IFN-γ Regulation in Anti-CD4 Antibody–Induced T Cell Unresponsiveness

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Abstract. The anti-rat CD4 mAb RIB5/2 is very potent in inducing allospecific tolerance in vivo. It is interesting that the unresponsiveness is breakable by exogenous IL-2 applied during the induction phase of tolerance. The molecular mechanisms underlying anti-CD4 antibody–mediated inhibition of allospecific T cell activation and how this is antagonized by exogenous IL-2 were investigated. Anti-CD4 treatment, in vivo, completely abrogated IL-2 production by allosreactive T cells. In contrast, anti-CD4–treated alloactivated T cells showed similar IFN-γ mRNA expression as untreated alloactivated T cells but did not secrete any protein. Thus, the anti-CD4 antibody cannot prevent IFN-γ mRNA expression but is interfering with posttranscriptional mechanisms that control IFN-γ production during alloactivation of T cells. Addition of IL-2 but not IL-15 to anti-CD4–treated alloactivated T cells restored IFN-γ protein production without leading to enhanced IFN-γ mRNA expression. Further investigations revealed a diminished activation of translation initiation factor eIF2α in anti-CD4–treated T cells, which was restored by exogenous IL-2. As activated eIF2α is essential for the translation of IFN-γ mRNA, the results may explain the reversibility of anti-CD4–induced unresponsiveness by exogenous IL-2. Furthermore, these results not only shed further light onto the molecular mechanisms of tolerance induction but also reveal the possible weaknesses of anti-CD4 antibody–induced unresponsiveness.

We previously showed that RIB5/2-induced unresponsiveness does not completely prevent activation and infiltration of T helper type 1 (TH1) T cells into the graft (8). We could demonstrate that donor-reactive T cells do persist and are detectable in the graft even after 300 d (8). Furthermore, we could show recently that anti-CD4–treated alloreactive T cells in vitro are less sensitive to apoptosis induced by either growth factor withdrawal (e.g., IL-2) or activation-induced cell death (13). Thus, the anti-CD4 treatment does not lead to the depletion or apoptosis of alloreactive T cells. It is interesting that RIB5/2-induced unresponsiveness in vivo is breakable by exogenous IL-2 applied during the induction phase of tolerance. This might have a negative impact on clinical tolerance induction protocols, as after transplantation (Tx), reactivation of preexisting alloreactive memory T cells or virus-specific T cells may result in IL-2 production, which may interact with tolerance induction. IL-2 has multiple biologic functions. It acts as growth factor for antigen-activated T cells by inducing the expression of cell-cycle proteins (14). IL-2 also enhances the expression of cytokines, e.g., IFN-γ in T cells and natural killer (NK) cells (15–17). In T cells, the engagement of the TCR/CD3 complex induces IFN-γ expression. Recently, it was shown that IL-2 receptor (IL-2R) signaling is required for the differentiation of IFN-γ-secreting effector T cells and induction of cytotoxic lymphocyte activity (18). IFN-γ itself profoundly affects a variety of immune responses, including upregulation of MHC I and MHC II expression, proliferation and...
differentiation of lymphocyte populations, augmentation of adhesion molecule expression on endothelial cells, and induction of immunomodulatory proteins such as TNF-α (19, 20). All of these processes enhance and sustain an inflammatory response. In the case of Tx, an enhanced inflammatory response may lead to acute graft destruction and loss. Furthermore, IFN-γ is essential for the development of chronic rejection (21). Here we investigated how IL-2 can influence the anti-CD4-mediated inhibition of alloimmune T cell activation, in particular their IFN-γ production in vivo and in vitro.

Materials and Methods

Grafting Techniques
We performed allogeneic kidney Tx of dark agouti (DA) (RT1avl) or Wistar Furt (WF) (RT1a) donor kidneys to Lew rats (RT1d) or BDIX (RT1b) recipients, respectively. The recipients were anesthetized, nephrectomized bilaterally, and given left donor renal allografts.

Adhesion molecule expression on endothelial cells, and induc-
tion (21). Here we investigated how IL-2 can influence the

Anti-CD4 mAb (RIB5/2; 20 mg/kg body wt per d) was injected intraperitoneally at day 1, 0, 1, 3, 5 post-Tx. Grafts were removed at different time points after Tx, and the graft infiltrating cells were collected after collagenase treatment and Ficoll gradient centrifugation.

Mixed Lymphocyte Reaction
Single-cell suspension of rat lymph nodes was prepared in PBS by forcing the organs through a 70-µm nylon mesh, and cells were resuspended in complete medium (DMEM supplemented with 1 mM pyruvate, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 × 10−3 µl-mercaptoethanol). Responder rat spleen or lymph node cells (2.5 × 106) were cultured with 2.5 × 106 irradiated stimulator (30 gray) cells. At day 5, either 2 µCi (3H)TdR/well was added and 1H thymidine incorporation was measured after an additional 10 h of culture, or the cells were harvested for PCR, ELISA, and flow cytometry analysis.

ELISA
Cell culture supernatants from mixed lymphocyte reaction were analyzed for IL-2 and IFN-γ secretion at the time points indicated using a commercially available kit (Biosource, Solingen, Germany).

mAb and Reagents
Human recombinant (hr) IL-2 was purchased from Cetus Corporation (Emeryville, CA). mIL-15 was purchased from REPROTECH (London, UK). Neutralizing anti-rat CD25 mAb was obtained from Serotec (Düsseldorf, Germany), and murIL-15 Re/Fc Chimera was obtained from R&D Systems (Minneapolis, MN). The following kinase inhibitors were used: Rapamycin and Ly294002 (Cell Signaling Technology, Beverly, MA).

Real-Time Reverse Transcription–PCR analysis
Cultured cells were analyzed daily for the expression of mRNA coding for IL-2 and IFN-γ by using quantitative real-time reverse transcription–PCR (RT-PCR; TaqMan, Perkin-Elmer Applied Biosystems, Rodgau-Jügesheim, Germany). Total RNA was prepared using the Miniprep Kit (Stratagene, Heidelberg, Germany) and reverse transcribed into cDNA by the murine leukemia reverse transcriptase (Life Technologies BRL, Gaithersburg, MD). The cDNA was then analyzed for cytokine gene expression with TaqMan-PCR, as described previously. Primers and probes were designed using the Primer Express Version 1.0. The probes were labeled with 6-carboxytetramethyl-rhodamin at the 5’ end and with the quencher 6-carboxytetramethyl-rhodamin at the 3’ end. PCR reagents were obtained from Perkin-Elmer. Amplification reactions (25 µl) contained 1 µl of the cDNA sample, 10× TaqMan buffer A (2.5 µl), 400 nM dUTP, 200 µM dATP, dCTP, dGTP, 6 mM MgCl2, 0.625 U AmpliTaq Gold, 0.25 U AmplErase uracil N-glycosylase (UNG), 5 PM/µl probe, sense and antisense primer). The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Reactions were performed using the Model 7700 Sequence Detector (TaqMan, Perkin-Elmer Applied Biosystems). The cycle number at which the reporter fluorescence reaches the threshold (Ct value) was used as a quantitative measurement of the target copies found in any sample. As a reference, rat β-actin or CD3 cDNA was used as standard, and mRNA concentrations of the below-mentioned genes and cytokines were compared with the sample content of β-actin or CD3 mRNA as indicated in figure legends. The sequences of the oligonucleotides used are as follows: β-actin sense 5’-GTACAAACCT-CTTTCAGCCTCCT-3’, β-actin antisense 5’-TTGTCGACGAGCA-GGCC-3’, β-actin probe 5’-CGCCACAGTGTCGGCAGGGTAC-3’, CD3 sense 5’-CAAAAGAAACATACATGGCGAGGCG-3’, CD3 antisense 5’-CTTCCTGGTGCCGATTG-3’, CD3 probe 5’-AGGGTTGGCTG-GGCTTCTTCTTGTTG-3’, IFN-γ sense 5’-AACAGTGAAGGCCA-AAAAAGGATGCCATT-3’, IFN-γ antisense 5’-TTCCATGACA-GCTTTGTGCTGG-3’, IFN-γ probe 5’-CGCCAAATGTTCAAGGT-GAACCAACC-3’, IL-2 sense 5’-CCTCCCCATGTTGCACG-3’, IL-2 antisense 5’-TCATTATTTCAGGGCGACTGAAGATG-3’, IL-2 probe 5’-CAATTCTGGGCCTGTTGGGAA-3’. Sense and antisense oligonucleotides were purchased from Metabion (Munich, Germany). The probes were purchased from Eurogentec (Seraing, Belgium).

Immunohistology
Cryostat sections (4 µm) of kidney transplants were harvested at day 5 post-Tx and fixed in acetone. For determination of cytokines, the sections were stained by a peroxidase antiperoxidase complex method as described in detail elsewhere (22). The following murine mAb were obtained from Sera-Lab (Accurate Chemicals, Westbury, NY): mAb directed against T cells (TCRα/β, R73), IL-2 (1D10), and IFN-γ (DB-10). Labeled cells within 20 high-power fields (HPF; ×400/section per rat) were counted with the aid of an ocular grid micrometer.

Flow Cytometric Analysis of T Cells
Cell suspensions were washed with PBS containing 2% FCS and 0.02% sodium azide. Samples were incubated with 4 µg/ml FITC-labeled anti-α,β-rat T cell receptor antibody and/or PerCp-labeled anti-rat CD8 antibody (Pharmlingen, San Diego, CA) for 15 min at 4°C. The samples were then fixed using a lysis solution (Becton Dickinson, San Jose, CA) for 15 min at room temperature, stained with 4 µg/ml PE-labeled anti-rat IFN-γ antibody (Pharmlingen) in 0.2% Saponin/PBS for 30 min, and washed with 0.2% Saponin/PBS. IFN-γ expression in T cells was determined by three-color flow cytometry on a FACScnt (Becton Dickinson).

Western Blot Analysis
Cells were solubilized in lysis buffer for Western blot (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.27 M sucrose, 0.1 mM microcystin, 0.1% 2-mercaptoethanol, and protease inhibitor cocktail).
with repeated dispersion through a needle. The samples were run on 4 to 12% SDS-PAGE gels and then blotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with one of the following: antibodies to ERK, phospho-ERK, p38, phospho-p38, phospho-p70S6, phospho-40S6, or phospho-eIF2α (Cell Signaling Technology, Beverly, MA) and β-actin antibody (Santa Cruz, Santa Cruz, CA).

Statistical Analyses

Data were analyzed using the statistical software SPSS (SPSS GmbH Software, München, Germany) and are reported as mean ± SD. Data for cytokine expression were analyzed using one-way ANOVA with Tukey correction except in instances in which only two groups were present, in which case t test was used. Differences were considered significant at P < 0.05.

Results

Influence of Nondepleting Anti-CD4 mAb RIB5/2 on IL-2 and IFN-γ mRNA Expression in Graft-Infiltrating Cells after Allogeneic Kidney Tx

The nondepleting anti-CD4 antibody RIB5/2 has been shown efficiently to prevent rejection of allografts. Multiple treatment with the anti-CD4 antibody (5 × 20 mg/body wt) around the time of Tx leads to indefinite graft survival (7, 8). By contrast, the average rejection time of untreated or control mAb-treated BDIX recipients of Wistar Furth (WF) grafts is 8 d (Figure 1A). We analyzed IL-2 and IFN-γ mRNA expression in grafts from control mAb- and anti-CD4 mAb (RIB5/2)–treated recipients on day 5 after Tx using real-time RT-PCR. Grafts from anti-CD4 mAb–treated recipients showed reduced IL-2 mRNA expression (P = 0.0042) as compared with grafts from control mAb–treated recipients (Figure 1B). In contrast, induction of IFN-γ mRNA expression was not or only marginally reduced. Importantly, cytokine mRNA expression was normalized to the amount of infiltrating T cells (CD3) and, therefore, is not confounded by treatment effects that could have influenced the migration or proliferation of alloresponsive T cells. The oligonucleotide panel (primers + probe) used to quantify IFN-γ mRNA recognizes a sequence that comprises regions from exons 3 and 4 and therefore detects only spliced IFN-γ mRNA. Cross-reactivity with genomic DNA or unspliced mRNA was not observed. In addition, the mRNA was reverse transcribed on the basis of its content of poly A. It therefore is likely that the IFN-γ mRNA measured was fully processed. Thus, anti-CD4 mAb–induced unresponsiveness does not prevent IFN-γ mRNA expression in alloreactive T cells in vivo.

Effects of the Nondepleting Anti-CD4 mAb RIB5/2 on IL-2 and IFN-γ Expression in Alloreactive T Cells In Vitro

To study the effects of the anti-CD4 mAb (RIB5/2) on IFN-γ production in more detail, we performed mixed lymphocyte reactions (MLR). Addition of RIB5/2 to allogeneic MLR completely blocked the induction of IL-2 expression with levels of IL-2 mRNA expression (day 2, P = 0.031) and cytokine production (day 2/3, P < 0.01) being similar to syngeneic cultures (Figure 1C). In contrast, the anti-CD4 antibody did not prevent induction of IFN-γ mRNA expression in alloreactive T cells. Despite similar levels of mRNA expression, there were strongly decreased levels of IFN-γ protein (day 3/4, P = 0.003; day 5, P = 0.017) detectable in the supernatants of anti-CD4–treated allogeneic cultures (Figure 1D). Importantly, immunohistochemical analysis of kidney grafts at day 5 post-Tx revealed similar results. Whereas rejecting grafts from control mAb–treated recipients showed dense mononuclear cell infiltration with many cells staining positive for IL-2 and IFN-γ, cytokine-positive cells could hardly be detected (<10% of rejecting controls) in grafts from anti-CD4 mAb (RIB5/2)–treated recipients (data not shown).

To investigate whether the abolished IFN-γ production in RIB5/2-treated alloreactive T cells is due to diminished protein secretion, we performed intracellular cytokine staining at day 4 of the MLR. As shown in Figure 3, approximately 70% of CD4+ T cells in untreated allogeneic cultures expressed IFN-γ, whereas only 30% of anti-CD4–treated cultures were IFN-γ positive. The high percentage of IFN-γ–producing T cells in our experiments was not unexpected, as similar results have been reported before (23). Similar results were observed in CD8+ T cells (data not shown), suggesting that anti-CD4 mAb treatment inhibits indirectly also the activation and subsequent IFN-γ protein production of CD8+ T cells. Therefore, the abolished IFN-γ production of anti-CD4 mAb–treated T cells is only partially due to a block in its secretion.

IFN-γ Production in Anti-CD4 mAb–Treated Alloactivated T Cells Can Be Restored by Exogenous IL-2 but not by IL-15

We have previously shown that treatment with recombinant IL-2 (200.000 U/d intraperitoneally) starting on day 2 after Tx almost completely abolished the anti-CD4 mAb–mediated effects (24). IL-2 has been shown to enhance IFN-γ production of activated T cells and NK cells (15–17). An overview about IL-2–induced signal transduction pathways with a focus on events that control key components of the posttranscriptional machinery is shown in Figure 2. Here, we examined whether exogenous IL-2 also restores IFN-γ protein production in anti-CD4 mAb–treated cultures. We used an IL-2 concentration of 1 U/ml, which did not increase the proliferation of anti-CD4–treated alloreactive T cells (data not shown). IL-2 was added to RIB5/2-treated cultures on day 2, when the TCR-driven IFN-γ message induction was already detectable. As shown in Figure 3A, addition of IL-2 to anti-CD4–treated cultures did not increase IFN-γ mRNA expression but led to a complete restoration of IFN-γ protein production (day 4, P = 0.02; day 5, P = 0.03).

IL-2 also increased the number of IFN-γ+ CD4+ T cells in RIB5/2-treated alloreactive T cells to approximately 75% (Figure 3B). Thus, IL-2 completely restored protein production (translation and secretion) of IFN-γ in alloactivated CD4+ T cells, suggesting that IL-2 expression is the primary target of anti-CD4 mAb treatment.

Signaling pathways used by the IL-2 and IL-15 receptor largely overlap as both receptors share the β and γc subunit (18). Therefore, we investigated whether IL-15, like IL-2,
influences IFN-γ protein production in alloactivated T cells.

Addition of recombinant IL-15Rα/Fc fusion protein to otherwise untreated alloactivated T cells did not prevent IFN-γ protein production (Figure 3C). Furthermore, unlike IL-2, rIL-15 could not restore IFN-γ protein production in RIB5/2-treated alloactivated T cells.

**Different Effects of Neutralizing Anti-CD25 Antibody and Rapamycin on IFN-γ Regulation of Alloreactive T Cells**

To demonstrate further the importance of IL-2 in IFN-γ production, we analyzed the effect of a neutralizing anti-CD25 antibody on IFN-γ mRNA induction and protein synthesis. Surprisingly, anti-CD25 mAb treatment of allo activated T cells...
cells, similar to the anti-CD4 antibody blocked IFN-γ protein production (day 3/4, \( P = 0.03 \); day 5, \( P = 0.0044 \); day 6, \( P = 0.0019 \)), whereas IFN-γ mRNA expression remained unaffected (Figure 4).

One of the signal transduction pathways induced by IL-2 is controlled by target of rapamycin (TOR) kinase. TOR regulates IL-2–induced T cell proliferation as well as phosphorylation and deactivation of the translational repressor 4E-BP. Thus, one explanation for the restoration of IFN-γ protein production by IL-2 may be a TOR-dependent deactivation of 4E-BP. To test this hypothesis, we added the TOR inhibitor rapamycin to allogeneic cultures. Rapamycin did not prevent IFN-γ expression, mRNA induction, and protein synthesis of alloactivated T cells (Figure 4). Rapamycin-treated allogeneic cultures showed a slightly delayed kinetic of IFN-γ mRNA and protein expression; however, maximal levels were similar to untreated allogeneic cultures. This delayed IFN-γ production may result from an inhibition of T cell proliferation. Thus, although IL-2 controls IFN-γ protein synthesis in alloreactive T cells, IL-2–induced activation of TOR is not involved in this process.

**Phosphatidylinositol 3 Kinase Is Involved in IL-2–Mediated Control of IFN-γ Protein Production during T Cell Activation**

One of the key components of the IL-2R signal transduction complex is the phosphatidylinositol (PI3) 3 kinase. Activation of PI3 kinase has been shown to be important for T cell proliferation and IL-2–induced gene expression (14). To assess the involvement of PI3 kinase in IL-2–mediated IFN-γ production, we added the PI3 kinase inhibitor Ly294002 to alloactivated T cells on day 2. Similar to anti-CD4 mAb treatment, Ly 294002 decreased IFN-γ protein production by alloactivated T cells (day 3, \( P < 0.001 \); day 4, \( P = 0.0024 \); day 5, \( P = 0.012 \)) but did not inhibit IFN-γ mRNA expression (Figure 5).

**Abolished eIF2α Dephosphorylation in RIB5/2-Treated Alloactivated T Cells**

During T cell stimulation, translation efficiency is mainly enhanced by an increase in the rate of translation initiation (25, 26). The translation initiation factor eIF2α facilitates the binding of the initiator tRNA (Met-tRNA) to the ribosome. The activity of eIF2α is regulated via phosphorylation/dephosphorylation. Phosphorylation of serine 51 in the α-subunit by certain kinases, e.g., protein kinase R, results in deactivation of eIF2α (26, 27). Stimulation of cells with growth factors such as insulin leads to the activation of phosphatases 1 and 2A in a PI3 kinase–dependent manner, which in turn are able to dephosphorylate and thereby activate eIF2α (28). Therefore, we investigated whether the lack of growth factor (IL-2) production in anti-CD4–treated T cells leads to diminished activation of eIF2α.

To assess the role of eIF2α in IL-2–mediated IFN-γ translation, we examined its phosphorylation in untreated and RIB5/2-treated alloactivated T cells in the presence or absence of recombinant IL-2 or IL-15. As shown in Figure 5, phosphorylation of eIF2α was reduced in alloactivated T cells compared with untreated cultures, indicating an increased activity of eIF2α after T cell stimulation. In contrast, the anti-CD4 mAb RIB5/2 inhibited the dephosphorylation of eIF2α in alloactivated T cells. Importantly, dephosphorylation of eIF2α was restored in anti-CD4–treated cultures by addition of IL-2, whereas exogenous IL-15 had no effect. These data suggest that reduced dephosphorylation-induced activation of the translation initiation factor eIF2α, as a result of a lack of IL-2 production, might be one mechanism for the inhibition of IFN-γ protein synthesis in anti-CD4–treated alloreactive T cells.

**Discussion**

Our results demonstrate that anti-CD4 treatment of alloactivated T cells with the nondepleting antibody RIB5/2 in vitro and in vivo blocked both IL-2 mRNA expression and protein synthesis. In contrast, alloantigen-induced IFN-γ protein production was inhibited by anti-CD4 treatment, although IFN-γ mRNA expression remained unchanged both in vitro and in vivo. Thus, the anti-CD4 antibody cannot prevent TCR-induced IFN-γ mRNA expression but interferes with posttranscriptional mechanisms that control IFN-γ protein production.
Figure 3. IFN-γ protein expression of anti-CD4–treated alloactivated T cells can be restored by exogenous IL-2. (A) IFN-γ transcription and secretion during untreated allogeneic MLR (■), anti-CD4–treated allogeneic MLR (○), and anti-CD4–treated allogeneic MLR plus 1 U/ml recombinant IL-2 added 2 d after onset of culture (●). Data are shown as mean ± SD of three independent experiments. *P < 0.05. (B) Intracellular IFN-γ expression of CD4-positive T cells at day 4 during MLR. (C) IFN-γ transcription and secretion during untreated allogeneic MLR (■), allogeneic MLR treated with 1 μg/ml mrIL-15 Ra/Fc Chimera (○), anti-CD4–treated allogeneic MLR (●), and anti-CD4–treated allogeneic MLR plus 1 U/ml mrIL-15 (○). Data are shown as mean ± SD of three independent experiments.

Figure 4. Different effects of neutralizing anti-CD25 antibody and rapamycin on IFN-γ regulation in alloreactive T cells. IFN-γ mRNA expression and secretion of untreated allogeneic MLR (■), rapamycin-treated allogeneic MLR (50 ng/ml; □), and anti-CD25 antibody–treated MLR (10 μg/ml; cross) was analyzed using real-time PCR and ELISA. Data are shown as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.
during T cell activation. We could further demonstrate that the lack of IL-2 production and subsequently diminished activation of the translation initiation factor eIF2α in anti-CD4–treated alloactivated T cells is responsible for the dramatically reduced IFN-γ protein synthesis.

In agreement with previous reports, we show that RIB5/2 treatment prevented IL-2 production of alloactivated T cells (11, 12). Surprisingly, we found that anti-CD4 treatment inhibited IFN-γ protein production but did not prevent IFN-γ mRNA expression in alloactivated T cells in vivo and in vitro. The latter finding is in opposition to previously reported observations, which demonstrated that IFN-γ production was completely abolished after anti-CD4 treatment (11, 12, 29). However, most studies determined only IFN-γ protein but not mRNA expression. Moreover, these studies did not correct IFN-γ expression levels for the reduced T cell infiltration into the graft after anti-CD4 mAb treatment. Thus, cytokine mRNA expression in whole-organ grafts could appear as decreased by the mere reduction of graft-infiltrating T cells when using non–T cell–specific housekeeping genes such as β-actin or HPRT for normalization, even though cytokine transcription at the single-cell level is unchanged. To account for such effects, we used CD3 as housekeeping gene in our real time RT-PCR analysis. For example, when we used β-actin as housekeeping gene, IFN-γ transcription in organ grafts of untreated recipients at day 2 after Tx was 10-fold higher than in grafts of anti-CD4–treated recipients. This was due to a delayed infiltration of alloreactive T cells because CD3 transcription was also 10-fold higher in organ grafts of untreated recipients. In contrast, 5 d after Tx, organ grafts of untreated and anti-CD4–treated recipients showed similar levels of CD3 and IFN-γ mRNA expression (data not shown). The Discrepancies between Previous and Our Findings Could Also Be Due to the Use of Different Anti-CD4 Antibodies. Because our method to quantify specific mRNA is based on total RNA extraction, we cannot differentiate between nuclear and cytoplasmic RNA. However, the primers and probe for detection of IFN-γ are designed in such a way that only spliced mRNA can be detected. Furthermore, oligo-dT was used for cDNA synthesis. The observation that not only secreted but also intracellular IFN-γ is strongly reduced in anti-CD4–treated MLR therefore suggests that anti-CD4 treatment prevents either the
export of fully processed mRNA into the cytoplasm or protein translation.

Furthermore, we analyzed the effect of the nondepleting anti-CD4 antibody RIB5/2 on TNF-α mRNA expression and cytokine production during an allogeneic MLR. Although anti-CD4–treated cultures showed a slightly delayed kinetic of TNF-α production, RIB5/2 did not prevent induction of TNF-α transcription or translation, and peak TNF-α protein levels were not significantly different from that in untreated allogeneic cultures (data not shown). Therefore, anti-CD4 treatment does not generally inhibit the translation of proinflammatory gene transcripts.

We further demonstrate that the lack of IL-2 production in RIB5/2-treated alloreactive T cells is responsible for the impaired IFN-γ protein synthesis. Addition of IL-2 to anti-CD4–treated cultures restored IFN-γ protein production without increasing mRNA expression. Treatment of alloactivated T cells with a neutralizing anti-CD25 antibody also resulted in reduced IFN-γ protein synthesis. Restoration of IFN-γ protein synthesis in RIB5/2–treated alloactivated T cells was IL-2 specific and could not be restored by addition of IL-15. This is somehow surprising because both cytokines share the IL-2Rβ and the common γ receptor chain. Only the α chain is cytokine specific but not believed to deliver any signal. However, several reports demonstrated differences in the function of both cytokines (30–33).

IL-2 has been shown to enhance IFN-γ production by activated T cells and NK cells by activating the p38 mitogen-activated protein (MAP) kinase and p42/p44 ERK kinase (15–17). Although we could demonstrate that specific MAP kinase inhibitors can prevent IFN-γ protein synthesis during T cell activation, the impaired protein production in RIB5/2–treated cultures is unlikely to be due to diminished p38 or 42/44 MAP kinase activation, because we found no differences in their activation between untreated and anti-CD4–treated alloactivated T cells (data not shown).

Further analysis of IL-2–stimulated signal transduction pathways revealed a possible involvement of PI3 kinase in the control of IFN-γ protein synthesis. PI3 kinase initiates several pathways that lead to increased protein synthesis in activated T cells (14, 34). It mediates the activation of the kinases PDK1/2, which in turn phosphorylate and activate Akt kinase. Akt is important for the activation of TOR and p70S6 kinase (35). However, as demonstrated here, activation of TOR is not important for IFN-γ protein synthesis in alloactivated T cells. Stimulation of cells with growth factors such as insulin or IL-2 leads to the activation of phosphatases 1 and 2A, which in turn dephosphorylate and thereby activate eIF2α (28). This is one mechanism by which growth factors initiate protein synthesis after cell stimulation. The efficiency of mRNA molecules to enter the translational machinery depends on their ability to bind to eIF2α. IFN-γ mRNA is able to bind eIF2α with high affinity, which correlates with its ability to compete in translation (27). It is interesting that RIB5/2 treatment of alloactivated T cells prevented the dephosphorylation of the translation initiation factor eIF2α. Addition of IL-2 to anti-CD4–treated cultures restored alloactivation-induced dephosphorylation of eIF2α and also IFN-γ protein production. In contrast, IL-15 restored neither eIF2α dephosphorylation nor IFN-γ protein production. Our results are in accordance with a very recent publication by Ben-Asouli et al. (36), who showed that sustained eIF2α phosphorylation and deactivation inhibited the translation of human IFN-γ mRNA, suggesting that eIF2α activation is important for IFN-γ protein synthesis. We therefore hypothesize that IL-2R signaling via activation of PI3 kinase may lead to the activation of phosphatase 1 and 2A, which in turn dephosphorylate and activate the translation initiation factor eIF2α. The lack of IL-2 production in anti-CD4–treated alloactivated T cells prevents the dephosphorylation of eIF2α and subsequently leads to reduced IFN-γ protein synthesis in activated T cells.

IFN-γ is important for a protective immunity. However, excessive production of IFN-γ after Tx may lead to graft destruction and graft loss. Preventing the protein synthesis of IFN-γ may be one mechanism by which anti-CD4 antibodies induce allospecific tolerance in vivo. Conversely, the restoration of IFN-γ protein production by IL-2 may also explain the reversibility of unresponsiveness by exogenous IL-2.

To our knowledge, this is the first report to link a therapy that induces T cell unresponsiveness with posttranscriptional mechanisms that are operational during T cell activation. These findings help to understand the underlying mechanisms that are operational during tolerance induction. Our results are of special importance when translating tolerance-inducing therapies into the clinical practice, where considerable IL-2 production induced by reactivation of preexisting alloreactive memory T cells or infection-reactive T cells may interfere with induction of unresponsiveness.

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References


