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IL-2 and IL-15 Regulate CD154 Expression on Activated CD4 T Cells

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The cellular and humoral immune system is critically dependent upon CD40-CD154 (CD40 ligand) interactions between CD40 expressed on B cells, macrophages, and dendritic cells, and CD154 expressed primarily on CD4 T cells. Previous studies have shown that CD154 is transiently expressed on CD4 T cells after T cell receptor engagement in vitro. However, we found that stimulation of PBLs with maximal CD28 costimulation, using beads coupled to Abs against CD3 and CD28, led to a very prolonged expression of CD154 on CD4 cells (>4 days) that was dependent upon autocrine IL-2 production. Previously activated CD4 T cells could respond to IL-2, or the related cytokine IL-15, by de novo CD154 production and expression without requiring an additional signal from CD3 and CD28. These results provide evidence that CD28 costimulation of CD4 T cells, through autocrine IL-2 production, maintains high levels of CD154 expression. This has significant impact on our understanding of the acquired immune response and may provide insight concerning the mechanisms underlying several immunological diseases.

Materials and Methods

Cells, culture, and activation

PBMC were obtained from healthy human volunteer donors using standard procedures. Phagocytic cells were depleted by incubation with uncoated Dynal beads, two beads per cell for 2 h at 37°C, followed by magnetic depletion (monocytes and macrophages efficiently phagocytose the beads). Cells were stimulated with Dynal beads coupled to anti-CD3 (OKT-3) and anti-CD28 (9.3) Abs (11), three beads per cell. Pure CD4 T cells were obtained by reacting cells with Abs against human CD8 (10 μg/ml), CD14 (1 μg/ml), CD16 (1 μg/ml), and CD20 (10 μg/ml) followed by two rounds of magnetic depletion with sheep anti-mouse-coated Dynal beads, six beads per cell. Neutralizing anti-human IL-2, IL-4, and IFN-γ Abs were used at 10 μg/ml and obtained from PharMingen (San Diego, CA). IL-2 was obtained from Boehringer Mannheim (Indianapolis, IN).

Flow cytometry

Cells were labeled with PE-coupled anti-human CD154 Ab (Becton Dickinson, Mountain View, CA) or PE-coupled IgG1 isotype control Ab and FITC-coupled anti-human CD4 (Immunotech, Westbrook, ME), washed, and resuspended in 1% paraformaldehyde. For intracellular CD154 staining, cells were first surface labeled as described above, but with a nonconjugated anti-human CD4 Ab (Immunotech, Westbrook, ME), washed, and resuspended in 1% paraformaldehyde. For intracellular CD154 staining, cells were first surface labeled as described above, but with a nonconjugated anti-human CD4 Ab or control Ab. Cells were then fixed and permeabilized using a Becton Dickinson intracellular staining kit and labeled with PE-coupled anti-human CD4 Ab or isotype control Ab. Data acquisition and flow cytometric analysis were performed on a Becton Dickinson FACSCalibur using CellQuest software.

RT-PCR

RNA was purified from 10^6 cells using Trizol. RT-PCR was run with a CD154 primer set: (CTCGAAAGTGAACACTGTCACCAGGATGTAGAACACATA) or (GTTGATCTAGAACAGGATGCGAACAAACTAA) and a GADPH primer set: (CGGTCATACGTCTGGATGACACATGGGCACCAGTGGTGACACA) using Titan one-step RT-PCR (Boehringer Mannheim) with 35 PCR cycles: 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s.

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Results

CD3/CD28 activation of CD4 cells induce prolonged CD154 expression through a calcineurin-independent pathway

Expression of CD154 on CD4 T cells has mainly been studied after TCR or ionomycin/PMA stimulation, which has revealed a very transient expression of CD154 (<24 h) that is dependent on calcineurin/NF-AT activation (12–14). To study CD154 expression under more physiological conditions, we used Abs against CD3 and CD28 coupled to beads (3x28 beads) as a form of surrogate APC. These beads are more potent than soluble or plate-bound Abs, probably due to their topological resemblance to cells, facilitating polarized interactions; an important factor in cell activation that has recently emerged from several excellent studies (15–17).

Fig. 1A shows primary/naive human PBLs stimulated with 3x28 beads. As expected, there was a rapid induction of surface CD154 expression on CD4 T cells. The level of surface CD154 was comparable or slightly higher than previously reported with plate-bound anti-TCR Abs with or without anti-CD28 costimulation (13, 18, 19). Effector cells, generated by 12–14-day expansion of cultures stimulated with 3x28 beads at day 0, also rapidly induced CD154 expression on CD4 cells upon restimulation with 3x28 beads; however, the level of induction was substantially higher than in naive cells (Fig. 1A). CD154 expression on both activated naive and effector CD4 cells did not decline as expected and was still expressed at high levels 4 days after the initial stimulation (Fig. 1A). Fig. 1B shows a schematic representation of CD154 expression on CD4 cells from PBL cultures stimulated with 3x28 beads at day 0 and restimulated at day 14. A similar CD154 profile was seen when purified CD4 cells were stimulated (data not shown), suggesting that CD8 T cells, B cells, or NK cells are not positively involved in the regulation of CD154 surface expression. It has previously been shown that large numbers of CD40-positive cells can induce down-modulation of CD154 on CD4 cells (20, 21); however, we did not see this effect in our PBL cultures, possibly due to the low amount of B cells present (3–11%).

A potential explanation for the prolonged CD154 expression could be that it is caused by repeated engagement between CD4 cells and 3x28 beads. However, after 2–4 days of stimulation, CD154 expression on both naive and effector CD4 cells was very resistant to cyclosporin A (CsA) treatment. In contrast, CsA efficiently inhibited CD154 expression when present before the 3x28 bead stimulation (Fig. 1C). Similar results were seen with the non-CsA-related calcineurin inhibitor FK-506 (data not shown). This suggests that a signal pathway distinct from CD3/CD28-induced NF-AT activation is, at least in part, responsible for the sustained CD154 expression.

IL-2 and IL-15 induce CD154 expression on previously activated CD4 cells

One of the main responses to CD28 costimulation of T cells is autocrine production of IL-2; intriguingly, TCR engagement with-
shown). To rule out that withdrawal of IL-2 for 24 h was directly toxic for the cells, we took the activated naive or effector CD4 cells previously cultured without 3x28 beads and IL-2 and restimulated them with 100 U/ml IL-2 for 24 h. This could readily reinduce CD154 expression, an effect not seen with 50 ng/ml IL-4 or 1000 U/ml IFN-γ (Fig. 2B). Beyond emphasizing the functional integrity of the IL-2-deprived cells, this also demonstrates that IL-2 by itself could induce, and not only sustain, CD154 expression on activated CD4 T cells. Reactivation with IL-2 did not change the constitutive high HLA-DR expression or induced expression of 4-1BB or CCR5 (data not shown), demonstrating that IL-2 re-stimulation did not unspecifically alter expression of other surface molecules. Subsequent titration showed that 5–10 U/ml of IL-2 was necessary for maximal CD154 reinduction on naive or effector cells and that 1–2 U/ml induced half the maximal CD154 expression (Fig. 2C and data not shown).

Interestingly, 100 ng/ml of IL-15 could reinduce CD40L expression in the absence of IL-2 (Fig. 2B) and could also sustain CD40L in the absence of 3x28 beads and IL-2 (data not shown). IL-2 and IL-15 share many biological activities and their receptors use the same β- and γ-chain, but have individual α-chains (23). In contrast to IL-2, IL-15 is mainly produced by activated professional APCs including monocytes, macrophages, and dendritic cells; it is therefore tempting to speculate that IL-15 produced during T cell-APC interaction might also participate in the regulation of CD40L expression in vivo.

It is well known that resting CD4 cells have virtually no CD25 expression and low IL-2Rα-chain before re-stimulation. Our in vitro generated effector CD4 cells had low, but significant, CD25 expression and CD122 (IL-2Rβ-chain) expression. Furthermore, we found that CD25 expression and low IL-2R β-chain before re-stimulation (Table I). To investigate the relative importance of 3x28 beads and IL-2 at different intervals after activation, we incubated cells in the presence or absence of 3x28 beads and/or IL-2 (as described in Fig. 2A) from days 0 to 1, 1 to 2, 2 to 3, and 3 to 4. As shown in Fig. 2, D and E, IL-2, without prior 3x28 bead stimulation, had no effect on CD154 expression on naive cells and only a limited effect on effector cells. In line with this, the 3x28

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Influence of IL-2 on CD154 expression. A, Naive (left) or effector (right) PBL were stimulated for 3 days with 3x28 beads and then cultured for 24 h with I) 3x28 beads (three beads per cell) and 100 U/ml IL-2; II) 3x28 beads and 10 µg/ml blocking anti-IL-2 Ab; III) 100 U/ml IL-2; and IV) 10 µg/ml blocking anti-IL-2 Ab. CD154 surface expression on CD4 cells; isotype control staining (shaded), CD154 staining (full line), MFI for CD154 staining is included. The experiment was repeated five times with similar results. B, Naive (●) or effector PBL (□) were stimulated for 3 days with 3x28 beads and then cultured for 24 h without 3x28 beads and IL-2 (as described above for A, 4) and then restimulated with PBS, 100 U/ml IL-2, 50 ng/ml IL-4, 1000 U/ml IFN-γ, or 100 ng/ml IL-15 for 24 h. CD154 level on CD4 cells, as MFI corrected for isotype control Ab staining. The experiment was repeated three times with similar results. C, Titration of antibody, such as in the Table I. CD25 and CD122 expression on naive and effector CD4 cells after stimulation with 3x28 beads

<table>
<thead>
<tr>
<th>Day (days of culture)</th>
<th>CD25 Naive Cells</th>
<th>CD122 Naive Cells</th>
<th>CD25 Effector Cells</th>
<th>CD122 Effector Cells</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>167.0</td>
<td>381.4</td>
<td>177</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>861</td>
<td>17</td>
<td>823</td>
<td>7</td>
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<td>9</td>
</tr>
<tr>
<td>4</td>
<td>799</td>
<td>8</td>
<td>723</td>
<td>16</td>
</tr>
</tbody>
</table>

*CD25 and CD122 expression are shown as MFI before (control) or after 1–4 days of stimulation.*
bead activation accounted for nearly all CD154 expression after 24 h, as the CD154 level was only very weakly affected by withdrawal of IL-2. However, gradually the 3x28 bead response weakened and IL-2 took over, most strikingly seen in effector CD4 cells where IL-2 induced 90% of the CD154 expression after 3–4 days of activation. Subsequent analysis showed that induction of IL-2 receptor α- and β-chain expression correlated with the ability of IL-2 to induce CD154 expression (Table I). However, there were no significant differences between IL-2 receptor levels on activated naive and effector CD4 cells, suggesting that the notable higher CD154 expression on effector cells may be due to enhanced responsiveness.

**IL-2 induces de novo production and expression of CD154**

To investigate how IL-2 regulates CD154 expression, we reinuced CD154 expression on effector CD4 cells previously rested for 24 h as described in Fig. 2B, but in the presence of various inhibitors. CD154 expression was measured after 6 h of 3x28 bead or IL-2 stimulation to limit the toxic effects of the inhibitors. Fig. 3A shows that pretreatment with actinomycin D, cyclohexamide, or brefeldin A, inhibitors of transcription, translation, and transport through the Golgi complex, respectively, blocked IL-2-induced re-expression of CD154 on previously activated effector CD4 cells, clearly demonstrating that IL-2 induces CD154 expression by de novo synthesis. Further analysis showed that rapamycin, an inhibitor of the IL-2-induced p70s6 kinase, significantly, but not dramatically, inhibited IL-2-induced CD154 expression (Fig. 3A). Rapamycin had only a limited effect on 3x28 bead-induced CD154 expression (when a blocking anti-IL-2 Ab was included to neutralize the effect of newly produced IL-2). As expected, CsA completely inhibited 3x28 bead-induced CD154 expression; however, IL-2-induced CD154 expression was also affected to some degree (Fig. 3A). Current knowledge in the field suggests that CsA does not inhibit IL-2-induced signal transduction. Thus, the most likely explanation would be that a basal level of calcineurin activity is involved in IL-2-induced CD154 expression. In conclusion, these results show that IL-2 induces de novo CD154 production by a distinct and until now unrecognized signal pathway.

Fig. 3B shows the CD154 mRNA level, assessed by RT-PCR, of 3-day 3x28 bead-activated naive or effector CD4 cells cultured in the presence or absence of 3x28 beads and/or IL-2 for 24 h. Generally, the CD154 mRNA level corresponded to the surface CD154 expression. However, although it is difficult to directly compare, the CD154 mRNA level did not seem to be reduced to the same extent as the surface expression in CD4 cells incubated for 24 h without 3x28 beads and IL-2. An explanation could be that the residual mRNA is either nonfunctional or requires complementation from other signals/factors to be translated, or that it is actively translated to CD154 protein that is sequestered within the cell. A previous study has shown that memory cells can store CD154 in intracellular vesicles (24). To investigate this in greater detail, we stained activated naive and effector CD4 cells for intracellular CD154 when incubated in the presence or absence of 3x28 beads and/or IL-2 for 24 h. Cells were reacted with a nonconjugated anti-CD154 Ab before intracellular staining to block surface CD154 staining. Fig. 3C shows that IL-2 and, to a lesser degree, 3x28 bead stimulation induced pronounced intracellular CD154 protein levels in effector CD4 cells. However, very low levels of preformed CD154 was observed after 24 h without IL-2 and 3x28 bead stimulation, indicating that the residual CD154 mRNA is not actively transcribed. This again suggests that IL-2-induced re-expression of CD154 on the cell surface is dependent on transcription and translation and is not simply a result of translocation from preformed intracellular stores.

**CD154 induced by IL-2 is functionally active**

To verify the functional capability of IL-2-induced CD154 on CD4 cells, a 24-h coculture experiment with Daudi B lymphoma cells was done. Daudi cells markedly up-regulate CD54 (ICAM-1) expression in response to CD40 engagement. Effector CD4 cells were purified by negative selection, stained with the fluorescent cell dye CFDA-SE, and fixed in 0.5% paraformaldehyde before coculture. As seen in Fig. 4, activated effector CD4 cells previously cultured for 24 h in the presence of 100 U/ml IL-2 alone strongly up-regulated CD54 expression on the Daudi cells, which
could be blocked by inclusion of a CD40-Ig fusion protein. Conversely, activated effector CD4 cells previously cultured without IL-2 or 3x28 beads for 24 h induced very weak CD54 expression on the Daudi cells. Essentially, the same results were observed with activated naive CD4 cells, but their weaker CD154 expression corresponded to a weaker induction of CD54 expression (data not shown). Control experiments showed that unstimulated naive CD4 cells had no significant effect on CD54 expression; in contrast anti-CD40 Abs coupled to beads induced robust CD54 expression on the Daudi cells (data not shown). These results demonstrate that the CD154 induced by IL-2 is functionally active.

Discussion

CD40 engagement of APC is essential for generation of effective humoral and cellular immunity. Of particular interest, recent data have demonstrated that CD40-activated APC can convert tolerogenic tumor-specific CD4 and CD8 cells to effective immunogenic cells (9, 10). Furthermore, CD40-activated APC acquires the capacity to stimulate CD8 cells in the absence of CD4 cells, suggesting that the essential CD4 help during CTL generation is mediated through CD154 expressed on activated CD4 cells (6–8). Thus, we now have a substantial knowledge concerning the importance of CD40 activation of APC. However, since most studies have used Abs reactive against CD40 for activation, an in depth knowledge of the regulation of the endogenously receptor for CD40, CD154, is lacking. Since CD40 is generally constitutively expressed on APC, regulation of CD154 expression might be crucial for modulation of the immune response.

In this study, we demonstrate that efficient Ag activation and CD28 costimulation of CD4 cells has a dual function in connection with CD154 regulation: 1) induction of transient CD154 expression by NF-AT-dependent signals, and 2) production of IL-2 and up-regulation of its high affinity receptor, which maintains CD154 expression by IL-2-dependent signals. IL-15 produced by activated APC might furthermore enhance CD154 expression. This will produce a highly regulated feedback mechanism and underscores the interdependence of CD28 and CD40 signal pathways during T cell-APC interaction.

These findings might be of possible clinical interest, since the serum level of IL-15 is elevated in patients with rheumatoid arthritis, a disease characterized by elevated CD40L expression on CD4 cells (25, 26). Furthermore, a previous study has shown a strong correlation between diminished CD154 and IL-2 mRNA levels in a large fraction of patients with common variable immunodeficiency (27). Given the present study, it is likely that the diminished CD154 is caused by the lack of IL-2, in particular since these patients have a normal CD154 gene.

With regard to regulation of CD154 expression on activated CD4 T cells in vivo, future studies with knockout mice lacking either CD25, IL-2, or IL-15Rs may give insights into the relative contribution and importance of IL-2 and IL-15, although interpretation of data obtained in knockout models may be complicated by the multitude of functions performed by IL-2 and IL-15.

One of the hallmarks of anergic T cells is their inability to produce IL-2 even after appropriate costimulation. Furthermore, anergic T cells have been shown to express significantly reduced levels of CD154 after activation (28), which is not surprising given the data in the current report. We would like to propose that anergic CD4 cells lack the ability to activate APC, at least in part, due to poor CD154 expression. Since CD40-activated APC have the ability to activate otherwise anergic/tolerant T cells, this establishes a sort of “catch 22,” which potentially is of fundamental importance for regulation of anergy/tolerance vs immunity.

References


