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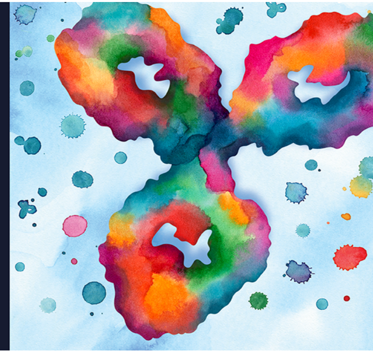

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# CD4$^+$CD25$^+$ T Cells Lyse Antigen-Presenting B Cells by Fas-Fas Ligand Interaction in an Epitope-Specific Manner[1]

**Wim Janssens, Vincent Carlier, Bo Wu,[2] Luc VanderElst, Marc G. Jacquemin, and Jean-Marie R. Saint-Remy[3]**

**Suppression by regulatory T cells is now acknowledged to play a key role in the down-regulation of T cell responses to foreign and self Ags. In addition to the naturally occurring CD4$^+$CD25$^+$ population, several subtypes of induced regulatory cells have been reported, but their mechanisms of action remain unclear. Conversely, cytotoxic CD4$^+$ cells that lyse cells presenting their cognate peptide have been described, but their potential role in immunoregulation remains to be delineated. A CD4$^+$ T cell line derived from BALB/c mice immunized with peptide 21–35, containing a major T cell epitope of a common allergen, *Dermatophagoides pteronyssinus* group 2 allergen, was found to lyse the Ag-presenting WEHI cell line via Fas-Fas ligand and only in the presence of the cognate peptide. Cytolytic activity was likewise shown for other T cell lines and occurred even after a single cycle of in vitro stimulation. Moreover, T cells that efficiently lysed WEHI cells were unresponsive to stimulation with their cognate Ag and were dependent on IL-2 for growth and survival, which was reflected in a constitutive expression of CD25 independently of activation status. Proliferating B cells were also killed by the CTLs. By lysing Ag-presenting B cells in an epitope-specific manner, the nonproliferating CTLs were shown to down-regulate the proliferation of bystander T cells. These data demonstrate that cytotoxic CD4$^+$CD25$^+$ T cells that lack proliferation capacities have the potential to down-regulate an immune response by killing Ag-presenting B cells. This could represent an important and specific down-regulatory mechanism of secondary immune responses in vivo. *The Journal of Immunology,* 2003, 171: 4604–4612.**

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Cytotoxic CD4$^+$ T cells have been reported in some studies involving both mice and humans. The cytotoxicity of CD4$^+$ T cells is usually restricted to CD4$^+$ cell lines and clones generated by long term culture (1), immunization with strong T cell activators such as keyhole limpet hemocyanin (KLH)[4] (2, 3), or mixed lymphocyte culture (4, 5). Researchers have therefore considered CD4$^+$ cytotoxicity to be of little physiological relevance. Only recently have cytotoxic CD4$^+$ cells been characterized in healthy individuals with expansion during chronic viral infection such as HIV and EBV (6, 7) and in patients with autoimmune diseases (8) as well as tumors (9). Although the mechanism of lysis by Fas-Fas ligand (FasL) interaction or granzyme degranulation has been well established, the potential role of such cells in immunoregulation remains to be delineated.

Regulatory CD4$^+$CD25$^+$ T cells are known to exert a suppressive activity on adjacent T cells in mice and humans, thereby down-regulating specific immune responses. Their roles in chronic inflammation and allergic disorders (10), in autoimmune and tumor diseases (11–13) are generally accepted, but their mechanism of action remains debatable (14). In vitro CD4$^+$CD25$^+$ T cells are activated by TCR recognition and require IL-2 for differentiation and survival, while being hyporesponsive, with minimal cytokine production (15–18). Naturally occurring CD4$^+$CD25$^+$ regulatory T cells are generated in the thymus during early stages of fetal and neonatal T cell development (19) and are fully functional at the time of export from the thymus. Such cells are polyclonal, are capable of recognizing various self-Ags (14, 20), and act via a cytokine-independent mechanism of cell-cell contact between T cells that is APC independent, at least in vitro (21). In addition to the naturally occurring CD4$^+$CD25$^+$ population, several subtypes of induced (or adaptive) regulatory cells have been described. Those cells are distinct from naturally occurring regulatory T cells in their requirement for further Ag-driven differentiation in the periphery in a specific immunological context. Induced regulatory T cells are allo- or autoantigen specific and exhibit mechanisms of action that vary according to the conditions under which they are induced (20). Tr1 cells, which act in vivo mainly by producing IL-10, are generated in vitro on immature dendritic cells (DC) or on spleen cells in the presence of IL-10 and/or IFN-$\alpha$ (22, 23). In vivo generation of respiratory DC has also been recently described (24). Th3 cells, which are generated in vivo after oral administration of Ag, down-regulate T cell immune responses and operate predominantly by production of TGF-$\beta$ (25). Finally, anergic T cells induced in vitro by anti-CD3 Abs or T-T cell presentation are reported to inhibit T cell responses in vivo via the down-regulation of costimulatory molecules on APC (26, 27).

In the present study we describe the generation of CD4$^+$ murine T cell lines endowed with Fas/FasL-mediated cytotoxic properties on B cells in an epitope- and MHC class II-restricted manner. CD4$^+$ T cells that efficiently lysed APC, constitutively expressed CD25$^+$ and behaved as regulatory cells in vitro by exerting a suppressive effect on their corresponding APC and bystander T cells.

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[4] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; CD40L, CD40 ligand; CD95L, CD95 ligand; DC, dendritic cell; Der p 2, *Dermatophagoides pteronyssinus* group 2 allergen; FasL, Fas ligand; HSA, human serum albumin; PI, propidium iodide; Tr, T regulatory; ICOS, inducible costimulator; GITR, glucocorticoid-induced TNF receptor family-related protein.

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In addition to Tr1, Th3, and anergic T cells, we propose that the cytotoxic CD4 cells described here belong to the broad spectrum of induced regulatory T cells, with a specific role in the down-regulation of secondary, B cell-mediated immune responses.

## Materials and Methods

### *Peptides and proteins*

15-Mer peptides derived from *Dermatophagoides pteronyssinus* group 2 allergen (Der p 2) were synthesized (purity, >85%) by Eurogentec (Herstal, Belgium). Sequences are: p21, CHGSEPCIIHRGKPF; p21(28), CHG SEPCNIHRGKPF; p21(27), CHGSEPSIIHRGKPF; and p71, NACHYMKC PLVKGQQ. Recombinant full-length Der p 2 is produced in *Pichia pastoris* in our laboratory as is the recombinant polypeptide B4, which contains four copies of p21 arranged in a linear sequence and linked by either two glycines or two serines. Human serum albumin (HSA) was supplied by CAF-DCF (Rode Kruis, Brussels, Belgium)

### *Generation of T cell lines*

Twelve-week-old BALB/C mice (H-$2^d$) were immunized by footpad injection of 50 $\mu$g of peptide or protein in CFA. On day 14, mice were boosted with the same dose of Ag in IFA. Ten days later popliteal lymph nodes were removed, T cells were obtained by Ficoll density gradient purification (Nycomed Pharma, Oslo, Norway) and enriched by negative selection using magnetic beads against CD11c, CD11b, and CD45R (Miltenyi Biotec, Bergisch Gladbach, Germany). Polyclonal T cell suspensions were subsequently expanded on syngeneic irradiated (4000 rad) spleen cells as a source of APC in the presence of the corresponding peptide or protein (18 $\mu$M/ml). After 1 wk, T cells were harvested and re-expanded on freshly prepared, irradiated APC. After a few cycles of stimulation, T cell lines were obtained that gradually lost their proliferation capacity during further cyclic expansion. To restore proliferation, 25 U/ml of IL-2 (BioSource, Nivelles, Belgium) was added twice per week for each cycle, and T cell numbers were increased by addition of PHA (10 $\mu$g/ml; Sigma-Aldrich, Steinheim, Germany) when required. G-121 was obtained by immunization and repetitive in vitro stimulation with p21–35. G-124 was obtained by immunization and repetitive in vitro stimulation with Der p 2. Polyclonal T cells specific for p71 were freshly prepared from BALB/c mice 9 days after immunization with Der p 2 (encompassing residues 21–35 and 71–86).

### *Cell culture*

T cells were cultured in enriched RPMI 1640 medium containing 10% FCS, 50 $\mu$M 2-ME, 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen, Merelbeke, Belgium), and 2.5 g/liter glucose (20%). WEHI 231 cells were purchased from the European collection of cell cultures (ECACC, Salisbury, U.K.) and were cultured in DMEM with 2 mM L-Gln, 10% FCS, 50 $\mu$M 2-ME, 100 U/ml penicillin, and 100 U/ml of streptomycin (Invitrogen). B cells were freshly prepared from spleen cells of BALB/c mice obtained by Ficoll density gradient purification (Nycomed Pharma) and were enriched by negative selection using magnetic beads against CD11c, CD11b, and CD90 (Miltenyi Biotec). B cells were cultured in enriched RPMI 1640 medium in the presence of 50 $\mu$g/ml of LPS (*Escherichia coli* 055:B5; Sigma-Aldrich).

### *Thymidine incorporation assay*

In the course of T cell line generation, T cells were cocultured on irradiated APC (4000 rad) at an APC/T ratio of 3/1 for the first cycle, which was progressively increased to 15/1 during further cyclic expansion. Aliquots of 200 $\mu$l of cocultures were seeded in 96-well plates and incubated for 72 h. Proliferation was assayed by the addition of 1 $\mu$Ci/well of [$^3$H]thymidine (ICN, Asse, Belgium) during the last 18 h of incubation. For WEHI cell or B cell proliferation assays, nonirradiated WEHI cells or LPS-activated B cells ($5 \times 10^4$ cells/well) were cocultured with CTLs ($5 \times 10^4$ cells/well) in 96-well plates for 32 h in the presence of various peptides or proteins used at the indicated concentrations and in the absence of LPS. Proliferation was assayed by the addition of 1 $\mu$Ci/well of [$^3$H]thymidine during the last 18 h. For blocking experiments with mAbs, target cells were preincubated with the mAb for 1 h, after which effector cells were added without removal of mAb. Each experiment was performed in triplicate, and results are expressed as the mean ± SD.

### *Thymidine release assay (JAM test) (28)*

WEHI cells were preincubated with 4.5 $\mu$Ci/ml for 10 h. After washing, WEHI cells ($1 \times 10^5$ cells/well) were cocultured with CTLs ($1 \times 10^5$ cells/well) in the presence or the absence of p21 (18 $\mu$M/ml). The release of [$^3$H]thymidine by target cells was evaluated after 4, 8, and 14 h by harvesting cells on glass fibers and counting the remaining radioactivity. The percentage of specific lysis was calculated as $100 \times$ ((cpm WEHI + T cells) − (cpm WEHI + T cells + p21))/(cpm WEHI + T cells). Each experiment was conducted in triplicate, and results are expressed as the mean ± SD.

### *Mouse Abs and FACS analysis*

WEHI, B, and T cells were stained with PE- and FITC-conjugated mAbs directed against lymphocyte surface molecules. Isotype-matched PE- or FITC-labeled Abs were used as negative controls. After staining and washing, cells were analyzed with a FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). The mAbs (BD PharMingen, Erembodegem, Belgium) used for cytofluorometry were PE-labeled anti-CD19, anti-CD4, anti-CD8a, anti-CD3, anti-CD25, anti-CD40 ligand (anti-CD40L), anti-CD95 ligand (anti-CD95L), anti-pan-NK, anti-CD28, anti-inducible costimulator (ICOS), and anti-CTLA-4 and FITC-labeled anti-CD4, anti-Ia/Ie, anti-CD11b, anti-CD14, anti-CD69, anti-CD95, and anti-V$\beta$1 to anti-V$\beta$17. For glucocorticoid-induced TNF receptor family-related protein (GITR) labeling, a biotinylated mAb against m-GITR/TNF receptor SF18 (R&D Systems, Abingdon, U.K.) was used in combination with streptavidin-PE (BD PharMingen). The mAbs used for blocking experiments included anti-IA/IE, anti-H-$2K^d$, anti-H-$2D^d$, anti-CD95, and anti-CD95L (BD PharMingen) and anti-hTGF-$\beta$ and anti-TNF-$\alpha$ (R&D Systems).

### *Cell death by FACS analysis*

Nonirradiated WEHI cells or LPS-activated B cells ($5 \times 10^4$ cells/well in each case) were cocultured for 24–40 h with CTLs ($5 \times 10^4$ cells/well) in 96-well plates. After labeling the APC with FITC- or PE-labeled Abs against CD19, cells were stained with propidium iodide (PI) or annexin V-FITC (BD PharMingen) for 15 min and were analyzed by FACS within 2 h. Alternatively, FITC-labeled cells were fixed, permeabilized, and stained with the TUNEL assay according to the manufacturer's protocol (Roche, Brussels, Belgium), before FACS analysis. Each experiment was conducted in triplicate, and results are expressed as the mean ± SD.

### *Cytokine measurement*

Aliquots of supernatants were collected after 32 h of WEHI$^-$ T cell coculture, with or without p21. IL-10 and TGF-$\beta$ were evaluated using the OptEIA mouse IL-10 ELISA kit (BD PharMingen) and the TGF-$\beta$1 Emax immunoassay system (Promega, Leiden, The Netherlands), respectively. IL-2, IL-4, IL-5, IFN-$\gamma$, and TNF-$\alpha$ levels were analyzed by fluorometry using the mouse Th1/Th2 cytokine CBA kit (BD Biosciences) in combination with a FACSCalibur and CBA software (BD Biosciences). All experiments were performed according to the manufacturer's instructions.

## Results

### *Upon recurrent stimulation, a CD4$^+$ T cell line lost its proliferation capacity and became IL-2 dependent*

In the course of studies of the regulation of immune responses toward Der p 2, an allergen derived from the house dust mite, *Dermatophagoides pteronyssinus*, we immunized BALB/c mice by sc injections of T cell epitope-containing peptides in CFA/IFA. This resulted in strong and specific B and T cell responses. CD4$^+$ T cell lines were derived from regional lymph nodes and were maintained in culture by repeated cyclic stimulation with spleen cells loaded with the corresponding peptides. One of these CD4$^+$ T cell lines, G-121, was specific for a T cell epitope encompassing residues 21–35 of Der p 2, but did not proliferate in the presence of the native protein, Der p 2 (29). Upon cyclic restimulation with a 21–35 synthetic peptide (p21) loaded on APC, G-121 gradually lost its proliferation capacity and became strictly dependent on IL-2 for expansion and survival (Fig. 1*a*). At this stage, cell numbers were only doubled after each stimulation cycle, but cell expansion was maintained for at least 30 cycles.

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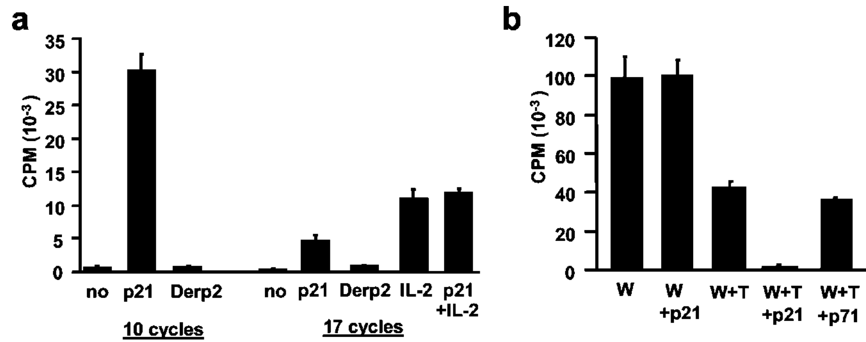


**FIGURE 1.** Proliferation of the G-121 T cell line. *a*, Thymidine incorporation assay showing the proliferation of G-121 T cells on irradiated spleen cells in the absence (no) or the presence of Ag (p21 or Der p 2, both at 18 $\mu$M/ml) and IL-2 (25 U/ml). Cocultures were seeded at a 15/1 APC/T cell ratio and were harvested 72 h later. The characteristics of the T cell line after 10 cycles of expansion are compared with those obtained after 17 cycles. Each bar represents the mean and SD of triplicate wells. *b*, Thymidine incorporation assay of nonirradiated WEHI cells (W) in the absence or the presence of nonproliferating G-121 T cells (T) and specific peptides (p21 and p71, both at 18 $\mu$M/ml). Cocultures were seeded at a 1/1 WEHI/T cell ratio and were harvested 32 h later. Each bar represents the mean and SD of triplicate wells.

*The G-121 CD4$^+$ T cell line lyses an APC line in the presence of its cognate peptide*

The loss of proliferative capacity could be due to either induced properties of the T cell line or lack of appropriate peptide presentation. The interactions between the T cell line G-121 and APC were therefore determined using WEHI cells, a tumor B cell line derived from BALB/c mice and commonly used for its Ag-presenting capacities (30). G-121 did not proliferate in the absence of IL-2. Thymidine incorporation was therefore used as an index of proliferation of nonirradiated WEHI cells. Fig. 1*b* shows that a significant reduction in proliferation was obtained upon addition of G-121. However, when WEHI cells were cultured in the presence of both G-121 and p21, WEHI cell proliferation was abolished. Substitution of p21 by an alternative 15-mer peptide containing another T cell epitope of Der p 2 (p71, residues 71–85) (29) had no effect on proliferation.

These findings were suggestive of a G-121- and Ag-dependent WEHI cell lysis. Inspection of cell cultures after short term incubation with p21 showed that a majority of cells were indeed lysed. Therefore, PI staining of the WEHI-T cell coculture was performed, which confirmed that after 40 h of coculture in the presence of p21, >90% of the CD19$^+$ WEHI cell population stained

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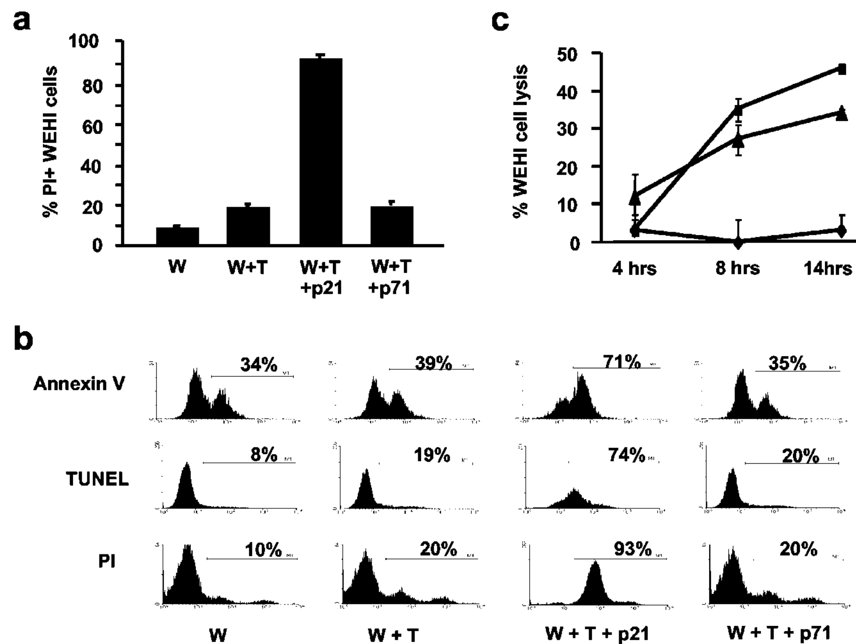


**FIGURE 2.** Apoptosis of WEHI cells following interaction with G-121 T cells. *a*, Evaluation of PI$^+$ WEHI cells (percentage) after coculture of WEHI and G-121 T cells in the presence or the absence of specific peptides (p21 and p71, both at 18 $\mu$M/ml). Cocultures were seeded at a 1/1 ratio and were harvested 40 h later. Cells were labeled with CD19-FITC and PI to assess the percentage of PI$^+$ cells in the CD19$^+$ gate by FACS analysis. Each bar represents the mean and SD of triplicate samples. *b*, Representative FACS profiles of cocultures of CD19$^+$ WEHI cells and CD4$^+$ G-121 T cells at a 1/1 ratio in the absence or the presence of specific peptides (p21 or p71, both at 18 $\mu$M/ml). Histograms of the gated CD19$^+$ WEHI population represent annexin V or TUNEL expression after 24-h coculture and PI expression after 40-h coculture. Numbers express the percentage of positive cells with regard to their negative controls. *c*, Thymidine release assay showing the percentage of WEHI cell lysis at different time points. Cocultures of thymidine-labeled WEHI cells with G-121 T cells at a 1/1 ratio in the absence (♦) or the presence of p21 (18 $\mu$M/ml; ■) are compared with cocultures of G-121 T cells with thymidine-labeled WEHI cells preloaded with p21 (18 $\mu$M/ml) and washed (▲). The percentage of specific lysis was calculated as explained in *Materials and Methods*. Data are the mean and SD of independent calculations from triplicate wells.

PI$^+$ compared with only 20% in the absence of p21 (Fig. 2*a*). Additionally, 24-h cocultures of WEHI cells and G-121 cells were checked for annexin V binding and TUNEL positivity, both specific and early apoptosis markers (31, 32). Addition of p21 to the coculture resulted in a clear increase in the number of annexin V- and TUNEL-positive cells in the CD19$^+$ gate compared with the annexin V binding or TUNEL staining observed in the absence of p21 (Fig. 2*b*). Finally, a thymidine release assay showed that WEHI cells incubated in the presence of G-121 were not killed during a 14-h incubation, but that addition of p21 in the culture medium resulted in 48% cell lysis over the same time period (Fig. 2*c*). The experiment was repeated by preloading WEHI cells with p21, washing, and subsequent addition of G-121. This resulted in a significant lysis of WEHI cells after only 4 h, which steadily increased with time (Fig. 2*c*). Taken together, these results show that the G-121 T cell line lyses APC only in the presence of its cognate peptide. In absence of the latter, the decrease in WEHI cell proliferation, as shown in Fig. 1*b*, is related to a mere reduction in WEHI cell proliferation, rather than to active peptide-specific killing.

FACS analysis of a 32-h coculture of WEHI and G-121 T cells with a specific gate on the surviving cell population revealed that in the presence of p21 almost all CD19$^+$ cells disappeared, while most CD4$^+$ cells remained alive (Fig. 3*a*). Moreover, no peptide-dependent increase in PI and annexin V staining could be observed in the CD4$^+$ population (data not shown). The efficiency of T cell-mediated WEHI cell lysis was further examined in experiments in which the T:E cell ratio varied from 1/1 to 25/1, using an assay system in which p21 was either added to the culture medium or used to load WEHI cells before coculture with the T cell line (Fig. 3*b*). Although the maximum lytic efficiency was observed at a 1/1 ratio in both cases, considerable lysis could still be observed under conditions in which a reduced number of T cells were added (86% at a 1/1 T:E cell ratio vs 69 and 39% at 5/1 and 25/1 T:E cell ratios, respectively)

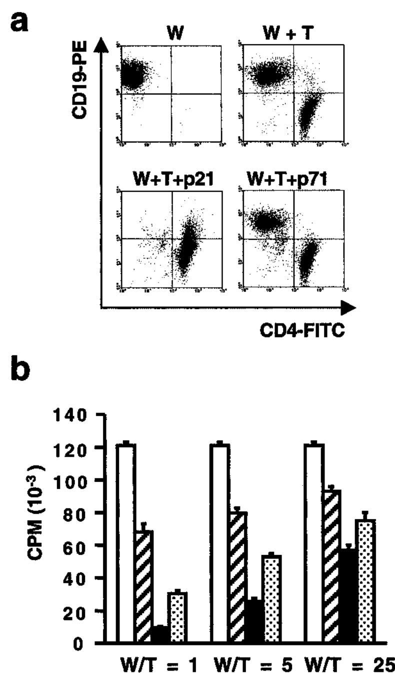


**FIGURE 3.** Efficient lysis of WEHI cells by G-121 T cells. *a*, Representative FACS profiles of cocultures of CD19$^+$ WEHI cells and CD4$^+$ G-121 T cells at a 1/1 ratio in the absence or the presence of specific peptides (p21 or p71, both at 18 $\mu$M/ml). Dot plots represent 32 h cocultures when gated on the surviving population as differentiated on the forward/side scatter plot. *b*, Thymidine incorporation assay of nonirradiated WEHI cells (W) cocultured with nonproliferating G-121 T cells (T) at different T:E cell ratios varying from 1 to 25. Pure WEHI cultures (□) are compared with cocultures of WEHI and T cells in the absence of p21(▨) or in its presence by either addition of p21 (18 $\mu$M/ml) to the medium (■) or preloading WEHI cells by overnight incubation with p21 (18 $\mu$M/ml; ▩). Each bar represents the mean and SD of triplicate wells.

*WEHI cell lysis depends on cognate interaction with p21 bound to MHC class II molecules*

We have previously demonstrated that p21 contained a promiscuous T cell epitope that was able to bind to MHC class II alleles of at least four different mouse haplotypes (33). Ile$^{28}$ in the p21 sequence was shown to act as a major MHC class II anchoring residue for each mouse strain. Fig. 4*a* shows that WEHI cell lysis by the T cell line depends on the dose of added peptide. To further delineate the specificity of the T cell epitope, two mutant peptides were prepared. Substitution of Ile$^{28}$Asn significantly decreased the peptide-dependent lysis of WEHI cells by the T cell line. In contrast, Cys$^{27}$Ser substitution, which has been shown to significantly increase T cell proliferation (B. Wu, unpublished observation), increased the capacity of the T cell line to kill WEHI cells.

Next, a recombinant polypeptide (B4) containing four copies of p21 was used to evaluate WEHI cell survival in the presence of the T cell line. Fig. 4*b* shows that B4-mediated lysis of WEHI cells is significantly enhanced over that obtained with p21. As this 6.5-kDa polypeptide requires processing for effective presentation (V. Carlier, unpublished observation), this result further strengthens the need for MHC class II loading. In the presence of the native protein Der p 2, no lysis was detectable, consistent with the inability of Der p 2 to induce proliferation of G-121 on irradiated APC even at early stages. Lastly, addition of Abs against MHC class II or MHC class I molecules demonstrated that anti-IA/IE Abs partially blocked the cytotoxic interaction between WEHI cells and the G-121 cell line, whereas the mixture of anti-H-2K$^d$ and anti-H-2D$^d$ Abs had no effect (Fig. 4*c*).

The G-121 CD4$^+$ T cell line therefore lyses, in a strictly specific manner, an active APC that has processed and presents the corresponding T cell epitope in the context of MHC class II molecules.

*Cytotoxic CD4$^+$ T cells constitutively express CD25 and do not produce IL-2 following specific activation*

The phenotype of the T cell line was analyzed by FACS (Fig. 5*a*). Ninety-five percent of the cells stained for CD3 and CD4 with a TCR positive for V-$\beta$-chain, subtype 8 (data not shown). After 1 wk of rest on irradiated APC, the T cell line became negative for CD69 and less positive for MHC class II. It remained negative for the activation markers CD40L, CD28, CTLA-4, and CD95L, but continued to be strongly positive for CD25 and ICOS, even after a second week of rest (data not shown). The CD4$^+$ T cell line was negative for CD14 and CD11b, excluding myeloid cells, as well as for a pan-NK marker and CD8, excluding common CTLs.

As G-121 is continuously expressing CD25 independently of its activation status, the cell line may belong to the CD25$^+$ T regulatory (Tr) cell repertoire. Since the cell line does not express GITR and CTLA-4 (Fig. 5*a*), two surface molecules that are deemed to be continuously expressed on naturally occurring regulatory T cells (20), it could represent a new subtype of induced regulatory cells. Moreover, following specific activation with p21, the T cell line generates high levels of TNF-$\alpha$ and IFN-$\gamma$, but no IL-2 (Fig. 5*b*). The absence of IL-2 production combined with IL-2 dependency for growth (Fig. 1*a*) are additional characteristics of CD4$^+$CD25$^+$ regulatory T cells (16, 18). Cytokines such as IL-10 and TGF-$\beta$, which are regarded as major regulatory cytokines (23,

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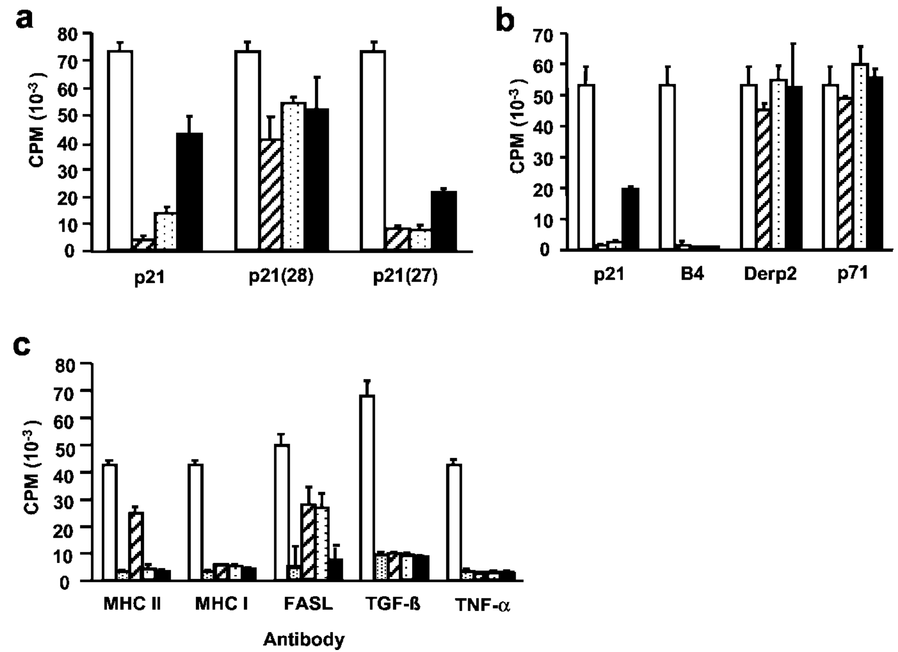



**FIGURE 4.** Cytotoxic CD4$^{+}$ T cells lyse WEHI cells in an Ag- and MHC class II-specific manner. Thymidine incorporation assays representing counts per minute as a parameter for the lysis of nonirradiated WEHI cells by G-121 T cells. Cocultures were seeded at 1/1 T:E cell ratio and were harvested 32 h later. Data shown are the mean and SD from triplicate wells. *a*, p21 and two p21 mutants were added at various concentrations during the coculture (from *left* to *right*: 0, 30, 3, and 0.3 $\mu$M/ml, respectively). *b*, The capacity of p21 to induce WEHI cell lysis in cocultures was compared with those of B4, Der p 2, and another unrelated 16-mer peptide of Der p 2 (p71). Bars represent different concentrations of corresponding proteins (from *left* to *right*: 0, 30, 3, and 0.3 $\mu$M/ml, respectively). *c*, The influence of different blocking Abs to the coculture of T cells is illustrated. □, Cocultures without p21, but with addition of the corresponding Ab at 10 $\mu$g/ml. The other bars represent cocultures in the presence of p21 (18 $\mu$M/ml) with addition of blocking Abs at various concentrations (from *left* to *right*: 0, 10, 1, and 0.1 $\mu$g/ml, respectively).

25), could not be detected, further distinguishing G-121 from other induced regulatory T cells.

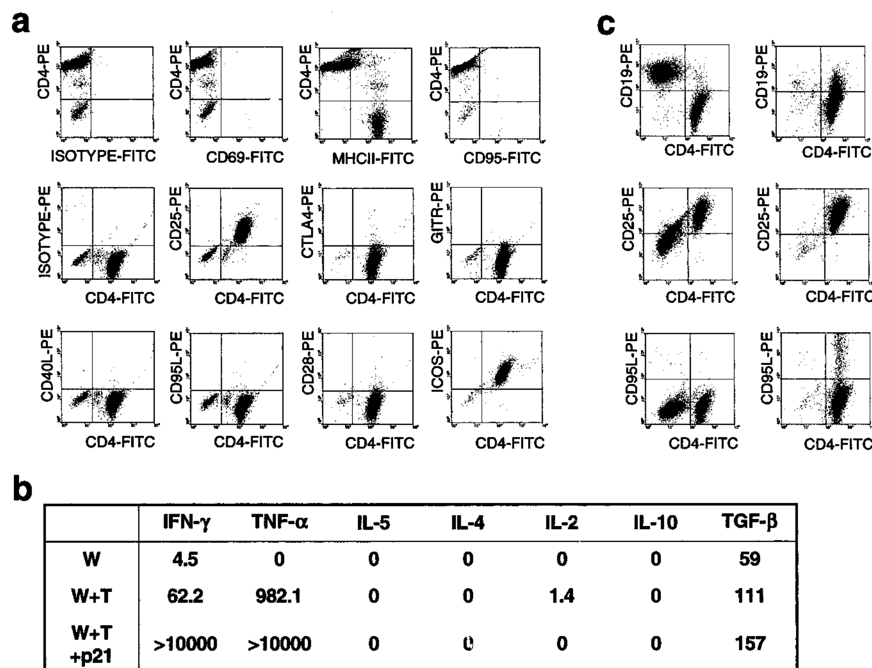
*WEHI cell lysis depends on Fas-FasL interaction*

FACS analysis performed on a 32-h coculture of WEHI cells with the G-121 T cell line showed that the CD19$^{+}$ WEHI cell population is completely destroyed upon addition of p21 (Fig. 5*c*). G-121 constitutively expresses CD25, while the CD95L marker is up-regulated only in the presence of p21, suggesting possible involvement of Fas-FasL interaction in the cytolytic process. This was confirmed by the addition of blocking Abs to CD95L in a WEHI cell proliferation assay, which resulted in a partial restoration of proliferation (Fig. 4*c*).

Some CD4$^{+}$CD25$^{+}$ T cells carry surface TGF-$\beta$, which is potentially involved in regulatory functions (34). TNF-$\alpha$, which is produced by G-121, may act in synergy with Fas-FasL-mediated cytotoxicity (35). Addition of a blocking Ab to TGF-$\beta$ or TNF-$\alpha$ did not, however, restore WEHI cell proliferation (Fig. 4*c*), thereby excluding the involvement of TGF-$\beta$ and TNF-$\alpha$ in the cytolytic process.

*Cytolytic activity is present at early cell line stages and increases during repetitive stimulation, with concomitant IL-2 dependency and loss of proliferation*

We next investigated whether APC lysis required several cycles of in vitro T cell stimulation and/or whether the cytotoxic properties could be observed with other T cell lines. To this end, another CD4$^{+}$ T cell line (G-124) was derived from BALB/c mice by immunization with Der p 2 in CFA, and repeated cyclic stimulations with Der p 2 were conducted as described for G-121. The




| | IFN-γ | TNF-α | IL-5 | IL-4 | IL-2 | IL-10 | TGF-β |
|---|---|---|---|---|---|---|---|
| W | 4.5 | 0 | 0 | 0 | 0 | 0 | 59 |
| W+T | 62.2 | 982.1 | 0 | 0 | 1.4 | 0 | 111 |
| W+T +p21 | >10000 | >10000 | 0 | 0 | 0 | 0 | 157 |

**FIGURE 5.** The cytotoxic CD4$^{+}$ T cells permanently express CD25$^{+}$ and, following activation, up-regulate FasL and produce high levels of TNF-$\alpha$ but no IL-2. *a*, Representative FACS profiles of the G-121 T cell clone after 1 wk of rest on irradiated spleen cells. The CD4$^{+}$ CD25$^{+}$ T cell population is contaminated with a population of irradiated APC expressing only high levels of MHC class II molecules. *b*, Cytokines (picograms per milliliter) assessed after 32 h of coculture of WEHI and G-121 T cells with or without addition of p21. Values are the means of triplicate wells. *c*, FACS profiles of a 32-h coculture of CD19$^{+}$ WEHI cells and CD4$^{+}$ T cells when gated on the surviving population as differentiated on the forward/side scatter plot. Dot plots on the *left* illustrate cocultures in the absence of p21, while those on the *right* were obtained in the presence of p21 (18 $\mu$M/ml).

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G-124 T cell line was assayed at different time points for its capacity to lyse APC in a thymidine incorporation assay for WEHI cell proliferation. Fig. 6*a* shows that WEHI cell proliferation was impaired in the presence of the cognate Ag, but not of a control Ag, HSA. The cytolytic properties were observed after a single cycle of in vitro expansion and were fully expressed after as little as three stimulation cycles. Conversely, proliferation of the T cell line induced by Ag presentation on irradiated APC gradually diminished and became strictly dependent on IL-2 (Fig. 6*b*). The expression of CD25 persisted for at least 10 days of rest following stimulation (data not shown).

These results indicate that the cytotoxicity of the $CD4^+CD25^+$ G-121 cell line is not an artifact of long-term culture, nor is it limited to p21 specificity. As cytotoxicity was also shown in $CD4^+$ cell lines generated against FIS, a 13-mer peptide from sperm whale myoglobulin encompassing a universal T cell epitope (data not shown), we believe that the cytotoxicity of $CD4^+CD25^+$ cells is not restricted to Der p 2 or derived peptides.

### *$CD4^+CD25^+$ CTLs also lyse B cells*

WEHI cells were found to be very susceptible to Fas-mediated cytolysis. Addition of an agonistic Ab against Fas lysed WEHI cells more efficiently than polyclonal LPS-activated B cells, although the expression levels of Fas on both cell types were comparable (data not shown). We therefore evaluated the cytotoxic properties of the G-121 T cell line on polyclonal B cells. Coculture experiments of LPS-activated B cells with nonproliferating cytotoxic G-121 T cells are shown in Fig. 7*a*. The proliferation of LPS-activated B cells was slightly increased in the presence of G-121 cells. However, when p21 was added to the coculture, the proliferation of B cells was completely abolished, while addition of p71, which has no homology with p21, had no effect (Fig. 7*a*, *left panel*). To demonstrate that lysis was independent of soluble peptide, B cells were preloaded with Ag for 12 h and carefully washed before starting the coculture. In accordance with the lysis observed when soluble p21 was added to the coculture, a peptide-specific lysis of preloaded B cells was demonstrated (Fig. 7*a*, *right panel*).

To show that the impaired proliferation of the B-T cell coculture was generated by peptide-specific lysis of the B cell population, 24-h cocultures were subjected to FACS analysis and stained for CD19 and PI. In accordance with our findings for WEHI cells, a significant increase in $PI^+$ B cells was demonstrated only in the

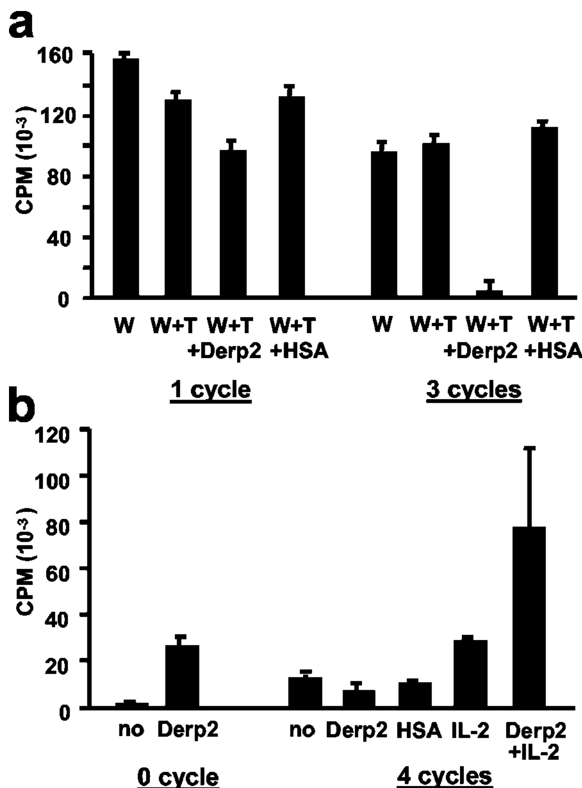


**FIGURE 6.** Proliferation and cytotoxic properties of a T cell line (G-124) of distinct specificity and at early stages. *a*, Thymidine incorporation assay of nonirradiated WEHI cells (W) in the absence or the presence of G-124 T cells (T) and specific proteins (Der p 2 and HSA, both at 18 $\mu$M/ml). Cocultures were seeded at a 1/1 WEHI/T cell ratio and were harvested 32 h later. Each bar represents the mean and SD of triplicate wells. Characteristics of T cells obtained after one cycle of in vitro expansion are compared with those obtained after three cycles. *b*, Thymidine incorporation assay showing the proliferation of G-124 T cells on irradiated spleen cells in the absence (no) or the presence of Ag (HSA or Der p 2, both at 18 $\mu$M/ml) and IL-2 (25 U/ml). Characteristics of the polyclonal T cells are compared with those of T cells obtained after four cycles of in vitro expansion. Each bar represents the mean and SD of triplicate wells. Cocultures were seeded at a 3/1 APC/T cell ratio and a 5/1 APC/T cell ratio for zero and four cycles, respectively, and were harvested 72 h later.

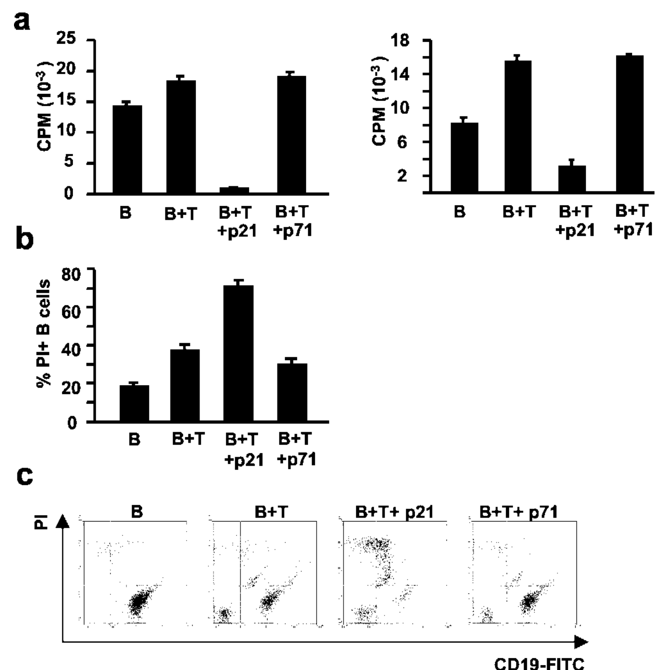


**FIGURE 7.** CTLs lyse B cells. Thymidine incorporation assay showing counts per minute as a parameter for the lysis of LPS-activated B cells. *a*, In the *left panel*, B cells were activated by LPS (50 $\mu$g/ml) for 36 h before starting coculture with T cells. Cocultures were then seeded in the absence of LPS and in the presence or the absence of p21 (18 $\mu$M/ml) and/or p71 (18 $\mu$M/ml) at a 1/1 B cell/T cell ratio and were harvested 48 h later. On the *right*, B cells were activated by LPS (50 $\mu$g/ml) for 36 h, the last 12 h of which B cells were preloaded with peptide p21 or p71 (18 $\mu$M/ml). Cocultures of preloaded B and T cells were then seeded in the absence of soluble peptide and LPS at a 1/1 B/T cell ratio and were harvested 48 h later. Data are the mean and SD of triplicate wells. *b*, Evaluation of $PI^+$ B cells (percentage) after coculture of LPS-activated B cells and G-121 T cells in the presence or the absence of specific peptides (p21 or p71, both at 18 $\mu$M/ml). Cocultures were seeded at a 1/1 ratio and were harvested 24 h later. Cells were labeled with CD19-FITC and PI, and were assessed by FACS for the percentage of $PI^+$ cells in the $CD19^+$ gate. Each bar represents the mean and SD of triplicate samples. *c*, Representative FACS profiles of 24-h coculture of LPS-activated B cells and G-121 T cells at a 1/1 ratio in the absence or the presence of specific peptides (p21or p71, both at 18 $\mu$M/ml).

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presence of p21 (Fig. 7, *b* and *c*). These experiments confirm that the cytotoxic $CD4^+CD25^+$ T cell line lyses primary B cells as well as the WEHI cell line. Finally, lysis of primary DC was also tested. Bone marrow-derived DC obtained after 6 days of culture in the presence of GM-CSF and IL-4 were not lysed by CTLs in the presence of p21. FACS analysis of a 24-h coculture of CD11c and cytotoxic CD4 cells demonstrated that, independently of the addition of T cells or p21, 40% of the CD11c population stained positively for PI (data not shown). Resistance of DC to the cytotoxic effects of G-121 could still be related to this particular subset of DC derived from bone marrow. The underlying mechanisms and interactions with DC are currently under investigation.

### *Ag-specific lysis down-regulates the interaction and proliferation of bystander T cells on B cells*

The cytotoxic G-121 cell line expressed some properties of $CD4^+CD25^+$ regulatory T cells. Because regulatory T cells are characterized by their potential to down-regulate bystander T cell-mediated immune responses, we investigated whether the peptide-specific lysis of APC could prevent the proliferation of adjacent T cells. To address this question, LPS-activated B cells were cocultured with proliferating polyclonal T cells from Der p 2-immunized mice with or without cytotoxic G-121 T cells. To prevent direct T-T cell interaction due to the presence of soluble Ag, B cells were preloaded with Ag for 12 h and carefully washed before starting the coculture. When polyclonal T cells were cocultured on LPS-activated B cells unloaded or preloaded with p21, p71, or with p71 and p21 (both encompassing a major T cell epitope of Der p 2), a nonspecific and an Ag-specific proliferation were shown (Fig. 8). Proliferation in the absence of Ag reflected the growth of B and T cells by CD40-CD40L interaction. The Ag-specific proliferation was observed in three separate experiments and was found to be significant (by Mann-Whitney test, $p < 0.05$). When cytotoxic $CD4^+$ cells were added to the coculture, T cell proliferation on p71-loaded B cells was not affected. However, when B cells were preloaded with peptide 21, proliferation of polyclonal T cells and B cells was significantly reduced, which was not overcome or prevented by loading B cells with p21 and p71 (Fig. 8).

This suppressive effect of the CTLs on adjacent T-B cell interaction demonstrates the regulatory potential of the cytotoxic $CD4^+$ cell line. Because no soluble peptide was added at any time of the experiment, thereby excluding direct T cell-mediated T cell lysis, the down-regulation of polyclonal T cells occurred indirectly via Ag-specific lysis of Ag-presenting B cells.

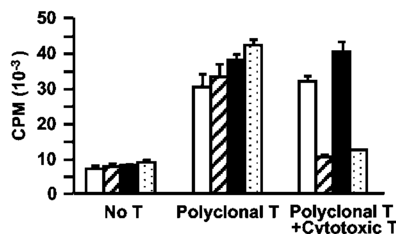


**FIGURE 8.** CTLs down-regulate the proliferation of bystander T cells. Thymidine incorporation assays showing counts per minute as a parameter for the suppression of B-T cell interaction. B cells were activated by LPS (50 $\mu$g/ml) for 36 h, the last 12 h of which B cells were preloaded with no peptide (□), peptide p21(18 $\mu$M/ml; ▨), peptide 71 (18 $\mu$M/ml ■), or the combination of peptide p21 and peptide 71 (18 $\mu$M/ml each; ▩). Cocultures of preloaded B and T cells were then seeded at a 1/1 B/T cell ratio in the absence of soluble peptide and LPS and were harvested 48 h later. The proliferation of B cells without T cells was compared with the proliferation of B cells cocultured with polyclonal T cells that were freshly derived from Der p 2-immunized mice, with or without cytotoxic G-121 T cells. All data are the mean and SD of triplicate wells.

## Discussion

The present data demonstrate that cytotoxic $CD4^+CD25^+$ T cells generated from peripheral T cells of mice immunized with an allopeptide lyse an activated Ag-presenting B cell line as well as polyclonal B cells via a Fas-FasL-dependent mechanism. This occurs in an Ag-specific and MHC class II-restricted manner. Following cognate interaction, $CD4^+CD25^+$ cells up-regulate FasL expression through which they transduce a death signal inducing target APC apoptosis.

Cytotoxic $CD4^+$ T cells have been previously described in mice, with both in vitro and in vivo activity, making use of FasL expression to induce lysis of Fas-sensitive target cells (3, 5, 36). However, most of these T cell clones have been generated under experimental conditions, leaving doubt about their physiological relevance. Moreover, in most of these studies, cytotoxicity could only be shown when an excess of $CD4^+$ cells was added to the APC (1, 2, 4). Instead, we show here that specific stimulation with a soluble peptide derived from a common allergen can generate $CD4^+$ T cells with cytotoxic properties in the early stages of cell line development and at physiological T:E cell ratios. The CTLs described here survive the cytotoxic interaction with Ag-presenting B cells, leaving the potential to further interact with other B cells in the setup of a secondary immune response.

The capacity of $CD4^+$ T cells to lyse WEHI cells increased by Ag-specific cyclic stimulation on irradiated APC in the absence of any exogenous cytokine. Meanwhile, $CD4^+$ T cell lines became unresponsive to stimulation on irradiated APC with high levels of Ag and dependent on IL-2 for growth and survival. Cytotoxicity could not be demonstrated on freshly prepared polyclonal T cells, which does not exclude its presence. It is possible that the cytotoxic effect remained undetected due to the threshold sensitivity of our assay. Upon repetitive in vitro stimulation, the number of specific polyclonal CTLs may have increased with a concomitant detection of Ag-specific cytotoxicity. Another explanation is that by cyclic stimulation the Ag specificity of the T cell line improved, resulting in a better "fitting" of the cognate interaction reaching a certain threshold to induce a cytotoxic signal to APC (37). In accordance with activation-induced cell death, only a high affinity interaction would generate a death signal, thereby down-regulating the immune response (38). Finally, there is evidence showing that the dose of Ag used to stimulate naive $CD4^+$ cells is an important factor in determination of the phenotype and function of $CD4^+$ cells (39): low doses of Ag induce CD40L on the T cell surface with concomitant IgM production upon B cell interaction, while high doses of Ag up-regulate FasL expression on the T cell surface, thereby eliciting B cell apoptosis. In accordance with these findings, high dose and repetitive stimulation of $CD4^+$ cells in vitro might lead to the induction of permanent cytotoxic properties on Ag-specific $CD4^+$ cells.

Increased cytotoxicity was associated with IL-2 dependency. The precise relationship between IL-2 and cytotoxicity remains unclear, although it has been demonstrated that IL-2 induces Fas/FasL-mediated cytotoxicity in $CD4^+$ Th1, but not Th2, clones (40). As regulatory T cells are totally absent in IL-2R-deficient mice, it is obvious that IL-2 signaling is important in the development and function of all subtypes of regulatory T cells (41, 42). Whereas cytotoxic $CD4^+$ T cells described in the literature are not known to express CD25, the IL-2 dependency of our particular cytotoxic $CD4^+$ cells was reflected in the constitutive expression of CD25 independently of activation status, suggesting a role for this subtype in T cell-mediated regulation. Most regulatory T cells

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do express CD25, but the expression is not necessarily associated with regulatory function, as nonregulatory effector cells are also $CD25^+$ during activation. Furthermore, regulatory functions have been demonstrated in $CD4^+CD25^-$ T cell populations (43). The CTLs reported here, however, fulfill the criteria of regulatory $CD4^+CD25^+$ cells insofar as they constitutively express CD25 after a resting period and proliferate only in the presence of IL-2 (15–17). They are activated by TCR recognition and express a cytokine profile that differs from that of Th1 or Th2 cells (14). They do not produce IL-2, but require IL-2 for survival and expansion (18, 44). By lysing APCs in an Ag-specific manner, CTLs have the potential to down-regulate a specific immune response. Moreover, we demonstrated in vitro that activated cytotoxic $CD4^+$ cells could also down-regulate the interaction and proliferation of nonrelated T cells with B cells via the lysis of the Ag-presenting B cells. Bystander suppression of immune responses to unrelated Ags (for instance, p71) is an additional property of regulatory T cells (21, 45).

The specificity of the T cell lines reported here is directed toward a peptide derived from an ubiquitous allergen. The peptide presents no known sequence or structural homology with other proteins or, in particular, with autoantigens. Such an exquisite specificity and absence of homology suggest that the $CD4^+CD25^+$ cells have been induced and are not part of the naturally occurring regulatory T cells selected in the thymus and from which they would have been expanded (46, 47). Besides, surface molecules such as GITR and CTLA-4, which are permanently expressed on naturally occurring regulatory T cells (20), could not be detected on cytotoxic $CD4^+CD25^+$ T cells. Therefore, the cytotoxic $CD4^+CD25^+$ T cells reported here subscribe to a new subtype of induced regulatory cells along with Tr1, Th3, and anergic T cells. Recently, it has been suggested that Tr1 cells are related to Th2 cells and that allergy might develop from failure to develop Tr1 cells or modified Th2 cells (48). Because cytotoxic $CD4^+$ cells preferentially develop in Th1 lineages, we suggest that regulatory cytotoxic $CD4^+$ cells might be the counterpart in Th1 immune responses of Tr1 cells in Th2 responses. Upon specific signaling, a Th1 as well as a Th2 immune response might then be reversed into their specific down-regulatory components, thereby preventing an excessive immune activation.

Fas-FasL interaction is one of the main apoptosis pathways (49), a key mechanism in down-regulating immune responses. The prevention of the cytotoxic activity of the G-121 T cell line by a specific Ab to FasL indicates that Fas-FasL interaction is the main mechanism of acute lysis. To our knowledge this mechanism has never been associated with $CD4^+CD25^+$ regulatory T cells. Naturally occurring $CD4^+CD25^+$ T cells do not down-regulate an immune response by lysis of APC. Upon activation via the TCR, naturally occurring regulatory T cells act through T-T cell interaction in a cytokine- and APC-independent manner. Although the molecular mechanism of this T-T cell contact is not entirely clear, it is not based on deletion (14, 21). However, this does not formally exclude the possibility that $CD4^+CD25^+$ T cells in addition can exert epitope-specific inhibitory effects on APC or B cells. Because naturally occurring regulatory T cells of nonmanipulated mice are polyclonal and self-Ag specific, TCR-transgenic mice are needed to further investigate these questions. We recently cloned the $\alpha$- and $\beta$-chains of the p21-specific TCR to produce such mice.

Although the G-121 T cell line produces large amounts of TNF-$\alpha$ that can act in synergy with Fas-FasL to induce apoptosis (35), the contribution of this cytokine is unlikely, since the addition of blocking anti-TNF-$\alpha$ Abs had no influence on the immediate lysis of APC. However, TNF-$\alpha$ remains an interesting cytokine in this context, because it inhibits cell growth and cytokine production, two properties in common with those of regulatory T cells (50).

With regard to the increasing heterogeneity of the $CD4^+$ T cell population with regulatory properties, one would assume that different populations may coexist in the periphery, where they contribute to the maintenance or induction of tolerance. Because B cells are considered potent APCs in secondary immune responses and because repetitive stimulation with high levels of Ag impaired the proliferation of $CD4^+$ T cells while their cytotoxicity improved, we hypothesize that cytotoxic suppression might be an important down-regulatory mechanism in tolerance induction by high doses of allergen. One report supports this hypothesis by describing the induction in vivo of cytotoxic $CD4^+$ T cells by oral administration of Ag at high doses (51).

In this context, the permanent expression of ICOS on the cytotoxic T cell line while CD28 and CTLA-4 are absent, is of interest. It is known that ICOS is induced on the T cell surface by Ag stimulation and provide costimulatory signals in secondary immune responses, which are less dependent on CD28 than in primary responses (52). Moreover, ICOS is expressed on germinal center T cells located in close proximity to B cells, which seems to promote the collaboration between T and B cells to produce a proper humoral response (53). Because ICOS signaling is essential in T cell-dependent B cell responses (54), it strengthens the hypothesis that $ICOS^+$ $CD4^+CD25^+$ CTLs are important in the down-regulation of secondary immune responses. Besides, ICOS-ICOSL interaction has also been associated with regulatory functions. For instance, it is crucial in the induction and activity of Ag-specific Tr1 cells obtained by respiratory exposure to allergens (55), and the density of its surface expression appears to modulate the production of proinflammatory to regulatory cytokines (56).

These data support the concept that the cytotoxicity of $CD4^+CD25^+$ T cells could be an important down-regulatory mechanism of secondary immune responses. By the lysis of B cells presenting a specific Ag abundantly on their surface, regulatory cytotoxic cells would not only down-regulate the Ag-specific secondary immune response, but also suppress activation of bystander T cells.

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