Interleukin 10 (IL-10) and Viral IL-10 Strongly Reduce Antigen-specific Human T Cell Proliferation by Diminishing the Antigen-presenting Capacity of Monocytes via Downregulation of Class II Major Histocompatibility Complex Expression

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Summary

Interleukin 10 (IL-10) and viral IL-10 (v-IL-10) strongly reduced antigen-specific proliferation of human T cells and $CD4^+$ T cell clones when monocytes were used as antigen-presenting cells. In contrast, IL-10 and v-IL-10 did not affect the proliferative responses to antigens presented by autologous Epstein-Barr virus-lymphoblastoid cell line (EBV-LCL). Inhibition of antigen-specific T cell responses was associated with downregulation of constitutive, as well as interferon γ- or IL-4-induced, class II MHC expression on monocytes by IL-10 and v-IL-10, resulting in the reduction in antigen-presenting capacity of these cells. In contrast, IL-10 and v-IL-10 had no effect on class II major histocompatibility complex (MHC) expression on EBV-LCL. The reduced antigen-presenting capacity of monocytes correlated with a decreased capacity to mobilize intracellular Ca^{2+} in the responder T cell clones. The diminished antigen-presenting capacities of monocytes were not due to inhibitory effects of IL-10 and v-IL-10 on antigen processing, since the proliferative T cell responses to antigenic peptides, which did not require processing, were equally well inhibited. Furthermore, the inhibitory effects of IL-10 and v-IL-10 on antigen-specific proliferative T cell responses could not be neutralized by exogenous IL-2 or IL-4. Although IL-10 and v-IL-10 suppressed IL-1α, IL-1β, tumor necrosis factor α (TNF-α), and IL-6 production by monocytes, it was excluded that these cytokines played a role in antigen-specific T cell proliferation, since normal antigen-specific responses were observed in the presence of neutralizing anti-IL-1, -IL-6, and -TNF-α mAbs. Furthermore, addition of saturating concentrations of IL-1α, IL-1β, IL-6, and TNF-α to the cultures had no effect on the reduced proliferative T cell responses in the presence of IL-10, or v-IL-10. Collectively, our data indicate that IL-10 and v-IL-10 can completely prevent antigen-specific T cell proliferation by inhibition of the antigen-presenting capacity of monocytes through downregulation of class II MHC antigens on monocytes.

Murine IL-10 (m-IL-10)[1] cytokine synthesis inhibitory factor (CSIF) is produced by Th2 cells and inhibits the synthesis of cytokines, particularly of IFN-γ, by Th1, but not Th2 cells (1). This inhibitory activity on cytokine production by IL-10 was found to be indirect and required the presence of APC (1, 2). In addition, it was demonstrated that cytokine production by Th1 clones was affected only when macrophages, but not purified conventional or $Ly1^+$ B cells, were used as APC (2). It was shown that m-IL-10 has multiple biological activities. IL-10 has mast cell growth factor activity in combination with IL-3 and/or IL-4 (3), and T cell growth factor activity on mature and immature mouse thymocytes in the presence of IL-2 and IL-4 (4). In addition, m-IL-10 increased the expression of class II MHC antigens on small murine splenic B cells and enhanced the viability of these cells (5). Furthermore, m-IL-10 was shown to be produced by murine $Ly\text{-}1^+$ B cells and B cell lymphomas and may act as an autocrine growth factor for these cells (6).

Recently, the cDNA encoding for human IL-10 was cloned from a cDNA library made from a tetanus toxin (TT)-specific

[1] *Abbreviations used in this paper:* CSIF, cytokine synthesis inhibitory factor; FMF, flow microfluorimetry; hsp, heat shock protein; m, murine; TT, tetanus toxoid; v, viral.

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$CD4^+$ T cell clone that produced multiple cytokines after activation, including IL-2, IFN-γ, IL-4, and IL-5, indicating that this clone does not fit the Th1 or Th2 subsets (7). Human IL-10 and m-IL-10 exhibit extensive sequence homology to a previously uncharacterized open reading frame in the EBV genome, BCRF-1 (7, 8). It was shown that both IL-10 and the protein product of BCRF-1, designated viral-IL-10 (v-IL-10), exhibited cytokine synthesis inhibitory activity on antigen-specific mouse T cell clones and human PBMC activated by antigen and macrophages or PHA and anti-CD3 mAbs, respectively (7, 9). However, v-IL-10 had little murine mast cell stimulatory activity or thymocyte growth factor activity, and it did not affect class II MHC expression on mouse splenic B cells (3, 5).

IL-10 affected cytokine synthesis of murine Th1 clones, but not that of Th2 clones, and the effects of IL-10 on murine Th0 clones, which are able to produce IL-2, IL-4, IL-5, and IFN-γ (10), have not yet been evaluated. Human $CD4^+$ T cell clones generally do not display the strict Th1 and Th2 lymphokine production profiles observed for murine $CD4^+$ T cell clones (11, 12), and are, in this aspect, more comparable to murine Th0 cell clones. However, recently we have shown that $CD4^+$ T cell clones specific for the house dust mite–derived allergen *Der p* I isolated from atopic patients produce very high quantities of IL-4 and IL-5, whereas the levels of IFN-γ and IL-2 production varied from low to normal (Yssel et al., manuscript submitted for publication). On the other hand, $CD4^+$ T cell clones specific for the 65-kD heat shock protein (hsp) of *Mycobacterium leprae (M. leprae)* had a "Th1-like" lymphokine profile, since these clones produced high levels of IFN-γ and very low, or sometimes even undetectable, levels of IL-4 and IL-5 (13).

In the present study, the effects of IL-10 and v-IL-10 on antigen-specific human T cell responses were investigated. It was shown that IL-10 and v-IL-10 strongly blocked the antigen-specific proliferative responses of both T cells and $CD4^+$ Th1- or Th2-like T cell clones. The inhibitory effects on antigen-specific T cell proliferation were only observed when monocytes, but not when EBV-LCL, were used as APC. In addition, it is shown that IL-10 and v-IL-10 have strong downregulatory effects on both constitutive and IL-4- or IFN-γ-induced class II MHC expression on monocytes, indicating that the strongly reduced proliferative T cell responses towards antigens are due to inhibition of antigen-presenting capacity of monocytes through downregulation of class II MHC antigens on these cells.

Materials and Methods

Cells and Reagents. The TT-specific T cell clone 827 (14), the T cell clones NP12, NP14, and NP44, which are specific for the major allergen in house dust, *Der p* I (Yssel et al., manuscript submitted for publication), and the 65-kD *M. leprae* hsp-specific T cell clones HY06, Rp1511, R2F10, and CAAp1515 (13, 15) have all been described previously. T cell clones (2×10^5/ml) were stimulated at 2-wk intervals by a feeder cell mixture consisting of 10^6/ml irradiated (4,000 rad) allogeneic PBL, 10^5/ml irradiated (5,000 rad) cells of the EBV-transformed B cell line (EBV-LCL) JY, and 0.1 μg/ml purified PHA (Wellcome Diagnostics, Beckenham, Kent, UK) as described (14) and expanded by addition of rIL-2. The EBV-LCL HSY, which was established from the same donor as T cell clones 827 and HY06 (14), NPR, which was derived from the same donor as T cell clones NP12, NP14, and NP44 (Yssel et al., manuscript submitted for publication), and PZB (15), which was HLA-DR identical to the tuberculoid leprosy patient from whom the T cell clones Rp1511 and R2F10 were derived, have been described. T cell clones and EBV-LCL were cultured in Yssel's medium (14) supplemented with 1% human AB^+ serum. TT was generously provided by Dr. Bizzini (Institute Pasteur, Paris, France). The *Der p* I cDNA clone was kindly provided by Dr. W. Thomas (Princess Margaret Hospital, Perth, Australia). Recombinant *Der p* I antigen was expressed in *Escherichia coli* and purified (16). *M. leprae* antigen (CD104) was a kind gift of Dr. R. J. W. Rees (London, UK). The *Der p* I–specific peptide (amino acids 89–117) and *M. leprae* 65-kD hsp peptides (amino acids 3–13 and 418–427) were made by solid phase peptide synthesis and checked by analytical reverse phase HPLC and amino acid analysis (17). *M. leprae*–specific peptides were kindly provided by Dr. D. C. Anderson (University of Washington, Seattle, WA).

Recombinant IL-10 and v-IL-10 were expressed in *E. coli* and purified (16). Purified human r-IL-4 and IFN-γ were provided by Schering-Plough Research (Bloomfield, NJ). The neutralizing mAb 19F1 recognizing both h-IL-10 and v-IL-10 has been described (J. Abrams et al., manuscript in preparation).

Isolation and Culture of Human Monocytes. Human peripheral blood monocytes were isolated from 500-ml blood samples from normal donors as described previously (18, 19). The monocyte preparation was over >95% pure, as judged by nonspecific esterase staining, and contained >98% viable cells. Monocytes were cultured in Yssel's medium (14) containing HSA supplemented with 1% pooled heat-inactivated human AB^+ serum. This culture medium was endotoxin free as determined by the Limulus amebocyte lysate assay (< 0.2 ng/ml endotoxin). The monocytes were cultured at a concentration of 4×10^6 cells/ml in teflon bags (Jansen MNL, St. Niklaas, Belgium) and, where indicated, IFN-γ (100 U/ml), IL-4 (100 U/ml), or TT (0.5 μg/ml) was added.

Proliferation Assays. Cloned T cells were used 9–12 d after stimulation with feeder cells. T cells (2×10^4 cells/well were incubated with irradiated (5,000 rad) autologous EBV-LCL (2×10^4 cells/well) or HLA-DR-matched human monocytes (10^4/well) in the presence of antigen or antigenic peptides in 200 μl in round-bottomed (Linbro; Flow Laboratories, McLean, VA) or flat-bottomed plates (Falcon; Becton Dickinson & Co., Lincoln Park, NJ), respectively. TT was added at a concentration of 0.5 μg/ml, *M. leprae* antigen (CD104) at 1 μg/ml, peptide amino acids 3–13 or peptide amino acids 418–427 of 65-kDa *M. leprae* hsp at 0.5 μg/ml, *Der p* I and the *Der p* 1–derived peptide 89–117 at concentrations ranging from 10 to 0.01 μg/ml. Cells were incubated for 72 h at 37°C and 5% CO_2, pulsed with [^{3}H]TdR for 4 h, and harvested as described previously (14). The results are expressed as cpm of [^{3}H]TdR incorporation and represent the mean of triplicate cultures.

Immunofluorescence Analysis. Cells (10^5) were incubated in V-bottomed microtiter plates (Flow Laboratories) with 10 μl of purified mAb (1 mg/ml) for 30 min at 4°C. After two washes with PBS containing 0.02 mM sodium azide and 1% BSA (Sigma Chemical Co., St. Louis, MO), the cells were incubated with 1:40 dilution of FITC-labeled $F(ab')_2$ fragments of goat anti-mouse antibody (Tago, Inc., Burlingame, CA) for 30 min at 4°C. After three additional washes, the labeled cell samples were analyzed by flow microfluorimetry (FMF) on a FACScan® (Becton Dickinson & Co.,

Sunnyvale, CA). The anti-MHC class II mAbs PdV5.2 (HLA-DR/DP/DQ) (20), Q5/13 HLA-DR/DP (21), SPV-L3 (HLA-DQ) (14), and anti-CD3 (SPV-T3b) (14) were described previously).

Ca^{2+} Flux Measurements. T Cells at 10^7/ml were loaded with Indo-1 by incubation with its acetoxymethyl ester (Molecular Probes, Junction City, OR) at a concentration of 1 μM for 45 min at 37°C. Cells were washed once and resuspended in fresh medium at 10^6 cells/ml and stored at room temperature in the dark until tested. Calcium levels were measured by FMF with a FACStar® plus (Becton Dickinson & Co.) equipped with a Coherent Innova 90 argon ion laser emitting 200 mW at 351/364 nm. Violet fluorescence emission (Ca^{2+} bound Indo-1) was detected through a 20-nm band pass 405-nm filter (Omega Optical, Brattleboro, VT). Blue fluorescence emission (free Indo-1) was measured through a 22-nm bandpass 485-nm filter (Omega Optical) after separation with 50:50 beam splitter. Cells were analyzed at 37°C at typical rates of 900 cells.

Results

h-IL-10 and v-IL-10 Inhibit Antigen-specific Proliferation of T Cells. To determine the effect of IL-10 on antigen-specific proliferation of human T cells, purified T cells obtained from donors immunized with TT were stimulated with TT and autologous purified monocytes. In Fig. 1, it is shown that both IL-10 and v-IL-10 inhibited TT-specific proliferative responses of the T cells in a dose-dependent fashion. Significant inhibitory effects were already observed at IL-10 concentrations of 10 U/ml, whereas maximal inhibition was obtained at 100 U/ml. These results indicate that specific proliferative responses of human T cells to soluble antigen are reduced by IL-10 and v-IL-10 when monocytes are used as APC. Furthermore, it is shown in Table 1 that preincubation of the monocytes for 24 h with IL-10 (100 U/ml) or v-IL-10 (100 U/ml), followed by incubation of these cells in the presence of purified T cells and TT (in the absence of IL-10), resulted in 50% reduction in the proliferative responses, as compared to the TT-specific proliferative responses of these T cells when cocultured with monocytes preincubated in medium alone. Addition of IL-10 or v-IL-10 to the cultures of T cells, TT, and monocytes preincubated with IL-10 or v-IL-10 abolished the TT-specific proliferative responses of purified T cells almost completely. These results indicate that IL-10 primarily acts on the antigen-presenting capacity of monocytes.

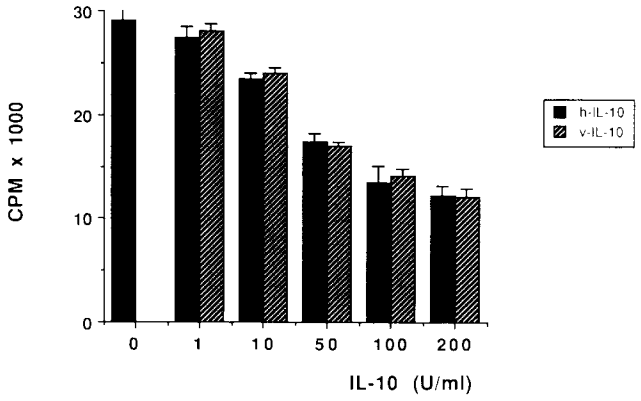

Figure 1. The effects of IL-10 and v-IL-10 on the proliferative responses of peripheral blood T cells to TT presented by autologous monocytes. Lymphocytes (5 × 10^4/well) and monocytes (2 × 10^4/well) were isolated by centrifugal elutriation and cultured in the presence of increasing concentrations of IL-10 and v-IL-10 for 72 h.

Table 1. *Tetanus Toxoid-specific Proliferative Responses of Peripheral Blood T Cells upon Culture with Autologous Monocytes, Preincubated with IL-10 and v-IL-10*

Culture condition	[^{3}H]TdR incorporation		
	–	+ IL-10	+ v-IL-10
	cpm × 10^{-3}		
T cells	3.4	1.4	1.3
T cells + monocytes	29.1	13.5	12.9
T cells + monocytes (preincubated in medium)	49.5	14.2	13.9
T cells + monocytes (preincubated with IL-10)	23.4	6.1	6.8
T cells + monocytes (preincubated with v-IL-10)	22.8	5.6	7.1

Peripheral blood T cells and monocytes were isolated by centrifugal elutriation and cultured in the absence or presence of IL-10 (100 U/ml) and v-IL-10 (100 U/ml). Monocytes (2 × 10^4/well) were added directly to the T cells (5 × 10^4/well), or after preincubation for 24 h in medium, IL-10 (100 U/ml), or v-IL-10 (100 U/ml). TT was added at 0.5 μg/ml).The SD was <10% in all tests.

IL-10 and v-IL-10 Reduce Proliferative Responses of Antigen-specific T Cell Clones. IL-10 and v-IL-10 also inhibited in a dose-dependent way the proliferative responses of the TT-specific T cell clone 827, the *Der p* I-specific T cell clone NP14, and the T cell clone HY06, which is specific for the antigenic peptide 3–13 of the 65-kD *M. leprae* hsp, when monocytes were used as APC. As shown in Fig. 2, 10 U/ml of IL-10 or v-IL-10 was sufficient to reduce antigen-specific responses by 50–70%, and maximal inhibition was observed at 100 U/ml. However, IL-10 and v-IL-10 did not affect antigen-specific proliferative responses of these and several other antigen-specific CD4$^+$ T cell clones when autologous EBV-LCL were used as APC. The anti-IL-10 mAb 19F1 completely neutralized the reduced responsiveness observed in the presence of IL-10, demonstrating the specificity of the inhibition (Table 2). IL-10 and v-IL-10 also inhibited the proliferative responses towards antigenic peptides that do not require antigen processing as shown (Fig. 1, Table 2). The proliferation of the *Der p* I-specific T cell clones NP12, NP14, and NP44 in response to both the whole *Der p* I and the *Der p* I-derived peptide 89–117 was strongly reduced by IL-10 and v-IL-10. Similar results were obtained with T cell clone Rp1511, which is specific for the 65-kD hsp of *M. leprae*. Proliferation of this clone in response to both the whole *M. leprae* antigen, or the 65-kD hsp derived peptide 3–13 is blocked for 90% by IL-10 (100 U/ml) and v-IL-10 (100 U/ml). The prolifera-

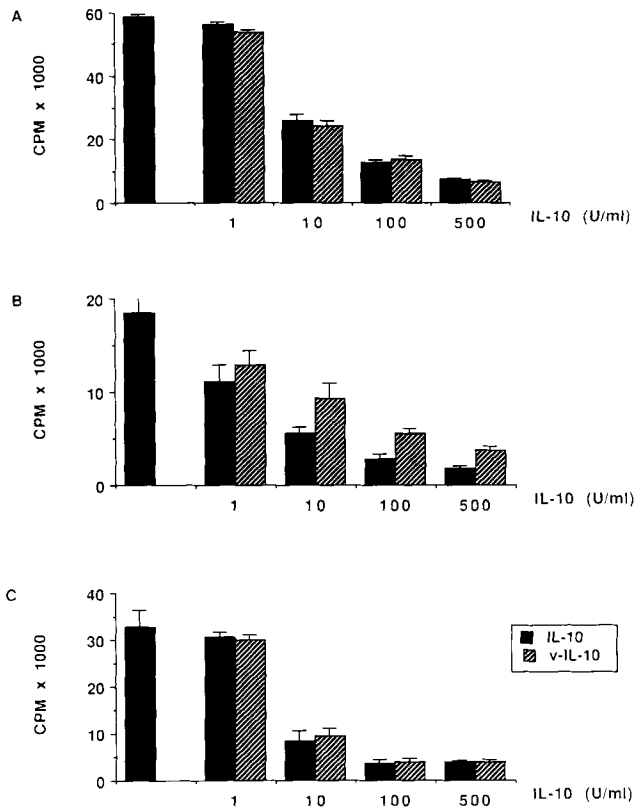

Figure 2. The effects of IL-10 and v-IL-10 on the proliferative responses of CD4+ T cell clones to antigens presented by HLA-DR-matched monocytes. T cell clones (2 × 10^4/well) and HLA-DR-matched monocytes (10^4/well) were cultured for 72 h in the presence of increasing concentrations of IL-10 or v-IL-10. (*A*) TT-specific T cell clone 827 and HLA-DR3+ monocytes. (*B*) *Der p* I-specific T cell clone NP14 and HLA-DRw11+ monocytes. (*C*) *M. leprae*-specific T cell clone HY06 and HLA-DR3+ monocytes.

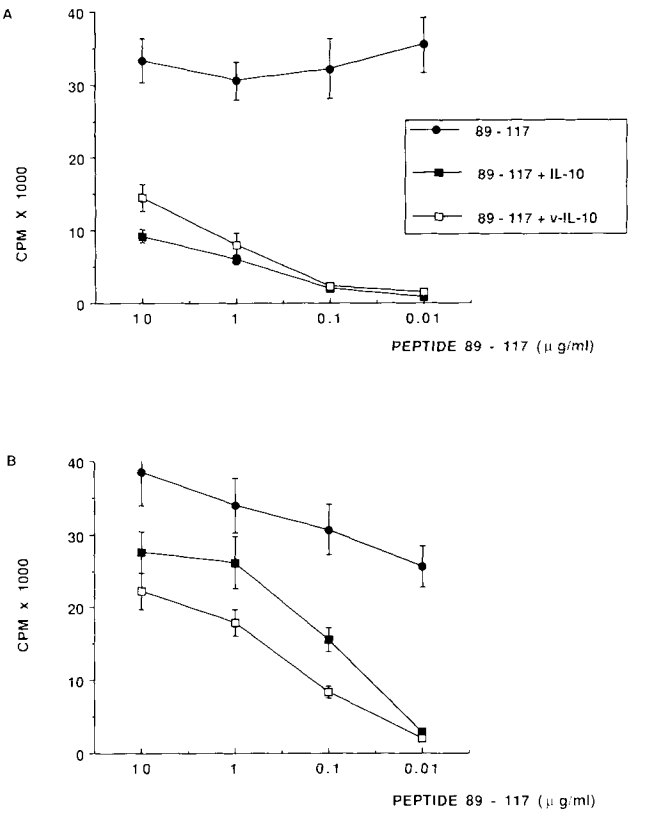

Figure 3. Proliferative responses of the *Der p* I-specific CD4+ T cell clones NP14 and NP44 to different concentrations of antigen presented by HLA-DR-matched monocytes in the presence of IL-10 and v-IL-10. T cell clones (2 × 10^4/well) NP-14 (*A*) and NP-44 (*B*) and HLA-DRw11 monocytes (10^4/well) were cultured in the presence of IL-10 (100 U/ml) or v-IL-10 (100 U/ml) at 10, 1, 0.1, and 0.01 μg/ml of the *Der p* I-derived peptide 89–117 for 72 h.

tive responses of the CD4+ T cell clones HY06, CAAp1515, and R2F10, which are specific for the 65-kD hsp-derived peptides 3–13 or 418–427 and were established from different donors, were also strongly inhibited by IL-10 and v-IL-10. Again, IL-10 and v-IL-10 had no affect on the antigen-specific proliferative responses when the peptides were presented by autologous EBV-LCL.

The Inhibition of T Cell Proliferation by IL-10 and v-IL-10 Is Dependent on Antigen Concentration. In Fig. 3, it is shown that the degree of reduction of the proliferative responses of the *Der p* I-specific T cell clones NP14 and NP44 by IL-10 and v-IL-10 depended on the antigen concentration. NP14 and NP44 activated by the *Der p* I-derived peptide 89–117 at concentrations varying from 0.01 to 10 μg/ml, and monocytes as APC showed the same degree of proliferation (Fig. 3). Although IL-10 and v-IL-10 significantly reduced the proliferative responses at peptide concentrations of 10 μg/ml, the reduction of the proliferative responses increased further at lower peptide concentrations. More than 90% reduction of the proliferative responses was observed when the *Der p* I-derived peptide was used at 0.01 μg/ml, a concentration that still resulted in maximal proliferative T cell responses. These results indicate that the reduced proliferation observed in the presence of IL-10 is particularly pronounced when the antigen concentration becomes more limited.

IL-10 and v-IL-10 Strongly Downregulate Class II MHC Membrane Expression on Monocytes but Not on EBV-LCL. The results obtained thus far suggest that IL-10 and v-IL-10 reduce the effectiveness of monocytes as APC. Antigen-presenting capacity of APC has been shown to depend, among other factors, on the density of class II MHC molecules accommodating the relevant antigenic peptides (22). Therefore, the effects of IL-10 and v-IL-10 on constitutive HLA-DR and HLA-DQ expression on monocytes were investigated. HLA-DR expression on human monocytes increases during culture of these cells, even after incubation in teflon culture bags, which prevents adhesion of the cells (Fig. 4) (23). In Fig. 4, it is furthermore shown that IL-10 strongly downregulated this increased HLA-DR expression on human monocytes. IL-10 (100 U/ml) reduced the constitutive HLA-DR expression by 90% after 40 h of culture. These results were confirmed by using several anti-class II MHC mAbs, specific for HLA-DR/DP or HLA-DR alone (not shown). Dose-response studies indicated that inhibitory effects on HLA-DR expression were already observed at IL-10 concentrations of 1 U/ml. The con-

Table 2. *Proliferative Responses of CD4⁺ Human T Cell Clones to Antigen Presented by HLA-DR-matched Monocytes or Autologous EBV-LCL in the Presence of IL-10 or v-IL-10*

T cell clone	Antigen stimulation	[³H]TdR Incorporation						
		Monocytes				EBV-LCL		
		–	v-IL-10	IL-10	IL-10 + αIL-10	–	v-IL-10	IL-10
		cpm × 10⁻³						
827	TT	20.1	4.4	6.1	18	8.3	8.4	8.7
NP12	r-*Der* p I	60.1	15.2	18.5	57.5	30.5	29.4	29.5
	89-117	60.1	16.8	18.5	57.5	65	59	60
NP14	r-*Der* p I	20.5	4.5	4.0	22.1	15.5	16.3	13
	89-117	35.7	2.9	3.5	33.5	12.5	12.9	14.1
NP44	r-*Der* p I	25.9	3.7	3.2	22.9	14.5	12.3	12.5
	89-117	35.1	5.1	5.0	36.2	20.5	21.9	18.7
RP1511	*M. leprae*	79.8	11.4	12.8	ND	ND	ND	ND
	2-12	82.9	9.4	8.9	ND	54.2	52.8	54.3
HY-06	2-12	22	1.0	1.2	20	8.7	8.1	9.1
CAAP1515	2-12	9	.7	.5	ND	7	6.2	5
R2F10	2-12	46	5.8	6	ND	150	146	160

T cell clones (2 × 10⁴/well) and monocytes (10⁴/well) or autologous EBV-LCL (2 × 10⁴) were cultured in the presence of IL-10 (100 U/ml) or v-IL-10 (100 U/ml) for 72 h. Neutralizing anti-IL-10 mAb 19F1 was added at a concentration of 10 μg/ml. r-*Der* p I and the *Der* p I–derived peptide 89-117 were added at concentrations of 1 μg/ml. *M. leprae* and *M. leprae* 65-kD hsp-derived peptide 3-13 were added at 1 and 0.5 μg/ml, respectively. The SD was <10% in all tests.

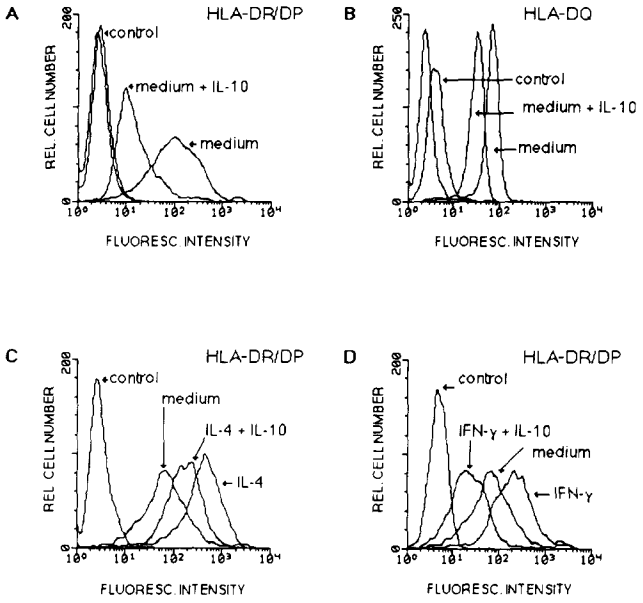

Figure 4. Effects of IL-10 on HLA-DR/DP and HLA-DQ expression on monocytes. Monocytes (4 × 10⁶/well) were cultured in the absence or presence of 100 U/ml IL-10 in medium (*A* and *B*), IL-4 (100 U/ml) (*C*), or IFN-γ (100 U/ml), (*D*) for 40 h and analyzed for expression of (*A*) HLA-DR/DP (Q5/13), (*B*) HLA-DQ (SPV-L3), (*C*) HLA-DR/DP, and (*D*) HLA-DR/DP.

stitutive expression of HLA-DQ, which is much lower than that of HLA-DR, was also strongly downregulated by IL-10. Identical results on downregulation of class II MHC expression were obtained with v-IL-10 (not shown). Interestingly, IL-10 and v-IL-10 also strongly blocked the enhanced HLA-DR and HLA-DQ expression induced by IL-4 (100 U/ml) or IFN-γ (100 U/ml). The dose-response curves shown in Fig. 5 for HLA-DR indicate that IL-10 seems to be more effective in counteracting the class II MHC enhancing effect of IFN-γ than that of IL-4. In contrast, IL-10 and v-IL-10 failed to affect constitutive HLA-DR and HLA-DQ expression on various EBV-LCL, as shown for NPR in Fig. 6.

IL-10 Affects the Capacity of Monocytes to Present Antigens as Reflected by Reduced Ca^{2+} Fluxes in the T Cell Clones. If the reduction in class II MHC expression on monocytes by IL-10 accounts for the strongly reduced antigen-specific proliferative responses, this would imply that we are dealing with a reduced capacity to induce T cell activation. If this conclusion is correct, reduced stimulatory capacity of monocytes as APC should be reflected in reduced induction of Ca^{2+} mobilization in the responding T cell clones. In Fig. 7, it is shown that Ca^{2+} fluxes induced in T cell clone 827 were suppressed by ~70% when monocytes preincubated with TT and IL-10 were used to activate the T cell clone. Ca^{2+} fluxes induced by an anti-CD3 mAb were used as con-

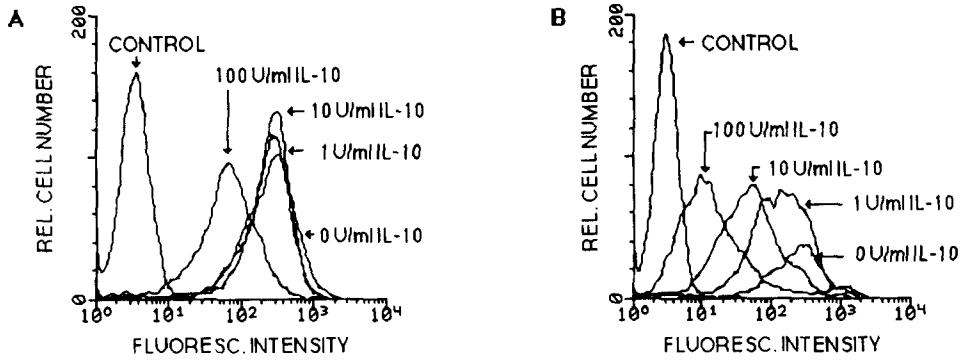

Figure 5. Effects of IL-10 on HLA-DR/DP and HLA-DQ expression on human monocytes cultured in the presence of IL-4 or IFN-γ. Monocytes (4 × 10^6/well) were cultured in the presence of (*A*) IL-4 (100 U/ml), or (*B*) IFN-γ (100 U/ml), and 0, 1, 10, and 100 U/ml IL-10, and analyzed for expression of HLA-DR/DP (Q5/13).

trols. In the presence of IL-10, but in the absence of monocytes, no changes were observed in the Ca^{2+} fluxes of the T cell clone in response to anti-CD3 mAbs. However, when monocytes, preincubated with TT in the presence of IL-10, were added to the T cell clones, strong reductions in both the plateau levels of the Ca^{2+} fluxes and the percentage of positive cells were observed, as compared to the Ca^{2+} fluxes induced by monocytes that were preincubated with TT alone. It has to be noted that Ca^{2+} fluxes could be measured at the earliest 3–4 min after addition of the monocytes to the T cells, because a 2-min centrifugation step was required in order to establish optimal T cell/APC contacts.

Reduced Antigen-specific T Cell Proliferation Induced by IL-10 and v-IL-10 Cannot be Neutralized by Exogenous IL-2 or IL-4. The results thus far suggest that the reduced proliferative responses of the T cell clones observed in the presence of IL-10 and v-IL-10 are related to a reduced capacity of monocytes to present antigen, which leads to an incomplete activation of the T cell clone. Since IL-10 has cytokine synthesis inhibitory (CSI) activity (de Waal Malefyt et al., manuscript in preparation), the effects of IL-2 and IL-4 on the inhibition of antigen-specific proliferative responses were examined. In Fig. 8, it is shown that IL-10 and v-IL-10 inhibited the antigen-specific response of T cell clone 827 to TT with monocytes as APC by 85–90%. The addition of increasing amounts of IL-2 to these cultures resulted in enhanced proliferative responses, both in the presence or absence of IL-10, until a plateau was reached at concentrations of 1,000 U/ml. Addition of IL-2 at concentrations of up to 100 U/ml, which is sufficient to saturate high affinity IL-2Rs, had no effect on the inhibition of antigen-specific T cell proliferation by IL-10. Even at IL-2 concentrations of >1,000 U/ml, still 30–40% inhibi-

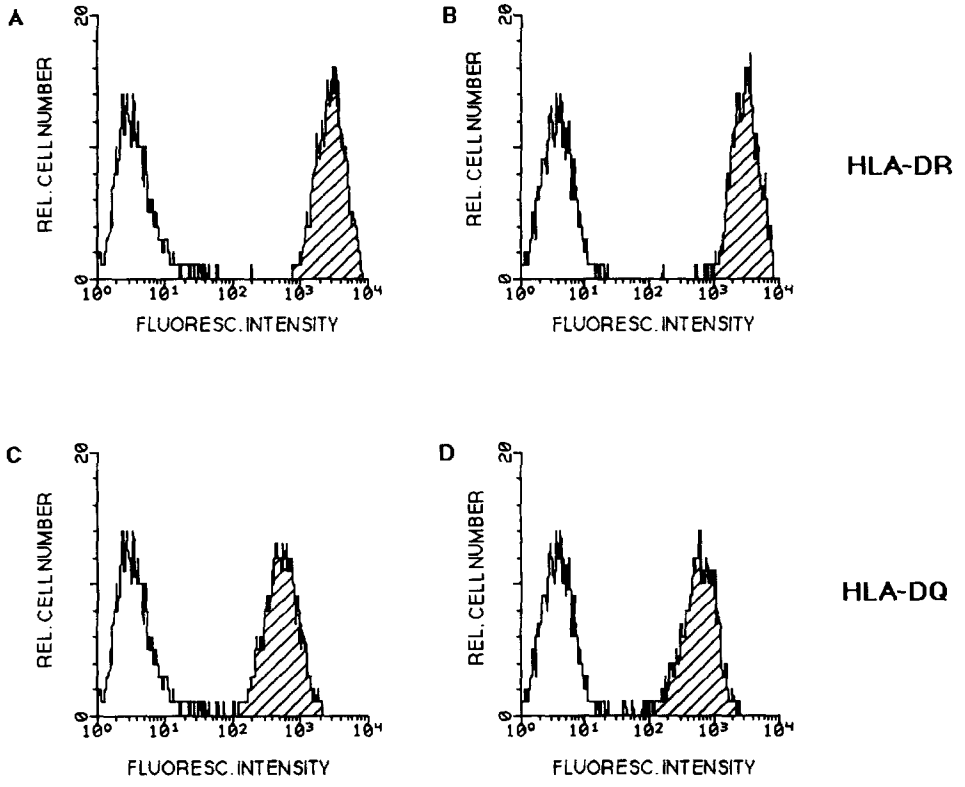

Figure 6. The effects of IL-10 on HLA-DR/DP and HLA-DQ expression on EBV-LCL. EBV-LCL NPR (5 × 10^5/well) were cultured in the absence (*A* and *C*), or in the presence (*B* and *D*) of IL-10 (100 U/ml) and analyzed for expression of (*A* and *B*) HLA-DR (Q5/13) and HLA-DQ (SPV-L3).

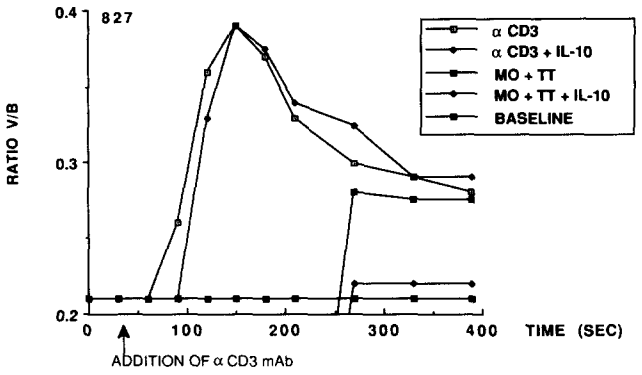

Figure 7. Induction of Ca^{2+} fluxes in T cell clone 827 by anti-CD3 mAb and to antigen presented by monocytes, preincubated with IL-10. Intracellular Ca^{2+} concentrations were measured in T cell clone 827 in response to anti-CD3 mAb, anti-CD3 mAb + IL-10 (100 U/ml), monocytes preincubated with TT (0.5 μg/ml), and monocytes preincubated with TT and IL-10 (100 U/ml). Anti-CD3 mAbs were added at 0 min. T cells and monocytes were centrifuged for 2 min to establish cell-cell contacts, gently resuspended, and analyzed on the FACS®.

tion was observed. These results indicated that inhibition of IL-2 production, due to the CSI activity of IL-10, could only partially account for the inhibition of antigen-specific proliferation of T cell clone 827. Furthermore, it is shown in Fig. 8 that monocytes and high concentrations of IL-2 were able to induce proliferation of the T cell clones in the absence of antigen. These responses were also inhibited by IL-10. However, the proliferative responses of T cell clone 827 to IL-2 in the absence of monocytes were, as expected, not affected by IL-10. Identical results were obtained with v-IL-10 (not shown). Addition of increasing amounts of IL-4 to the T cell clone 827 activated by TT and monocytes as APC completely failed to affect the antigen-specific proliferative responses, either in the absence or in the presence of IL-10 and v-IL-10 (not shown). This indicated that IL-4 was not involved in the IL-10-mediated inhibition of antigen-specific proliferation of this T cell clone.

Expression of IL-2Rα or IL-2Rβ chains of T cell clones that were cultured for up to 72 h in the presence of IL-10 or v-IL-10 was not modulated (not shown). Collectively, these data indicate that reduced antigen-presenting capacity of monocytes caused by IL-10-mediated downregulation of class II MHC expression leads to incomplete activation of the T cell clones that can only be partially rescued by very high concentrations of exogenous IL-2, but not by IL-4.

Discussion

IL-10 and its viral counterpart, v-IL-10, can completely prevent the specific proliferative responses of $CD4^+$ T cells and $CD4^+$ T cell clones towards various protein antigens and antigenic peptides. Reduced antigen-specific proliferative responses were observed only when monocytes, and not when autologous or allogeneic EBV-LCL carrying the appropriate restriction elements, were used as APC. These results are in line with observations made in murine systems where Fiorentino et al. (2) showed that IL-10 inhibited IFN-γ production by mouse T helper cell clones when monocytes, but not when B cells, were used as APC. Interestingly, the reduced responsiveness was partially restored by exogenous IL-2, but only at very high concentrations of >1,000 U/ml, suggesting that the reduced proliferation observed in the presence of IL-10 and v-IL-10 under the present culture conditions was not a mere consequence of inhibition of endogenous IL-2 production, due to CSI activity of IL-10 or v-IL-10. This observation is compatible with murine studies that have indicated that m-IL-10 (CSIF) had only weak inhibitory effects on IL-2 production by Th1 cells after activation by antigen presented by macrophages (1). In addition, these studies showed that m-IL-10 still suppressed the production of IFN-γ by Th1 clones when these experiments were performed in the presence of saturating quantities of IL-2 (1). IL-10 and v-IL-10 also re-

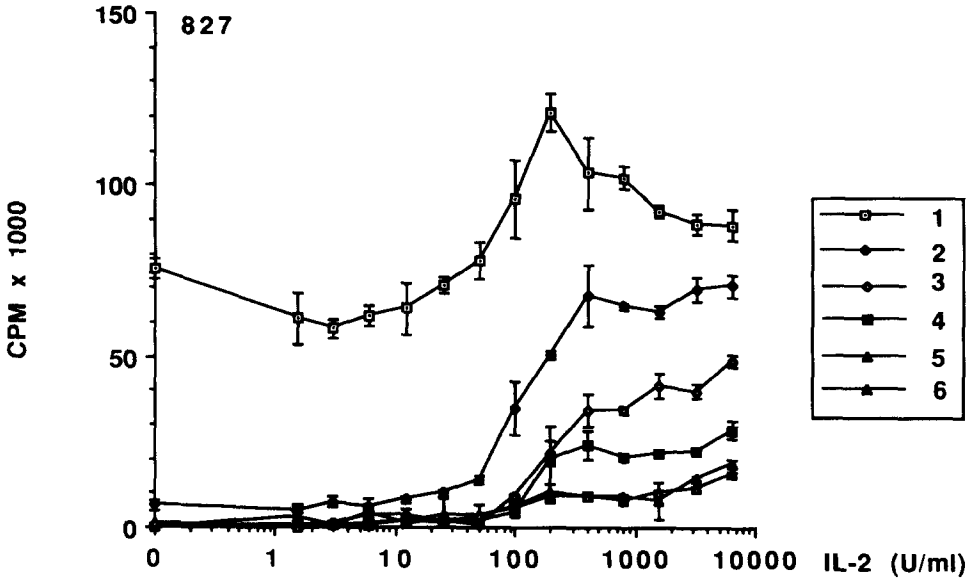

Figure 8. Effects of IL-2 on the reduced antigen-specific proliferative responses of $CD4^+$ T cell clone 827 induced by IL-10. T cell clone 827 (2×10^4/well) was cultured with increasing amounts of IL-2 under the following conditions: (*1*) with monocytes (10^4/well) and TT (0.5 μg/ml); (*2*) with monocytes, TT, and IL-10 (100 U/ml); (*3*) with monocytes in the absence of TT; (*4*) with monocytes and IL-10 in the absence of TT; (*5*) with TT in the absence of monocytes; and (*6*) with TT and IL-10 in the absence of monocytes.

duced the proliferative responses to antigenic peptides, which do not require processing (24, 25), indicating that the inhibitory effects of IL-10 and v-IL-10 are not related to inhibition of antigen processing by the monocytes. Comparable data were obtained in murine studies that demonstrated that m-IL-10 also reduced cytokine production after activation by superantigens that do not require processing (2). Murine studies furthermore indicated that m-IL-10 suppressed lymphokine production by Th1 cells (1). Human $CD4^+$ T cell clones cannot generally be divided in Th1 or Th2 subsets (11, 12). However, the *Der p* I–specific T cell clones have been shown to produce very high levels of IL-4 and IL-5 and low to normal levels of IL-2 and IFN-γ. Because of these aberrant IL-4/IFN-γ production ratios, these clones were designated as "Th2-like" cells (Yssel et al., manuscript submitted for publication). In contrast, the *M. leprae*–specific T cell clones produced unusually high levels of IFN-γ, and undetectable or very low levels of IL-4 and IL-5, and were therefore considered to represent "Th1-like" cells (13). Although these clones do not fully represent murine Th1 or Th2 cells, it is of interest to note that IL-10 and v-IL-10 inhibited the proliferation of both the *Der p* I– and the *M. leprae*–specific clones equally well.

IL-10 and v-IL-10 strongly downregulated constitutive class II MHC expression on human monocytes. IL-10 and v-IL-10 not only downregulated constitutive class II MHC expression, but also blocked the class II MHC enhancing effects of IFN-γ and IL-4 in a dose-dependent fashion. IL-10 and v-IL-10 failed to modulate class I MHC expression on monocytes and did not affect the levels of CD11a, CD11b, CD11c, CD18, CD54 (ICAM-1), CD58 (LFA-3), CD14, CD44, VLA-4, VLA-5, VLA-6, CD23, and CD25 (results not shown). These results indicate that the reduction in the antigen-presenting capacity of monocytes is not due to inhibition of intercellular adhesion molecules that either directly (26, 27), or by mediating T cell APC contacts, play an important role in T cell activation and proliferation. The observation that the APC function of EBV-LCL was not affected by IL-10 and v-IL-10 tested over a wide concentration range and after prolonged incubation times (up to 4 d), together with the fact that IL-10 and v-IL-10 failed to downregulate class II MHC expression on these cells, suggested that the reduction in antigen-specific proliferative responses of $CD4^+$ T cells and T cell clones could be associated with the strong reduction in surface class II MHC expression on the monocytes. Harding & Unanue (28) recently have shown that ~300 class II MHC-specific antigenic peptides complexes on APC representing only 0.1–0.2% of the restricting class II MHC molecules were sufficient to induce T cell proliferation. Their studies furthermore indicated that most class II MHC molecules were not occupied by the antigenic peptide, but probably by self or other antigenic peptides (28). Such a very low percentage of class II MHC molecules accommodating the specific peptides may account for the observation that the proliferative responses were reduced by >90%, whereas there was still considerable surface class II MHC expression on the monocytes. In addition, it was shown recently that antigenic peptides need to associate with newly synthesized HLA-DR molecules before cell surface expression (29, 30). Recycling of class II MHC molecules (31), or association of peptides with "empty" class II MHC molecules expressed on the cell surface, do not significantly contribute to the expression of immunogenic class II MHC peptide complexes (29–31). This suggests that class II MHC synthesis and cell surface expression of new MHC class II/peptide complexes is particularly important for antigen presentation. It is possible that this process is downregulated by IL-10. This notion is further supported by the observation that reduction of the proliferative responses was more pronounced when the antigen concentration became limiting. From these observations, it can be concluded that IL-10 and v-IL-10 in these culture systems do not act as suppressor factors, but that the reduction in antigen-specific T cell proliferation in the presence of monocytes as APC merely reflects a strong reduction in the antigen-presenting capacity of these cells. This contention was compatible with the observations that the $[Ca^{2+}]_i$ fluxes were strongly reduced when the TT-specific T cell clones were stimulated by monocytes preincubated with TT in the presence of IL-10 or v-IL-10 and that neither IL-2-induced proliferation nor the expression of IL-Rα and IL-2Rβ chains on the T cell clones was affected by IL-10 and v-IL-10.

More recently, we have demonstrated that IL-10 and v-IL-10 strongly inhibit the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and granulocyte/macrophage (GM)-CSF (31a). IL-1α, IL-1β, and also IL-6, TNF-α and GM-CSF have been shown to have costimulatory effects on antigen-specific T cell proliferation (32–35). However, it was excluded that the strong reduction in antigen-specific proliferative responses was due to inhibition of the production of these monokines, because reduced responsiveness could not be neutralized by addition of saturating concentrations of these monokines. In addition, it was demonstrated that under the present culture conditions, antibodies against these monokines did not decrease the antigen-specific proliferative T cell responses (not shown). It was also ruled out that IL-10 induced the production of TGF-β (36), or other unknown soluble suppressor factors by monocytes. Supernatants of cultures in which strong reduction in T cell proliferation was obtained in the presence of monocytes, TT, and IL-10, failed to affect the specific proliferative responses of T cell clones induced by TT presented by autologous EBV-LCL (not shown). Furthermore, freshly isolated monocytes added in relatively high concentrations to cultures of TT-specific T cell clones, TT, and autologous EBV-LCL as APC failed to reduce the proliferative responses of these clones in the presence of IL-10, indicating that IL-10 did not induce these "bystander" monocytes to release factors suppressing antigen-specific T cell proliferation. In addition, supernatants of monocytes cultured in the presence or absence of IL-10 and tested in the presence of the neutralizing anti-IL-10 mAb failed to enhance the TT-specific proliferative responses of T cell clones suboptimally activated by TT and autologous EBV-LCL. This argues against the idea that IL-10 could suppress the production of a putative soluble monocyte factor, which has costimulatory activity on antigen-specific T cell proliferation.

Collectively, our data indicate that both-10 and v-IL-10

through downregulation of class II MHC molecules on monocytes strongly reduce the antigen-presenting capacity of these cells, thereby reducing, or preventing, optimal antigen-specific proliferative T cell responses.

Recently, we have shown that monocytes stimulated by LPS produce IL-10. However, IL-10 is produced late (24–36 h) after activation (31a), whereas IL-1α, IL-1β, IL-6, and TNF-α are generally maximally produced after 8–12 h of activation (37). These kinetics of IL-10 production by monocytes, together with its inhibitory effects on proinflammatory monokine production and its downregulatory effects on class II MHC molecules, suggest that IL-10 in an autocrine fashion may play a role in damping immune responses.

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