Autocrine IL-12 Is Involved in Dendritic Cell Modulation Via CD40 Ligation

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Ligation of CD40 on dendritic cells (DC) triggers production of IL-12. Using an adoptive transfer model we have previously shown that rIL-12 acts directly on DC to enhance presentation of an otherwise poorly immunogenic tumor peptide. Using the same experimental model, we now describe a similar adjuvanticity of CD40 ligation on peptide presentation by DC. We also explore the possibility that the IL-12 resulting from CD40 ligation directly affects the APC function of DC, mediating or contributing to the adjuvant effect of CD40 ligation. CD40 engagement in vitro and rIL-12 at concentrations in the range induced by CD40 ligation were equally effective in priming DC for presentation of the tumor peptide in vivo. Remarkably, the copresence in vitro of neutralizing Ab to IL-12, but not to TNF-α, IL-1β, or IFN-γ, ablated the enhancing effect of CD40 engagement on the APC function of DC. These data suggest a major role for autocrine IL-12 in DC modulation via CD40 ligation. The Journal of Immunology, 1999, 163: 2517–2521.

It is well established that IL-12 drives the development of cell-mediated immune responses (1, 2). The identification of the cells and mechanisms responsible for IL-12 production and of the cellular targets at the site of T cell priming is a critical issue that still needs to be clarified. Previous evidence indicates that appropriate stimuli can induce APC to release IL-12 and that the cytokine will act on bystander T cells that recognize Ag on physically distinct APC (3). Thus, IL-12 appears to function in a paracrine fashion, because IL-12 production and Ag presentation can be carried out by different cells. In addition, IL-12 production by APC is induced by the interaction between CD40 on the APC and CD40 ligand expressed on T cells after activation (4). CD40 ligand is the most potent stimulus in up-regulating costimulatory molecules (5) and cytokines such as IL-12 (6), TNF-α (7), and IL-1β (8).

We have previously shown that IL-12 primes dendritic cells (DC)1 in vitro for effective presentation of a poorly immunogenic tumor peptide upon transfer of the DC to recipient hosts (9–11). Nonameric P815AB represents a minimal core peptide recognized by CTL in vitro and is part of a protein encoded by gene P1A which, silent in most normal tissues except testis and placenta, is instead expressed by murine mastocytoma cells (11). We have previously shown that P815AB fails to initiate class I-restricted reactivity in vivo, presumably as a result of a poor ability to recruit CD4+ cells to the afferent induction of the response initiated by host transfer with P815AB-pulsed DC (9). However, the poor immunogenicity of the peptide under such priming conditions can be overcome by preexposure of the DC to rIL-12 (10).

The aim of the present study was to investigate whether CD40 engagement on DC may also prime the cells for effective presentation of the tumor peptide. By comparing CD40 ligation and rIL-12, we found that the former stimulus was highly effective in priming DC, and that rIL-12 concentrations in the range of those induced by CD40 cross-linking would likewise be an effective stimulus for DC priming. Most interestingly, the presence of Ab to IL-12 during cross-linking in vitro blocked the adjuvant effect of CD40 modulation in the DC. In contrast, no effect was afforded by neutralization of TNF-α, IL-1β, or IFN-γ. These data suggest an important role for autocrine IL-12 in the adjuvant effect of CD40 engagement on DC.

Materials and Methods

Mice and reagents

Male DBA/2J (H-2b) mice were obtained from Charles River Laboratories (Calco, Milan, Italy) and were used at the age of 2–4 mo. Murine rIL-12 was a generous gift from Dr. B. Hubbard (Genetics Institute, Cambridge, MA). IL-12 was 98.8% pure, as assessed by SDS-PAGE, and endotoxin contamination was <0.9 EU/mg on Limulus amebocyte assay. The specific activity of the purified rIL-12 preparation, measured as ability to stimulate proliferation in human PHA-activated blasts, was 3.1 × 108 U/mg. Endotoxin was removed from all solutions containing rIL-12 or anti-CD40 Abs with Detoxi-gel (Pierce Chemical, Rockford, IL), resulting in endotoxin contamination below the detection limit (0.05 EU/ml) of the assay (Coatest Endotoxin, Chromogenix, Mölndal, Sweden) (12). Rat anti-mouse IL-12 p40 mAb C17.8 and hamster anti-mouse IL-12 p35 (clone Red-T) were from Genzyme (Cambridge, MA). C17.8 mAb was conjugated to biotin using conventional methods. Hamster anti-murine CD40 (HM40-3) was from PharMingen (San Diego, CA) and goat anti-hamster IgG was from Pierce Chemical. Affinity-purified sheep anti-mouse IL-12 polyclonal Ab was generously provided by the Immunology Department of Genetics Institute, and the control Ab (sheep IgG) was purchased from Pierce Chemical. Neutralizing rat anti-mouse TNF-α (MP6-XT3, PharMingen), hamster anti-mouse IL-1β (Genzyme), and rat anti-mouse IFN-γ (XMG1.2) (10) mAbs were also used. The nonameric P815AB peptide (LPYLGWLVF) was synthesized and purified as described (13).

DC preparation and in vitro treatments

DC were prepared from collagenase-treated spleens (collagenase type IV, Sigma Chemical, St. Louis, MO), as described (12). Briefly, DC were purified using a positive selection column and CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered cells were routinely >98% N418. For CD40 cross-linking (14), DC were incubated on
ice for 10 min in PBS plus 10% mouse serum, for 20 min with hamster anti-mouse CD40 mAb (5 μg/ml), and then overnight at 37°C with goat anti-hamster Ab (5 μg/ml) in Iscove’s medium plus 10% FCS. For IL-12 induction (9, 10), cells were incubated overnight with different concentrations of rIL-12. With both procedures, cells were washed between and after the incubations prior to peptide loading (5 μM, 2 h, at 37°C), irradiation, and i.v. injection (3 × 10^7/mouse) into recipient hosts, as described (9, 10).

Detection of IL-12 p70 in DC culture supernatants

Culture supernatants of DC (2 × 10^6/ml) recovered at the end of the cross-linking procedure were assayed for IL-12 p70 contents by ELISA using anti-p35 and biotinylated anti-p40 mAbs (12). The sensitivity limit of this assay was ~0.015 ng/ml. In measuring IL-12 induction by rIL-12 with or without anti-CD40 Abs, the overnight cultures were washed and incubated for 24 h in fresh medium alone. IL-12 p70 titers being measured at 1 and 24 h.

Skin test assay

An assay system for measuring the class I-restricted DTH response was employed in which 50 μg of P815AB peptide in 30 μl of 6% DMSO in saline were inoculated into the left hind footpads of mice transferred with DC 2 wk earlier (15). The right hind footpad received the same volume of vehicle. The DTH reaction was recorded 24 h later, when the animals were killed, their hind feet were cut off at the hair line, and weights were recorded as a measure of swelling, edema, and cellular infiltration. Results were expressed as the increase in footpad weight over that in the vehicle-injected counterpart. Data are the means ± SD for at least six mice per group. The statistical analysis was performed using Student’s paired t test by comparing the mean weight of experimental footpads with that of control, vehicle-injected counterparts (9, 10, 15). The data reported are from representative experiments, and experiments with similar results were performed three to six times.

Results

IL-12 production by CD40-modulated dendritic cells

In a preliminary series of experiments, we wanted to examine DC production of IL-12 upon ligation of CD40 and to determine the quantitative expression of this production. Highly purified DC cultures were established from the spleens of DBA/2 mice, and the cells were subjected to overnight cross-linking with hamster anti-mouse CD40 Ab plus goat anti-hamster Ab (14). The release of IL-12 p70 was measured by ELISA at the end of the overnight cross-linking (Table I). In three different experiments, the range for IL-12 production under the adopted conditions was 0.41–5.64 ng/ml of p70 heterodimer. The basal production of unstimulated DC was 0.06–0.18 ng/ml.

Effect of CD40 engagement on peptide presentation by DC

We have previously shown that transfer of DC sequentially exposed to IL-12 and a tumor peptide (P815AB) initiates cell-medi-
is known to synergize with CD40 ligation (16) were assayed for possible interference with the effect of CD40 cross-linking. Equal concentrations (10 μg/ml) of each of these mAbs or anti-IL-12 Ab were added to the DC cultures in concurrence with CD40 stimulation. The recovered cells were then loaded with the peptide and used in the in vivo assay. Fig. 3 shows that neutralization of TNF-α, IL-1β, or IFN-γ did not affect the APC function of CD40-modulated DC. Again, the response was completely blocked by the use of anti-IL-12 Ab during CD40 stimulation.

Effect of rIL-12 and CD40 engagement on production of IL-12
Similarly to CD40 engagement, rIL-12 induces the production of IL-12 by DC cultures (12). We therefore became interested in ascertaining the combined effects of rIL-12 and CD40 cross-linking on this production. We measured p70 titers in culture supernatants of DC treated overnight with anti-CD40 Abs, rIL-12 (100 ng/ml), or a combination of both and then washed extensively prior to incubation in medium alone for an additional 24 h. Supernatants were harvested at 1 and 24 h. No IL-12 was found in any group at 1 h, thus excluding the presence of residual cytokine bound or internalized by the cultures. Under the adopted conditions of testing, the production of IL-12 by CD40-modulated DC was rather limited, approximately one-third of that induced by rIL-12 alone (Table II). Remarkably, coexposure to anti-CD40 Abs and rIL-12 led to a 3.6-fold increase in this production.

Effect of rIL-12 and CD40 engagement on peptide presentation by DC
The finding of synergic effects between anti-CD40 Abs and rIL-12 on IL-12 production prompted us to investigate the combined effects of the two modalities of DC activation on P815AB presentation in vivo. Using an experimental model similar to that adopted in Fig. 1, DC exposed to different concentrations of rIL-12 and/or CD40 cross-linking were loaded with P815AB and then injected into recipient hosts that were assayed at 2 wk for footpad reactivity to the tumor peptide. Fig. 4 shows that CD40 ligation apparently increased the ability of rIL-12 to trigger footpad reactivity to P815AB. However, with the lowest IL-12 concentration (i.e., 0.1

### Table II. IL-12 production by DC treated with anti-CD40 and rIL-12

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-12 p70 (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.05 ± 0.01b</td>
</tr>
<tr>
<td>Anti-CD40 Abs</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>rIL-12</td>
<td>1.44 ± 0.06</td>
</tr>
<tr>
<td>Anti-CD40 Abs + rIL-12</td>
<td>5.23 ± 0.36</td>
</tr>
</tbody>
</table>

*DC were treated overnight with anti-CD40 Abs, rIL-12 at 100 ng/ml, or a combination of both. After washing, cultures were incubated in fresh medium alone for an additional 24 h, and IL-12 p70 levels were measured in supernatants by ELISA at 1 and 24 h. The 1-h IL-12 titers were consistently below the detection limit of the assay.

*b Data are the means ± SD of replicate determinations in one of three experiments with similar results.
ng/ml), the extent of the footpad response resulting from concomitant CD40 ligation was not significantly different from that of the latter treatment alone.

Discussion

The identification of the cells responsive to IL-12 at the site of T cell priming is an important issue that awaits clarification. In a series of original experiments Murphy and colleagues (3) demonstrated that Listeria monocytogenes can stimulate macrophages to produce IL-12 and that this cytokine can act on T cells recognizing Ag on different APC. This suggests a major role for IL-12 acting in a paracrine fashion at the site of T cell priming.

DC are professional APC specialized in Ag capture, migration to secondary lymphoid organs, and T cell priming (17, 18). One major function of DC is the production of IL-12 (19). This production is up-regulated via MHC class II and CD40 signaling and down-regulated by Th2 cytokines (20). A recent report from our laboratory has shown that not only are DC a major cell type producing IL-12 but they also express a high affinity receptor for IL-12 (12). Signaling through this receptor leads to nuclear localization of NF-κB and changes in DC function, including expression of a greater amount of fully mature class II molecules despite a decline in class II biosynthesis. Activation of DC through IL-12 exposure also confers increased APC function on these cells, resulting in effective presentation of a tumor peptide following transfer of peptide-loaded DC into recipient hosts (9–11).

Because DC respond to stimulation via CD40 signaling by producing very high levels of bioactive IL-12 (5), the possibility exists that the endogenously produced IL-12 affects T cell activation not only in a paracrine fashion but also via direct effects on the CD40-modulated DC. In addition, because activation of the IL-12R and CD40 engagement appear to share signaling pathways in APC (12, 21), it is possible that IL-12 mediates, contributes to, or interferes with the modulation of DC activity via CD40 ligation.

Transfer of DC exposed in vitro to IL-12 and the tumor peptide P815AB confers CD8+ cell-mediated reactivity on prospective recipients of an intrafootpad challenge with the peptide, which is accompanied by increased resistance to challenge with tumor cells expressing the otherwise poorly immunogenic rejection Ag P815AB (11). To investigate possible effects of CD40 engagement on the APC function of DC, we resorted to this experimental model by exposing DC to CD40 cross-linking prior to peptide loading in vitro. We found that this maneuver resulted in an adjuvanticity comparable with that induced by treatment with rIL-12 (Fig. 1). To ascertain whether endogenous IL-12 is involved in the adjuvancytive association with CD40 cross-linking, we measured IL-12 concentrations in supernatants of CD40-modulated DC and used the same range of rIL-12 concentrations for DC activation (Table I and Fig. 1). We found that these concentrations were fully capable of activating DC, thus suggesting that endogenous IL-12 might contribute to the effects of CD40 engagement.

In addition to IL-12, the cytokines TNF-α (7) and IL-1β (8) are also known to be induced by CD40 signaling. IFN-γ, on the other hand, is known to synergize with CD40 engagement in modulating DC activity (16). It appeared therefore of interest to analyze the effects of IL-12 neutralization on the adjuvancytative association with CD40 engagement and to compare these effects with those of Abs to TNF-α, IL-1β, or IFN-γ. We found that the presence of anti-IL-12 Ab, but not of anti-TNF-α, anti-IL-1β, or anti-IFN-γ mAbs, completely blocked the adjuvant effect of CD40 stimulation in the priming to P815AB (Figs. 2 and 3). This suggests that, at least in terms of improved APC function, the endogenous production of IL-12 acting in an autocrine fashion is responsible for most of the effect resulting from CD40 signaling. Alternatively, although less likely, a basal production of IL-12 by DC, as observed in Table I, might be a necessary cosustimulus for modulation of DC. If so, the mere engagement of CD40 would be an insufficient stimulus for increasing the APC function of DC.

Although the present data seem to indicate that IL-12 mediates the effect of CD40 cross-linking in the model of peptide presentation in vivo, indirect evidence suggests that the two mechanisms of DC modulation, IL-12-mediated activation and CD40 engagement, may be operationally distinct relative to other DC functions. Considering that rIL-12 induces the production of IL-12 by DC (12), we have tested the effect of rIL-12 exposure and CD40 cross-linking, either alone or in combination, on the production of IL-12. We have found that the combined modality of DC activation resulted in IL-12 levels much higher than either maneuver alone (Table II). Furthermore, we have been able to detect DC functions that are differentially affected by CD40 modulation and rIL-12, including B7-1 expression. While boosted by CD40 ligation, this expression is not modified by CD40 exposure to rIL-12 (Ref. 12 and our unpublished observations). On the other hand, the combined modality of DC activation (CD40 cross-linking plus IL-12 treatment) resulted in only a limited increase in the effect of rIL-12 alone on tumor peptide presentation in vivo (Figs. 1 and 4). This could be due to the remarkable potency of IL-12 in the activation of the in vivo response, with nearly maximal responses being induced by relatively low IL-12 concentrations. In order to clarify this issue, we are currently investigating the combined effects of CD40 ligation and rIL-12 on a series of in vivo functions, including resistance to challenge with P815AB-expressing tumor cells and generation of CTL activity.

In addition to previous evidence that the CD40–CD40 ligand interaction represents an important means by which T-T help occurs via APC activation (5, 22, 23) and a temporal bridge between CD4+ and CD8+ cells (12, 24), our data suggest that bidirectional influences take place between T cells and APC via IL-12 release at the site of T cell priming. The IL-12 released as a consequence of CD40 engagement may condition not only the developing Th response, but also the DC to differentiate to a state in which they may express a greater amount of fully mature class II molecules (see Footnote 4). Improved presentation of class II-restricted epitopes of P815AB to CD4+ cells is, in fact, a likely mechanism through which rIL-12 primes DC for effective presentation of the tumor peptide (10). As a whole, the present findings suggest a new role for CD40 ligation in regulating DC function and may be relevant to the design of therapeutic strategies using cultured DC.

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References


