Brief Definitive Report

Anti–Interleukin 10 Receptor Monoclonal Antibody Is an Adjuvant for T Helper Cell Type 1 Responses to Soluble Antigen Only in the Presence of Lipopolysaccharide

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Abstract

Soluble foreign antigen usually leads to a transient clonal expansion of antigen-specific T cells followed by the deletion and/or functional inactivation of the cells. As interleukin (IL)-10 is a key immunoregulatory cytokine, we questioned whether neutralization of IL-10 during priming with soluble antigen could prime for a subsequent T helper cell type 1 (Th1) effector recall response. By using an adoptive transfer model to track the fate of antigen-specific T cell receptor (TCR)-transgenic $CD4^+$ T cells, we show that administration of soluble ovalbumin (OVA) protein, but not $OVA_{323–339}$ peptide antigen, together with an anti–IL-10 receptor (R) mAb led to the enhancement of a Th1 response upon rechallenge. Lipopolysaccharide (LPS) present in the protein was necessary for priming for Th1 recall responses in the presence of anti–IL-10R mAb, as removal of LPS abrogated this effect. Moreover, addition of LPS to the peptide did not itself allow priming for recall Th1 effector responses unless endogenous levels of IL-10 were neutralized with an anti–IL-10R mAb. A significant increase in OVA-specific IgG1 and IgG2a isotypes was observed when the protein antigen was administered with anti–IL-10R mAb; however, this was not the case with peptide antigen administered together with anti–IL-10R and LPS. Our data, showing that LPS receptor signaling and neutralization of endogenous immunosuppressive cytokines is essential for Th1 priming, has important implications for the design of relevant vaccines for effective in vivo immunotherapy.

Key words: IFN-γ • soluble antigen • immunosuppression • cytokines • IL-10

Introduction

Several factors may determine whether encounter of antigen in a primary response will lead to the clonal expansion of specific antigen receptor–expressing lymphocytes and their differentiation into specific memory effector cells (for review see references 1 and 2). Soluble foreign antigen usually leads to a transient clonal expansion of antigen-specific T cells, followed by the deletion and/or functional inactivation of the cells (for review see references 1 and 2). In some cases, soluble antigen can lead to subsequent unresponsiveness to an immunizing regimen of antigen in adjuvant (for review see references 1 and 2). It has been suggested that the dose and form of antigen, the route of administration of antigen, the delivery of appropriate costimulatory signals, and the genetic background of the host may determine whether an antigen primes for an appropriate memory effector response (for review see references 1–3).

Several mechanisms have been proposed to explain the abortive immune response initiated by soluble antigen, including deletion or anergy (for review see references 1 and 2). In addition, soluble antigen does not lead to activation of the innate immune response to produce inflammatory mediators as induced by infectious organisms or adjuvants, such as CFA (containing mycobacteria) or LPS (endotoxin) required for effective priming of Th1 responses (4, 5). Alternatively, soluble antigen intraperitoneally (3, 6, 7) has been proposed to result in a Th1→Th2 switch, with abrogation of cell-mediated immune Th1 responses, characterized by $CD4^+$ T cell proliferation, IL-2 and IFN-γ produc-

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tion, and switching to IgG2a. Under such circumstances, Th2 responses with IL-4 production and IgE remained intact or were elevated (3, 6, 7). However, other reports of soluble antigen–induced tolerance have not been interpreted as a Th1→Th2 switch, as IL-4–producing $CD4^+$ T cells could not be detected (8). A mechanism for regulation of organ-specific autoimmune pathology has also been suggested to result from a switch of a cell-mediated Th1-type response to a Th2 response (9). However, recent studies suggest that active tolerance to self- and gut antigens may not be so simple and that other regulatory cells may exist that produce TGF-β and/or, in some cases, IL-10 (10–14).

IL-10 inhibits the production of Th1-specific cytokines by its effects on the APC and downregulates inflammatory cytokines such as IL-12 (15, 16), as well as the expression of costimulators (17) and class II MHC (18). Most importantly, IL-10 has been shown to inhibit the maturation of dendritic cells (DCs), which are the principle APCs involved in the initiation of an immune response (19). There is evidence that IL-10 plays an important role in mucosal immune regulation as well as preventing more generalized immunopathologies. Mice with a targeted disruption of the IL-10 gene ($IL\text{-}10^{-/-}$ mice) developed enterocolitis (20) and showed increased sensitivity to LPS-induced shock (21). In addition, $IL\text{-}10^{-/-}$ mice showed enhanced disease as compared with wild-type mice when experimental autoimmune encephalomyelitis was induced by $MOG_{35–55}$ in CFA (22), suggesting a role for IL-10 in protection from the development of autoimmunity.

In this study, we investigate whether neutralization of IL-10 allows exogenous soluble peptide or protein antigens to prime for Th1 effector responses. We show that neutralizing endogenous IL-10 with an anti–IL-10R mAb can render soluble peptide or protein antigen immunogenic for Th1 recall responses, provided that there is LPS present to activate the innate immune response.

Materials and Methods

Animals. Mice transgenic for the DO11.10 α/β TCR (23) on a BALB/c genetic background were identified at age 4–6 wk by staining peripheral blood leukocytes with the anti-TCR clonotype-specific mAb KJ1-26, as previously described by Kearney et al. (24). These mice were heterozygous for the TCR α and β transgenes. Mice transgenic for the DO11.10 α/β TCR were backcrossed on a RAG-deficient ($RAG^{-/-}$) BALB/c background. Female nontransgenic BALB/c mice between 8 and 10 wk old were purchased from Taconic Farms, Inc.

Culture Medium, Antigens, Antibodies, and other Reagents. cRPMI 1640 (BioWhittaker) supplemented with 10% FCS (Hyclone), 2-ME (0.05 mM; GIBCO BRL), L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes buffer (10 mM), and sodium pyruvate (1 mM) was used as culture medium.

The antigenic OVA peptide (OVA) from chicken ovalbumin ($OVA_{323–339}$) was synthesized free of endotoxin (Biosynthesis, Inc.). OVA was purchased from Calbiochem (28 EU/mg of endotoxin/LPS).

Anti–IL-10R mAb was provided by K. Moore (DNAX, Palo Alto, CA; reference 25), and an isotype-matched control was supplied by J. Abrams (DNAX). mAbs used for flow cytometric analysis included anti–mouse CD4–Cy5, L-selectin–PE (PharMingen), and anticlonotype mAb for transgenic DO11.10 TCR, KJ1-26 (26). Additional anticytokine mAbs for immunoassay and flow cytometry, including anti–mouse IL-10 and IFN-γ reagents, were purified as previously described (27).

Adoptive Transfer and Immunization. The adoptive transfer was performed as previously described by Kearney et al. (24). In brief, a single spleen cell suspension from DO11.10 transgenic mice was injected intravenously into unmanipulated syngeneic BALB/c recipients such that 4–5 $\times$ 10^6 $KJ1\text{-}26^+CD4^+$ T cells were adoptively transferred. Mice were primed 2 d after adoptive transfer with either $OVA_{323–339}$ (7 or 200 μg) or OVA (200 μg or 5 mg) subcutaneously at the base of the tail (similar trends were obtained with both doses of antigen but results are shown for higher doses, as higher numbers of antigen-specific $CD4^+$ T cells were visualized). Mice were rechallenged subcutaneously 12 d after priming with OVA (100 μg) emulsified in CFA (Difco Labs.). Mice were analyzed at indicated time points after rechallenge. In some experiments, mice were injected intraperitoneally with anti–IL-10R (0.5 mg) mAb (25) weekly throughout the experiments, starting at the day of priming.

Preparation of T Cells and APCs. $CD4^+$ T cells were enriched by positive selection using MiniMACS™ separation columns (Miltenyi Biotec) to achieve 98% $CD4^+$ T cells. Cells were then set up in culture at 2 $\times$ 10^5 per well and restimulated with $OVA_{323–339}$ (1 μM) and irradiated syngeneic splenic APCs (5 $\times$ 10^5 per well). Supernatants were collected at 24 h for the measurement of IL-2 and at 48 h for the measurement of IFN-γ, IL-4, and IL-10 by immunoassay (27).

Cytokine Assays. IFN-γ was detected using a two-site sandwich ELISA, with a lower limit of sensitivity of 100 pg/ml. The ELISA for IL-2 has been described previously (27), with a limit of sensitivity of 195 pg/ml.

OVA-specific Serum Isotype ELISAs. For analysis of OVA-specific IgG1 and IgG2a, 96-well plates (Fisher Scientific) were coated with whole OVA (Sigma-Aldrich), 10 μg/ml in PBS. Plates were blocked with 20% FCS, and serum samples were added at appropriate dilutions. Samples were developed by sequential incubation with biotinylated IgG1 or IgG2a isotype–specific mAb (PharMingen), streptavidin–horseradish peroxidase (Caltag Labs.), and substrate (Kirkegaard & Perry Laboratories, Inc.). OVA-specific IgE and IgA isotype titers were determined as previously described (28). Plates were read at 450 nm and analyzed based on OVA-specific isotype standards. Data shown are OVA-specific isotype titers in nanograms per milliliter.

Removal of LPS. To deplete the LPS (otherwise known as endotoxin) from the OVA protein antigen preparation (activity detected by limulus amebocyte assay; BioWhittaker), OVA at 10 mg/ml was adsorbed on a Detoxi-Gel™ Endotoxin Removing Gel (Pierce Chemical Co.) according to the manufacturer's instructions to reduce endotoxin levels to below the level of 5 EU per 5 mg of protein.

Results and Discussion

Neutralization of IL-10 during Priming with Soluble OVA Protein but Not $OVA_{323–339}$ Peptide Antigen Leads to the Development of a Th1 Effector Recall Response. To determine whether neutralization of IL-10 could allow soluble pep-

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tide and protein antigen to prime for a Th1 effector immune response, BALB/c mice, which had been previously transferred with OVA-specific $CD4^+$ T cells (KJ1-26^+ $CD4^+$) from DO11.10 mice, were primed subcutaneously with soluble $OVA_{323–339}$ peptide or protein in the presence or absence of an IL-10R mAb (25). Mice were then rechallenged using OVA protein in CFA, and purified $CD4^+$ T cells from lymph nodes were analyzed for their ability to produce enhanced levels of IFN-γ as a result of appropriate priming. As previously shown (24), $CD4^+$ T cells obtained from mice that had received soluble $OVA_{323–339}$ before challenge produced significantly less IL-2 in vitro in response to $OVA_{323–339}$ presented by irradiated APCs than $CD4^+$ T cells obtained from mice that had received PBS (Fig. 1, top). This correlated with significantly reduced numbers of antigen-specific $CD4^+$ T cells and reduced [^{3}H]thymidine incorporation in vitro in response to specific antigen (data not shown). Immunizing mice with $OVA_{323–339}$ peptide antigen in the presence of an anti–IL-10R mAb did not enhance the production of IL-2 (Fig. 1) nor the number of antigen-specific T cells and their [^{3}H]thymidine incorporation (data not shown). Treatment with soluble OVA protein antigen before rechallenge with OVA in CFA led to a small but reproducible decrease in IL-2 production (<50% in more than three experiments; Fig. 1, top). A small reduction in the number of antigen-specific $CD4^+$ T cells was observed and reduced [^{3}H]thymidine incorporation in vitro in response to specific antigen (data not shown). The presence of a mAb directed against the IL-10R during priming with soluble protein (in contrast to peptide) antigen enhanced the levels of IL-2 produced by $CD4^+$ T cells almost to the level produced by $CD4^+$ T cells from mice that were pretreated with PBS before the OVA in CFA challenge. This increase could be accounted for completely by an increase in KJ1-26^+CD4^+ T cells, as on a per cell basis, IL-2 levels were identical in the presence or absence of anti–IL-10R mAb (Fig. 1, bottom).

$CD4^+$ T cells from mice that had previously received soluble protein or peptide antigen produced similar or lower levels of IFN-γ upon restimulation in vitro relative to the levels obtained from unprimed mice that had only been given the OVA in CFA challenge (Fig. 1). This showed that soluble antigen did not prime for an effector Th1-type response and in some instances (not shown) actually led to suppressed levels of IFN-γ production upon rechallenge with CFA plus OVA. Addition of the anti–IL-10R mAb could not convert the soluble $OVA_{323–339}$ peptide into a priming regimen for a Th1 response and the production of IFN-γ (Fig. 1), even when the peptide was administered repeatedly to compensate for its shorter half-life in vivo (data not shown). In contrast, simultaneous administration of the anti–IL-10R mAb with the soluble OVA protein led to effective priming for a Th1 effector response, with highly elevated and prolonged levels of IFN-γ production (Fig. 1, top). This increase not only resulted from an increased number of antigen-specific $CD4^+$ T cells producing IFN-γ but also reflected an increase in the amount of IFN-γ produced per antigen-specific $CD4^+$ T cell (Fig. 1, bottom). A higher level of fluorescence intensity for intracellular IFN-γ production in a greater percentage of the cells as shown by flow cytometry was also observed (data not shown). We have previously shown that this increase in fluorescence intensity for cytokine staining correlates with maximal IFN-γ production from committed Th1 cells stimulated appropriately (O'Garra, A., unpublished data). Early production of IL-10 during the pri-

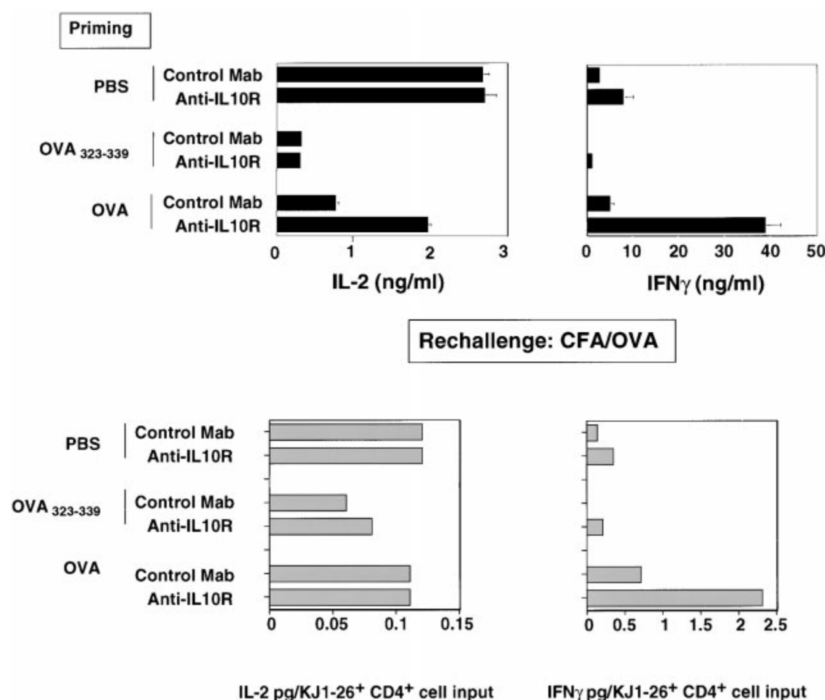

Figure 1. Treatment of OVA-primed mice with anti–IL-10R mAb before rechallenge potentiates the production of IFN-γ. $CD4^+$ T cells were purified from the draining lymph nodes of mice primed and rechallenged as indicated in Materials and Methods and stimulated in vitro with $OVA_{323–339}$ and APCs. Supernatants were harvested after 24 (IL-2) or 48 h (IFN-γ) of culture.

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mary response to protein antigen (data not shown) could possibly explain the rapid downregulation of IFN-γ by the inhibitory effects of IL-10 on APCs (15) and may account for the poor ability of soluble protein antigen to prime for subsequent effector immune responses.

Anti–IL-10R mAb Is an Adjuvant for Priming Th1 Effector Recall Responses Only in the Presence of LPS. As the OVA protein preparation but not the $OVA_{323–339}$ peptide preparation contains low amounts of LPS (28 EU/mg), we determined if LPS together with the anti–IL-10R mAb was required for the OVA protein to prime for a Th1 effector recall response. To investigate this, we first purified the LPS-free OVA protein using a Detoxi-Gel™ Endotoxin Removing Gel to <5 EU/5 mg and then compared its ability to prime for a subsequent Th1 recall response with the original OVA protein preparation. As shown in Fig. 2, when the contaminating LPS was removed from the OVA protein (referred to as *OVA), anti–IL-10R mAb had little effect on priming for a Th1 recall response, in contrast to its effects with the original OVA preparation. Furthermore, addition of similar amounts of LPS (140 EU [14 ng]) to the $OVA_{323–339}$ peptide resulted in successful priming for a Th1 recall response only in the presence of the anti–IL-10R mAb. Thus, triggering of the innate immune response by low levels of LPS is not sufficient to allow soluble peptide or protein antigens to prime for Th1 recall responses unless the suppressive effect of IL-10 is neutralized. Additionally, these data show unequivocally that the ability of IL-10 to regulate the immune response only comes into play when there is simultaneous activation of the innate immune response.

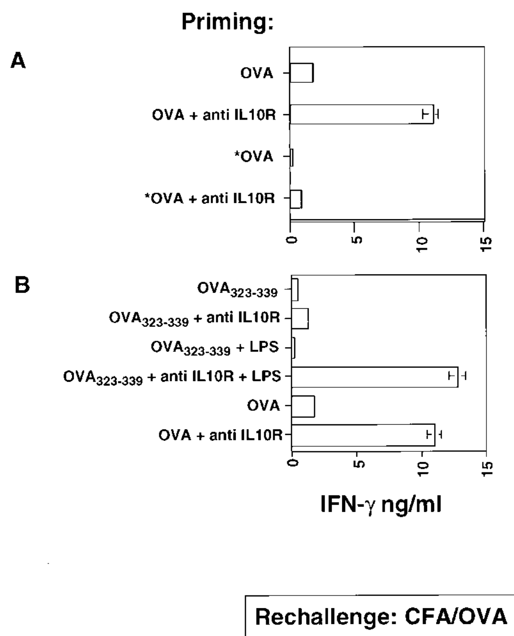

Figure 2. Neutralization of IL-10 allows priming for Th1 recall responses by soluble protein or peptide antigen only when low levels of LPS are present. $CD4^+$ T cells were purified from the draining lymph nodes of mice primed with OVA protein, OVA protein that had been purified as free of LPS (*OVA), or $OVA_{323–339}$ peptide antigen in the presence or absence of LPS (140 EU [14 ng]) plus or minus anti–IL-10R mAbs as shown. Mice were then rechallenged with OVA and CFA as indicated in Materials and Methods and stimulated in vitro with $OVA_{323–339}$ and APCs. Supernatants were harvested after 24 (IL-2) or 48 h (IFN-γ) of culture. Results were reproducible in greater than three experiments.

Anti–IL-10R mAb Plus LPS Enhances Antigen-specific IgG1 and IgG2a Levels to Protein but Not Peptide Antigen. In addition to the enhanced production of IFN-γ by $CD4^+$ T cells, soluble OVA protein administered together with anti–IL-10R mAb also led to a significant enhancement of OVA-specific IgG1 and IgG2a antibodies upon rechallenge (Fig. 3). This enhancement was abrogated upon removal of LPS from the protein antigen (data not shown). Soluble $OVA_{323–339}$ peptide, when delivered with or without anti–IL-10R mAbs, did not induce OVA-specific IgG antibody responses. Furthermore, although addition of similar amounts of LPS to the $OVA_{323–339}$ peptide resulted in successful priming for a Th1 recall response in the presence of the anti–IL-10R mAb, this did not lead to priming for IgG2a responses (data not shown), in keeping with a requirement for cross-linking of the antigen for generation of antibody production from B cells.

The effects of neutralizing IL-10 may result indirectly from enhancement of $CD4^+$ T cell help by releasing inhibition of APC function in a primary immune response (15, 16). A dominant property of IL-10 is its ability to downregulate the APC function of macrophages and/or DCs (15). These effects include the expression of costimulators such as CD80 and CD86 (17), the trafficking of peptide-bearing class II MHC molecules (18), and the inhibition of production of inflammatory mediators such as IL-12, which are required for the development and maintenance of Th1 responses (16, 29). DCs, the principal APCs in-

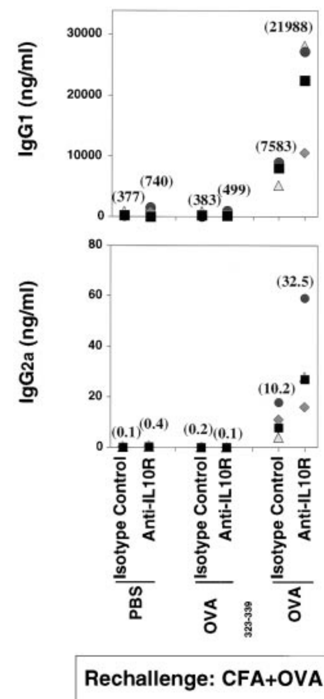

Figure 3. Anti–IL-10R mAb plus LPS enhances antigen-specific IgG1 and IgG2a levels with protein and not peptide antigen. Mice were primed and rechallenged as indicated in Materials and Methods. Serum was collected 7 d after rechallenge with OVA in CFA. The average concentration in nanograms per milliliter for each isotype is indicated in parentheses. Each symbol represents data from an individual mouse. Results were reproducible in greater than three experiments.

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volved in the initiation of an immune response, capture foreign antigens encountered in peripheral tissues, process the antigens into peptides bound to MHC molecules, and migrate to lymphoid organs for presentation of the MHC–peptide complexes to lymphocytes (19). Furthermore, on the one hand, proinflammatory agents such as LPS are strong inducers of DC maturation (30, 31), whereas IL-10 inhibits DC maturation (for review see reference 19). Thus, it is likely that a delicate balance between proinflammatory factors and IL-10 is critical in the tight regulation of the transition of resting/immature DCs to mature DCs and hence the initiation of an immune response. It has been recently reported that immature DCs sequester intact antigens in lysosomes and that neither peptides nor proteins are converted to peptide–MHC class II complexes until a maturation stimulus such as LPS is received (32, 33), a component of Gram-negative bacterial pathogens (34). Furthermore, it has also been shown that LPS promotes accumulation of antigen-bearing DCs in the T cell areas of lymphoid tissue (35). Thus, it is likely that IL-10 can act as a limiting factor at this stage to regulate the magnitude of an immune response by inhibiting the maturation of DCs, as well as possibly inhibiting the migration of initiating APCs. The results in this study may have implications for the breakdown of tolerance to autoantigens, which has been suggested to be initiated by infection. This may also require a genetic predisposition of the host for a defect in the production or signaling capacity of immunoregulatory molecules such as IL-10. This would result in a lower threshold of reactivity of autoreactive T cells in the context of activation of inflammatory molecules of the innate immune response, as exemplified by the findings of Bettelli et al. (22) in the induction of EAE.

In summary, we show that anti–IL-10R can render soluble peptide or protein antigen immunogenic for subsequent Th1 recall responses, provided that there is a signal such as LPS present to activate the innate immune response. These findings have important implications for the design of relevant vaccines for in vivo immunotherapy and suggest that, in addition to triggering T cells through the TCR via specific antigen, this will require signaling through Toll receptors as well as neutralization of endogenous suppressive molecules such as IL-10.

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