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CUTTING EDGE

Cutting Edge: Lipopolysaccharide Induces IL-10-Producing Regulatory CD4<sup>+</sup> T Cells That Suppress the CD8<sup>+</sup> T Cell Response

Joke M. M. den Haan,* Georg Kraal,* and Michael J. Bevan†

TLR ligands are potent activators of dendritic cells and therefore function as adjuvants for the induction of immune responses. We analyzed the capacity of TLR ligands to enhance CD8<sup>+</sup> T cell responses toward soluble protein Ag. Immunization with OVA together with LPS or poly(I:C) elicited weak CD8<sup>+</sup> T cell responses in wild-type C57BL/6 mice. Surprisingly, these responses were greatly increased in mice lacking CD4<sup>+</sup> T cells indicating the induction of regulatory CD4<sup>+</sup> T cells. In vivo, neutralization of IL-10 completely restored CD8<sup>+</sup> T cell responses in wild-type mice and OVA-specific IL-10 producing CD4<sup>+</sup> T cells were detected after immunization with OVA plus LPS. Our study shows that TLR ligands not only activate the immune system but simultaneously induce Ag specific IL-10-producing regulatory Tr1 cells that strongly suppress CD8<sup>+</sup> T cell responses. In this way, excessive activation of the immune system may be prevented. The Journal of Immunology, 2007, 178: 5429–5433.

Dendritic cells (DCs) express pathogen recognition receptors, including the TLRs that allow them to be activated by microbial derived molecules. Activation of DCs by TLR ligands potentiates the up-regulation of costimulatory molecules, MHC class II molecules and the production of cytokines and chemokines (1, 2). This combined action leads to an enhanced capacity of TLR-activated DCs to stimulate naive CD8<sup>+</sup> and CD4<sup>+</sup> T cells which can result in autoimmunity (3–5). In the absence of microbial stimulatory agents CD8<sup>+</sup> T cell responses are dependent on “help” provided by CD4<sup>+</sup> T cells. This help consists of cytokines produced by helper CD4<sup>+</sup> T cells that promote memory CD8<sup>+</sup> T cell responses. In addition, helper CD4<sup>+</sup> T cells “license” the DCs by CD40L-CD40 interaction (6). Licensed DCs specifically attract CD8<sup>+</sup> T cells via chemokine release and have increased capacity to activate naive CD8<sup>+</sup> T cells (7, 8). In contrast, CD4<sup>+</sup> T cell help is often unnecessary for effector CD8<sup>+</sup> T cell responses elicited by pathogens due to direct activation of DCs by pathogen recognition receptors (6).

In contrast to the helper CD4<sup>+</sup> T cells, regulatory CD4<sup>+</sup> T cells can down-regulate CD8<sup>+</sup> T cell responses. The naturally occurring CD25<sup>+</sup> CD4<sup>+</sup> Treg cells arise in the thymus and express the Foxp3 transcription factor (9). These CD25<sup>+</sup> Foxp3<sup>+</sup> regulatory CD4<sup>+</sup> T cells have been shown to suppress CD8<sup>+</sup> T cell priming and expansion in vitro as well as in vivo (10–12). Stimulation via TLRs counteracts suppressive effects of CD25<sup>+</sup> CD4<sup>+</sup> T cells by inducing IL-6 production by DCs, causing effector T cells to become insensitive to the suppressive activity of regulatory T cells (13). The ability of TLR ligands both to activate DCs and simultaneously to alleviate the suppression by regulatory T cells can explain the proinflammatory effects of TLR ligands and their capacity to stimulate strong adaptive immune responses.

We analyzed whether the TLR ligands LPS and poly(I:C) could function as adjuvant for CD8<sup>+</sup> T cell priming in vivo and the role of CD4<sup>+</sup> T cells in this process. We discovered that LPS induces Ag-specific suppressor CD4<sup>+</sup> T cells that inhibit CD8<sup>+</sup> T cell priming via IL-10. This strongly implies that microbial activation of DCs not only results in proinflammatory adaptive immune responses, but also in the induction of regulatory T cells that down-regulate these same responses, thereby preventing overstimulation.

Materials and Methods

Mice, immunizations, and in vivo depletions

C57BL/6 mice and MHC class II-deficient mice were purchased from Taconic Farms, Charles River Laboratories, and The Jackson Laboratory. All mouse experiments were performed with the approval of the Institutional Animal Care and Use Committee at the University of Washington or at the Free University Medical Center in Amsterdam with approval of the Free University Animal Experiments Committee. Six- to 10-wk-old mice were immunized with 500 µg of OVA (Calbiochem) together with 30 µg of LPS (026:B6; Sigma-Aldrich) or 100 µg of poly(I:C) (Sigma-Aldrich). To deplete CD4<sup>+</sup> cells, 200–300 µg of GK1.5 mAb was injected i.p. on day −4, −2, and +3 of the day of immunization. The efficiency of depletion was minimally 98.5% for CD4<sup>+</sup> cells, 25–50 µg of PC61 mAb was injected i.p. on day −4

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3 Abbreviations used in this paper: DC, dendritic cell; Treg, regulatory T cell.
or ∼3 of the day of immunization. The efficiency of depletion was determined with staining with Abs 7D4 and PC61 that recognize non-competing epitopes on CD25. No residual staining with PC61 could be detected whereas the efficiency of depletion analyzed with 7D4 was 91%. Neutralization of IL-10 in vivo was achieved by injections with JES5–2A5 on day −6 (300 µg), −2 (300 µg), day 0 (500 µg), and day +4 (300 µg), of the day of immunization.

Intracellular cytokine staining

Intracellular cytokine staining was performed essentially as described (14); 3 × 10⁵ splenocytes/well were plated in 96-well plates in the presence of 1 µg/ml brefeldin A (GolgiPlug, Cytofix/Cytoperm kit; BD Pharmingen) and 0.1 µg/ml OVA257–264 peptide for 5 h at 37°C. Following the incubation, cells were stained with CD11a-FITC and CD8-PE, permeabilized and stained for intracellular cytokine expression using IFN-γ-allophycocyanin (BD Pharmingen) and reagents provided in the Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD Pharmingen).

Cytotoxicity assay

Splenocytes (50 × 10⁶) were restimulated in 20 ml of complete IMDM together with 2.5 × 10⁶ irradiated embryonic fibroblasts transfected with OVA257–264, H-2Kb, and CD80 (15). Five days after restimulation a cytotoxicity assay was performed following standard procedures using EL-4 cells coated with or without OVA257–264 as target cells.

IL-10 ELISA

Splenocytes were restimulated in 96-well flat bottom plates at 0.75 × 10⁶/well in 200 µl of complete IMDM with 100 µg/ml OVA protein (Calbiochem or Seikagaku), with 1 µg/ml OVA257–264 or with 100 µg/ml OVA323–339 or OVA262–276 peptide. After 4 days of culture, supernatants were tested for IL-10 content by ELISA according to the manufacturer’s instructions (BD Biosciences). To deplete B220⁺, CD8⁺, or CD4⁺ cells from total splenocytes, cells were incubated for 20 min with Abs RA3-6B2, 53.6.7 or GK1.5, respectively. The cells were further depleted using anti-rat Dynal beads following the manufacturer’s instructions (Dynal Biotech).

Results

TLR ligands stimulate increased CD8⁺ T cell responses in the absence of CD4⁺ T cells

Intravenous injection of protein Ag generally results in induction of T cell tolerance, because the Ag is then presented by non-activated DCs, which precludes priming of T cell responses. To determine whether TLR ligand stimulation of DCs would result in efficient CD8⁺ T cell responses, we immunized mice i.v. with OVA either alone or in the presence of TLR-4 and -3 ligands LPS or poly(I:C) (Fig. 1, A and B). There was no detectable CD8⁺ T cell response to OVA alone, but immunization with OVA plus LPS, or OVA plus poly(I:C), activated OVA-specific CD8⁺ T cells in wild-type C57BL/6 mice as well as in MHC class II-deficient mice, which lack CD4⁺ T cells. Apparently, activation of splenic DCs by these TLR ligands, as can be detected by up-regulation of costimulatory molecules (data not shown), is sufficient for CD8⁺ T cell priming and makes CD4⁺ T cell help unnecessary. Surprisingly and consistently, compared with wild-type mice, higher CD8⁺ T cell responses were observed in MHC class II-deficient mice and in mice that were depleted of CD4⁺ T cells by opsonizing Ab (Fig. 1C). CD8⁺ T cells activated in the absence of CD4⁺ T cells could be efficiently restimulated in vitro and showed strong cytotoxic activity (Fig. 1D), illustrating that these cells did not exhibit the “helpless” phenotype of CD8⁺ T cells destined to die after restimulation (15). Together these experiments indicated that CD4⁺ T cells in wild-type mice exerted a significant

FIGURE 1. TLR agonists promote higher CD8⁺ T cell responses in the absence of CD4⁺ T cells. A, C57BL/6 and MHC class II-deficient mice were immunized with 500 µg of OVA either alone or plus 30 µg of LPS or 100 µg of poly(I:C). Seven days after immunization the OVA-specific CD8⁺ T cell response was determined by intracellular IFN-γ staining. FACS plots are gated on CD8⁺ T cells, and the number displayed indicate the percentage of CD11a⁻IFN-γ-producing cells. B, Average OVA-specific CD8⁺ T cell response in C57BL/6 and MHC class II-deficient mice. The percentage of CD11a⁻IFN-γ-producing cells out of total CD8⁺ T cells is depicted. Error bars display SEM, n = 3, and results are representative of two independent experiments for poly(I:C) and five independent experiments for LPS. C, C57BL/6, MHC class II-deficient and CD4-depleted C57BL/6 mice were immunized with OVA plus LPS, and on day 7 the frequency of OVA specific CD8⁺ T cells was assessed by IFN-γ staining. The percentage of CD11a⁻IFN-γ-producing cells out of total CD8⁺ T cells is depicted. Results are representative of five independent experiments with 2–4 mice. D, Splenocytes from C were restimulated in vitro and their cytotoxic capacity evaluated by ⁵¹Cr release assay. The average cytotoxicity of two mice is depicted. Error bars display SEM.
suppressive effect on CD8$^+$ T cells after priming with OVA plus LPS or poly(I:C).

**In vivo suppression of CD8$^+$ T cells is mediated via IL-10**

In several different experimental systems CD25$^+$ Tregs have been shown to suppress CD8$^+$ T cell responses (10, 11). To investigate whether CD25$^+$ CD4$^+$ T cells suppressed the CD8$^+$ T cell response, we depleted mice of CD25$^+$ cells and immunized with OVA plus LPS. Although the CD25$^+$ CD4$^+$ T cells were efficiently depleted (Fig. 2A), the OVA-specific CD8$^+$ T cell response was only partially increased compared with control IgG-treated mice (Fig. 2B), suggesting that in addition to CD25$^+$ CD4$^+$ T cells other types of regulatory CD4$^+$ T cells were responsible for the CD8$^+$ T cell suppression. Ag-specific regulatory CD4$^+$ T cells can be elicited in the periphery by antigenic stimulation in the presence of high concentrations of IL-10 (16, 17). These T1 cells do not express CD25 or Foxp3 and mediate their suppression via IL-10 (18, 19). They have been shown to suppress effector CD4$^+$ T responses in a number of models, but less is known of their effects on CD8$^+$ T cells (20). To determine whether IL-10 was important for the CD8$^+$ T cell suppression, we neutralized IL-10 in vivo by injecting a neutralizing Ab. When IL-10 was neutralized we observed a strong CD8$^+$ T cell response in wild-type mice after immunization with OVA plus LPS that was equivalent to that seen in the absence of CD4$^+$ T cells (Fig. 2C). These results together indicated that IL-10 was necessary for the CD8$^+$ T cell suppression and that in addition to CD25$^+$ CD4$^+$ Tregs other regulatory CD4$^+$ T cells were involved.

**LPS induces Ag-specific Tr1 in vivo**

To determine whether Ag-specific Tr1 cells were induced, splenocytes from wild-type mice and MHC class II-deficient mice previously immunized with OVA plus LPS were restimulated with OVA in vitro, and supernatants were analyzed for IL-10. OVA-induced IL-10 was detected in cultures derived from wild-type mice but not in those from MHC class II-deficient mice (Fig. 3A) and IL-10 production in wild-type splenocytes was completely abolished after removal of CD4$^+$ T cells (Fig. 3B). This strongly suggested that Ag recognition by CD4$^+$ T cells resulted in IL-10 production. Finally, we tested the known MHC class I and II epitopes from OVA for IL-10 production and found that only restimulation with the MHC class II epitope OVA262–276 resulted in strong IL-10 production (Fig. 3C). Together these experiments show that OVA-specific IL-10-producing CD4$^+$ T cells are induced by immunization with OVA plus LPS.

**Discussion**

The present study shows that immunization with TLR ligands induces Ag-specific IL-10-producing CD4$^+$ T cells that strongly suppress CD8$^+$ T cell responses in vivo. A number of studies have previously shown that TLR ligands can function as adjuvant for CD8$^+$ T cell responses especially in synergy with each other or when combined with anti-CD40 ligation (21–23). Our data indicate that although TLR ligands are strong adjuvants for the induction of CD8$^+$ T cell responses, they simultaneously initiate a suppressive mechanism via IL-10 producing CD4$^+$ T cells.

IL-10 is a well-known anti-inflammatory cytokine that suppresses DC Ag presentation and the production of proinflammatory cytokines such as IL-12 p70 (24). Boonstra et al. (25) demonstrated that macrophages and DCs produced significant amounts of IL-10 after stimulation with TLR ligands and that IL-12 p70 production was strongly suppressed by this endogenous IL-10 production. Especially the splenic CD8$^+$ T cell set was highly regulated by IL-10. This strongly suggested that Ag recognition by CD4$^+$ T cells resulted in IL-10 production. Finally, we tested the known MHC class I and II epitopes from OVA for IL-10 production and found that only restimulation with the MHC class II epitope OVA262–276 resulted in strong IL-10 production (Fig. 3C). Together these experiments show that OVA-specific IL-10-producing CD4$^+$ T cells are induced by immunization with OVA plus LPS.

**FIGURE 2.** Suppression of CD8$^+$ T cell priming is mediated via IL-10. A, C57BL/6 mice were treated with control rat IgG or PC61 Ab. On the day of immunization, blood of depleted mice was analyzed. FACS profiles show CD4$^+$ gated cells stained with anti-CD25 Abs 7D4 and PC61. One representative mouse of each group is shown. B, MHC class II-deficient mice and C57BL/6 mice treated with control rat IgG or depleted of CD25$^+$ cells were immunized with OVA plus LPS, and on day 7 the number of OVA-specific CD8$^+$ T cells was determined by intracellular IFN-γ staining. The percentage of CD11a$^+$ IFN-γ-producing cells out of total CD8$^+$ T cells is depicted. n = 11 from four separate experiments, error bars display SEM. C, C57BL/6 treated with control rat IgG, or depleted of CD4$^+$ cells or treated with neutralizing anti-IL-10 Ab were immunized with OVA plus LPS and the number of OVA-specific CD8$^+$ T cells was determined by intracellular IFN-γ staining. The percentage of CD11a$^+$ IFN-γ-producing cells out of total CD8$^+$ T cells is depicted. n = 6 from two separate experiments and error bars display SEM.
blockade of IL-10 function in vivo may be of therapeutic interest for the development of vaccines based on CD8⁺ T cell immunity.

In addition to the autocrine suppressive effects of IL-10 on APCs, high levels of IL-10 also are known to lead to the generation of suppressive Tr1 cells, which in turn are characterized by the production of IL-10. Tr1 cell generation has been demonstrated in several human and murine in vitro and in vivo model systems (20). Here we show that TLR4 signaling by LPS results in Tr1 induction and CD8⁺ T cell suppression in vivo. Similarly, Bordetella pertussis stimulates IL-10 production and Tr1 generation via TLR4 signaling (29). In this model, IL-10 production and Tr1 generation was essential for limiting inflammatory pathology in the lungs after B. pertussis infection. Also in other infectious models, IL-10 was found to prevent exacerbation of inflammation and disease (30). Apparently, IL-10 and Tr1 cells serve as a protective strategy for the host to prevent excessive damage by the host adaptive response.

TLR ligands are being evaluated in many vaccination studies because of their excellent capacity to activate DCs. We now show that TLR ligands not only function as adjuvant for CD8⁺ T cell priming, but that they also induce IL-10 producing Tr1 cells which in turn results in suppression of CD8⁺ T cell activation. These observations should be taken into account when considering TLR containing adjuvants for the priming of CD8⁺ T cell responses for vaccination purposes.

Disclosures

The authors have no financial conflict of interest.

References


