BROAD PROGRAMMING BY IL-2 RECEPTOR SIGNaling FOR EXTENDED GROWTH TO MULTIPLE CYTOKINES AND FUNCTIONAL MATURATION OF ANTIGEN-ACTIVATED T CELLS

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Broad Programming by IL-2 Receptor Signaling for Extended Growth to Multiple Cytokines and Functional Maturation of Antigen-Activated T Cells

Thomas R. Malek, Aixin Yu, Paul Scibelli, Mathias G. Lichtenheld, and Elaine K. Codias

Coincident production of IL-2 and induction of high-affinity IL-2R upon TCR engagement has precluded a clear distinction for the biological outcome of signaling through TCR/costimulatory molecules vs the IL-2R. Using a novel transgenic mouse on the IL-2Rβ−/− genetic background, this study has separated the relative outcome of signaling through the TCR and IL-2R. We show that stimulation through the TCR and CD28 or CD40 ligand directly leads to T cell activation and several rounds of proliferation in an IL-2-independent fashion. However, this stimulation is insufficient for extended T cell growth to multiple cytokines or differentiation into CTL or IFN-γ-secreting effector T cells. IL-2 is required for these functions in part by regulation of cyclin D3 and granzyme B. Somewhat less efficiently, IL-4 stimulation of these transgenic T cells redundantly rescued many of these activities. These data demonstrate a fundamental requirement for IL-2 and perhaps other common γ-chain-dependent cytokines to promote selective gene expression by Ag-activated T cells for their subsequent growth and differentiation into effector T lymphocytes. The Journal of Immunology, 2001, 166: 1675–1683.

Interleukin-2 exerts multiple biological functions, including acting as a T cell growth factor, sensitizing activated T cells to activation-induced cell death (AICD), promoting CTL, NK, and lymphokine-activated killer activity, and enhancing Ig secretion, by binding to high-affinity receptors comprised of α, β, and common γ-chain (γc) subunits (1, 2). Signaling pathways by the IL-2R and IL-15R largely overlap as both receptors share the β and γc subunits (3). Along with the IL-2R and IL-15R, IL-4R, IL-7R, and IL-9R also use γc as a subunit, leading to signaling redundancy inasmuch as each activates Janus kinase-1 and Janus kinase-3 through their unique subunit and γc, respectively (2, 4, 5). With the exception of the IL-4R, which activates STAT6, all the other γc-dependent cytokines activate STAT5a, STAT5b, and STAT3. This overlap in signal transduction results in functional redundancy among these receptors with respect to stimulation of T cell growth and sensitizing T cells to AICD (6, 7).

In most models concerning the activation of mature T lymphocytes, TCR and costimulatory signals move resting T cells to the G1 phase of the cell cycle while cytokine signals, especially IL-2, promote DNA synthesis and subsequent cell division. The primary data supporting this scenario is that IL-2 very efficiently promotes the growth of Ag-activated T cells (8, 9). IL-2 regulates the expression cell cycle proteins (10–12), and anti-IL-2 and IL-2R Abs block Ag- or mitogen-induced T cell proliferation (13, 14). In the latter case, except under limiting culture conditions, anti-IL-2 blockade does not usually completely block Ag receptor-induced proliferation. This result typically has been suggested to reflect the inefficiency of the Abs, due to the high-affinity interaction of IL-2 with its receptor. Alternatively, such findings might represent IL-2-independent T cell growth, and several recent studies support this notion (15–17). In any case, the dynamics of T cell activation and the typical experimental systems in use do not allow clear separation of the outcome of TCR and costimulatory T cell activation vs IL-2-dependent effects.

Attempts to discern the role of IL-2 on peripheral T cell function using T lymphocytes from IL-2- or IL-2R-deficient mice is complicated by their severe lethal autoimmunity and imbalance in hemostasis that typically imparts intrinsic T cell functional defects (18–20). Recently, we have developed a transgenic (Tg) mouse on the IL-2Rβ−/− genetic background in which physiological levels of IL-2Rβ was targeted to the thymus (16). These animals are long-lived and do not exhibit the autoimmune syndrome characteristic of IL-2 or IL-2R deficiency, but their peripheral T cell compartment remains nonresponsive to IL-2. Therefore, this experimental system offers the opportunity to not only examine the contribution of IL-2 to peripheral T cell function without the complication of the severe immunomodulatory pathology, but also furnishes a genetic approach to separate the outcome of signaling through the TCR and costimulatory molecules vs the IL-2R. In this study, we have examined this issue with these IL-2Rβ-deficient T cells with respect to their growth, death, and differentiation into effector cells in vitro.

Materials and Methods

Mice

Production of Tg mice that expressed wild-type IL-2Rβ under the control of the proximal lck promoter has been previously described (16). These mice were backcrossed to IL-2Rβ−/− or Tg−/− IL-2Rβ−/− littermates were used. C57BL/6 mice, which occasionally were used as controls, and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME).
Antibodies

Cy-Chrome-CD2Ra (53.6.7), Cy-Chrome CD4 (GK 1.5), PE-CD4 (GK 1.5), PE-IL-2Rβ (TMb1), FITC-CD8β (53.5.8), biotin-anti-rat IgG2a (R7/130), anti-CD28 (37.51), anti-CD40 ligand (CD40L) (MR1), anti-Bcl-2 (3F11), anti-hamster IgG (G70-204, G94-56), and PE-streptavidin were obtained from BD Pharmingen (San Diego, CA). FITC-CD4 (GK 1.5), biotin-anti-Bcl-x (SAβ6) (21), CTLA4Ig (7D4), and anti-CD2 (145-2C11) were purified and conjugated, as necessary, in our laboratory. Purified anti-IL-7Rα (A7R34) (23) was kindly provided by S. Nishikawa (Kyoto University, Kyoto, Japan). Anti-Bcl-x (2A1) was kindly provided by L. Boise (University of Miami, Miami, FL). Rat Ig was obtained from Sigma (St. Louis, MO). Abs to p27 (no. SC-1641), cyclin D2 (no. SC-1641), cyclin D3 (no. SC-1641), and β-actin (no. SC-8432) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-donkey anti-rabbit Ig and HRP-sheet anti-mouse Ig were obtained from Pharmacia (Arlington Heights, IL).

Cell culture

T cells were prepared by negative selection by, first, depletion of B cells on anti-mouse Ig-coated plates, followed by further depletion by incubation at 37°C for 45 min with anti-CD24 (J11D), anti-NK1.1 (PK136) and anti-MHC class II (M5.114) and rabbit C (Accurate Chemicals and Scientific, Westbury, NJ). To enrich for CD4+ T cells, anti-CD8 (H2D.2) was also included in the Ab and C depletion. Viable cells were purified by centrifugation through Lympholyte M (Accurate Chemicals and Scientific). Accessory cells (AC) were prepared by treatment of normal spleen cells with anti-Thy-1.2 and C at 37°C for 45 min and washed three times with HBSS. For proliferation assays of primary T cells, spleen cells (2 × 10^6/well), unfractionated lymph node cells (1 × 10^6/well) or purified T cells (1 × 10^6/well) were cultured with complete medium in flat-bottom 96-well plates as previously described (16) with soluble (1 μg/ml) or plate-bound (treated with 3 μg/ml in PBS) anti-CD3, plate-bound anti-CD28 (treated with 3 μg/ml in PBS), AC (1 × 10^6/well), 10 ng/ml PMA, 50 U/ml IL-2, 500 U/ml IL-4, 25 μg/ml anti-IL-2, 25 μg/ml CTLA4Ig, 25 μg/ml anti-CD40L, or 25 μg/ml rat Ig, as indicated, unless otherwise specified, for 48 h. [3H]Thymidine was added during the last 4 h. For molecular analysis, anti-CD3-redirected cytokine-dependent proliferation, cell growth, and cytokine secretion assays, spleen cells were cultured at 2 × 10^5/well in 24-well flat-bottom culture plates as previously described (16) with anti-CD3, 350 U/ml IL-4, or 50 ng/ml IL-7 for 48 h, as indicated. Allogeneic CTL were prepared exactly as described (16). As required, supernatant fluids were collected, the cells were harvested and washed with PBS, and total RNA (16) or Nonidet P-40 extracts were prepared (24). For Western blot analysis, membranes were incubated previously described. In Western blot analysis, membranes were incubated with the following: anti-Bcl-2, followed by mouse anti-hamster Ig and HRP-sheet anti-mouse Ig; anti-p27, anti-cyclin D3, anti-Bcl-x, or anti-β-actin, followed by HRP-sheet anti-mouse Ig; or anti-cyclin D2, followed by HRP-donkey anti-rabbit Ig.

IL-4 binding assay

The indicated cells (10 × 10^6/tube) were incubated in duplicate in 1 ml of complete medium (16) with 50,000 cpm [3H]-labeled IL-4 (70 μCi/μg) for 30 min at 4°C. The cells were washed and the cell-associated radioactivity was determined by counting in a γ scintillation counter. Over 95% of the binding was determined to be specific as assessed by inhibition with 200 ng/ml unlabeled IL-4.

Results

IL-2/IL-2R-independent proliferation by peripheral T cells

Wild-type transgenic IL-2Rβ was expressed in the thymus of IL-2Rβ−/− mice, and these mice will be referred to as Tg IL-2Rβ−/−. Spleen cells from Tg IL-2Rβ−/− mice generated substantial proliferative response to soluble anti-CD3 or PMA and IL-4, but essentially no response to PMA and IL-2 (16). The anti-CD3-induced response was not inhibited by a mixture of mAbs to IL-2 and γc and was generally ~50% of control response by IL-2Rβ−/− littermate spleen cells (16). By contrast, proliferation, cytokine production, and CTL responses by Tg IL-2Rβ−/− T cells paralleled that observed for control IL-2Rβ−/− littermate T cells (Ref. 16, and data not shown), and for simplicity these data are not included in this report. A time course study indicated that both the Tg IL-2Rβ−/− and IL-2Rβ−/−/− littermate spleen cells generated similar proliferative responses to anti-CD3 after 24 h in culture (Fig. 1A), but the response by the Tg IL-2Rβ−/− cells was ~50% lower after 48 h in culture. After that time, both responses substantially decreased, although a somewhat more sustained response was seen for T cells from the Tg IL-2Rβ−/− mice.

To determine whether the reduced proliferation by the Tg IL-2Rβ−/− T cells was a failure of activated T cells to fully transfix the cell cycle and divide, spleen cells from control and Tg IL-2Rβ−/− mice were labeled with CSFE and cultured with anti-CD3 for 48 h. As the cells divide, the fluorescence intensity of the CSFE-labeled cells decreases proportionally (27). For the recovered cells, a similar reduction in CSFE staining was noted for T cells from both types of mice, with a greater decrease in CD8+ cells (Fig. 1B). The level of decrease in CSFE staining is consistent with two to three cell divisions after 48 h in culture. Therefore, although the magnitude of the T cell proliferation to anti-CD3 by Tg IL-2Rβ−/− cells is reduced at 48 h, the responsive cells showed near normal capacity to divide during the first 2 days of the response.

The notion that at least some T cell proliferation is independent of IL-2 in our Tg model is at odds with the observation that spleen cells from Tg-negative IL-2Rβ−/− mice are extremely hyporesponsive to activation by anti-CD3 (16). These poor responses also extend to activation by PMA and ionomycine (18), suggesting that splenic T cells from IL-2Rβ−/− mice contain an intrinsic defect for proliferation. However, the spleens of IL-2Rβ−/− mice are known to be infiltrated by a large number of potentially suppressive granulocytes. Therefore, we tested whether this low response extended to lymph node T cells from the IL-2Rβ−/− mice (Fig. 1C). Unlike the spleun, unfractionated lymph node T cells demonstrated readily measurable proliferative responses to anti-CD3,

Cytokine assays

IL-2 was measured in the CTLL bioassay (28) in the presence of anti-IL-4 (11B11). IFN-γ and IL-4 were measured by ELISA using kits from BD Pharmingen according to the manufacturer’s instructions.

Molecular analysis

Northern blot (29) and Western blot (30) analyses were performed as previously described. In Western blot analysis, membranes were incubated with the following: anti-Bcl-2, followed by mouse anti-hamster Ig and HRP-sheet anti-mouse Ig; anti-p27, anti-cyclin D3, anti-Bcl-x, or anti-β-actin, followed by HRP-sheet anti-mouse Ig; or anti-cyclin D2, followed by HRP-donkey anti-rabbit Ig.
liferated to anti-CD3, which by necessity was independent of IL-2Rβ-dependent signaling by IL-2 or IL-15.

**IL-2-independent T cell proliferation depends upon TCR and costimulatory signals**

To determine which cell surface molecules are required for T cell proliferation by Tg IL-2Rβ/−/− T cells, initially the proliferation by purified splenic T cells to soluble and plate-bound anti-CD3 was compared (Fig. 2A). As expected, purified T cells from both control and Tg IL-2Rβ/−/− mice failed to respond after stimulation with either soluble or plate-bound anti-CD3, indicating that engagement of only the TCR was insufficient for T cell proliferation. The addition of AC or cross-linking with plate-bound anti-CD28 resulted in vigorous proliferation with the strongest responses in cultures supplemented with AC. In both cases the responses by the Tg IL-2Rβ/−/− T cells was again about 50% of the control responses.

To further examine the role of IL-2 as well as CD28 and CD40L costimulation in proliferation by T cells from Tg IL-2Rβ/−/− mice, we assessed the capacity of anti-IL-2, CTLA4Ig, and anti-CD40L to block such responses (Fig. 2B). Anti-IL-2 inhibited T cell proliferation by control T cells by ~50% while exerting minimal effect on Tg IL-2Rβ/−/− T cells. Therefore, the magnitude of proliferation by the control T cells in the presence of anti-IL-2 was essentially similar to Tg IL-2Rβ/−/− T cells cultured in the absence of anti-IL-2. When unfractionated spleen cells were stimulated with soluble anti-CD3, the resulting T cell proliferation from IL-2Rβ/−/− control mice was partially blocked by anti-IL-2, CTLA4Ig, or anti-CD40L whereas only CTLA4Ig and anti-CD40L partially blocked the responses by the Tg IL-2Rβ/−/− T cells. However, a mixture of CTLA4Ig and anti-CD40L substantially (~80%) inhibited the proliferation by both types of T cells. Thus, these data demonstrate that T cell proliferation by Tg IL-2Rβ/−/− T cells during the first 2 days in culture is dependent upon the TCR and costimulation through CD28 and CD40L, but is independent of IL-2.

**IL-2Rβ and IL-4R redundantly promote cytokine responsiveness by activated T cells**

After 48 h in culture, most T cells from Tg IL-2Rβ/−/− mice are activated based on proliferation, IL-2 secretion, cell division (Figs. 1, 2, and 6), and expression of CD69 and CD25 (Ref. 16 and Fig. 4). As expected, these T blasts were unresponsive when restimulated with IL-2 or IL-15. By contrast, these Tg IL-2Rβ/−/− T blasts were responsive to IL-4 and IL-7 (Fig. 3A) and, surprisingly, hyporesponsive to IL-4 and IL-7 (Fig. 3A). The poor responses by Tg IL-2Rβ/−/− T blasts to IL-4 or IL-7 was especially evident during longer-term cultures (Fig. 3B). Thus, in the absence of IL-2β signaling, not only were these anti-CD3-activated T cells nonresponsive to IL-2, but they were also hyporesponsive to IL-4 and IL-7.

Since IL-4 and IL-7 redundantly function as T cell growth factors, we tested whether these cytokines might prime T cell blasts to subsequently respond to these cytokines by adding exogenous IL-4 or IL-7 during the first 48-h culture. Inclusion of IL-4 and, to a much lesser extent, IL-7 during culture with anti-CD3 resulted in enhanced T cell proliferation by Tg IL-2Rβ/−/− T blasts after re-culture with IL-4 and IL-7 for 24 h (Fig. 3A). It was also possible to grow Tg IL-2Rβ/−/− T cells, albeit less efficiently than the IL-2Rβ/−/− littermate T cells, provided that T cells were primed with anti-CD3 and exogenous IL-4 and re-cultured with IL-4 (Fig. 3B). Collectively, these data demonstrate that sustained cytokine responsiveness requires that activated T cells receive cytokine signals that may be redundantly provided by IL-2 or IL-4.
**IL-4 sensitizes Tg IL-2Rβ−/− T cells to AICD**

Although Tg IL-2Rβ−/− peripheral T cells do not respond or proliferate to IL-2, it is possible to grow these cells with IL-4 for at least 5 days (Table I). When such unfractionated or CD4-enriched T cells were recultured with Con A or anti-CD3 (not shown) and IL-4, the majority of the cells died. The levels of AICD were very similar to identically treated control cells. Thus, IL-4 functioned as a growth factor for Tg IL-2Rβ−/− T cells and sensitized them to AICD.

**IL-2-dependent and -independent regulation of cytokine receptors, anti-apoptotic and cell cycle proteins**

To begin to define the molecular basis by which the IL-2R and IL-4R redundantly promote T cell growth, the expression of several cytokine receptors, cell cycle regulatory proteins, and anti-apoptotic proteins was determined for T blasts from control littermate and Tg IL-2Rβ−/− mice. As expected, IL-2Rβ was selectively not detected for Tg IL-2Rβ−/− T blasts, and the levels of IL-2Rα by the Tg IL-2Rβ−/− cells were markedly diminished (Fig. 4A), consistent with the role of IL-2 to up-regulate IL-2Rα (31). When compared with T blasts from littermate control mice in four independent experiments, IL-2Rα was typically 3- and 16-fold lower on CD4 and CD8 T blasts, respectively. Interestingly, Tg IL-2Rβ−/− T blasts also showed diminished levels of IL-7Rα (Fig. 4A). This finding indicates that IL-2Rβ signaling is required for maintaining expression of IL-7Rα and may partially explain the generally poor proliferative responses by these T blasts when re-challenged with IL-7 (see Fig. 3). In contrast, Tg IL-2Rβ−/− T blasts expressed normal levels of γc and IL-4R, the latter as assessed by radiolabeled IL-4 binding (Fig. 4B). Therefore, the relatively poor response to IL-4 re-stimulation by these T blasts is not due to failed IL-4R expression.

Unless exogenous IL-4 was added with anti-CD3 at culture initiation, we have never been successful at inducing proliferation by

**FIGURE 3.** Cytokine-induced proliferation and extended growth by activated T cells from Tg IL-2Rβ−/− mice. Spleen cells from mice of the indicated genotypes were cultured for 48 h with anti-CD3 without exogenous cytokines or with exogenous IL-4 or IL-7, as indicated by 1st on the x-axis. A, Cytokine-induced proliferation. The activated T cells were harvested, washed, and recultured with an optimal concentration of IL-2, IL-4, or IL-7, as indicated for 24 h. Data represent the x ± SE for four mice per group, except for cells precultured in IL-7, which represents the x ± range of two mice per group. B, Cytokine-dependent cellular expansion. The activated T cells were harvested and recultured for four days without anti-CD3, but with cytokines as indicated by 2nd on the x-axis. The bars indicate the number of cells harvested from the respective cultures on day 6. Where an asterisk appears, the culture was performed, but the bars are not visible because the values are very small. Data are the x ± SE of three experiments.
the T blasts upon re-stimulation with defined cytokines, unfracti- 
tionated cytokine-containing supernatants, or anti-CD3 and AC 
(data not shown), and upon reculture for an additional 24 h, these 
T cells typically appeared dead (not shown). This failure to survive 
is not the result of impaired expression of Bcl-2 or Bcl-x (Fig. 4  
C). In fact, the levels of Bcl-x in the Tg IL-2R"/" T blasts were  
greater than those seen for littermate control cells. However, when 
the expression of several cell cycle regulatory proteins were ex-
amined, Tg IL-2R"/" T blasts selectively expressed reduced lev - 
els of p27 and especially cyclin D3 (Fig. 4  C). After normalizing 
the band intensities to those of β-actin, densitometric analysis of 
this and two other experiments revealed that Tg IL-2R"/" cells  
contained on average 3.3-fold less cytoplasmic cyclin D3 when  
compared with the control T blasts. Thus, IL-2Rβ signaling is  
required to properly maintain expression of cyclin D3. The addi-
tion of exogenous IL-4 to the Tg IL-2Rβ"/" T cells did not ob-
viously affect the expression p27 or cyclin D2, but slightly in-
creased (1.4-fold) the levels of cyclin D3, but these levels were  
still lower than the control T cells. This result suggests that IL-4  
up-regulation of cyclin D3 may also partially account for the ca-
pacity of IL-4 to enhance subsequent IL-4 responsiveness by these  
activated T cells.

IL-2Rβ and IL-4R redundantly promote generation of T effector  
cell function

We previously reported that Tg IL-2Rβ"/" T cells were greatly  
impaired in differentiation into CTL (Fig. 5, A and B) (16). How-
ever, addition of IL-4 to these T cells while they were stimulated  
with allogeneic spleen cells or anti-CD3 substantially enhanced  
their levels of alloreactivity (Fig. 5 A) or redirected (Fig. 5 B) CTL  
activity. IL-7 was much less efficient in promoting CTL activity by  
Tg IL-2Rβ"/" T cells while IL-12- and IL-6-containing superna-
tants (not shown) did not mediate such a function. Northern blot  
analysis indicated that Tg IL-2Rβ"/" T blasts lacked granzyme B  
mRNA and expressed reduced levels of perforin mRNA (Fig. 5  C).  
Addition of exogenous IL-4 to anti-CD3 Tg IL-2Rβ"/" T cells  
induced expression of some granzyme B mRNA and somewhat  
enhanced the expression of perforin mRNA, although the levels of  
these mRNAs were still lower than seen in the control T cells. In

Table I. Tg IL-2Rβ"/" T cells cultured in IL-4 are sensitive to AICD*  

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Genotype</th>
<th>T Cells</th>
<th>Percent Dead</th>
<th>Percent Specific AICD</th>
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</thead>
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<tr>
<td>1</td>
<td>IL-2Rβ&quot;/&quot;</td>
<td>Unfractionated</td>
<td>42</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>Tg IL-2Rβ&quot;/&quot;</td>
<td>21</td>
<td>85</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>IL-2Rβ&quot;/&quot;</td>
<td>CD4</td>
<td>27</td>
<td>81</td>
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<tr>
<td>4</td>
<td>Tg IL-2Rβ&quot;/&quot;</td>
<td>30</td>
<td>80</td>
<td>50</td>
</tr>
</tbody>
</table>

* Purified unfractionated or CD4" (78–81% CD4") T cells were cultured for  
AICD as described in Materials and Methods.  

**Percent specific AICD = percent dead (+ Con A) – percent dead (– Con A).
any case, the ability of IL-4 to enhance CTL activity by Tg IL-2Rβ−/− T cells is at least partially accounted for by the regulation of two genes required for CTL function.

Cytokine production by anti-CD3-activated Tg IL-2Rβ−/− T cells was also assessed. After 24 h in culture, anti-CD3 induced generally similar levels of IL-2 by T cells from Tg IL-2Rβ−/− and IL-2Rβ−/− littermate mice (Fig. 6A). By contrast, after 48 h, the levels of IL-2 always increased for the Tg IL-2Rβ−/− T cells while they were always reduced and often undetectable for the IL-2Rβ−/− littermate T cells. This reduction in IL-2 is consistent with its consumption by the activated IL-2Rβ−/− T cells. By contrast, the increase in IL-2 at 48 h by the Tg IL-2Rβ−/− T cells indicates that these cells were unable to use the secreted IL-2.

Unlike IL-2Rβ−/− littermate T cells, those from Tg IL-2Rβ−/− mice did not secrete IFN-γ (Fig. 6B). Inclusion of exogenous IL-4 during the culture diminished the levels of IFN-γ secreted by the control T cells, as expected, while it did not significantly affect the amount of IFN-γ secreted by the Tg IL-2Rβ−/− T cells. Thus, these data indicate that signaling through IL-2Rβ is required for IFN-γ production in vitro.

After 48 h in culture with anti-CD3, the percentage of IL-2Rβ−/− and Tg IL-2Rβ−/− T cells that were CD4 was 31.5 ± 3.6 and 43.5 ± 5.8, respectively, and the percentage that were CD8 was 42.0 ± 3.6 and 16.3 ± 3.4, respectively, as determined in three experiments; the inclusion of exogenous IL-4 during this culture did not significantly alter these percentages (not shown). The lower fraction of activated Tg IL-2Rβ−/− CD8 is in part due to the lower number of CD8 T cells in the spleens of these mice that is typically reflected as a CD4:CD8 ratio of 4:1 rather than the 2:1 ratio of normal mice. In any case, the failure of anti-CD3 to induce IFN-γ cannot be attributed to lack of activation or survival of Tg IL-2Rβ−/− CD4− T cell.

To further explore the capacity of Tg IL-2Rβ−/− T cells to differentiate into cytokine producing effector cells, the anti-CD3 plus exogenous IL-4 stimulated T cells were further cultured for 2–3 days with only exogenous IL-4. These cells were then restimulated with anti-CD3 and AC, but without exogenous cytokines, and the resulting supernatant fluids were tested for IFN-γ and IL-4. Both the IL-2Rβ−/− and Tg IL-2Rβ−/− IL-4-expanded T cells produced IFN-γ, although on average, the level secreted by the Tg IL-2Rβ−/− T cells was 3.6-fold lower (Fig. 6C). Thus, Tg IL-2Rβ−/− T cells do not express an absolute defect in the production of IFN-γ. Both the IL-2Rβ−/− and the Tg IL-2Rβ−/− T cells produced equivalent levels of IL-4 (Fig. 6D). Therefore, these culture conditions were not sufficient to completely polarize cytokine production into a Th1 or Th2 pattern. The higher amount of IFN-γ secreted by the IL-2Rβ−/− T cells may be due to greater numbers of CD8 cells whose IFN-γ production is not as readily down-regulated by IL-4 (32). These effector cells produced modest levels of IL-2 with the highest amount by the Tg IL-2Rβ−/− T cell (average 3.1 U/ml), which was about 4-fold higher than detected by the IL-2Rβ−/− T cells (16). Thus, collectively these data demonstrate that in the absence of IL-2Rβ function, IL-4 redundantly functions to promote CTL and IFN-γ secretion by anti-CD3-activated T cells.

**Discussion**

Based on the results of this study, several new observations are apparent concerning the role of the IL-2R during activation, proliferation, and effector cell differentiation of peripheral T cells. Our data indicate that engagement of TCR and costimulatory signals results in activation and proliferation that is independent of IL-2 or other γc-dependent cytokines. The cornerstone for this conclusion is that highly purified Tg IL-2Rβ−/− T cells proliferated in response to anti-CD3 and anti-CD28 cross-linking or anti-CD3 in the presence of AC. It is further supported by the failure of anti-IL-2 or a mixture of anti-IL-2 and γc mAbs (16) to inhibit this proliferation. Furthermore, IL-2 secretion by Tg IL-2Rβ−/− T cells increased over time. Thus, unlike control littermate T cells, IL-2 was
not consumed at the time when maximal T cell proliferation occurred. We also showed that lymph node T cells from IL-2Rγc−/− mice, despite their severe pathobiology, proliferated when directly stimulated through the TCR and CD28 to levels nearly comparable to that usually detected for Tg IL-2Rβ−/− T cells, but lower than observed for normal T cells. Similar results were previously noted for IL-2−/− lymph node T cells (15, 33). The expression of several genes known to be regulated by IL-2 were also absent or reduced in the anti-CD3-activated Tg IL-2Rβ−/− T cells. The two most highly IL-2-dependent targets were IL-2Rα, especially on CD8+ T cells, and granzyme B, while the levels of cyclin D3, IL-7Rα, and perforin were also consistently reduced. Lastly, the observations that TCR stimulation led to expansion of lymph node Tg IL-2Rβ−/− T cells in vivo (16) and the capacity for Ag to induce substantial clonal expansion of TCR Tg γc−/− T cells upon adoptive transfer to RAG−/− recipient mice (34) further demonstrate that TCR activation in vivo leads to proliferation independent of IL-2 and all other γc-dependent cytokines.

Past studies have shown that Con A or anti-CD3-induced proliferation by splenic T cells from IL-2−/−, IL-2Rα−, and IL-2Rβ-deficient mice were highly variable, and these responses were often only 5–10% of control lusitammate T cells (7, 18, 19, 33). This finding has lead to the conclusion that T cell proliferation in vitro is largely IL-2 driven, although a portion is independent of IL-2/IL-2R. It is evident from the anti-IL-2 blocking experiments that ~50% of the anti-IL-2-induced proliferation by normal T cells under optimal stimulatory conditions was dependent upon IL-2. Importantly, the magnitude of the proliferation of the Tg IL-2Rβ−/− T cells in the absence or presence of anti-IL-2 or anti-γc (16) was typically 50% of the control responses by normal lusitammate T cells. These findings indicate that the proliferation by the normal T cells is comprised of two components, one independent of IL-2 and other γc-dependent cytokines which is larger than previously appreciated, and a second IL-2-dependent response. Therefore, the typical poor polyclonal-induced proliferation by IL-2−/−, IL-2Rα−, and IL-2Rβ-deficient T cells is likely the result of intrinsic cell defects due to the accompanying autoimmune syndrome. The substantial blockade of the IL-2-independent T cell proliferation by the Tg IL-2Rβ−/− T cells by the combination of anti-CD40L and CTLA4lg demonstrates that this IL-2-independent response is largely driven by engagement of the TCR and either CD28 or CD40L. Recently, human T cells have also been shown to proliferate in an IL-2-independent fashion that is dependent upon CD28 (17), and proliferation has also been induced in T cells from IL-2−/− and IL-2γc−/− mice after cross-linking CD3 and CD28 (15, 35). The simplest interpretation of these data is that signaling through the TCR and these costimulatory molecules is sufficient to directly induce T cell proliferation. In fact, some of the signal transduction pathways linked to TCR signaling (reviewed in Ref. 36), e.g., MAP kinase and PI-3-kinase pathways, have been shown to promote cellular proliferation in numerous other cell types. Alternatively, we cannot rule out that engagement of TCR, CD40L, and/or CD28 may have induced another cytokine or cell-cell molecular interaction that drives this proliferation. So far, we have been unsuccessful in stimulating proliferation by these anti-CD3-activated T cells with crude cytokine-containing supernatants or defined cytokines (data not shown) suggesting that the anti-CD3-induced response by the Tg IL-2Rβ−/− T cells in not driven by soluble mediators.

Regardless of what precisely mediated this IL-2Rβ-independent proliferation, it should be stressed that the growth of such T cells is limited to two to three cell divisions. Thus, TCR and costimulatory activation in the absence of IL-2 signal transduction resulted in an aborted response. Strikingly, these T cells were not competent for cytokine-dependent expansion mediated by IL-4, even though they expressed normal levels of this cytokine receptor. Furthermore, in the absence of exogenous cytokines, Tg IL-2Rβ−/− T cells did not differentiate into effector T cells, as assessed by their minimal CTL activity and lack of IFN-γ secretion. Therefore, these findings indicate that IL-2 functions in a much broader role than a T cell growth and survival factor and is responsible for programming activated T cells for extended cytokine-dependent T cell growth and differentiation into effector cells.

Consistent with the results herein, many studies have shown an important role for IL-2 in multiple T cell functions in vitro, yet paradoxically, the peripheral T cell compartment in these Tg IL-2Rβ−/− mice, with only minor exceptions, is outwardly normal (16). Therefore, some of the T cell activities that require IL-2 in vitro may be provided by redundant activity in vivo, perhaps by other γc-dependent cytokines. For example, the addition of exogenous IL-4 during the first 2 days with anti-CD3 yielded activated Tg IL-2Rβ−/− T cells that expanded upon subsequent culture with IL-4, were sensitive to AICD, and differentiated to CTL as well as IL-4- and IFN-γ-secreting cells. Thus, IL-4 redundantly functioned in the absence of IL-2Rβ signaling for these activities in vitro and is a candidate to provide such an activity in vivo. However, usually the capacity of IL-4 to rescue these functions by the Tg IL-2Rβ−/− T cells was still suboptimal when compared with
the responses by littermate control cells. Therefore, it remains to be determined whether IL-4 serves such a redundant function in vivo.

The induction of granzyme B by IL-4 at least partially accounts for the ability of this cytokine to promote CTL differentiation by activated Tg IL-2Rβ/−/− T cells, and identifies granzyme B as a key IL-2-regulated gene, as suggested by others (37). Although IL-4 has been shown to function as a T cell growth factor (38) and sensitizes T cells to AICD (6, 7), our study showed a requirement for IL-4 to promote these functions by anti-CD3-activated T cells in the absence of IL-2β signaling. The lower levels of cyclin D3 and p27 by anti-CD3 stimulated Tg IL-2Rβ/−/− T blasts may partially explain the inability to sustain their growth to γc-dependent cytokines such as IL-4. Other studies have shown that IL-2 induces cyclin D3 by a STAT5-dependent mechanism (11, 39). The levels of cyclin D3 in Tg IL-2Rβ/−/− T blasts were somewhat enhanced by IL-4, raising the possibility that proteins that directly regulate the cell cycle are key targets by which γc-dependent cytokines redundantly function to induce a cytokine responsive state. We also noted that Bcl-x levels were actually enhanced in the Tg IL-2Rβ/−/− T blasts. This result is consistent with the requirement of TCR and costimulatory signals for the expression of Bcl-x (40) and indicates that IL-2 signaling normally functions to reduce expression of this anti-apoptotic protein, which will ultimately promote AICD. Thus, IL-2 signaling during the course of TCR-mediated activation will be responsible for both the increase and decrease of gene expression in a highly regulated fashion.

In anti-CD3-induced activation and proliferation by normal T cells, it is very difficult to separate the outcome of TCR/costimulatory signals from IL-2 function as the production of IL-2 depends on costimulation. With respect to cytokine-secreting effector T cells, it has been generally concluded that IL-2 functions primarily to support the growth of Ag-activated T cells while IL-12 or IL-4 primarily act to induce Th1 or Th2 cytokines, respectively. Based on this notion, it was somewhat unexpected that anti-CD3 failed to induce IFN-γ secretion by Tg IL-2Rβ/−/− T cells in the absence of exogenous cytokines as this response was accompanied by substantial cell proliferation and cell division. Therefore, IL-2 may play a more direct role beyond just proliferation, at least in vitro, for the differentiation of effector IFN-γ-secreting T cells. Consistent with our findings, in some in vitro systems, IL-2 appeared to play a necessary role to promote both IFN-γ and IL-4 secretion by developing T helper cells (41). IL-2 has been reported to induce IL-12Rβ2 expression on T cells in the absence of IL-2β signaling using IL-2R-deficient T cell without intrinsic functional defects. Thus, further study of T cells from these Tg IL-2Rβ/−/− mice should help clarify the precise contribution by which IL-2β signaling regulates T cell activation in vitro and in vivo without complication of immune suppressive autoimmune disease.

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