IL-12 Is Required for Anti-OX40-Mediated CD4 T Cell Survival

Carl E. Ruby, Ryan Montler, Rongxui Zheng, Suyu Shu and Andrew D. Weinberg

*J Immunol* 2008; 180:2140-2148; doi: 10.4049/jimmunol.180.4.2140

http://www.jimmunol.org/content/180/4/2140

**References**

This article cites 53 articles, 31 of which you can access for free at: http://www.jimmunol.org/content/180/4/2140.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
IL-12 Is Required for Anti-OX40-Mediated CD4 T Cell Survival

Carl E. Ruby,* Ryan Montler,* Rongxui Zheng,† Suyu Shu,† and Andrew D. Weinberg1*

Engagement of OX40 greatly improves CD4 T cell function and survival. Previously, we showed that both OX40 engagement and CTLA-4 blockade led to enhanced CD4 T cell expansion, but only OX40 signaling increased survival. To identify pathways associated with OX40-mediated survival, the gene expression of Ag-activated CD4 T cells isolated from mice treated with anti-OX40 and -CTLA-4 was compared. This comparison revealed a potential role for IL-12 through increased expression of the IL-12R-signaling subunit (IL-12Rβ2) on T cells activated 3 days previously with Ag and anti-OX40. The temporal expression of IL-12Rβ2 on OX40-stimulated CD4 T cells was tightly regulated and peaked ~4–6 days after initial activation/expansion, but before the beginning of T cell contraction. IL-12 signaling, during this window of IL-12Rβ2 expression, was required for enhanced T cell survival and survival was associated with STAT4-specific signaling. The findings from these observations were exploited in several different mouse tumor models where we found that the combination of anti-OX40 and IL-12 showed synergistic therapeutic efficacy. These results may lead to the elucidation of the molecular pathways involved with CD4 T cell survival that contribute to improved memory, and understanding of these pathways could lead to greater efficacy of immune stimulatory Abs in tumor-bearing individuals. The Journal of Immunology, 2008, 180: 2140–2148.
Materials and Methods

Mice

Four- to 6-wk-old male and female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories and used at 6–10 wk of age. BALB/c and C57BL/6 IL-12-deficient (p35–/−), DO11.10 (OVA323–339), OT-II (OVA323–339), OT-I (OVA325–334), and OT-II IL-12Rβ2-deficient mice were bred and maintained at the Earle A. Chiles Research Institute Animal Facility (Portland, OR). The DO11.10 STAT4-deficient mice were a gift from Dr. K. Murphy (Washington University, St. Louis, MO). All animal studies were approved by an internal animal care committee.

Adoptive transfer and immunization

Spleens and lymph nodes (LNs)3 from DO11.10, OT-I, OT-II IL-12Rβ2-deficient, or DO11.10 STAT4-deficient mice were harvested and processed by crushing between two frosted glass microscope slides and RBC lysed with ACK (InVitrogen Life Technologies). The percentage of DO11.10 T cells or OT-II IL-12Rβ2-deficient T cells were identified by FACS by using anti-OVA (Sigma-Aldrich) and 50 μg of anti-OX40 (OX86) or 100 μg of anti-CTLA-4 (9H10), or IgG control (Sigma-Aldrich). The following day, mice were given a second injection of anti-OX40 or CTLA-4 or rat IgG. The blocking CTLA-4 Ab does not signal through CTLA-4, but rather interferes with the ligation of CD80/86 to CTLA-4, increasing the duration of CD28 stimulation, and also blocks potential downstream intra-T cell molecular events responsible for the differences between these two immune-enhancing strategies could provide insights into the pathways mediating OX40-enhanced survival of CD4 T cells. However, only OX40 engagement led to a substantial increase in long-term T cell survival (7, 17). The molecular events responsible for the differences between these two immune-enhancing strategies could provide insights into the pathways mediating OX40-enhanced survival of CD4 T cells. Ag-activated T cells (DO11.10) were purified directly ex vivo from mice stimulated with anti-OX40 or -CTLA-4. RNA was isolated from the purified cells to determine differences in gene expression between the two groups via Affymetrix gene array analysis. At least a 3-fold increase or decrease in 163 gene products from Ag-specific CD4 T cells; however, only OX40 engagement led to a substantial increase in long-term T cell survival (11, 27); however, IL-12Rβ2 mRNA was increased 17-fold upon OX40 engagement (Table I). Further analysis of the raw hybridization numbers revealed that IL-12Rβ2

Results

OX40 engagement induced the expression of IL-12Rβ2

Previous studies have shown that both OX40 engagement and CTLA-4 blockade effectively boosted the early expansion of Ag-specific CD4 T cells; however, only OX40 engagement led to a substantial increase in long-term T cell survival (7, 17). The molecular events responsible for the differences between these two immune-enhancing strategies could provide insights into the pathways mediating OX40-enhanced survival of CD4 T cells. Ag-activated T cells (DO11.10) were purified directly ex vivo from mice stimulated with anti-OX40 or -CTLA-4. RNA was isolated from the purified cells to determine differences in gene expression between the two groups via Affymetrix gene array analysis. At least a 3-fold increase or decrease in 163 gene products from Ag-specific CD4 T cells; however, only OX40 engagement led to a substantial increase in long-term T cell survival (11, 27); however, IL-12Rβ2 mRNA was increased 17-fold upon OX40 engagement (Table I). Further analysis of the raw hybridization numbers revealed that IL-12Rβ2

In vitro T cell cultures

Splenocytes (1–5 × 10⁸) from wild-type, OX40−/−, or t-bet−/− mice were harvested and cultured with 1 μg/ml anti-CD3 for 24 h. Cultures were then treated with 20 μg/ml anti-OX40 (chicken-anti-mouse OX40 IgY) or Ig control (IgY) for an additional 48 h and analyzed.

Intracellular cytokine staining

Splenic T cells were obtained as described above and stimulated for 6 h in vitro with 2.5 or 5 μg/ml OVA peptide 323–339 or control peptide, SIINFEKL, in RPMI 1640 containing 10% FBS and 1 mg/ml Golgi Stop (BD Biosciences/BD Pharmingen). The cells were harvested and stained with FITC-CD4 and KJ-126. Cells were made permeable with CytoPerm/PermWash buffers and stained with allophycocyanin-IFN-γ (BD Biosciences/BD Pharmingen; Calbiochem).

Determination of annexin V+ cells

Spleens from adoptively transferred and immunized mice were harvested 4 days after immunization and a total of 1–2 × 10⁶ CD4+ cells were plated in wells of a 24-well plate in 1 ml of medium and incubated overnight at 37°C. Cells were harvested 24 h later and stained according to the manufacturer’s instructions with FITC-CD4, KJ-126, and PE-annexin V (BD Biosciences/BD Pharmingen; Calbiochem).

Immunoblot analysis

LNs were isolated 4, 6, 7, and 8 days after immunization and cells were incubated with biotinylated anti-KJ-126 on ice. DO11.10 T cells were pulsed (≥90%) with antibiotin microbeads via the AutoMacs cell sorter (Miltenyi Biotec). Lysates were prepared, run on polyacrylamide gels (1.5 × 10⁵ cell equivalents/lane or equivalent microgram quantities of protein), and transferred onto nitrocellulose membranes. Abs for phospho-Akt, Akt, Bcl-xL, (Cell Signaling Technology) phospho-STAT4, and STAT4 (Santa Cruz Biotechnology) were used to detect these proteins. NIH ImageJ software (National Institutes of Health, Bethesda, MD) was used to analyze the phospho-Akt/Akt data. Data were presented as percent of a ratio of phosphoprotein vs total protein.

Tumor models

The MCA205 sarcoma tumor cell line H12, a stable clone from the 3-methylcholanthrene-induced parental tumor, was suspended in HBSS (1–3 × 10⁶) and injected i.v. into 6- to 8-wk-old female C57BL/6 mice to establish pulmonary metastases. Eighteen days after tumor inoculation, mice were sacrificed and metastatic tumor nodules on the surface of the lung were counted following counterstaining with India ink.

The TRAMP C1 prostate cancer line was derived from the transgenic adenocarcinoma of the mouse prostate (TRAMP) (23) and was shown to be MHC class II negative. Male C57BL/6 mice were challenged with 7.5 × 10⁵ TRAMP C1 cells suspended in HBSS by s.c injection on the right flank. Following tumor challenge, mice were monitored for tumor growth and sacrificed if tumors were ulcerated or if tumor growth reached 150 mm³.

Electrofusion of dendritic cells (DCs) and tumor cells

DCs were generated from the spleens of mice injected with eight daily i.p. injections of Flt-3 ligand, as previously described (24), and were enriched by CD11c microbeads following the manufacturer’s instructions (Miltenyi Biotec). Cells were cultured for 24 h in medium containing 10 μg/ml of both GM-CSF and IL-4. DCs and irradiated MCA205 (H12) tumor cells were electrofused as previously described (25, 26).

Statistical analysis

For all experiments, a Student t test (two-tailed) was used to compare means of selected groups. For analysis, values of p < 0.05 were considered significant and were expressed as follows: *, p < 0.05; **, p < 0.001; and ***, p < 0.0001, if not specifically stated. Experiment repetition is indicated in the figure legends.

2 Abbreviations used in this paper: LN, lymph node; TRAMP, transgenic adenocarcinoma of the mouse prostate; DC, dendritic cell.
Materials and Methods

BALB/c mice were immunized and the kinetics of IL-12Rβ2 expression were analyzed; IL-12Rβ2−/− mice were treated with 20 μg/ml anti-OX40 or IgG for 24–48 h, and CD4+ CD25− T cells were analyzed for the expression of IL-12Rβ2 and CD25. Data are representative of at least three separate experiments.

To verify the gene array data, IL-12Rβ2 protein levels were measured on Ag-activated transgenic CD4+ T cells stimulated in vivo with Ag, in combination with anti-OX40, anti-CTLA-4, or rat IgG. Four days following immunization, expression of IL-12Rβ2 was measured on Ag-activated transgenic T cells from peripheral blood, draining LNs, and spleens. T cells isolated from anti-OX40-treated mice expressed the IL-12Rβ2 protein, with the greatest levels found in the Ag-draining LNs (Fig. 1A). In contrast, IL-12Rβ2 was not detected on Ag-specific CD4 T cells from either the rat IgG or anti-CTLA-4-treated mice at any time in either immune compartment (Fig. 1A and data not shown). Although the levels detected in the OX40-treated group appear low, they are in agreement with our gene array data where low—but detectable—levels of IL-12Rβ2 mRNA were observed. The kinetics of IL-12Rβ2 cell surface expression peaked 4 days after Ag stimulation and then decline thereafter until undetectable by day 7 (Fig. 1B). These results demonstrate that OX40 engagement produced a window of IL-12Rβ2 expression that occurred after initial expansion, but before contraction.

The specific requirements for IL-12Rβ2 expression on OX40-activated CD4 T cells were also investigated. Wild-type CD4+ T cells activated with anti-CD3 and incubated with a control Ig failed to express IL-12Rβ2, but these cells when treated with anti-OX40 displayed a marked increase in IL-12Rβ2 expression (Fig. 1C).

transcript levels were “present” at low levels in the OX40 agonist group vs completely “absent” in the CTLA-4 blockade group.

Table I. Differential gene expression between anti-OX40 stimulation and anti-CTLA-4 blockade in Ag-specific CD4+ T cells

<table>
<thead>
<tr>
<th>Gene No.</th>
<th>Gene/Protein Name</th>
<th>Fold Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_008354</td>
<td>IL-12Rβ2</td>
<td>+17.0</td>
</tr>
<tr>
<td>NM_009820</td>
<td>RUNX2</td>
<td>+6.5</td>
</tr>
<tr>
<td>M64404</td>
<td>IL-1Ra</td>
<td>+5.2</td>
</tr>
<tr>
<td>NM_008367</td>
<td>CD25</td>
<td>+3.5</td>
</tr>
<tr>
<td>NM_009743</td>
<td>Bcl-xL</td>
<td>+1.5</td>
</tr>
<tr>
<td>Decreased</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-007464</td>
<td>Survivin</td>
<td>−1.1</td>
</tr>
<tr>
<td>NM_177410</td>
<td>Bcl-2</td>
<td>−1.5</td>
</tr>
<tr>
<td>X05719</td>
<td>CTLA-4</td>
<td>−3.5</td>
</tr>
<tr>
<td>XM_284241</td>
<td>CD27</td>
<td>−4.0</td>
</tr>
<tr>
<td>U89491</td>
<td>Eph1</td>
<td>−5.2</td>
</tr>
<tr>
<td>U32395</td>
<td>Mad4</td>
<td>−6.0</td>
</tr>
</tbody>
</table>

*DO11.10 T cells from the draining LNs were collected 4 days after immunization and enriched (>90%). Total RNA was extracted from the enriched DO11.10 T cells and then hybridized to an Affymetrix MOE 2.0A chip. The probed arrays were scanned and then analyzed by Affymetrix software.

FIGURE 1. Anti-OX40 induces the expression of IL-12Rβ2 on activated CD4+ T cells in vivo and in vitro. A, A total of 3 × 10^6 OVA-specific DO11.10 CD4+ T cells were adoptively transferred into recipient mice and 1 day later mice were immunized s.c. with 500 μg of OVA and 50 μg of anti-OX40, IgG, or 100 μg of anti-CTLA-4. A second s.c. injection of these Abs was given 24 h after the first injection. Four days after immunization, spleens and LNs were harvested and the cells were gated on the OVA-specific CD4 T cells (KJ-126−) and analyzed for the expression of IL-12Rβ2 (open histograms) or isotype control (gray histograms) by FACS. B, LNs from anti-OX40-treated mice were harvested and the cells were gated on the OVA-specific CD4 T cells (KJ-126−) and analyzed for the expression of IL-12Rβ2 expression that occurred after initial expansion, but before contraction.

To verify the gene array data, IL-12Rβ2 protein levels were measured on Ag-activated transgenic CD4+ T cells stimulated in vivo with Ag, in combination with anti-OX40, anti-CTLA-4, or rat IgG. Four days following immunization, expression of IL-12Rβ2 was measured on Ag-activated transgenic T cells from peripheral blood, draining LNs, and spleens. T cells isolated from anti-OX40-treated mice expressed the IL-12Rβ2 protein, with the greatest levels found in the Ag-draining LNs (Fig. 1A). In contrast, IL-12Rβ2 was not detected on Ag-specific CD4 T cells from either the rat IgG or anti-CTLA-4-treated mice at any time in either immune compartment (Fig. 1A and data not shown). Although the levels detected in the OX40-treated group appear low, they are in agreement with our gene array data where low—but detectable—levels of IL-12Rβ2 mRNA were observed. The kinetics of IL-12Rβ2 cell surface expression peaked 4 days after Ag stimulation and then decline thereafter until undetectable by day 7 (Fig. 1B). These results demonstrate that OX40 engagement produced a window of IL-12Rβ2 expression that occurred after initial expansion, but before contraction.

The specific requirements for IL-12Rβ2 expression on OX40-activated CD4 T cells were also investigated. Wild-type CD4+ T cells activated with anti-CD3 and incubated with a control Ig failed to express IL-12Rβ2, but these cells when treated with anti-OX40 displayed a marked increase in IL-12Rβ2 expression (Fig. 1C).
cells deficient in OX40 or t-bet (required for IL-12Rβ2 expression (28)) did not express IL-12Rβ2 upon anti-OX40 stimulation. These data—as well as results from a CD4 T cell peripheral tolerance model that showed OX40 stimulation enabled T cells to become sensitive to IL-12 stimulation (29)—suggest a potential role for IL-12Rβ2 in some of the OX40-mediated effects on T cells.

**IL-12 signaling is critical for OX40-enhanced survival of CD4 T cell survival**

The kinetics of IL-12Rβ2 expression following OX40 engagement suggested that IL-12 might play a role in OX40-mediated CD4 T cell survival. To determine whether IL-12R signaling directly affected CD4 T cell survival, OVA-specific TCR-transgenic CD4 T cells (OT-II) were backcrossed to IL-12Rβ2-deficient mice. The initial expansion of the IL-12Rβ2-deficient OT-II T cells following OVA and anti-OX40 administration was indistinguishable from wild-type OT-II; however, as the population of Ag-specific T cells contracted, significantly more wild-type OT-II T cells survived compared with the IL-12Rβ2-deficient OT-II T cells (Fig. 2A). Additional studies were conducted using IL-12 (p35)-deficient mice as recipients of wild-type DO11.10 T cells as a second approach. This approach allowed us to verify the OX40-IL-12 survival hypothesis in a different strain of mice with a different genetic mutation that has disrupted IL-12 signaling. In the absence of IL-12, survival of DO11.10 T cells in anti-OX40-immunized mice was severely diminished compared with wild-type mice (Fig. 2B) and similar to what was observed with IL-12Rβ2-deficient OT-II T cells. These differences in survival were evident 6 days after immunization, which was manifested as accelerated CD4 T cell contraction in IL-12-deficient hosts (Fig. 2C), and persisted for 60 days after immunization (Fig. 2D and data not shown). In these experiments, we saw similar levels of the Ag-specific CD4 T cells in the three compartments that were analyzed: PBL, LN, and spleen; however, this probably does not account for all the trafficking events involved. Finally, we have also shown anti-OX40 can enhance the survival of Ag-activated CD8 T cells (30); yet, the IL-12-specific survival, shown here, appeared to be specific for CD4 T cells, as survival of OX40-stimulated Ag-specific CD8 T cells was unaffected in the absence of IL-12 (data not shown).

The effector function, assessed by IFN-γ production, of anti-OX40-stimulated Ag-specific CD4 T cells was unaffected by the absence of IL-12. DO11.10 T cells isolated 4 days following immunization and anti-OX40 from IL-12-deficient mice produced similar levels of IFN-γ when compared with wild-type controls (Fig. 2E). Although this data seems to be at odds with the premise that IL-12 drives a Th1 response, previous studies have shown that IL-12 (p35)-deficient mice infected with lymphocytic choriomeningitis virus produce equivalent levels of IFN-γ when compared with wild-type mice (31). Furthermore, a recent study demonstrated IL-12Rβ2 did not play a significant role in the differentiation of Ag-specific CD4 T cells following OX40 engagement (32). Therefore, in anti-OX40-stimulated CD4 T cells, IL-12 appears to be more important for survival than Th1 differentiation.

The experiments described above have demonstrated that anti-OX40-stimulated Ag-specific CD4 T cells primed in the absence of IL-12 signaling contract rapidly and fail to survive. Thus, to determine whether the CD4 T cells were reversibly affected by the absence of IL-12, they were adoptively transferred into IL-12-deficient hosts to test whether their survival could be rescued. DO11.10 T cells from OVA- and anti-OX40-immunized IL-12-deficient hosts were harvested 4 days after immunization, and equal numbers of OVA-specific T cells were transferred into wild-type or IL-12-deficient recipients. Fourteen days after immunization, LNs were harvested and the survival of OVA-specific T cells was measured. If IL-12 was required during Ag priming (days 0–4), we would expect that T cells would fail to survive in both recipients. This was not the case, as the number of DO11.10 T cells was significantly greater in IL-12-sufficient mice compared with IL-12-deficient mice, demonstrating that IL-12 signaling several
days after Ag priming can rescue T cells primed in the absence of IL-12 (Fig. 3A). The converse was observed when OVA-specific T cells primed for 4 days in IL-12 intact mice were transferred into IL-12-deficient hosts. The numbers of OVA-specific T cells were significantly decreased compared with OVA-specific T cells transferred to intact hosts (Fig. 3B). Furthermore, data in Fig. 1A showed that IL-12Rβ2 expression was undetectable 7 days following immunization and OX40 stimulation, suggesting that the biological effect of IL-12 had to occur after priming but before day 7. To test this hypothesis, OVA-specific T cells were primed in wild-type mice and, 8 days later, were transferred into IL-12-deficient and -sufficient hosts. The number of DO11.10 T cells in LNs harvested 10 days later was the same in IL-12-deficient and -sufficient hosts (Fig. 3C). These results suggest that OX40-enhanced CD4 T cell survival is dependent on the timeframe/kinetics determined by IL-12Rβ2 expression on Ag-specific CD4 T cells.

**STAT4 signaling is involved in OX40-mediated survival**

To determine the molecular signaling events by which IL-12 mediates anti-OX40 survival, we investigated two known IL-12-mediated signaling pathways, Akt and STAT4. The Akt-signaling pathway has been shown to be activated by IL-12 and has been linked to OX40-mediated survival (12, 20); STAT4 signaling, mediated by IL-12, is involved with Th1 differentiation (18, 19). Phosphorylation of both proteins is required for downstream signaling and was analyzed in Ag-specific T cells isolated from Ag and anti-OX40 immunized wild-type or IL-12-deficient recipients. There was no difference in the level of phospho-Akt between wild-type and IL-12-deficient mice over the course of 4–7 days after immunization (Fig. 4A). In contrast, levels of phospho-STAT4 in Ag-specific T cells from IL-12-deficient mice were greater than in IL-12-deficient mice (phospho-STAT4/total STAT4). The greatest difference occurred 7–8 days postimmunization, when Ag-specific CD4 T cells in the absence of IL-12 experienced accelerated contraction (Fig. 4, B and C). Interestingly, substantial levels of phospho-STAT4 were still detected in OVA-specific CD4 T cells isolated from IL-12-deficient mice, possibly due to other inflammatory cytokines signaling through STAT4 (e.g., IFN-α/IFN-β and/or IL-23) (31, 33, 34).

**Differences in STAT4 phosphorylation in OVA-specific T cells from anti-OX40-treated IL-12-deficient mice suggest IL-12-mediated STAT4 activation could be important in OX40-mediated survival.** To address the role of STAT4 in OX40-mediated T cell survival, we transferred STAT4-deficient DO11.10 T cells into wild-type mice and compared their lifespan to STAT4-sufficient DO11.10 T cells following OVA and anti-OX40 immunization. DO11.10 CD4 T cells were enriched (>90%) and lysates from the DO11.10 CD4 T cells were analyzed by immunoblot. A, Phospho-Akt (PO4-Akt) and total Akt (Akt). B, Phospho-STAT4 (PO4-STAT4) and total STAT4 (STAT4). C, The ratio of phospho-STAT4 to total STAT4 was measured by image analysis (NIH Image J software) in three independent experiments.
ex vivo examination of annexin V. The STAT4-deficient and wild-type DO11.10 T cells for these experiments were harvested from mice (wild-type and IL-12-deficient) 4 days after immunization. In addition, 3 x 10^6 Wt DO11.10 CD4 T cells were adoptively transferred into Wt or IL-12-deficient mice (IL-12^/-^). All mice were then immunized with OVA and anti-OX40 as previously described. A, Four and 7 days after immunization, LNs were harvested and the frequency of DO11.10 CD4 T cells determined (n = 3–4). B, Four days after immunization, spleens were harvested and the frequency and number of DO11.10 CD4 T cells determined. Then, 1 x 10^6 cells were cultured overnight in medium, and 24 h later the DO11.10 CD4 T cells were analyzed for the expression of annexin V. C, Intracellular production of IFN-γ and IL-4 by splenocytes from Wt and STAT4-deficient DO11.10 CD4 T cells harvested 4 days after immunization. Data representative of two to three independent experiments.

FIGURE 5. The effect of STAT4 on anti-OX40-stimulated Ag-specific CD4 T cell survival and function. A total of 3 x 10^6 OVA-specific DO11.10-STAT4-deficient (STAT4^/-^) CD4 T cells were adoptively transferred into wild-type (Wt) mice. In addition, 3 x 10^6 Wt DO11.10 CD4 T cells were adoptively transferred into Wt or IL-12-deficient mice (IL-12^/-^). All mice were then immunized with OVA and anti-OX40 as previously described. A, Four and 7 days after immunization, LNs were harvested and the frequency of DO11.10 CD4 T cells determined (n = 3–4). B, Four days after immunization, spleens were harvested and the frequency and number of DO11.10 CD4 T cells determined. Then, 1 x 10^6 cells were cultured overnight in medium, and 24 h later the DO11.10 CD4 T cells were analyzed for the expression of annexin V. C, Intracellular production of IFN-γ and IL-4 by splenocytes from Wt and STAT4-deficient DO11.10 CD4 T cells harvested 4 days after immunization. Data representative of two to three independent experiments.

IL-12 synergizes with anti-OX40 to enhance antitumor immunity
The critical role for IL-12 in OX40-mediated survival of Ag-specific CD4 T cells described above suggests that the combination of OX40 and IL-12 signaling could drive enhanced immunity in tumor-bearing hosts. We therefore tested the combination of anti-OX40 and IL-12 in several tumor models. First, in a pulmonary metastases model (MCA205), mice were vaccinated with DC:tumor
Data representative of two to three independent experiments. were challenged with 7.5 vaccination combined with both anti-OX40 and IL-12 led to a OX40 or IL-12 alone did not reduce lung metastases; however, Moreover, the combination of fusion cell vaccination with anti-
fusion cells alone showed no antitumor efficacy (data not shown).

The efficacy of the combined treatment of anti-OX40 and IL-12 was also examined in the s.c. tumor model, TRAMP-C1 prostate tumor (23). In this model, anti-OX40 was injected 3 and 7 days posttumor inoculation, as previously described (9). The TRAMP C1 tumor was resistant to anti-OX40 monotherapy and to therapies that combined anti-OX40 with androgen ablation, rIL-2, or irradiated GM-CSF-secreting tumor as a vaccine (data not shown). In addition, IL-12 monotherapy, six daily injections starting 4 days after tumor transplantation, failed to mediate any tumor-free survival (Fig. 6B). The combination of IL-12 treatment with anti-OX40 treatment showed a synergistic therapeutic effect in mice challenged with s.c. TRAMP-C1 tumors (Fig. 6B). To determine whether the protective tumor immunity induced by anti-OX40 and IL-12 was dependent on CD4 T cells, we depleted CD4 T cells 1 day before tumor inoculation and treatment. The ability of the combined treatment to protect against TRAMP-C1 tumors was completely ablated following CD4 T cell depletion (Fig. 6B). The data from these two tumor models—as well as results from a third tumor model, CT26 colon carcinoma (data not shown)—demonstrate the ability of IL-12 to dramatically enhance anti-OX40-mediated tumor therapy.

Discussion

Our results demonstrate that the improved survival of Ag-activated CD4 T cells observed following OX40 engagement requires signal-
ing of the proinflammatory cytokine IL-12. Survival via an IL-12-specific signal is due to the up-regulation of the signaling chain of the IL-12R, IL-12Rβ2, on CD4 T cells 4–6 days following OX40 engagement, though the exact mechanism behind the up-
regulation of IL-12Rβ2 by OX40 has yet to be determined. The cooperation of OX40 and IL-12 signaling appears to rely in part on STAT4 activation, which suggests STAT4-mediated transcription not only accounts for the differentiation of Ag-specific effector CD4 T cells, but is also critical for survival. These results may help to explain the enhanced immunotherapeutic effects of combined OX40 stimulation and IL-12 treatment in several tumor models.

The results presented here are the first to describe the involve-
ment of IL-12 signaling in the mechanism of OX40-mediated sur-
vival of CD4 T cells. Initial OX40 engagement has been shown to activate the transcription factor NF-κB, which is capable of induc-
ing the expression of a number of survival proteins (37, 38). The OX40-mediated NF-κB activation most likely occurs during cell surface expression of OX40, between 24 and 72 h after TCR prim-
ing. This “early” survival protein induction could then be main-
tained by other signals and/or potentially shift to other “long-term” survival proteins and pathways. These proteins may include sever-
al antiapoptotic proteins such as Bcl-2, Bcl-xL, and pAkt that have been previously linked to this survival phenomenon (11–13).

The survival signal mediated by IL-12 in OX40-stimulated T cells coincided with the temporary expression of IL-12Rβ2 on the Ag-specific CD4 T cells. The surface expression of IL-12Rβ2 on effector T cells occurred near the start of contraction (4–6 days postimmunization), thereby placing the timing of IL-12-mediated survival between a first set of survival signals primarily mediated by CD28 and IL-2 during the Ag priming and a final set of signals that maintains long-term memory likely through IL-7 signaling (39, 40). The limited window for IL-12-mediated effector T cell survival demonstrate that IL-12 is not involved in the maintenance of long-lived memory CD4 T cells, which was also observed in a study measuring fungal Ag-specific T cells in IL-12-deficient and -sufficient mice, yet the T cells in the absence of IL-12 signal-
ing failed to survive. Thus, it appears IL-12 facilitates a critical OX40-mediated survival signal that is upstream or independent of some of the antiapoptotic proteins previously described (11–13).

The survival signal mediated by IL-12 in OX40-stimulated T cells coincided with the temporary expression of IL-12Rβ2 on the Ag-specific CD4 T cells. The surface expression of IL-12Rβ2 on effector T cells occurred near the start of contraction (4–6 days postimmunization), thereby placing the timing of IL-12-mediated survival between a first set of survival signals primarily mediated by CD28 and IL-2 during the Ag priming and a final set of signals that maintains long-term memory likely through IL-7 signaling (39, 40). The limited window for IL-12-mediated effector T cell survival demonstrate that IL-12 is not involved in the maintenance of long-lived memory CD4 T cells, which was also observed in a study measuring fungal Ag-specific T cells in IL-12-deficient hosts (41).

A role for IL-12 survival signaling may not be specific for OX40 and may include other members of the TNFR family. Recently, CD27 stimulation of CD4 T cells was also shown to induce the IL-12Rβ2 protein (42). CD27, a costimulatory member of the TNFR family, has the ability to increase the survival of Ag-primed CD4 T cells (43), like OX40, and IL-12 signaling may play a role in CD27-mediated survival. In contrast, it could be convenient to also speculate that the IL-12Rβ2 induced by CD27 and OX40 may have a different function, due to the differential expression of these two TNFRs, as CD27 expression occurs within 24 h of initial TCR priming, and OX40 24–72 h after TCR priming. Additional studies would be needed to clarify the role of IL-12 in CD27-stimulated T cells and if other members of the TNFR family also induce IL-12Rβ2.

IL-12 signaling in OX40-stimulated CD4 T cells promotes T cell survival that appears to be mediated in part by STAT4. Indeed,
decreased levels of phospho-STAT4 were found in anti-OX40-treated T cells isolated from IL-12-deficient hosts, and STAT4-deficient T cells were rapidly deleted following Ag and anti-OX40 priming. STAT4-deficient effector T cells also failed to produce IFN-γ, which suggest that STAT4 also plays a role in effector cell differentiation. Our data point to two possible divergent roles for STAT4 in OX40-stimulated CD4 T cells: 1) IL-12-mediated activation of STAT4 drives survival and 2) early activation of STAT4 during the priming phase by other cytokines (e.g., IFN-α/IFN-β) may be required for effector cell differentiation (31, 44). The STAT4-specific differentiation signals delivered to OX40-stimulated T cells appear to preclude the STAT4-survival signals initiated by IL-12. Potential survival signals mediated by IL-12/STAT4, may include IL-18R and the short-lived kinase pim-1. IL-12 and STAT4 have been directly linked to expression of IL-18R, which has been shown to “strengthen/extend” T cell responses and directly signals through the transcription factor NF-κB for a possible second round of survival signaling (45, 46). The survival kinase pim-1 is also induced by IL-12/STAT4 signaling and pim-1 can directly inactivate the proapoptotic protein Bad (47, 48). Additional experiments are required to fully understand the timing, survival proteins induced and roles played by innate cytokines in STAT4 activation, survival, and differentiation.

OX40 signals have been demonstrated to be critical in the generation of both Th1 and Th2 responses (3, 49), yet a role for IL-12 in Th2 survival seems unlikely. The CD4 T cell responses shown here appear to be primarily skewed toward a Th1 response, as assessed by a greater IFN-γ/IL-4 ratio (Fig. 5C). Therefore, this study demonstrated the mutual dependence of OX40 and IL-12 for optimal survival of CD4 T cells that differentiate into Th1 cells, but IL-12 most likely would not influence the survival of CD4 T cells under conditions that favor Th2 differentiation. OX40-mediated Th2 survival may then originate from OX40-40L expressing thymic stromal lymphopoietin-activated DCs and/or CD4+CD3− accessory cells found in B cell areas (50–52).

Successful tumor regression using the combination of OX40 engagement and IL-12 suggest effective antitumor responses in vivo may require critical “danger” signals (e.g., CpG, dsDNA, or LPS). A theory as to why some tumors do not elicit potent immune priming may be the lack of these “danger” signals, which would result in the lack of proinflammatory cytokines and costimulatory molecules, such as IL-12 and OX40 ligand. Restoration of these signals in tumor-bearing mice may help boost adaptive and innate immunity, which are likely missing in the nonimmunogenic tumor environment. The effects of IL-12 and OX40 signaling, observed in animal studies, could translate to increased success of immunotherapy in patients with cancer by increasing the longevity of tumor Ag-specific T cell responses. In fact, a recent clinical study in cancer patients identified a correlation between the persistence of tumor-specific T cells and clinical responses in patients with melanoma (10). The studies presented here also highlight differences in the modes of action of anti-CTLA-4 and anti-OX40 which are both currently in clinical trials for tumor immunotherapy (53) (A. D. Weinberg, unpublished observation).

In conclusion, the data described here identify a novel pathway showing that IL-12 is essential for OX40-mediated CD4 T cell survival in vivo. The dependence of OX40 on IL-12 suggest that this interaction is an important link between the innate and adaptive arms of immunity. Ultimately, these findings could be exploited to enhance immunotherapy in patients with cancer or chronic pathogens and could help to elucidate the exact molecular pathways involved with CD4 T cell survival that lead to improved memory T cell survival.

Acknowledgments
We thank Drs. Walter Urba and Anthony Vella for their critical review of this manuscript, and Dr. Kenneth Murphy for providing the STAT4−/− DO11.10 mice.

Disclosures
The authors have no financial conflict of interest.

References


