fails to penetrate the intestinal mucosa in sufficiently high concentrations to block ICOS/B7RP-1 interactions in this tissue.

To date, the role of ICOS in the development of CD8\(^+\) T cell responses and/or CD4\(^+\) Th1 cell responses in infection has been studied in two models (15, 16). After infection with Leishmania mexicana, ICOS-deficient mice demonstrated impaired Ab production, and CD4\(^+\) T cells from infected mice secreted diminished amounts of cytokines. The production of both IgG1 and IgG2a as well as the secretion of both IL-4 and IFN-\(\gamma\) were impaired, indicating that ICOS signaling was involved in the regulation of both the Th2 and Th1 responses against this pathogen (15). Comparable results were observed after infection of mice with Nippostrongylus brasiliensis (16). Treatment with an ICOS-\(\alpha\) fusion protein reduced the production of Th1 and Th2 cytokines. However, IFN-\(\gamma\) production by CD8\(^+\) T cells was not affected. The ICOS-\(\alpha\) fusion protein was also used to analyze T cell responses against lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (16). During LCMV infection, the ICOS-\(\alpha\) treatment had only a marginal effect on the Th1 cell response and no influence on the CD8\(^+\) T cell response. The Th1 cell response against vesicular stomatitis virus was more sensitive to ICOS-\(\alpha\) treatment, but again the CD8\(^+\) T cell response was not impaired (16). Currently, we have no explanation for the strong impairment of the CD8\(^+\) T cell responses in Listeria infection compared with the limited effect on CD8\(^+\) T cells in other infection models. This discrepancy is reminiscient of CD28 costimulation, which influences CD8\(^+\) T cell responses against L. monocytogenes but not against certain virus strains (reviewed in Ref. 24).

In conclusion, this report describes a novel role of ICOS in the regulation of protective CD8\(^+\) T cell responses in a biologically relevant model of a food-borne infection with a bacterial pathogen.

Acknowledgments

We thank Dr. Robert Hurwitz for assistance in the preparation of tetramers; Dr. Kerstin Bonhagen for help with the FACS staining; Manuela Stäber for purification and labeling of Abs; John S. Whoriskey and Thomas Horan for construction, expression, and purification of fusion proteins; and Dr. Brenda G. Yoshinaga for help in editing of the manuscript.

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