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Interleukin-10 Down-Regulates MHC Class II αβ Peptide Complexes at the Plasma Membrane of Monocytes by Affecting Arrival and Recycling

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Summary

Interleukin-10 (IL-10) inhibits antigen-specific T cell responses when human monocytes are used as antigen-presenting cells. This is correlated with a down-regulation of MHC class II molecules on the surface of the monocyte. Here we show that IL-10 does not affect MHC class II transcription, polypeptide synthesis, subunit assembly, or antigenic peptide loading. Instead, newly synthesized mature MHC class II molecules are localized to the MHC class II loading compartment but are prevented from reaching the plasma membrane. In addition, treatment of monocytes with IL-10 leads to an accumulation of internalized MHC class II complexes in intracellular vesicles. These results indicate that IL-10 affects antigen presentation by regulating MHC exocytosis and recycling.

Introduction

Interleukin-10 (IL-10), an 18 kDa homodimeric cytokine (Vieira et al., 1991), is secreted by activated T cells (Yssel et al., 1992), B cells (Matthes et al., 1993), and monocytes (de Waal Malefyt et al., 1991a) and binds to specific receptors that are expressed on all haematopoietic cells (Liu et al., 1994). Although it is a cofactor for the growth and differentiation of B cells (DeFrance et al., 1992; Rousset et al., 1992), mast cells (Thompson-Snipes et al., 1991), and murine T cells (MacNeil et al., 1990), IL-10 also has strong immunosuppressive and antiinflammatory activities (reviewed by Moore et al., 1993).

In humans, IL-10 has been shown to inhibit the proliferation of and IL-2 production by activated human T cells (de Waal Malefyt et al., 1991b) and is able to induce a state of antigen-specific nonresponsiveness (Groux et al., 1996). The effects of IL-10 on human monocytes include inhibition of lipopolysaccharide-induced proinflammatory cytokine secretion (de Waal Malefyt et al., 1991a; 1993) as well as down-regulation of constitutive or cytokine-induced expression of cell adhesion and costimulatory molecules such as CD54 (ICAM-1), CD80, and CD86 (Kubin et al., 1994; Willems et al., 1994). In addition, IL-10 decreases the plasma membrane expression of major histocompatibility complex (MHC) class II glycoproteins (de Waal Malefyt et al., 1991a, 1991b). Down-regulation of MHC class II on monocytes correlates with a decrease in proliferation and cytokine production by antigen-specific T cell clones when IL-10–treated monocytes are used as antigen-presenting cells. We inferred that the down-regulation of MHC class II antigen complexes by IL-10 prevented, rather than inhibited, the activation of T cells. In the present investigation, we have examined the effects of IL-10 on plasma membrane expression of MHC class II glycoproteins from freshly isolated human monocytes and have studied the mechanism of its action.

The biosynthesis and transport of MHC class II molecules is a tightly regulated process that involves the intersection of secretory and endocytic pathways (reviewed by Cresswell, 1994) to load newly synthesized MHC class II molecules with peptides generated for the most part from antigens degraded in the endosomal/lysosomal organellar system. During biosynthesis, the immature MHC class II complex comprises αβ heterodimers that associate in the endoplasmic reticulum with a nonpolymorphic third glycoprotein, designated the invariant chain (Ii). Ii facilitates the proper folding of the αβ subunits and targets the MHC class II complex to the endosomal/lysosomal system. There it is degraded by resident proteases, leaving a roughly 20–amino acid residue intermediate that is eventually removed from the αβ heterodimer in a reaction catalyzed by another nonpolymorphic MHC class II heterodimeric molecule, human leukocyte antigen-DM (HLA-DM) (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995). HLA-DM is located in lysosome-like MHC class II–containing compartments (MIICs) (Sanderson et al., 1994) where the dissociation of Ii enables peptides to occupy a groove in the MHC class II molecule. A lag period of approximately 1–3 hr transpires before the mature MHC class II complex arrives at the plasma membrane (Neefjes and Ploegh, 1992), where it serves as a ligand for clonotypic T cell receptors. At the cell surface, MHC class II molecules can recycle (Reid and Watts, 1990; Pinet et al., 1995; Cella, 1997) and exchange their antigenic peptides (Pinet et al., 1995; Cella, 1997; Zhong, 1997).

Recently, immature dendritic cells derived from human monocytes following culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were shown to increase their cell surface expression of MHC class II through a process involving decreased internalization coupled with increased synthesis, when stimulated with lipopolysaccharide or tumor necrosis factor-α (TNFα) (Cella, 1997). In a related study, mouse bone marow–derived dendritic cells cultured in the presence of GM-CSF were shown to increase their cell surface expression of MHC class II through a process involving decreased internalization, coupled with more efficient exocytosis (Pierre, 1997). Therefore, the relative level of cell surface MHC class II expression can be regulated posttranslationally, both by exocytosis and by recycling.

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Here we show that IL-10 acts on these same post-translational processes and interferes with the exocytosis of newly synthesized, as well as the recycling of internalized MHC class II molecules in human monocytes. Of particular interest is that the effect imposed by IL-10 on the newly synthesized pool of MHC class II occurs relatively late in the biosynthetic pathway, subsequent to the association of MHC class II with antigenic peptides. Vesicular transport of MHC class II molecules can thus be negatively regulated by the IL-10 signaling pathway.

Results

IL-10 Affects Cell Surface MHC Class II Expression

The effect of IL-10 on constitutive or IFNγ-induced cell surface MHC class II expression by human monocytes was initially examined by immunofluorescence and flow cytometry. IFNγ was used as a potent monocyte activator that strongly up-regulates MHC class II expression (Basham and Merigan, 1983). Human monocytes used in these experiments were isolated by negative selection from buffy coats of healthy donors and were 85%–95% positive for CD14. Cells were stained after a 15 hr culture at 37°C either in the absence of or in the presence of IFNγ and IL-10, or after an equivalent period of time at 4°C. Since monocytes are able to produce low levels of IL-10 in culture (de Waal Malefyt et al., 1991a), control cultures were supplemented with a neutralizing antibody to IL-10 (monoclonal antibody [MAb] 12G8). Three anti-MHC class II antibodies were used (Figure 1). Two MAbs were directed against conformational epitopes of HLA-DR (L243 and Tü36), and one antibody was directed against an HLA-DRβ chain epitope (Q5/13). In addition, an antibody to a conformational determinant on MHC class I molecules (G46-2.6) was included as a control.

Following culture with 12G8, the level of HLA-DR expression was increased, as assessed by the binding of all three HLA-DR antibodies. In contrast, monocytes cultured in the presence of IL-10 showed a dramatic decrease in HLA-DR expression, to one third of the initial level of HLA-DR. IFNγ induced an even stronger enhancement of MHC class II expression, which was also significantly inhibited by IL-10. These results indicate that the IL-10–mediated loss of HLA-DR staining is not dependent on the epitope recognized by the detecting antibody and therefore reflects a genuine down-regulation of HLA-DR from the plasma membrane. Cell surface levels of HLA-DQ and HLA-DP followed the same pattern of expression as HLA-DR (data not shown). In addition, IL-10 is not responsible for a general down-regulation of cell surface molecules, since the levels of MHC class I and CD14 on monocytes remain unaffected (Figure 1, de Waal Malefyt et al., 1993) and levels of CD16, CD64, and TNFα p55 receptors actually increase (te Velde et al., 1992).

We next examined the kinetics by which IL-10 affected constitutive and IFNγ-induced MHC class II expression (Figure 2). The cell surface levels of MHC class II increased on control and IL-10–treated monocytes up to approximately 4 hr (Figure 2A). Of note, the levels of

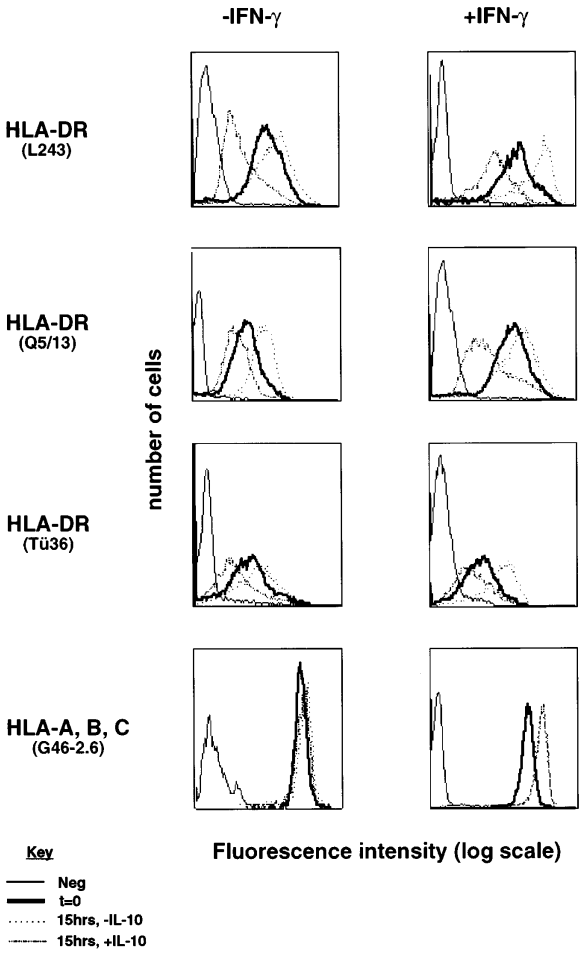

Figure 1. Effect of IL-10 on Cell Surface MHC Class II Expression by Human Monocytes

Human monocytes were isolated from peripheral blood and cultured for 15 hr at 4°C (t = 0) or at 37°C in the presence of a neutralizing antibody to IL-10 (12G8, −IL-10) or IL-10. Identical cultures were set up with the addition of recombinant human IFNγ. Cells were stained by indirect immunofluorescence using Tü36 and Q5/13 (anti-HLA-DR) and a horse anti-mouse IgG-PE second antibody. G46-2.6 (anti-MHC class I) and L243 (anti-HLA-DR) antibodies were directly conjugated to PE. Stained cells were analyzed by flow cytometry. Neg, isotype-matched controls.

MHC class II expression on IL-10–treated cells did not further increase. Instead, MHC class II expression decreased until 16 hr, from which time forward they remained constant. By 16 hr of culture in the presence of IL-10, the mean fluorescence intensity of MHC class II on the surface of the monocytes represented only 15% of the initial level. In contrast, a sustained increase over time in MHC class II cell surface expression was observed on monocytes cultured in the presence of the anti-IL-10 MAb, until a plateau was reached at 16 hr. Spontaneous up-regulation of MHC class II on human monocytes cultured in vitro has been reported previously (Smith and Ault, 1981). The increased expression of MHC class II upon in vitro culture of monocytes could not be attributed to stimulation through the Fc receptor (Barcy et al., 1995) by the anti-IL-10 MAb, since

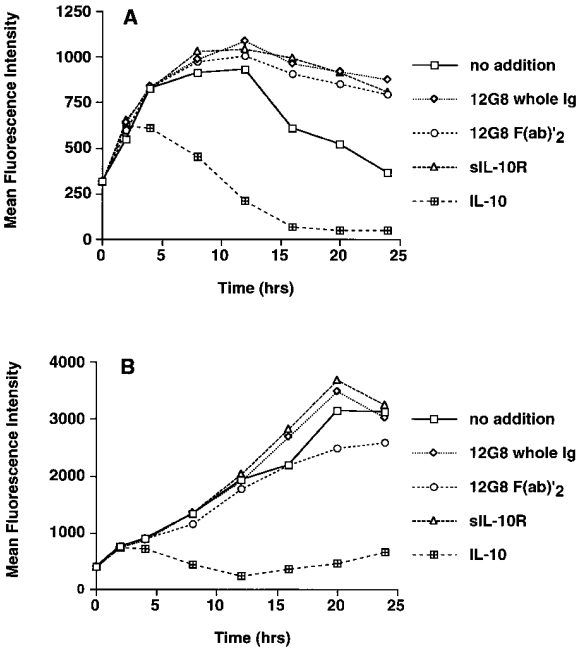

Figure 2. Time Course of IL-10 Mediated Down-Regulation of MHC Class II on Human Monocytes

Monocytes were cultured in medium alone, or with 10 μg/ml anti-IL-10 whole Ig, or 30 μg/ml anti-IL-10 F(ab)′$_2$, or 50 μg/ml recombinant soluble IL-10 receptor, or 400 U/ml IL-10.

(A) Cells were removed from culture at the indicated times and stained with L243-PE (anti-HLA-DR) and analyzed by flow cytometry.

(B) Identical cultures were set up with the addition of 400 U/ml recombinant human IFNγ.

F(ab)′$_2$ fragments of the same antibody, as well as the addition of a soluble IL-10R, did not prevent the up-regulation of MHC class II. Without the addition of the anti-IL-10 MAb, F(ab)′$_2$ fragments of the anti-IL-10 MAb, or soluble IL-10 receptors, endogenously produced IL-10 could down-regulate MHC class II expression after 12 hr.

When monocytes were cultured in the presence of IFNγ and either the anti-IL-10 MAb, anti-IL-10 F(ab)′$_2$, or soluble IL-10 receptors (Figure 2B), their MHC class II expression increased steadily to high levels up to 20 hr. Similarly, MHC class II expression on monocytes cultured with IFNγ and IL-10 also increased during the first 2–4 hr. However, during the following 8–12 hr of incubation MHC class II levels were strongly reduced. These kinetics indicate that a lag period of 2–4 hr exists between the binding of IL-10 to its receptor and inhibition of constitutive or IFNγ-induced MHC class II cell surface expression.

IL-10 Does Not Affect Transcription or Translation of MHC Class II Subunits

To examine whether IL-10 exerted its effects on MHC class II expression at the transcriptional level, RNA was extracted from monocytes cultured in the absence or presence of IFNγ and either the anti-IL-10 MAb or IL-10 and analyzed for the expression of HLA-DRA, Ii, HLA-DMA, and HLA-DMB transcripts. The levels of steady-state mRNA expression of these four genes were not affected by IL-10 in cultures either with or without IFNγ (Figure 3A). As expected, the expression levels of HLA-DRA, Ii, HLA-DMA, and HLA-DMB mRNA were up-regulated in the presence of IFNγ.

Subsequently, the synthesis of MHC class II α, β, and Ii polypeptides was addressed. MHC class II was immunoprecipitated from metabolically labeled monocytes using anti-MHC class II antibodies (DA6.147 and L243) or an anti-Ii antibody (rabbit anti-Ii). As a control for equal loading, an MHC class I (W6/32) antibody was used. Cells had been preincubated in the presence of the anti-IL-10 MAb or IL-10 for 8 hr, a period sufficient to observe a substantial decrease in cell surface MHC class II. Immediately following a 20 min pulse in the absence (Figure 3B) or presence (Figure 3C) of IFNγ, the MHC class II αβIi complex was present in both the anti-IL-10 MAb–treated and the IL-10–treated monocytes. When adjusted for loading, very similar levels of immunoprecipitated complexes were present in both cultures (Figure 3D). These results indicate that translation and subunit assembly of the MHC class II complex is not affected by IL-10.

IL-10 Does Not Affect the Generation of SDS-Stable Compact Class II Complexes

Failure to bind peptide into Ii-dissociated MHC class II complexes has been reported to prevent plasma membrane transport of newly synthesized MHC class II complexes as a result of aggregation and eventual degradation (Germain and Rinker, 1993). Peptide loading leads to a conformational change of MHC class II complexes, biochemically characterized by increased resistance to denaturation by SDS (Germain and Hendrix, 1991; Neefjes and Ploegh, 1992). Therefore, we examined the fraction of peptide-loaded MHC class II complexes in IL-10–treated monocyte cultures by their ability to retain subunit association in the presence of SDS. We had previously determined by pulse chase analysis and immunoprecipitation that the Ii had dissociated from MHC class II complexes in monocytes cultured in the absence or presence of IL-10 by 2 hr after synthesis (data not shown).

Figure 4A illustrates that immediately after a 20 min pulse, MHC class II complexes in control and IL-10–treated monocytes dissociate into the α and β subunits, since these complexes have not entered the peptide loading compartment. However, after 2 hr of chase, SDS-stable compact MHC class II complexes are present. Most importantly, the fraction of SDS-resistant dimers is very similar in monocytes cultured in the presence of IL-10 or the anti-IL-10 MAb. Analysis of SDS-stable MHC class II complexes from monocytes incubated with IFNγ (Figure 4B) also reveals that the fraction of SDS-stable dimers is comparable in control and IL-10–treated cells when MHC class II is expressed at high levels.

IL-10 Affects Accumulation of MHC Class II at the Plasma Membrane

To address whether newly synthesized MHC class II complexes could accumulate at the plasma membrane, we used an antibody capture assay (Krangel et al., 1979).

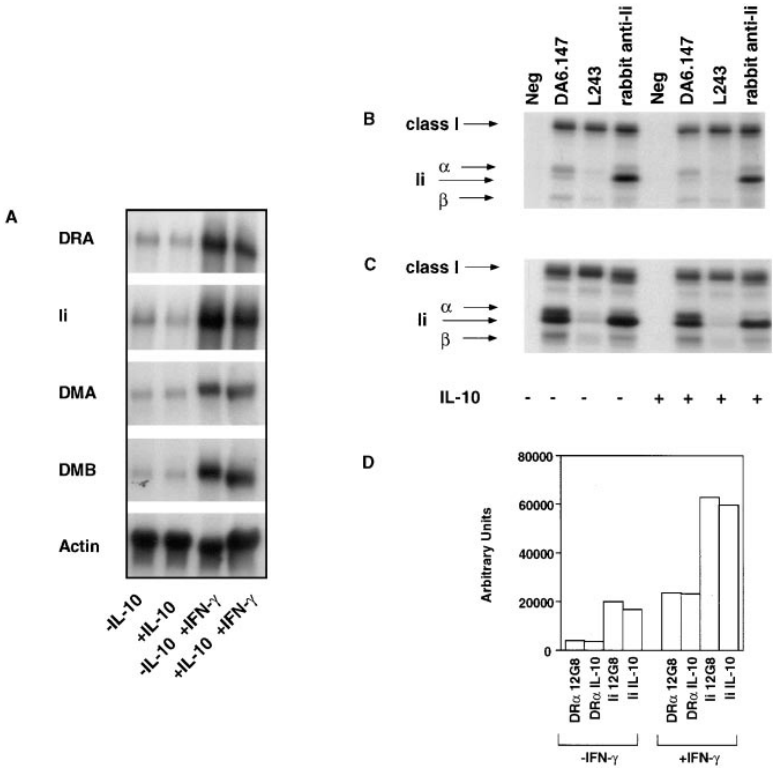

Figure 3. Examination of IL-10 and/or IFNγ on MHC Class II Steady-State mRNA and Synthesis of MHC Class II Subunits from Monocytes

Monocytes were cultured for 15 hr in the presence of 10 μg/ml anti-IL-10 MAb (−IL-10) or 400 U/ml IL-10. Identical cultures were set up with the addition of 400 U/ml recombinant human IFNγ.

(A) Total RNA was extracted, electrophoresed, blotted to Nytran membrane, and hybridized to ^{32}P-labeled cDNA probes.

(B) Human monocytes were preincubated with 10 μg/ml anti-IL-10 MAb (−IL-10) or 400 U/ml IL-10 for 8 hr, and pulsed for 20 min with [^{35}S]methionine + cysteine.

(C) Identical cultures were set up with the addition of 400 U/ml recombinant human IFNγ. Cells were detergent extracted and antigens were immunoprecipitated with the indicated antibodies from equal amounts of trichloroacetate-precipitable radioactivity. Precipitates were analyzed by SDS–polyacrylamide gel electrophoresis. Included in all precipitation experiments was an anti-MHC class I antibody (W6/32), which was used to compensate for loading differences.

(D) The intensities of the HLA-DRα bands from the DA6.147 and rabbit anti-Ii precipitates of control and IL-10–treated monocyte lysates were quantitated by phosphorimage analysis and adjusted for differences in loading. The relative amount of Ii that is coprecipitated in αβIi complexes from DA6.147 precipitates varies among donors.

In this experiment, antibody was added to metabolically labeled and chased cells in order for it to bind newly synthesized MHC class II arriving at the plasma membrane. Figure 5 shows that from monocytes cultured with the anti-IL-10 MAb and incubated either in the absence (Figure 5B) or in the presence (Figure 5D) of IFNγ, immediately following the pulse, no MHC class II could be isolated from the plasma membrane. However, MHC class II was present after 3 hr of chase, and especially in the case of IFNγ-treated cells, accumulated on the cell surface following a longer chase (Figure 5D). In contrast, a considerably smaller fraction of MHC class II was precipitated from the plasma membrane of IL-10–treated monocytes at all time points. Thus, although equal amounts of MHC class II molecules were synthesized in control and IL-10–treated monocytes (Figures 5A and 5C), less MHC class II was detected at the plasma membrane in the presence of IL-10, suggesting that IL-10 affects the accumulation of MHC class II at the plasma membrane.

MHC Class II Molecules Occupy MIIC-Like Compartments in IL-10–Treated Monocytes

We proceeded to characterize the subcellular localization of MHC class II in IL-10–treated monocytes by confocal laser scanning microscopy. To this end, monocytes were cultured for 12 hr with the anti-IL-10 MAb or IL-10, fixed in paraformaldehyde, permeabilized with saponin, and stained with L243, an anti-MHC class II MAb, in conjunction with antibodies to the MIIC markers CD63 and HLA-DM. The presence of HLA-DM in lysosome-like structures identifies a compartment through which the majority of newly synthesized MHC class II must traverse on their way to the plasma membrane (Bénaroch et al., 1995; Warmerdam et al., 1996). Figure 6 shows that MHC class II staining of control monocytes is predominantly localized at the plasma membrane, with few apparent vesicles staining in the interior of the cell. Of note, monocytes cultured in the presence of IL-10 show a punctate vesicular pattern of MHC class II expression localized to the interior of the cell, along with strongly reduced cell surface expression. Moreover, in the IL-10–treated cells, MHC class II localized to vesicles that costained with CD63 and HLA-DM. Similar results were obtained with cells cultured in the presence of IL-10 and IFNγ (data not shown). Close inspection of CD63 staining reveals the appearance of CD63$^+$ structures that do not colocalize with HLA-DR (Figure 6B, arrows), whereas the relative position of all HLA-DR$^+$ vesicles appears superimposable on those of all HLA-DM$^+$ vesicles (Figure 6D), implying that, at least in human monocytes, CD63 is more widely expressed in lysosome-like structures. Finally, the colocalization of HLA-DR with either of these two endosomal markers was not seen in control monocytes (Figures 6A and 6C), nor was MHC class I found to colocalize with CD63 in IL-10–treated monocytes (Figure 6E). These observations suggest that IL-10 causes an accumulation of class II in HLA-DM$^+$ lysosomal structures.

IL-10 Affects Recycling of MHC Class II Molecules

The pulse chase experiments indicated that IL-10 reduces the accumulation of MHC class II on the plasma

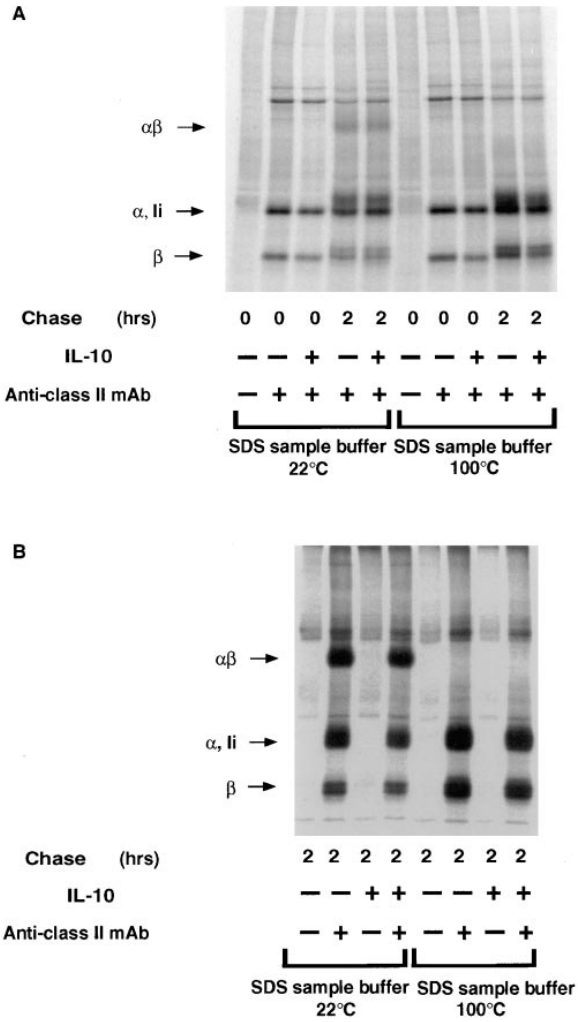

Figure 4. Formation of SDS-Stable MHC Class II Complexes in Monocytes Cultured in the Presence or Absence of IL-10 and IFNγ

(A) Monocytes were preincubated for 8 hr with 10 μg/ml anti-IL-10 MAb (-IL-10) or 400 U/ml IL-10 and pulsed for 20 min with [^{35}S]methionine + cysteine. (B) Identical cultures were set up with the addition of 400 U/ml recombinant human IFNγ. Cells were lysed immediately after labeling or were chased for 2 hr under conditions in which they were preincubated and then lysed. Extracts were precipitated with DA6.147, an anti-MHC class II MAb; eluted in nonreducing SDS-sample buffer, heated either at 22°C or 100°C; and analyzed by SDS-PAGE. The positions of the free MHC class II α, free MHC class II β, and free Ii, and the SDS-stable αβ complexes are indicated. Note that in nonreducing SDS-PAGE the Ii comigrates with HLA-DRα.

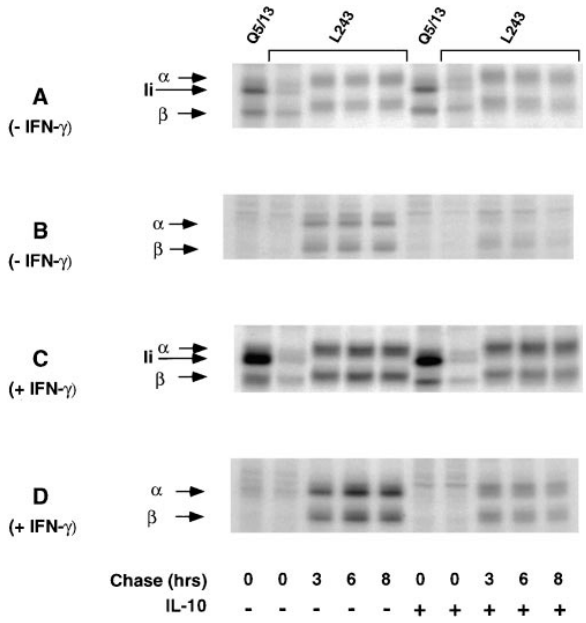

Figure 5. Antibody Capture of Accumulated MHC Class II Molecules on the Plasma Membrane of Monocytes Cultured in the Presence or Absence of IL-10 and IFNγ

Monocytes were preincubated in the presence of (A) 10 μg/ml anti-IL-10 MAb (−IL-10) or (B) 400 U/ml IL-10. (C and D) Identical cultures were set up with the addition of 400 U/ml recombinant human IFNγ. Cells were pulsed for 20 min with [^{35}S]methionine + cysteine and chased in the same conditions in which they were preincubated. At the times indicated, cells were divided into two equal portions. One portion was detergent extracted immediately (A and C) and immunoprecipitated with the conformation dependent anti-MHC class II antibody, L243. The other portion was incubated with anti-MHC class II MAb prior to detergent extraction (B and D). These cells were subsequently extracted in the presence of unlabeled monocytes containing a 5-fold excess of MHC class II, and the immunoprecipitates were analyzed by SDS-PAGE. In the case of the 0 chase time point, the first of the two lanes represents an additional aliquot of cells that were precipitated with the anti-β chain–specific class II MAb, Q5/13. This was done to ensure that after the pulse, although no class II could be precipitated from the cell surface, the material could be precipitated from inside the cell using this non–conformation-dependent antibody. Although there appears to be a difference in the electrophoretic mobility of the MHC class II subunits arriving at the plasma membrane in the presence of IL-10 as compared to that in the presence of the anti-IL-10 MAb (D), this difference is not reflected in the degree or kinetics of sialylation, which represents the terminal carbohydrate posttranslational modification (data not shown). Moreover, this apparent difference in glycosylation does not affect the arrival of MHC class II to the MIIC in the presence of IL-10 (see Figures 4 and 6).

membrane. We next studied whether IL-10 had an effect on the plasma membrane pool of MHC class II. Anti-MHC class II-F(ab)′–biotin conjugates (L243 F(ab)′-biotin) were bound to monocytes that had been preincubated in the presence of IL-10 or the anti-IL-10 MAb. Monocytes were returned to culture, and at 90 min aliquots of cells were removed, fixed, permeabilized, and analyzed by confocal microscopy for the presence of MHC class II. Figure 7 shows that MHC class II molecules are expressed at the plasma membrane in monocytes cultured with the anti-IL-10 MAb and maintained on ice (Figure 7A). This pattern of expression did not change when internalization was allowed to proceed (Figure 7B), although the relative intensity of surface staining decreased in accordance with the internalization of these molecules. In contrast, IL-10 caused an accumulation of MHC class II (Figure 7D) but not CD14 (Figure 7F) in internal structures, indicating that this cytokine specifically affects the dynamics of the plasma membrane pool of MHC class II.

To quantitate the effect of IL-10 on the plasma membrane pool of MHC class II, the kinetics of internalization was determined by flow cytometry on nonpermeabilized cells. Monocytes were cultured and stained as described above. Figure 7G shows that in control monocytes, there was a precipitous loss of MHC class II up

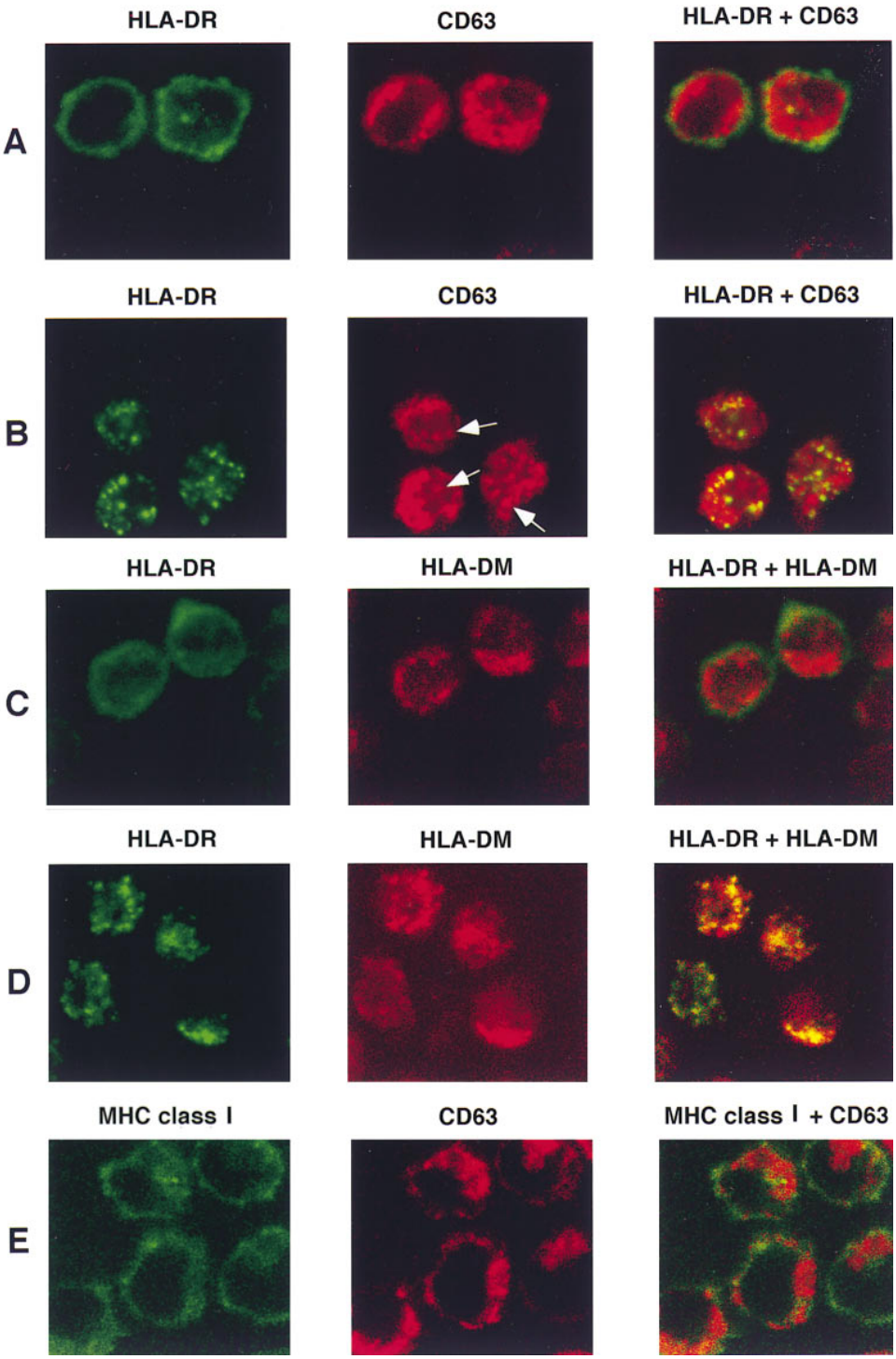

Figure 6. Intracellular Immunofluorescence Analysis of MHC Class II Expression from Monocytes Cultured in the Presence or Absence of IL-10

Monocytes were cultured for 12 hr in the presence of 10 μg/ml anti-IL-10 MAb (A and C) or 400 U/ml IL-10 (B, D, and E). At the end of culture, cells were washed; fixed; permeabilized with saponin; and stained with L243–fluorescein isothiocyanate, an anti-HLA-DR MAb (A–D), or W6/32-FITC (anti-MHC class I MAb) (E), together with either an anti-CD63 rabbit serum followed by a Texas Red–conjugated anti-rabbit antibody (A, B, and E) or an anti-HLA-DM antibody followed by a Texas Red–conjugated anti-mouse IgG (C and D). Cells were analyzed by confocal laser scanning immunofluorescent microscopy. Arrows in (B), $CD63^{+}$ structures that are negative for HLA-DR.

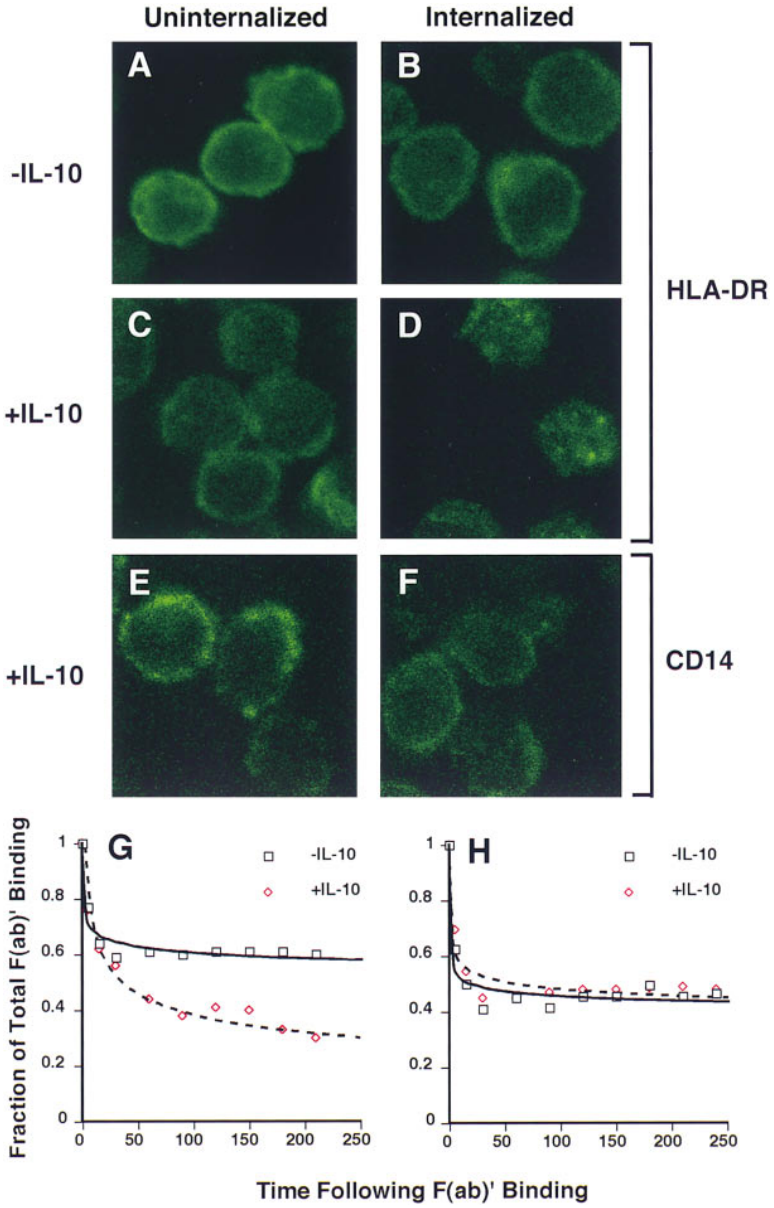

Figure 7. Internalization of MHC Class II Molecules from Monocytes Cultured in the Presence or Absence of IL-10

Monocytes were preincubated at 37°C for 6 hr in the presence of 10 μg/ml neutralizing antibody to IL-10 (A and B) or 400 U/ml IL-10 (C–F). Cells were washed at 4°C and incubated with either L243 F(ab)′ (anti-HLA-DR) MAb conjugated to biotin (A–D and G) or an anti-CD14 F(ab)′ biotin conjugate (E, F, and H). Cells were either kept on ice (A, C, and E) or returned to culture under the same conditions in which they were preincubated (B, D, and F). Following a 90 min incubation at 37°C, cells were fixed, permeabilized, incubated with streptavidin-FITC, and analyzed by confocal laser scanning immunofluorescent microscopy. Alternatively, aliquots of unpermeabilized cells were removed at the indicated time points (G and H), stained with avidin-PE, and analyzed by flow cytometry.

to 15 min following warming of the cells to 37°C. This drop corresponds to 30%–40% of total binding at time 0 and appears to be consistent with the decrease in intensity of plasma membrane staining observed by image analysis following internalization. After 15 min, the level of MHC class II remained relatively constant through 4 hr in culture. The shape of this binding curve is consistent with internalization of MHC class II followed by the attainment of an equilibrium of internalized and reexpressed molecules.

The plasma membrane dynamics of MHC class II in monocytes treated with IL-10 are notably different. Here, internalization proceeds for a longer period, although the rate of this process, as defined by the slope of the curve prior to its inflection, is not appreciably different from that in controls. Moreover, an equilibrium between internalized and returning molecules is not as evident as the L243 F(ab)′-biotin complexes continue to decrease from the surface in IL-10–cultured monocytes. The reduced reappearance of internalized class II is specific, since IL-10 did not affect CD14 in a comparable manner (Figure 7H). This suggests that IL-10 incubation inhibits the return of internalized MHC class II molecules to the plasma membrane, consistent with microscopic analysis of class II–bearing internal structures present in IL-10–treated but not control monocytes. Therefore, IL-10 appears to affect both the exocytosis and recycling of MHC class II in human monocytes.

Discussion

The ability of IL-10 to inhibit antigen-specific T cell clone proliferation when monocytes are used as antigen-presenting cells has been documented (de Waal Malefyt et al., 1991b). IL-10 down-regulates cell surface expression

of three important monocyte-encoded glycoproteins implicated in T cell stimulation (e.g., CD80, CD86, and CD58 [Kubin et al., 1994; Willems et al., 1994]), as well as MHC class II (de Waal Malefyt et al., 1991a, 1991b). Antibody blocking experiments show that of these cell surface molecules, anti-MHC class II antibodies block proliferation of T cell clones to the greatest extent (R. d. W. M., unpublished data). This illustrates the importance of IL-10–mediated down-regulation of MHC class II on the function of human monocytes.

The effects of IL-10 on gene regulation in monocytes have been described primarily for the transcription rate and mRNA stability of IL-1α, IL-1β, IL-6, IL-12, TNFα, IFNα, IL-8, MIP1α, GM-CSF, M-CSF, G-CSF, and CD80 (de Waal Malefyt et al., 1991a, 1993; Chin and Kostura, 1993; Gruber et al., 1994). We have shown that IL-10 does not affect the steady-state message levels of MHC class II, Ii, or HLA-DM. Instead, our results reveal a previously undescribed mechanism of MHC class II down-regulation mediated by IL-10 on human monocytes. This occurs through a posttranslational effect on the exocytosis of newly synthesized MHC class II molecules to the plasma membrane, along with a negative effect on the recycling of the plasma membrane pool of these molecules. Of particular note is our observation that IL-10 acts at a relatively late stage in MHC class II biosynthesis, following the loading of antigenic peptides in an endosomal compartment. This feature distinguishes the effects of IL-10 from those of monensin (Machamer and Cresswell, 1984), chloroquine (Nowell and Quaranta, 1985), concanamycin B (Bénaroch et al., 1995), and leupeptin (Neefjes and Ploegh, 1992), all of which affect MHC class II exocytosis prior to the generation of peptide-loaded complexes.

The rapid down-regulation of MHC class II by IL-10 is selective because IL-10 treatment of monocytes results in the up-regulation of cell surface CD16, CD64, and TNFα receptors (te Velde et al., 1992). Moreover, virtually no effects can be seen on the expression of surface MHC class I or CD14 (de Waal Malefyt et al., 1993) following a 15 hr incubation of monocytes in the presence of IL-10. Extended incubations up to 24 hr reveal only marginal effects on surface MHC class I levels (data not shown), possibly because of an effect on the expression of TAP (transporter associated with antigen processing) (Salazar-Onfray et al., 1997, Zeidler et al., 1997).

The effect of IL-10 on MHC class II expression is cell-type specific. We have examined MHC class II on activated T cells and resting B cells isolated from peripheral blood, as well as on B-lymphoblastoid cell lines, all of which express IL-10 receptors (Liu et al., 1994). IL-10 did not down-regulate surface expression of MHC class II molecules on these cells. However, IL-10–mediated inhibition of MHC class II expression on Langerhans cells (Péguet-Navarro et al., 1994) and macrophage-derived microglial cells (Frei et al., 1994) have been reported. Therefore, the effect of IL-10 on MHC class II biosynthesis appears to be a myeloid-restricted phenomenon, the specificity of which may be attributable to differences between myeloid and lymphoid cell types in signaling events downstream of the IL-10 receptor. Such differences in IL-10 signaling have been documented; in one example, they lead to differential assembly of Stat1α and Stat3 complexes in human T cells and monocytes (Finbloom and Winestock, 1995).

Our results support a model whereby IL-10 reduces the arrival of newly synthesized MHC class II at the plasma membrane. This idea is based on our observations that newly synthesized MHC class II is impeded from accumulating at the plasma membrane in monocytes cultured in the presence of IL-10. Although these observations are not sufficient to confirm our conclusion, the accumulation of MHC class II in HLA-DM–containing compartments in IL-10–cultured but not control-cultured cells, as seen by immunofluorescence and immunoelectron microscopy (data not shown), support this interpretation. Since HLA-DM identifies a compartment through with the majority of newly synthesized class II must traverse prior to their arrival at the plasma membrane (Bénaroch et al., 1995; Warmerdam et al., 1996), these observations indicate that IL-10 affects the biosynthetic pool of MHC class II.

Earlier studies by Reid and Watts (Reid and Watts, 1990) and more recent work by Pinet and Cella (Pinet et al., 1995; Cella, 1997) have shown that internalized MHC class II can be recycled. Our results also indicate that IL-10 affects the reexpression of internalized MHC class II. In fact, while 50% of the original amount of L243 F(ab)′-biotin remains on the surface in control monocytes following 12 hr incubation, those cultured in the presence of IL-10 have only 10% (data not shown). This suggests that the effect of IL-10 on the recycling pool of MHC class II also contributes to the overall down-regulation of these molecules, as depicted in Figure 2. The image analysis revealing internalized MHC class II trapped inside IL-10– but not control-cultured monocytes supports this interpretation. IL-10 may therefore be affecting factor(s) specific for trafficking from the MIIC or a factor that acts on both the MIIC and a recycling compartment.

The effect of IL-10 on a single labeled cohort of MHC class II is not absolute, whether we are measuring the accumulation at the plasma membrane or recycling. However, this impedance is cumulative, such that whatever small proportion of molecules that arrive on the plasma membrane in the presence of IL-10 are now subject to the effects of IL-10 upon their internalization. Meanwhile, the proportion of MHC class II that successfully recycle back to the surface are subject to the effects of IL-10 upon a subsequent round of internalization.

We have described a mechanism by which IL-10 down-regulates the cell surface expression of MHC class II molecules by affecting the appearance of mature MHC class II αβ peptide complexes on the surface of freshly isolated human monocytes. Our results, together with the recent observations on MHC class II exocytosis and recycling by monocyte-derived dendritic cells (Cella, 1997) demonstrate that MHC class II trafficking by professional antigen-presenting cells is a highly regulated and dynamic process that can be controlled by pro- and anti-inflammatory cytokines. Furthermore, this down-regulation of MHC class II exocytosis and

recycling contributes to the anti-inflammatory and immunosuppressive activities of IL-10.

Experimental Procedures

Cells and Antibodies

Monocytes were purified by negative selection from peripheral blood mononuclear cells of healthy donors. In brief, 3×10^8 ficol separated mononuclear cells were incubated on ice with a cocktail of MAbs (Becton-Dickinson, Mountain View, CA) consisting of αCD2 (Leu-5A), αCD3 (Leu-4), αCD8 (Leu 2a), αCD19 (Leu-12), αCD20 (Leu-16), αCD56 (Leu-19), αCD67 (IOM 67) (Immunotech, Westbrook, ME), and anti-glycophorin antibody (10F7MN, American Type Culture Collection, Rockville, MD). Antibody-bound cells were washed and then incubated with sheep anti-mouse IgG coupled magnetic beads (Dynal, Oslo, Norway) at a bead-to-cell ratio of 20:1. Antibody-bound cells were separated from monocytes by application of a magnetic field. The anti-IL-10 MAb 12G8 was produced at DNAX (Abrams, 1992). Soluble IL-10R (Liu et al., 1994) was a kind gift from K. Moore (DNAX). W6/32 (Barnstable et al., 1978) (American Type Culture Collection) and G46-2.6 (IgG_1, Pharmingen, San Diego, CA) antibodies react with a conformational epitope on human MHC class I molecules. DA6.147 (Guy et al., 1982), an anti-DRα MAb; Map.DM1, an anti-HLA-DM MAb; and rabbit anti-Ii were kindly provided by Peter Cresswell (Yale Medical School, New Haven, CT) and Frances Brodsky (University of California, San Francisco, CA). Tü36 (Shaw et al., 1985) and L243 (Lampson and Levy, 1980) detect a conformational determinant on HLA DR αβ and αβIi complexes. Q5/13 (Quaranta et al., 1981) reacts with an epitope on HLA-DRβ chains.

Cell Culture Conditions

Monocytes were seeded at 3×10^6/ml in Yssel's medium (Gemini Bioproducts, Calabasas, CA) containing 1% human AB serum. For metabolic labeling studies, preincubations with IL-10 were done for 8–10 hr using 400 U/ml IL-10. The neutralizing antibody to IL-10 (12G8) was used at 10 μg/ml. The $F(ab)'_2$ fragment of 12G8 was used at 30 μg/ml. Soluble IL-10 receptor was added to cultures at a final concentration of 50 μg/ml. Recombinant human IFNγ (R&D Systems, Minneapolis, MN) was added to cultures at a final concentration of 400 U/ml.

Immunofluorescence Analysis

First, 2×10^5 purified human monocytes were incubated in phosphate-buffered saline (PBS) containing 1% human serum on ice for 20 min. Cells were then pelleted at 200 × g and, for indirect immunofluorescence, resuspended (10 μg/ml) with Tü36 or Q5/13 or isotype-matched control antibodies. In situations where primary antibody was not directly conjugated to phycoerythrin (PE), a horse anti-mouse IgG-PE antibody (Vector Laboratories, Burlingame, CA) was used. Following an additional 20 min incubation on ice, cells were washed in PBS containing 1% human serum followed by two washes in PBS alone. For direct staining, cells were resuspended in L243-PE or G46-2.6-PE (anti-MHC class I). Cells were fixed in PBS containing 1% paraformaldehyde and analyzed on a FACScan flow cytometer (Becton Dickenson). Immunofluorescence of permeabilized cells was performed as previously described (Philips et al., 1992), with minor modifications. Cells were fixed for 20 min in 4% paraformaldehyde in PBS and permeabilized in PBS with 0.1% saponin and 2% dialyzed fetal calf serum. Analysis was performed on a Molecular Dynamics Multiprobe 2010 confocal laser scanning microscope.

Metabolic Labeling

Monocytes were prestarved at 5×10^6/ml in cysteine- and methionine-free Dulbecco's modified Eagle's medium (ICN, Costa Mesa, CA) containing 5% dialyzed human serum, 6 μg/ml deoxyribonuclease I (Sigma, St. Lous, MO), and either 10 μg/ml anti-IL-10 MAb 12G8 or 400 U/ml IL-10 (labeling media) for 1 hr at 37°C. Cells were pelleted, resuspended to 5×10^7/ml in labeling media containing 5 mCi Tran^{35}S-Label (specific activity ~1000 Ci/mmol; ICN). After 20 min, cells were chased by diluting to 3×10^6/ml in 37°C Yssel's medium containing 1% human serum and 2 mM each of unlabeled methionine and cysteine with the addition of either the anti-IL-10 MAb or IL-10. At various time points, cells were removed from 37°C and diluted in cold PBS.

Detergent Extraction and Immunoprecipitation

Immunoprecipitation from detergent extracts was performed as previously described (Koppelman and Cresswell, 1990). For samples that received antibody prior to detergent extraction, cells were washed three times with PBS and incubated with the anti-HLA-DR MAb L243 at 10 μg/ml for 1 hr on ice. Antibody-coated cells were washed three times in PBS and extracted in PBS containing 1% Nonidet P-40, 1 mg/ml bovine serum albumin, and unlabeled cells providing 5- to 10-fold excess of MHC class II molecules. Nuclei and cell debris were cleared by centrifugation, and the supernatant was incubated with Protein G-Sepharose beads for 30 min at 4°C.

mRNA Isolation and Northern Analyses

Total RNA was isolated from 10^7 monocytes using RNA STAT-60 (Tel-Test, Friendswood, TX). RNA was electrophoresed, transferred to membrane, and hybridized with probes labeled to high specific activity ($>10^8$ cpm/μg) by the hexamer labeling technique (Feinberg and Vogelstein, 1983). Filters were hybridized, washed under stringent conditions, and developed as previously described (de Waal Malefyt et al., 1989).

Preparation of $F(ab)'_2$, F(ab)′ and Biotinylated F(ab)′

$F(ab)'_2$ fragments of the anti-IL-10 MAb 12G8 were prepared using an Immunopure $F(ab)'_2$ Preparation Kit (Pierce, Rockford, IL). F(ab)′ fragments of the anti-HLADR MAb L243 were prepared using an Immunopure F(ab)′ Preparation Kit (Pierce). F(ab)′ fragments were biotinylated by dialyzing protein into 50 mM sodium bicarbonate (pH 8.5) and adding a 10-fold molar excess of NHS-SS-biotin (sulfosuccinimidyl 6-(biotinamido) hexanoate) (Pierce). The reaction proceeded for 2 hr at 4°C. Unreacted biotin was removed by centrifugation through a Centricon 30 (Amicon, Beverly, MA). The sample was diluted in 10 mM sodium phosphate, 150 mM sodium chloride and recentrifuged two successive times to remove unreacted biotin.

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