The Role of Interleukin-10 (IL-10) in IL-15-Mediated T-Cell Responses

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Interleukin-15 (IL-15) is a potent T-cell stimulating factor, which has recently been used for pre-clinical in vivo immunotherapy. Here, the IL-15 effect on CD3-stimulated peripheral human T cells was investigated. IL-15 induced a significant T-cell proliferation and upregulated CD25 expression. IL-15 significantly enhanced T-cell production of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and IL-10. Between 10- and 100-fold greater concentrations of IL-15 were necessary to reach a biological effect equivalent to that of IL-2. Blockade of IL-2 binding to the high-affinity IL-2 receptor did not affect the IL-15 effects, suggesting that IL-15 did not act by inducing endogenous IL-2. Exogenously administered IL-10 significantly reduced the IL-15 and IL-2-mediated IFN-

TNTERLEUKIN-15 (IL-15) has recently been cloned from the simian kidney epithelial cell line CV1/EBNA.¹ Although IL-15 shows no structural homology with IL-2, both cytokines exert functional similarities. Using specific receptor antibodies and cells transfected with different IL-2R subunits, the IL-15 signal has been shown to be transduced by an intact IL-2R β - γ chain complex, followed by activation of the same pattern of cytoplasmic kinases as are stimulated by IL-2.²⁻¹⁰ The ligand binding protein of IL-15, however, is distinct from the IL-2R α chain.¹¹ This may account for divergent functional activities such as activation of pre-Tcell lines by IL-2 but not IL-15, as well as a different capacity to induce cytokine production by T cells.^{2,8,10}

Adoptive immunotherapy with IL-2 has been used extensively in the treatment of adult renal cell carcinoma, melanoma, colon carcinoma, and pediatric sarcomas.¹²⁻¹⁵ More recently, IL-15 has been introduced into anticancer therapy. In mice dose-response curves showed that an about twofold dose of IL-15, compared to IL-2, was necessary to reach the same effect. However, the dose of IL-15 required to induce severe side effects such as pulmonary capillary leakage syndrome was six times higher than that required for IL-2.¹⁶ These differences might be caused by differential regulation of IL-2– and IL-15–induced cytotoxic effects. However, the mechanism by which IL-15 regulates cytotoxic cytokines remains widely elusive.

IL-10 is a negative regulator of T-cell activation and proliferation. Recently the inhibition of interferon- γ (IFN- γ) production in LPS treated MNCs has been shown to correlate with an inhibition of IL-12 production. Addition of exogenous IL-12 could partially overcome this inhibition.¹⁷ Besides these indirect effects, some minor directly inhibitory effects of IL-10 on T-cell function such as inhibition of IL-2 and other cytokines have also been described.^{18,19} In contrast to IL-2, IL-15 is produced by macrophages as well as by a variety of other cells and tissues, but not by T cells.^{20,21} IL-10 has been shown to be able to upregulate the expression of IL-15 in mouse macrophages,²² suggesting that the role of IL-10 in IL-15-mediated immune responses might be different from that in IL-2-mediated immune responses. To add to the understanding of the interaction between IL-15 and IL-10 in T-cell immunity, CD3-activated T cells were stimulated with both cytokines.

 γ and TNF- α production, whereas T-cell proliferation and CD25 expression were not affected. The inhibitory effects of exogenously administered IL-10 on T-cell cytokine production appeared indirect, and are likely secondary to decreased IL-12 production by accessory cells. Inhibition of endogenous IL-10 binding to the IL-10 receptor significantly increased IFN- γ and TNF- α release from T cells. These data suggest that endogenous IL-10 can regulate activated T-cell production of IFN- γ and TNF- α via a paracrine negative feedback loop. The observations of this study could be of relevance for the therapeutic use of IL-15 in vivo.

MATERIALS AND METHODS

Reagents. Recombinant human cytokines as well as cytokine enzyme-linked immunosorbent assay (ELISA) were purchased from R&D SYSTEMS (Minneapolis, MN), Biosource (Camarillo, CA), and Peprotech (Frankfurt a.M., Germany). Monoclonal antibodies (MoAbs) for fluorescence-activated cell sorting (FACS) analysis were supplied by Becton Dickinson (Heidelberg, Germany). Sheep erythrocytes were obtained from Behring (Marburg, Germany). For stimulation of T cells a purified anti-CD3 MoAb (clone X 35, mouse IgG2a; Immunotech, Hamburg, Germany) was used. Function of endogenous IL-12 or binding of endogenous IL-10 or IL-2 to their receptors were blocked by a neutralizing anti-IL-12 MoAb (clone 24910.1, mouse IgG1; R&D Systems), an inhibitory anti-IL-10R MoAb (clone 37607.11, mouse IgG1; R&D Systems) or an inhibitory antibody to the high-affinity IL-2R (clone 22722.2, mouse IgG1). All experiments with neutralizing or blocking MoAbs were controlled for by performing identical experiments with nonreactive control MoAbs (anti-CD20 MoAb [clone B9E9, mouse IgG2a, Immunotech], mouse IgG2a MoAb [clone 20102.1, R&D Systems], mouse IgG1 MoAb [clone 11711.11, R&D Systems]) prepared under identical conditions to experimental MoAbs.

Isolation and stimulation of T cells. T cells were obtained from healthy donor buffy coat preparations by ficoll density centrifugation and subsequent positive selection using neuraminidase-treated sheep erythrocytes as described previously.¹⁹ T-cell preparations contained

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90% to 95% CD3⁺ T cells, about 2% of each B cells, monocytes, or CD56⁺ natural killer (NK) cells as determined by immunofluorescence staining and FACS analysis. Using trypan blue staining, viability was checked to be >95% before stimulation. T cells were stimulated with anti-CD3 MoAb (100 ng anti-CD3/10⁶ cells) on ice for 30 minutes and resuspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum and penicilline/streptomycine at a density of 10⁶/mL. T cells were then stimulated as indicated with IL-15, IL-10, or combinations thereof.

In some experiments ultrapure T-cell preparations were used. Therefore, T cells were isolated from mononuclear cells (MNCs) by twofold rosetting. Thereafter, B cells, NK cells, and monocytes were depleted by incubation of the cell suspension with magnetic beadantibody-bound anti-CD20, anti-CD56, and anti-CD33 antibodies and cell extraction with a MiniMACS separation unit (Milteny, Berjind-Gladlach, Germany). FACS analysis of these T-cell fractions revealed T-cell purity to be greater than 97% with less than 0.5% of B cells or monocytes and about 1% NK cells. These T-cell fractions were then stimulated by immobilized anti-CD3 in the presence or absence of IL-2 or IL-15. Therefore 96-well flat-bottom full area plates (Falcon, Lincoln Park, NJ) were coated with 4 μ g/mL of anti CD3 diluted in carbonate buffer pH 9.8 according to the description of Taga et al.²³ After 1 hour at 37°C, excess antibody was removed and plates were washed and then stored at 4°C until usage. The purity of the T cells was further shown by only marginal CD25 expression and lack of significant T-cell cytokine production after incubation with unbound anti-CD3 MoAb (data not shown). In contrast, stimulation with immobilized anti-CD3-mediated significant T-cell activation as shown by significantly enhanced expression of CD25 (data not shown) and T-cell cytokine production (see Results). Taken together these data indicate that virtually no reactive antigenpresenting cells (APCs) were present in these T cells.

Cytokine ELISA. IL-10, IL-12 (heterodimer), tumor necrosis factor- α (TNF- α) and IFN- γ were detected by commercially available ELISA as has been described previously.¹⁹ Therefore cells were stimulated for 72 hours. Cell-free supernatants were procured and stored at -70° C until determination of cytokines.

FACS analysis. T cells were stained with fluorescence labeled anti-CD56, anti-CD3, anti-CD20, anti-CD33, anti-CD25, or isotype control antibodies suspended in phosphate-buffered saline (0.2 mol/L phosphate buffer pH 7.2 and 50 mmol/L NaCl) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (PBSBA). After 20-minutes incubation on ice, cells were washed in PBSBA and immunofluorescence was measured by FACScan (Becton Dickinson).

T-cell proliferation. T cells were cultured with medium or cytokines after anti-CD3 stimulation at a density of 10^5 /mL. After 66 hours, H³-thymidine (0.1 μ Ci) was added. Six hours later cells were procured and thymidine incorporation was determined by a beta counter.

Detection of β -actin, IL-10, and IFN- γ mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA isolation was performed by RNAzol preparation as described previously.24 Briefly, 1 \times 106 cells were lysed in 200 μL RNAzol (Wak-Chemie, Bad Homburg, Germany) and 20 µL chloroform. The suspension was mixed by vortexing and chilled on ice for 10 minutes. After centrifugation at 12,000g and 4°C for 15 minutes, the aqueous phase was transferred to an Eppendorf tube (Eppendorf, Hamburg, Germany) and reconstituted with an equal volume of isopropanol. RNA was precipitated at -20°C for 16 hours. Precipitates were pelleted at 12,000g and 4°C, then washed twice in 70% ethanol. Airdried pellets were resuspended in 20 µL RNase-free water. First strand cDNA synthesis was performed at 37°C for 60 minutes. Heatdenatured RNA of 10^6 cells was mixed with $11 \,\mu$ L Bulk-Mix (Moloney Murine Leukemia Virus reverse transcriptase, RNAguard, RNase/DNase-free BSA, deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate in aqueous solution; Pharmacia, Uppsala, Sweden), 1 μ L dithiotritol (DTT) (200 mmol/L aqueous solution, Pharmacia), 1 μ L random hexamer primers (random hexadeoxynucleotides at 0.2 $\mu g/\mu L$ in aqueous solution, Pharmacia). Diethylpyrocarbonate (DEPC)-treated water was added to a final volume of 50 μ L. First strand cDNA (1 μ L, 0.1 μ L, 0.01 μ L) was added to 45 μ L of PCR mix containing 5 μ L 10 × buffer (100 mmol/L Tris-HCl pH 8.3; 500 mmol/L KCl, 15 mmol/L MgCl₂; 0.01 % wt/vol gelatin), 2.4 mL deoxynucleotide triphospahte (2.5 mmol/L each, Pharmacia), 33.45 μ L sterile water, 2 μ L of each primer (10 μ mol/L), and 0.15 μ L of AmpliTaq DNA polymerase (Roche Molecular Systems, Inc, Grenzach-Wyhlen, Germany). The first-strand cDNA was amplified using a Biometra thermal cycler (Biometra, Göttingen, Germany) for 25 (β -actin) or 32 (IFN- γ and IL-10) cycles. The temperature profile used for cytokine mRNA amplification was 94°C for 30 seconds for denaturation, 62°C for 45 seconds for annealing, and 72°C for 50 seconds for primer extension. The temperature profile for amplification of β -actin mRNA was 94°C for 30 seconds for denaturation, 64°C for 40 seconds for annealing, and 72°C for 45 seconds for primer extension. Semiquantitative determination of PCR products was performed similar to previous description.^{25,26} Initially, nonsaturable PCR conditions were excluded by using three different concentrations of template (1, 0.1, 0.01 μ L) for the amplification of β -actin cDNA. PCR products were separated on ethidium bromidestained 1.6% agarose gels (Sigma Chemical Co, Munich, Germany). The gels were photographed under UV light and the band intensities were measured on polaroid films using a ELS 400-SM densitometer (Hirschmann, Munich, Germany). Subsequently, β -actin band intensities were used to extrapolate the RNA content of the different samples. cDNA was entered into the PCR at amounts that would yield similar β -actin band intensities and would thus allow direct comparison of the different cytokine PCR products. RT samples to which no cellular RNA had been added served as templates for RT-PCR negative control reaction. Cytokine specific primers and β -actin primers were synthesized by MWG-Biotec (Freiburg, Germany). All primers were RNA specific and the structure of β -actin primers has previously been reported.27 The structures of the IL-10 primers were : 5'ACA GCT GCA CCC ACT TCC 3'(sense), 5'CCC AGG GAG TTC ACA TGC g3'(antisense); IFN-y (5-GTT ACT GCC AGG ACC CAT ATG 3'(sense), 5'-GAC AGT TCA GCC ATC ACT TGG 3' (antisense). Primer sequences were compared with Genbank Heidelberg and with previously published data.28

Statistical analysis. The *t*-test was used for statistical evaluation of the data.

RESULTS

IL-15– and IL-2–induced T-cell proliferation and activation. Highly enriched T cells were stimulated with medium, anti-CD20, a nonspecific isotype control antibody, anti-CD3 alone, or anti-CD3 + IL-15 or IL-2 for 3 days. Stimulation with medium or isotype control antibodies did not induce significant T-cell proliferation. IL-15 and IL-2 induced a significant and dose-dependent increase of T-cell proliferation compared with basal levels or T cells activated by anti-CD3 alone (data not shown, P < .01). However, about 10 times higher IL-15 concentrations were necessary to reach the same degree of proliferation as with IL-2, which is consistent with previous reports.^{1,29} Addition of IL-10 at a high concentration (10 ng/mL) did not suppress IL-2– or IL-15–mediated T-cell proliferation, consistent with previously reported results.³⁰

Analysis of T-cell activation by measuring CD25 expression showed that stimulation of T cells with medium or



Fig 1. Effect of IL-15 on T-cell activation. FACS analysis of CD25 expression was performed with a PE-conjugated antihuman CD25 MoAb on T cells, which had been cultured as indicated with medium, anti-CD20, a nonspecific IgG2a isotype control antibody, anti-CD3, anti-CD3 + IL-15 (100 ng/mL, 10 ng/mL, 1 ng/mL, 0.1 ng/mL), anti-CD3 + IL-15 (100 ng/mL) + IL-10 (10 ng/ mL), anti-CD3 + IL-2 (100 ng/mL, 10 ng/mL, 1 ng/mL, 0.1 ng/mL and anti-CD3 + IL-2 (100 ng/mL) + IL-10 (10 ng/mL) for 72 hours. Control profiles using PE-conjugated mouse IgG-1 are shown on each panel (light areas). Shown are the results from one representative experiment. A total of four experiments has been performed. Depicted are cell number (Y-axis) and fluorescence intensity of the CD25 staining (X-axis).

isotype control antibodies did not induce significant T-cell activation (Fig 1). Stimulation with anti-CD3 in the presence or absence of IL-15 or IL-2 induced a significant increase in CD25 expression (Fig 1, P < .01). Again, about 10-fold higher concentrations of IL-15 were necessary to reach effects equivalent to those mediated by IL-2 (Fig 1). At a high concentration, exogenously administered IL-10 (10 ng/mL) did not suppress the IL-2– or IL-15–mediated CD25 expression on activated T cells.

IL-15– and IL-2–induced cytokine production by T cells. To analyze the effect of IL-15 on T-cell–derived cytokine production, T cells were cultured with the respective stimuli for 3 days. T-cell activation by IL-15 and IL-2 was accompanied by a significant and dose-dependent increase of IL-10, IFN- γ and TNF- α production by T cells (Fig 2, P < .01). However, between 10- and 100-fold higher concentrations of IL-15 were necessary to induce cytokine concentrations approximately equivalent to those yielded by IL-2 stimulation.

The role of endogenous IL-2 in IL-15–mediated cytokine production was further evaluated by using an inhibitory antibody against the high-affinity IL-2 receptor. This antibody could significantly reduce IL-2 (used at 1 or 10 ng/mL, P < .01) mediated induction of IL-10, IFN- γ , or TNF- α . However, this antibody did not significantly reduce IL-15–mediated cytokine production (Table 1).

Interaction between IL-15 or IL-2 and IL-10. In this study the effect of IL-10 on IL-15 or IL-2-mediated cytokine production by purified T cells was analyzed. Therefore, T cells



Fig 2. IL-15-induced cytokine production by T cells. (A) T cells were isolated by rosetting and then stimulated as indicated. After 72 hours cell-free supernatants were procured and assayed for IFN- $\gamma.$ Depicted are mean and SEM of six independent experiments. Significantly altered cytokine production induced by anti-CD3 + IL-15 (◊) or IL-2 (♦) compared to anti-CD3 alone (248 \pm 58 pg/mL) is indicated by * (P < .01) or ** (P < .05). (B) T cells were isolated by rosetting and then stimulated as indicated. After 72 hours cell-free supernatants were procured and assayed for TNF- α . Depicted are mean and SEM of six independent experiments. Significantly altered cytokine production induced by anti-CD3 + IL-15 (□) or IL-2 (■) compared to anti-CD3 alone (65 ± 9 pg/ mL) is indicated by * (P < .01) or ** (P < .05). (C) T cells were isolated by rosetting and then stimulated as indicated. After 72 hours cell-free supernatants were procured and assayed for IL-10. Depicted are mean and SEM of six independent experiments. Significantly altered cytokine production induced by anti-CD3 + IL-15 (△) or IL-2 (▲) compared to anti-CD3 alone (2.5 \pm 1.6 pg/mL) is indicated by * (P <.01) or ** (P < .05).

Table 1. The Role of Endogenous IL-2 in IL-15–Mediated Cytokine Production

	% Inhibition of Cytokine Production		
Culture Conditions	IFN-γ	TNF-α	IL-10
Anti-CD3 + IL-15 (100 ng/ mL) + anti-IL-2R	1 ± 9	1 ± 7.5	1 ± 7.6
+ anti-IL-2R Anti-CD3 + IL-2 (1 ng/mL)	64 ± 8*	68 ± 3.2*	50 ± 5.4*
+ anti-IL-2R	$85 \pm 9.5^*$	90 ± 3.4*	80 ± 4.8*

T cells were stimulated by anti-CD3 + IL-15 or IL-2 in the presence of an inhibitory MoAb against the high affinity-IL-2R or an isotype control antibody. % Inhibition of cytokine production in the presence of the inhibitory MoAb was calculated by (1 – [cytokine production in the presence of anti-IL-2R α]/[cytokine production in the presence of the isotype control antibody]) × 100%. Depicted are mean and SEM of six independent experiments. Significant inhibition (P < .01) of cytokine production is indicated by (*). were stimulated with anti-CD3 + IL-15 or IL-2 (each used at 100 ng/mL) in the presence or absence of IL-10. IL-10 significantly reduced the IFN- γ and TNF- α protein synthesis in a dose-dependent fashion (Fig 3, P < .01). At a concentration of 10 ng/mL IL-10 suppressed IFN- γ production by more than 90% and TNF- α production by more than 70%. At this concentration, IL-10 also inhibited IL-2 and IL-15 induced IL-10 and IFN- γ mRNA expression as determined by semiquantitative RT-PCR (data not shown). At lower concentrations of IL-2 or IL-15 (1 or 10 ng/mL, data not shown), IL-10 showed the same degree of inhibition of cytokine production as for concentrations of 100 ng/mL (Fig 3).

Previously IL-10 had been reported to suppress IFN- γ production by MNCs via inhibition of IL-12 synthesis, and very low concentrations of IL-12 had been shown to be able to induce significant cytokine release from MNCs.¹⁷ Therefore, we performed a series of experiments with ultrapure T cells to better understand the role accessory cell-produced IL-12 may have in mediating the observed suppression of T-cell cytokine production by IL-10.

T cells obtained after rosetting with sheep erythrocytes



Fig 3. Inhibitory effect of IL-10 on IL-2- or IL-15mediated cytokine production. (A) T cells were isolated by rosetting and then stimulated by anti-CD3 + IL-15 (100 ng/mL) in the presence or absence of IL-10 (0.1 to 10 ng/mL). Depicted are mean and SEM of four independent experiments showing inhibition of cytokine-induced IFN- γ or TNF- α production. % Inhibition of cytokine production by IL-10 was calculated by (1 - [cytokine production in the presence of IL-10]/[cvtokine production in the absence of IL-10]) \times 100%. (B) T cells were isolated by rosetting and then stimulated by anti-CD3 + IL-2 (100 ng/mL) in the presence or absence of IL-10 (0.1 to 10 ng/mL). Depicted are mean and SEM of four independent experiments showing inhibition of cytokine-induced IFN- γ or TNF- α production. % Inhibition of cytokine production by IL-10 was calculated by (1 - [cytokine production in the presence of IL-10]/[cytokine production in the absence of IL-10]) × 100%.

Table 2. The Role of Endogenous IL-12 in IL-2and IL-15-Mediated Cytokine Production

	% Inhibi	% Inhibition of Cytokine Production			
Culture Conditions	IFN-γ	TNF - α	IL-10		
Anti-CD3 + IL-15 (100 ng/mL) + anti-IL-12 Anti-CD3 + IL-2 (100	60.3 ± 4.4*	32.3 ± 4.9†	44.7 ± 6.1*		
ng/mL) + anti-IL-12	$74 \pm 2.3^*$	27 ± 4.41	36 ± 1.1*		

T cells were stimulated in the presence of accessory cells by anti-CD3 + IL-2 or IL-15 and a neutralizing anti-IL-12 antibody or an isotype control antibody. % Inhibition of cytokine production in the presence of the neutralizing MoAb was calculated (1 – [cytokine production in the presence of anti-IL-12]/[cytokine production in the presence of the isotype control antibody]) \times 100%. Depicted are mean and SEM of three independent experiments. Significant inhibition of cytokine production is indicated by * (*P* < .01) or † (*P* < .025).

contained a low number of APCs. Stimulation of four different T-cell populations with high concentrations of IL-2 and IL-15 (each 100 ng/mL) induced low but significant concentrations of the IL-12 heterodimer (P < .025), which is the biologically active form of this cytokine.³¹ IL-10 could reduce the IL-2–induced IL-12 production from 3.9 ± 0.8 pg/mL to below 1 pg/mL (P < .025). Similarily, IL-15–mediated IL-12 release was reduced from 3.0 ± 0.7 pg/mL to below 1 pg/mL (P < .025).

Using a neutralizing antibody against IL-12, the role of endogenous IL-12 in IL-2– and IL-15–mediated cytokine production was analyzed. At a concentration where this antibody inhibited at least 1 ng/mL of IL-12, IL-2, and IL-15–induced cytokine production was significantly reduced (Table 2). However, the extent to which the different cytokines were inhibited varied somewhat. The greatest inhibition was obtained for IFN- γ , while inhibition of IL-10 and TNF- α production was less pronounced (Table 2).

To analyze the role of endogenous IL-12 in the IL-10– induced suppression of IL-2– and IL-15–mediated cytokine production, two approaches were taken: (1) the inhibitory effect of IL-10 was assessed in the absence of endogenous IL-12 and (2) the effect of exogenous IL-12 on IL-10–mediated cytokine suppression was determined.

Using ultra-pure T cells, which were virtually completely void of APCs, IL-15 and IL-2 each induced significant (P < .01) amounts of IFN- γ and TNF- α in CD3-activated T cells compared to stimulation with anti-CD3 alone (data not shown). In addition, IL-15 stimulated the release of 978 ± 118 pg/mL of IL-10 from CD3-activated T cells compared with 389 ± 134 pg/mL after stimulation with anti-CD3 alone (P < .01). Similarly IL-2 stimulated also a significant IL-10 release from CD3-activated T cells (1,354 ± 223 pg/mL; P < .01). Under these experimental conditions, IL-2– and IL-15–mediated cytokine production were not significantly affected by anti-IL-12 or IL-10, suggesting that IL-12 is not produced by ultra-pure T-cell populations and that the suppressive effects of IL-10 on IL-2/15 activated T-cell cyto-kine production are not directly mediated.

IL-10 significantly reduced the IL-15–mediated IFN- γ and TNF- α production by T cells stimulated in the presence

of accessory cells (Fig 4A and B). IL-12 prevented the IL-10–induced suppression of IFN- γ and TNF- α by IL-15– activated T cells. The IL-12 effect on the IFN- γ production exceeded the effect on TNF- α release, probably because IL-12 participated more strongly in the production of IFN- γ than of TNF- α (Table 2). Similar results were obtained with IL-2 (data not shown).

Kinetics of IL-2– and IL-15–mediated cytokine release. Kinetic analyses of IFN- γ , TNF- α , and IL-10 production showed that IL-15– and IL-2–induced IL-10 release peaked on day 2, while maximum concentration of IFN- γ and TNF- α levels were only reached after 3 to 5 days, when IL-10 levels were already beginning to decline. In detail, on day 2 mean IL-10 response was above 85% of the maximum response compared to less than 60% of IFN- γ and TNF- α (P < .01, data not shown). In contrast, on day 5 mean IFN- γ and TNF- α release was above 80%, of the maximum response while mean IL-10 levels had already dropped to less than 65% (P < .01, data not shown).

The role of endogenous IL-10 in IL-2- and IL-15-mediated cytokine production. To evaluate the inhibitory potential of IL-15- and IL-2-induced IL-10 production the role of endogenously produced IL-10 on cytokine release was assessed. Stimulation of T cells by IL-15 or IL-2 in the presence of an IL-10R blocking MoAb significantly upregulated the release of IFN- γ and TNF- α (Table 3), suggesting that endogenous IL-10 could affect the IL-2/IL-15-mediated immune responses through negative feedback regulation. Similarly, blocking of IL-10 binding to its receptor by use of the inhibitory MoAb also lead to an increase of detectable IL-12. However, these differences were not statistically significant due to high interexperimental variation of the results. This might be explained by technical reasons, because IL-12 concentrations were detected at the lower detection limit of our assay. Since transforming growth factor- β (TGF- β) is another suppressive cytokine also produced by T cells, we investigated whether endogenous TGF- β release might also act on IL-2/IL-15-activated T-cell cytokine production. However, stimulation of T cells by anti-CD3 and IL-2 or IL-15 failed to induce immunoreactive TGF- β (data not shown). Consequently, a neutralizing anti-TGF- β MoAb did not affect cytokine release from activated T cells when compared with an isotype control antibody (data not shown).

DISCUSSION

In this study we report that IL-15– and IL-2–activated purified human T cells. Previously, Kanegane et al³² had observed that, similar to IL-2, IL-15 could induce proliferation and activation of memory and naive T cells, when stimulating a mononuclear cell fraction. We also showed that IL-10 could not suppress IL-2– or IL-15–mediated T-cell proliferation or CD25 expression. This is in agreement with previous observations that IL-10 downmodulates mitogen or anti-CD3 induced T-cell proliferation in the presence or absence of accessory cells via inhibition of endogenously produced IL-2.^{23,30} In these experiments addition of IL-2 could compensate the inhibitory effect of IL-10, suggesting that IL-2–mediated T-cell proliferation was not dependent on the secretion of accessory cell-derived factors such as IL-



Fig 4. Effect of IL-12 on IL-15-mediated cytokine production by T cells. (A and B) T cells were isolated by rosetting and then stimulated as indicated. Depicted are the increments in IFN- γ (A) or TNF- α (B) production obtained by stimulation with anti-CD3 + IL-15 (100 ng/mL) over stimulation with anti-CD3 alone of one representative experiment. At total of four experiments have been performed.

12, as has been demonstrated for anti-CD2–activated MNCs. $^{\rm 33}$

The comparison between IL-2– and IL-15–mediated Tcell activation revealed that between 10- to 100-fold greater concentrations of IL-15 were necessary to reach equivalent biological effects as with IL-2. These results are similar to those reported by Carson et al²⁹ who investigated NK-cell activation by IL-15 and IL-2. The difference in potency might be explained by the different number of IL-15/IL-2

Table 3. Effect of Endogenous IL-10 on Cytokine Production

Culture Conditions	Enhancement (%) of Cytokine Production			
	TNF - α	IFN-γ	IL-12	
Anti-CD3 + IL-15 (100 ng/mL) + anti-IL-10R	38 ± 13†	31 ± 9*	59 ± 51	
Anti-CD3 + IL-2 (100 ng/mL) + anti-IL-10R	53 ± 16†	52 ± 13*	108 ± 64	

In the presence of accessory cells T cells were stimulated by anti-CD3 + IL-15 or IL-2 and an inhibitory anti-IL-10R MoAb (1 μ g/mL) or a corresponding isotype control antibody (1 μ g/mL). Depicted is the enhancement of TNF- α and IFN- γ production upon inhibition of endogenous IL-10 binding to the IL-10R. Mean and SEM of six independent experiments are shown. % Enhancement was calculated as (1 – [cytokine production in the presence of an anti-IL-10R MoAb]/[cytokine production in the presence of an anti-IL-10R MoAb]/[cytokine production in the presence of the control antibody]. Stimulation of T cells with IL-2 in the presence of the control antibody revealed 185 ± 41 pg/mL TNF- α , 5.0 ± 1.1 ng/mL IFN- γ and 2.3 ± 1.4 pg/mL IL-12, while stimulation with IL-15 in the presence of the control antibody revealed 181 ± 35 pg/mL TNF- α , 4.8 ± 1.23 ng/mL IFN- γ , and 1.4 ± 0.15 pg/mL IL-12. Significant enhancement of cytokine production by blocking of IL-10 binding to its receptor is indicated by (* P < .01, † P < .025).

high-affinity receptors expressed on NK cells. Similarily, anti-CD3–stimulated T cells expressed 5 to 7 times less high-affinity IL-15 receptors than IL-2 receptors.³⁴ However, this might not completely explain the striking difference between IL-2 and IL-15 concentrations. In addition to a different number of high-affinity binding sites, Kumaki et al³⁵ also found that binding of IL-15 significantly reduced the binding capacity of IL-15 to B and T cells by about 80%.

Since activated T cells produce their own IL-2, we explored the role of endogenous IL-2 in IL-15–mediated T-cell activation. Stimulation of anti-CD3 activated T cells by IL-15 was not affected by an inhibitory MoAb to the high-affinity IL-2R, which significantly reduced the effect of IL-2, suggesting that the IL-15 effect on cytokine release from T cells was not mediated by inducing endogenous IL-2. These results are in keeping with those reported by Mori et al.³⁶ They showed that IL-15–mediated IL-5 production by human T-cell clones or stimulated PBMC was not dependent on IL-2 and that IL-15 could replace the function of IL-2.

In contrast to the effects on IL-15/IL-2–mediated T-cell proliferation and CD25 expression, IL-10 could significantly suppress IL-15– and IL-2–induced production of IFN- γ and TNF- α . IL-10 did not only inhibit protein release but also mRNA expression, suggesting that IL-10 downregulated protein de novo synthesis stimulated by IL-15 and IL-2. Taga et al³⁰ showed that in the presence of accessory cells IL-10 could inhibit both IFN- γ production and proliferation of T cells after anti-CD3 stimulation. However, IL-2 could restore T-cell proliferation, while IFN- γ production remained suppressed even in the presence of exogenously added IL-2. Thus, in contrast to IL-2–mediated T-cell proliferation, IL-2– as well as IL-15–induced cytokine production seem to be dependent on the production of certain accessory cell derived factors.

Therefore, we investigated the role of IL-12 in IL-2- and IL-15-mediated cytokine production by T cells and the role of endogenous IL-12 in the IL-10- mediated inhibition of IL-2 and IL-15 effects. We found that stimulation of T cells by IL-2 and IL-15 in the presence of accessory cells mediated low concentrations of IL-12 heterodimer, which is the biologically active form of IL-12.³¹ In addition, an anti-IL-12 antibody could significantly inhibit the IL-2- and IL-15mediated production of IFN- γ and TNF- α . In addition, anti-IL-12 also inhibited the release of IL-2- and IL-15-induced IL-10, although IL-12 had originally been described as a Th-1 cell stimulating cytokine.³⁷ However, Meyaard et al³³ showed that IL-12 could also facilitate the production of IL-10, a Th-2 derived cytokine, and that IL-2 and IL-12 had a synergistic effect on IL-10 release from T cells and T-cell clones.38

Armant et al³⁹ described that IL-2-mediated T-cell activation in the presence of APCs but not involving TCR stimulation is dependent on endogenous IL-12 production. The mechanism by which IL-12 is induced seems to be mediated by an interaction between CD40L and CD40. IL-2 has been shown to significantly upregulate IL-12 production, probably by enhancing T-cell-APC interaction. In this study we could show for the first time that IL-2- and IL-15-mediated stimulation of TCR-activated T cells is dependent on the production of endogenous IL-12. By two lines of evidence we could also show that the inhibition of this IL-12 production might be a major mechanism by which IL-10 suppresses IL-2-

and IL-15-mediated T-cell activation: (1) the significant inhibitory effect of IL-10 on IL-2/IL-15-activated T-cell cytokine production was not directly mediated because it was not observed in APC-free preparations and (2) addition of exogenous IL-12 prevented IL-10-induced suppression of cytokine production by IL-2/IL-15-activated T cells. However, it also appeared that IL-12 was not the only factor involved, because anti-IL-12 could not completely inhibit the IL-2- and IL-15-mediated cytokine production and exogenous IL-12 could not completely overcome the inhibitory effect of IL-10. D'Andrea et al¹⁷ found that IL-10 inhibited IFN- γ release by MNCs after stimulation with lipopolysaccharide or Staphylococcus aureus Cowan I. The inhibitory effect was closely related to a suppression of IL-12 production, but the inhibitory effect of IL-10 could be fully compensated only by a combination of IL-1 β and IL-12.

T cells produce substantial amounts of IL-10. Inhibition of endogenous IL-10 binding to the IL-10R significantly upregulates IL-15– and IL-2–mediated T-cell cytokine production. Since the inhibitory effect of IL-10 on T-cell cytokine production appeared to be indirect, involving decreased IL-12 production by accessory cells, these data suggest that endogenous IL-10 can regulate T-cell production of IFN- γ and TNF- α via a paracrine negative feedback loop.

This negative feedback loop by endogenous IL-10 might be important for the kinetics of cytokine responses induced by IL-15 and IL-2. IL-15– and IL-2–induced IL-10 production peaked earlier than IFN- γ and TNF- α . These results are in keeping with those of McHugh et al⁴⁰ who found that stimulation of peripheral blood mononuclear cells with phytohemagglutinin first induced an IL-10 secretion, followed by a release of IFN- γ and TNF- α . In contrast to its effect on T cells, IL-10 augmented IL-2-mediated proliferation of human NK cells, and exacted additive effects on cytotoxic activity. Production of IFN- γ , granulocyte-macrophage colony-stimulating factor, and TNF- α by IL-2-stimulated NK cells was significantly enhanced by IL-10. Thus, the induction of IL-10 by IL-2 and IL-15 during T-cell activation might not only exhibit a negative feedback control mechanism in T-cell immunity, but can also facilitate cross-talk with NK cells, which might lead to enhanced NK-cell activity.⁴¹

IL-15 has been used for anticancer activities in vivo and in vitro^{16,42} and it seems to exhibit a therapeutic index superior to IL-2.¹⁶ The cytotoxic effects of IL-2 and probably of IL-15 are mediated by cellular mechanisms including the activation of Fas and Perforin as well as by the release of soluble mediators such as IFN- γ and TNF- α .⁴³ The results of this study show that IL-15 could significantly induce the release of cytotoxic cytokines from T-cell receptor activated T cells via an IL-2–independent mechanism. In addition, exogenous and endogenous IL-10 inhibited IL-2– and IL-15–mediated T-cell derived cytokine production. In contrast, IL-12 prevented the inhibitory action of IL-10 on IL-2/IL-15–mediated T-cell cytokine production. Thus, future concepts of anticancer immunotherapy should consider the use of IL-15 alone or in combination with IL-12.

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