Effects of Immunoregulatory Cytokines on B7-1 and B7-2 Isoform Expression on Human Monocytes and B cells.

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ABSTRACT:

T cell activation and the generation of effective immune responses is critically dependent on APC-derived signalling. The relative expression of B7 isoforms on APC may be important in determining the nature and extent of the immune response, and immunoregulatory cytokines may mediate their effects through alterations in B7 isoform expression. The effects of a panel of cytokines on B7 isoform expression on resting and activated monocytes and B cells was evaluated. IL10 and IL4, which induce the development of Th2 type T cells, downregulated expression of B7-2 and modestly upregulated expression of B7-1 on unstimulated human monocytes. IFNγ, a potent inducer of Th1 type T cells, upregulated both B7-1 and B7-2 expression. TNFα downregulated B7-2 expression, but did not alter B7-1 expression. Addition of anti-IL10 antibodies did not abrogate the effects of TNFα on B7-2 expression. LPS had effects on B7 isoform expression on purified monocytes similar to those of IL10 in PBMC, namely marked B7-2 downregulation and modest B7-1 upregulation. None of the cytokines influenced the levels of expression of B7 isoforms on either resting or activated B cells. Thus cytokines that influence development of T helper type immune responses have differential effects on expression of individual B7 isoform on monocytes but not on B cells. These findings may have important implications in activation and control of immune responses in infections, autoimmunity and malignancies.

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LIST OF ABBREVIATIONS:

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
CD	Cluster of Differentiation
CMIR	Cell Mediated Immune Response
DC/LC	Dendritic Cell/Langerhans Cell
DTH	Delayed Type Hypersensitivity
EAE	Experimental Allergic Encephalomyelitis
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
HLA	Human Lymphocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL2R	Interleukin-2 Receptor
IMDM	Iscove's Modified Dulbecco's Medium
kD	Kilodalton
LPS	Lipopolysaccharide
MAb	Monoclonal Antibody
MCF	Mean Channel Fluorescence
MHC	Major Histocompatibility Complex
ng	Nanogram
NK	Natural Killer
NOD	Non-Obese Diabetic
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PMA	Phorbol Myristate Acetate
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T Helper
TNF	Tumour Necrosis Factor

INTRODUCTION:

The central role of the immune system is the differentiation of self from non-self such that protective immune responses against foreign material can be generated. T lymphocytes are the central regulatory cells in the immune system, influencing processes as diverse as cytotoxicity, immunoglobulin production by B cells and control of autoreactivity. The central interaction in an adaptive immune response is the presentation by an APC of an antigen contained in a self-MHC molecule to a T cell bearing a compatible TCR. This interaction can, under certain circumstances, induce T cell activation and proliferation, with local production of immunoregulatory cytokines culminating in the generation of specific immune responses. T cells universally express surface CD3 in conjunction with the T cell receptor, which is generated through alternate DNA splicing in a manner similar to that of immunoglobulin production by B cells. T cells can be further subdivided into two major subgroups: i) those which express surface CD4 and serve a mainly helper or immunoregulatory function; and ii) those which express surface CD8 and are mainly cytotoxic. T helper cells (Th) control immune responses, either by stimulating or suppressing immune activation, through their production and local secretion of cytokines. Cytokines, a large number of which have been described and whose functions are often varied and context dependent, are also produced by many other immune cells such as B cells and monocyte/macrophages. CD4+ and CD8+ T cells also differ in their requirements for activation by APC. CD4+ T cells require antigens to be bound in class II MHC on the APC surface, whereas CD8+ T cells require class I MHC for antigen presentation.

T cells are critically dependent on APC to become activated. APC functions are served by many different cell types, depending on tissue type: Langerhans cells, dendritic cells, B lymphocytes and monocyte/macrophages are all capable of fulfilling APC roles. Exogenous peptides (such as bacterial components), following phagocytosis by an APC, are internally processed and presented to CD4+ T cells bound in self MHC of class II type (i.e. HLA-DR, HLA-DP or HLA-DQ). Endogenous peptides, such as viral proteins produced by an infected cell, are presented to mainly CD8+ cytotoxic T cells by class I MHC (i.e. HLA-A, HLA-B or HLA-C).

T helper cells have been classified into three main subgroups by their cytokine production profiles: Th1, Th2 and the immature phenotype Th0 (see below). The exact mechanisms underlying the development of Th1 versus Th2 immune responses have been investigated recently. Immunoregulatory cytokines, antigen type, hormones and costimulatory molecule expression on APC may all play vital roles in controlling the T helper phenotype of the immune response generated. Immunoregulatory cytokines may regulate the expression of costimulatory molecules on the surface of APC, and thus control the expression of immunostimulatory cytokines and cytokine receptors such that an effective immune response can be generated. The type of cytokine released may have a direct effect on the expression of costimulatory molecules on APC (such as monocyte/macrophages, Langerhans cells, B cells, and dendritic cells). The following sections will review the role of cytokines and costimulatory molecules in the generation of Th1 versus Th2

immune responses, immunoregulatory cytokines, and the CD28/B7 costimulatory pathway.

T helper lymphocyte subsets and T helper immune responses.

Classification of human and murine cytokines into Th1 and Th2 types has helped in the elucidation of the mechanisms of resistance or susceptibility to infections (1,2). Distinct subsets of T helper cells were first determined by Mosmann in murine models on the basis of cytokine secretion patterns and effector functions (3). Th1 cells produce IL2, IFNγ and TNFβ (lymphotoxin) and primarily induce delayed-type hypersensitivity (DTH) and cell-mediated immune responses (CMIR). Th2 cells produce IL4, IL5, IL10 and IL13, and primarily induce humoral immune responses, including IgE mediated allergic responses. Undifferentiated Th0 cells produce both Th1 and Th2 cytokines, and serve as precursors for both Th1 and Th2 cell types (1,4). Selective induction of CMIR by intracellular pathogens (viruses, intracellular bacteria and protozoa) and humoral immune responses by extracellular pathogens (free viruses, extracellular bacteria and helminths) may be dependent upon the selective induction of Th1 or Th2 cell subsets respectively (1,4).

T helper subsets differ not only in the cytokines and immune responses they produce, but also in their responsiveness to cytokines in their microenvironments. Both Th1 and Th2 subsets respond to IL2, but Th2 cells are much more responsive to IL4, and are inhibited to a greater degree by IFNγ (5). IFNγ promotes differentiation of Th1 cells over Th2 cells, as does IL12, a cytokine whose production is under direct control of IFNγ (1). IL4 is believed to be the critical

cytokine controlling Th2 type responses (1). Despite the demonstrated effects of cytokines on Th subset generation in the primary immune response, these effects are much less pronounced at secondary challenge, although some effects can still be found. For example, IL12 can induce IFNγ secretion in established Th2 clones (6,7). These findings suggest that the dominant effects of cytokines on Th subset generation occurs during primary contact with the antigen, although some mutability of responses does exist (1).

T helper subsets also differ in their ability to supply B cell help. Th2 cells provide help in proportion to their number, while Th1 cells inhibit B cell immunoglobulin secretion at higher T to B cell ratios. This may serve as a mechanism to control immunoglobulin secretion (8). Th1 clones exhibit much greater macrophage induction (9), a finding in agreement with the greater role of macrophages in Th1 type responses. Cytokine class responses are reciprocally controlled: Th1 cells inhibit Th2 cells and vice versa (1,10,11). Overlap in cytokine functionality exists, as the Th1-type cytokine IFNγ is essential for the generation of opsonizing and complement activating antibodies which are against extracellular pathogens (12). HIV/AIDS may follow a pattern of gradual replacement of Th1 by Th2 type responses (1,12-22).

In vivo responses to pathogens can be classified into those in which the cytokine production pattern has been skewed towards a Th1 or Th2 profile, although cytokines of all type may be produced in varying amounts. In murine models, immune responses can be clearly differentiated into those in which Th1 responses predominate, involving phagocyte-mediated host responses to intracellular

microbes, and those in which Th2 responses predominate, involving phagocyte-independent host responses to microbes such as helminths (1). In humans, however, immune responses do not exhibit such polarization, although the Th1/Th2 paradigm can still be applied (1). Early experiments using cells from the same donors demonstrated that the nematode *Toxocara canis* and the intracellular bacterium *Mycobacterium tuberculosis* were found to induce predominantly Th1 and Th2 type immune responses, respectively, as predicted by murine Th1/Th2 models (23,24). *In vitro* studies of other human disease states have yielded similar data supporting a predominant role for either Th1 or Th2 class responses in the clearance of infections and establishment of disease states. Immune responses skewed towards Th2 profiles have been noted in lepromatous leprosy (25), atopic dermatitis (26), vernal conjunctivitis (27) and bronchial asthma (28). Th1 type cytokine profiles have been demonstrated in Hashimoto's autoimmune thyroiditis (29), contact dermatitis (30), tuberculoid leprosy (25), multiple sclerosis (31), Lyme's arthritis (32) and reactive arthritis (33). *In vivo* studies in asthma have demonstrated increased expression of Th2 type cytokines using *in situ* hybridization (34,35). Likewise, *in vivo* Th1 type cytokine expression patterns have been reported in multiple sclerosis (36) and in pancreas biopsy samples of insulin-dependent diabetes mellitus (37).

Although many cytokines influence the development of a particular T helper cell phenotype, cytokines that have been found to be particularly important include IL12 and IFNγ, which promote differentiation of naïve antigen specific Th0 cells into Th1 type cells, and IL4 and IL10 which induce the development of Th2 type cells (1,2,6,12,38-46). IL4 is a 129 amino acid monomeric polypeptide produced by

Th2 type T lymphocytes and mast cells whose main functions include B cell activation, stimulation of IgE class switching, and induction of Th2 type immunity (47). IFNγ is a 143 amino acid monomeric polypeptide produced by T cells and NK cells whose main functions include macrophage activation, enhanced MHC expression and protection against intracellular infections (47). Immunization of mice in the presence of anti-IL4 antibodies diminishes the amount of IL4 produced and number of IL4-producing cells at subsequent challenge, an effect which lasts up to 14 weeks (48). Mice normally resistant to leishmaniasis can be rendered vulnerable through administration of exogenous IFNγ, an effect which appears to involve a reduction in the number of IFNγ-producing cells rather than simply through neutralization of endogenous IFNγ (49).

Interleukin-10 (IL10) is a pleiotropic molecule possessing a broad spectrum of biological activities: it promotes development of naive T cells into cells with a Th2 phenotype, although to a lesser extent than does IL4, and has immunosuppressive effects including the downregulation of MHC class II expression on monocytes and suppression of T cell proliferative responses (1,50,51). IL10 exhibits 73% homology to murine IL10, and 83% homology to the product of an open reading frame of the Epstein-Barr virus genome known as BCRF1 or viral IL10 (52,53). Human IL10 is produced by a number of cell types, including Th0 cells, Th2-like CD4+ T cell clones, B cell lines derived from AIDS patients with Burkitt's lymphomas, activated monocytes and peripheral blood T cells (54-56). IL10 is also produced in trace amounts by Th1 cells (1). IL10 is a growth factor for normal and EBV transformed human B cells and a promoter of proliferation of IL2 activated cytotoxic T

cells (54,55,57). Human IL10 inhibits antigen-specific proliferation of Th0, Th1, and Th2-like T cells, as well as antigen-stimulated cytokine synthesis by PBMC and natural killer (NK) cells in a macrophage/monocyte dependent manner (50,58). IL10 has been associated with the immunopathogenesis of a number of diseases including septic shock, lymphoproliferative disorders and autoimmune diseases (1). Induction of IL10 expression has been postulated as a strategy for immune evasion in certain parasitic and EBV infections (52,59).

Natural killer cell stimulatory factor, or IL12, is a heterodimeric disulfide-linked polypeptide comprised of a heavy chain (p40) and a light chain (p35) (42,46). The heavy chain is inducible following stimulation, but IL12 biological activity is limited to the dimeric polypeptide (42,46). IL12 was first isolated from EBV-positive B cell lines (42). IL12 is involved in the regulation of cytokine production and proliferation of T cells and natural killer cells and IL12 enhances the proliferation and cytotoxic activity of T cells and NK cells, and stimulates IFNγ production (42,46). IL12 participates in the development of CD8+ T cells and Th1 type cells (42). IL12 has been implicated in protective immune responses to a number of parasitic infections and in antitumour and antimetastatic activities (42,46). IL12 induces differentiation of naive T helper cells into cells with Th1 phenotypes (42,45,46). IL12 deficient mice show deficiency in generation of Th1 responses, while IL5 deficient mice show deficiency in generation of Th2 responses (12). Finally, IL12 has recently been shown to induce IL10 production by T cells, indicating a potential negative feedback mechanism (60).

TNFα (cachectin), a trimeric polypeptide composed of 157 amino acid monomers, is produced by macrophages, NK cells, Th1 type T cells and to a lesser

extent by Th2 type T cells (61). Its main functions include induction of local inflammation and endothelial activation, and plays a central role in the pathogenesis of septic shock in Gram negative infections (47). *In vitro*, the main target cells of TNFα are monocyte/macrophages, with relatively minor effects on T cells. *In vivo*, TNFα can manifest both Th1 and Th2 characteristics depending on experimental conditions (62). TNFα production has been shown to be induced by IL12 (63), and TNFα induces IL10 production by monocytes (60).

Other factors which influence the maturation of Th0 cells towards Th1 or Th2 type responses include the nature of the antigen and the type of APC (1). Antigen processing by B cells skews the immune response to a Th2 pattern whereas processing by macrophages induces TH1 type responses. Certain antigens induce Th1 cell anergy exclusively, resulting in predominantly Th2 type responses (1,6,38-41,43,44,64).

Costimulatory molecules and cell activation.

The two signal hypothesis of Bretscher and Cohn (65) was first postulated in 1970, prior to the isolation and characterization of cytokines. In this model, the nature and type of an immune response to a presented antigen depends on the presentation by an APC to a T cell of a combination of two distinct signals. If both signal 1 and signal 2 are present, a normal proliferative response occurs. If signal 1 is present in the absence of signal 2, antigen-specific clonal anergy develops, such that the presented antigen is not recognized as foreign. Finally, if signal 2 is presented in the absence of signal 1, no discernible effect on immune function occurs. This theoretical model provides a simple and elegant means by which the

immune system can control the intensity of the immune response to a foreign or self antigen. Regulation of expression of the protein that mediates signal 2 would therefore be central to the regulation of an immune response in this model.

Recent insights into the role of costimulatory molecules in immune activation have supported Bretscher and Cohn's hypothesis (66). It is now clear that T cells require two distinct signals from APC to become activated. In applying the Bretscher/Cohn model to the CD28/B7 pathway, signal 1 is provided by the interaction of the MHC/antigen complex with the TCR, and confers antigen specificity. Signal 2 is provided by the interaction of T cells with APC via a variety of costimulatory molecules which induces clonal expansion (67,68). While CD40 and CD45 ligands have been suggested to have costimulatory functions (29), the most important costimulatory pathway is that provided by the interaction of a B7 molecule on APC with a member of the CD28/CTLA-4 family of molecules on T cells. As hypothesized, binding of the MHC/antigen complex with the TCR in the absence of a second signal mediated through CD28/B7 induces reversible, antigen-specific T cell anergy (67-69). Experimental data suggest that, beyond the lack of effect of signal 2 alone as originally hypothesized, CD28/B7 interaction in the absence of MHC/TCR interaction may play a negative regulatory role, inducing downregulation of CD28 expression on T cells (70). The mechanisms by which the immune system controls expression and function of the CD28/B7 regulatory pathway are thus critical in the control of immune responses.

Expression and function of CD28 and CTLA-4.

CD28 was discovered through binding studies involving MAb that stimulated T cells to a comparable degree as TCR stimulation. CD28 was thus determined to be an activation marker for lymphocytes before its role as the surface receptor for B7 isoforms was known (71,72). A second B7 receptor, CTLA-4, was discovered during a search for markers involved in T cell cytolytic activity (73); surface CTLA-4 expression has since been correlated with T cell cytolytic function (68). CD28 and CTLA-4 are homodimeric molecules composed of two 44 kD subunits. CD28 and CTLA-4 exhibit approximately 20% sequence homology in both mice and humans, with a carboxy terminus hexapeptide MYPPPY completely conserved between the molecules and between species such as mice, rats and humans (74). CD28 and CTLA-4 have been mapped to adjacent genomic locations in both mice and humans (73,75). Both CD28 and CTLA-4 are members of the immunoglobulin gene superfamily. Both possess a V_H-like region in their extracellular domains, and both have a small transmembrane region and a short intracytoplasmic tail (76). Like CD28, CTLA-4 binds to both major B7 isoforms with comparable avidity (77). However, compared to CD28, CTLA-4 binds either B7 isoform with greater avidity (77,78). Thus, in comparison to CD28, CTLA-4 represents a high avidity, low abundance, inducible B7 receptor (68). These findings suggest that CD28 and CTLA-4 are genetically linked and evolutionarily related (79).

CD28 is expressed at high levels on resting T cells. CD4+ T cells in non-activated state are >95% CD28+, whereas approximately 50% of resting CD8+ T cells are CD28+. In contrast, CTLA-4 is not expressed on resting T cells. Following T cell

activation, however, CTLA-4 is rapidly upregulated, although peak levels of CTLA-4 expression density do not exceed 2-3% those of CD28 (75,80-82).

Initial functional studies suggested that CD28 and CTLA-4 may exert overlapping costimulatory influences on T cell activation. Anti-CD28 MAb dramatically enhance T cell proliferation when combined with polyclonal activators such as PMA, PHA or anti-CD3 (71,83-88), and induction of T cells via CD28 receptors has been shown to amplify T cell cytolytic activity (89,90). Activation of T cells via anti-CD28 MAb signalling enhances IL2 secretion both by increasing the IL2 gene enhancer activity and by stabilizing IL2 mRNA (91-95). CD28-mediated signalling also increases IL2Rα (CD25) expression, and increases production of Th1 type cytokines such as IFNγ and IL-12 (72,96-98).

The initial molecular events following cell activation via CD28 have been investigated recently. Binding of B7 isoforms to CD28 triggers both Ca^{2+}-dependent (97,99) and Ca^{2+}-independent signalling pathways (79,100-103). Ca^{2+}-dependent pathways require a higher degree of CD28 aggregation than Ca^{2+}-independent pathways, and appear to induce higher levels of cell activation (104). Ca^{2+}-independent pathways were determined in part through experiments that demonstrated resistance of CD28-mediated signalling to Cyclosporin-A, which mediates its immunosuppressive effects through binding to cyclophilin and downregulation of calcineurin, an important mediator of Ca^{2+}-dependent signalling pathways (105). Both Ca^{2+}-dependent and Ca^{2+}-independent pathways involve the activation of protein tyrosine kinases (100,106). Ca^{2+}-independent pathways increase the half-life of cytokine mRNAs through post-

translational effects (98), and alter the transcriptional regulation of IL-2 mRNA (91-95), possibly through activation of the kinase PLCγ1 (79).

Although early investigations suggested that CD28 and CTLA-4 played overlapping roles, recent data have suggested that they may in fact play opposing roles in T cell activation. Binding of CD28 to T cells in the absence of costimulation or mitogen-induced activation renders the T cells less sensitive to CD28-costimulation in subsequent experiments, and leads to a transient downregulation of CD28 expression and hyporesponsiveness to CD28-mediated intracellular signalling (70). Experiments involving anti-CTLA-4 antibodies that trigger intracellular signalling through the CTLA-4 molecule have suggested that CTLA-4 may have a negative influence on immune activation depending on the state of cell activation. Anti-CTLA-4 MAb downregulated mouse mixed lymphocyte reactions, inhibited the proliferation of T cells activated with a combination of CD3 and CD28, and may induce antigen-specific apoptosis in previously activated cells (107). CTLA-4 knockout mice further support an immunosuppressive role for CTLA-4, since these mice were found to exhibit lymphoproliferative disorders and early lethality although their T cells were found to proliferate normally to TCR stimulation (108).

The role of CD28 in T cell activation appears to depend on the state of T cell maturation. In naïve T cells, anti-CTLA-4 MAb exert an agonist influence on T cells costimulated with CD3 and CD28, and resting T cells do not proliferate in response to CD28 signals alone, as predicted by the Bretscher/Cohn model, However, previously activated T cells are highly responsive to anti-CTLA-4 MAb activation alone, in the absence of a second source of stimulation (79). Thus the effect of signalling via the

CD28 and CTLA-4 surface molecules appears to depend on the state of cell activation, and is determined through an integration by the T cell of the signals provided by CD28 and CTLA-4.

Expression and function of B7 isoforms.

The ligand for CD28/CTLA-4 is B7, a family of surface proteins expressed on the surface of most APC, including B cells, monocyte/macrophages, Langerhans cells and dendritic cells (67). B7 isoforms exist in at least 3 forms, namely B7-1, B7-2 and B7-3. The B7-1 isoform, the first member of the B7 family to be discovered, was originally found as a B lymphoblast antigen recognized by the MAb BB-1 (109). Later, the B7 antibody, which also bound to B7-1, was reported (110). Analysis of the relative binding of these MAb, in addition to studies involving the FUN-1 and BU63 MAb (111,112), have led to the recognition of three distinct B7 isoforms, named B7-1 (also known as CD80), B7-2 (also known as CD86) and B7-3 (67,113,114). Both BB-1 and B7 MAb bind to B7-1, but only BB-1 binds to the putative B7-3 protein (114). BB-1 binds to a protein that maps to the human chromosome 12, whereas both B7-1 and B7-2 have been mapped to chromosome 3 (79). Thus B7-1 and B7-2 appear to be encoded on chromosome 3, with B7-3 encoded on chromosome 12. B7-2 was identified through the ability of CTLA-4Ig, a fusion protein that binds to and blocks all known B7 isoforms, to further block costimulatory reactions which were not blocked by anti-B7-1 MAb (67). Most experimental evidence published to date concerns B7-1 and B7-2; the exact role and amino acid sequence of B7-3 remain to be established.

B7 isoforms are 262 amino acid monomers, with two immunoglobulin-like extracellular regions (one resembling a V region and one a C region), a transmembrane domain and a short cytoplasmic tail (79). B7-1 molecules feature less cross-species sequence conservation than do CD28 and CTLA-4, with mouse and human B7-1 molecules approximately 45% conserved at the amino acid level, compared to 67% and 76% conservation for CD28 and CTLA-4 respectively (79). In addition, the intracytoplasmic regions of B7-1 exhibit very little cross-species homology, and the intracytoplasmic regions of human B7-1 and B7-2 are not similar, indicating that either B7-CD28 signalling is unidirectional from APC to T cell, or that different B7 molecules transmit different retrograde signals.

B7-1 and B7-2 exhibit similar binding avidities for both CD28 and CTLA-4: CTLA-4 is bound with higher avidity by both B7-1 and B7-2 compared to CD28. Slightly different dissociative kinetics were noted, with B7-1 being released more slowly than B7-2 (115). These results argued against a specific pairing pattern between B7 isoforms and CD28/CTLA-4. Investigations into a particular pattern of B7/CD28 pairing or preferential binding were prompted by kinetics studies. Temporal patterns of costimulatory molecule expression in immune activation raised the possibility of distinct roles for B7-1 and B7-2 (116). CD28 is constitutively expressed on a majority of T cells, whereas CTLA-4 is upregulated following activation. Likewise, B7-2 is constitutively expressed on non-dendritic cell APC and is very rapidly upregulated following APC activation, whereas B7-1 is expressed at relatively low levels and is upregulated more slowly. These findings led to the suggestion that the primary ligand pairs were B7-2/CD28 and B7-1/CTLA-4 (116).

Recent data have not supported this hypothesis (117-120). It is becoming clear that B7 mediated costimulation is more complex than initially believed.

Relative levels of expression for B7 isoforms vary between different APC, and are subject to different regulatory mechanisms. B7-1 was originally found on activated and virally transformed B cells and macrophages but not on resting B cells or monocytes (79). B7-1 was found to be upregulated on monocytes by IFNγ (121). In mice, B7-1 and B7-2 are expressed on a variety of cells, including B cells, T cells macrophages and dendritic cells. B cell activation upregulated B7-1 and B7-2 expression to peak levels after 18-42 hours of culture, with B7-2 being induced to higher levels than B7-1 (120). Expression of mRNA for B7-1 and B7-2 on monocytes and dendritic cells can be rapidly induced following activation (67,113,114). Neither B7-1 nor B7-2 is constitutively expressed on freshly isolated Langerhans and dendritic cells, but both can be induced in culture with B7-2 reaching higher density than B7-1 (67,113,114,120).

In addition to cell activation, immunoregulatory cytokines mediate control of B7 isoform expression on APC. The manner in which B7 isoform expression is altered by cytokines varies with the APC type. On Langerhans cells, B7-1 expression is downregulated by IL10 and IFNγ but is upregulated by IL1α, IL1β, IL4, GMCSF, and TNFα, and B7-2 expression is downregulated by IL10 but not by IFNγ (122,123). On monocytes and dendritic cells, IL10 has been shown to inhibit B7-2 expression (124,125). While the effect of B7 isoform expression on biological function is not known, increased expression of B7 isoforms on Langerhans cells and monocytes has been correlated with enhanced APC function (126,127). High levels

of B7-1 expressed on tumour cells transfected with B7-1 demonstrated increased susceptibility to NK-mediated lysis (128). IL10 has been shown to mediate its inhibitory effects by inhibiting the expression of B7 molecules on monocytes/macrophages (129). Dendritic/Langerhans cells generated through GMCSF and TNFα stimulation of CD34+ progenitor cells express both B7-1 and B7-2 (112).

The question of distinct functional roles for individual B7 isoforms has been studied recently. Initial functional studies suggested similar functions for B7-1 and B7-2. B7-1 and B7-2 transfected cells provide similar costimulatory signals to antigen-activated T cells (120,130-132). Early investigations suggested that B7-1 and B7-2 activate similar intracellular signalling pathways and stimulate similar degrees and types of cytokine secretion (77,133). Both B7-1 and B7-2 can be used under different conditions to induce development of T helper cells with Th2 phenotype (134,135). APC from B7-1 knockout mice (which express B7-2) are able to present alloantigens, indicating some redundancy of function, since cells expressing B7-1 has been found to effectively present alloantigens under other conditions (136).

Data indicating distinct roles for B7 isoforms in immune function have been accumulating in recent years. Experiments involving differential blocking of B7 isoforms have yielded conflicting results. Blocking of B7-1 during concanavalin A and alloantigen stimulation resulted in abrogation of proliferation, even though the APC in this model system co-expressed B7-2 (137). In another study, blocking B7-2 on APC which expressed both B7 isoforms resulted in decreased T cell proliferation

and Th1 type cytokine production, but did not alter upregulation of the activation antigens CD69 and IL2Rα on T cells (120). In a third study, CTLA-4Ig but not anti-B7-1 Ab blocked T cell proliferation in response to APC expressing both isoforms, suggesting B7-2 was the dominant B7 isoform in the interaction (117). Current concepts of the variable roles of B7 isoforms at different stages of immune activation, and differential use of B7 isoforms by different APC, may partially explain these discrepancies.

There is evidence to suggest that stimulation of cells via B7-1 and B7-2 induce different intracellular signalling pathways. In contrast to Levine and Lanier (77,133), Nuñes et al. found evidence suggesting differential activation of signalling pathways involving *ras* in T cells by B7 isoforms. CD28 cross-linking Ab upregulated *ras* expression, while B7-1 transfected APC did not induce *ras* expression (138). These data suggest that relative levels of activation and binding to CD28 vs. CTLA-4 by one or both B7 isoforms at different stages of immune activation may be critical in determining the type of cell activation pattern induced.

Different patterns of B7 isoform expression at different anatomic and physiologic locations may also indicate distinct functional roles. For example, germinal centre B cells exhibit restricted expression of B7-2, with very little B7-1 expression noted (111). The immunologic significance of this finding remains to be determined. Caux et al. (112) found that dendritic/Langerhans cells generated by incubation of CD34+ progenitor cells with GMCSF and TNFα are nearly 100% positive in expression of B7-1, but only 50-70% positive in expression of B7-2. However, despite its lower level of expression, B7-2 was found to be the major

functional B7 isoform, as specific B7-2 blockade resulted in 50-80% inhibition of DC/LC to T cell interaction whereas none of the anti-B7-1 Ab alone had this effect. B7-1 was suggested to play an adjunctive role, however, since combinations of B7-1 and B7-2 blockade had a greater negative effect on T cell stimulation. Also, in the same study, in contrast to the kinetics findings of other APC, B7-1 appeared earlier than B7-2 in the maturation of DC/LC. The potential for generalization from these findings in this highly specialized *in vitro* model is likely limited.

Evidence from *in vivo* models has also suggested distinct roles of B7 isoforms in immune function. Animal models of EAE and NOD mice have indicated that disease severity and T helper cell cytokine profile can be altered by specific blockade of B7 isoforms (see below).

The role of B7 isoforms in development of T helper cell phenotype.

Differential B7 isoform expression may be a critical factor in influencing the T helper phenotype of an immune response. The exact molecular mechanisms of induction of Th2 type T cells by IL4 and Th1 type T cells by IFNγ have not been delineated, but may be related to the ability of these cytokines to modulate B7 isoform expression on APC. IL4, IL10, IFNγ and TNFα are known to modulate the expression of vital cell surface molecules such as HLA class II antigens, cytokine receptors and adhesion molecules (51,124,139,140). The relative expression of B7 isoforms on APC may also be critical in determining the nature and extent of the immune response (128,134,141-144). Immunoregulatory cytokines may influence the development of the Th1 versus Th2 type immune responses by altering the levels

of B7 isoform expression on APC. In particular, IL10 and IL12 have been suggested to elicit their biological effects via B7 and CD28 molecules respectively (129,145). B7-2 but not B7-1 has been found to stimulate secretion of IL4, a cytokine central to a Th2 type response (134), and differential blockade of B7 isoforms in experimental leishmaniasis in mice alters the T helper cytokine pattern generated (144).

Two recent *in vivo* studies have also supported an influence of B7 isoforms on T helper phenotype. In experimental allergic encephalomyelitis, a murine model of multiple sclerosis and thus a condition believed to depend on exaggerated Th1 type immunity, differential blockade of the B7 isoforms B7-1 and B7-2 led to differences in cytokine profile and disease severity (142). Treatment with anti-B7-1 blocking Ab resulted in the generation of T cells with Th2 phenotype and a reduction in disease severity, both in terms of amelioration of established disease and less pronounced induction of new disease. In contrast, treatment with anti-B7-2 Ab resulted in the generation of an immune response with a Th1 profile, and increased severity of disease. These results suggest that skewing the balance of B7 isoform signalling towards B7-1 results in a predominantly Th1 type response, and vice versa. Also, costimulation was found to be physiologically important even in those animals with established disease, suggesting that the relative balance of B7-1 vs. B7-2 is important beyond the primary antigen contact (67).

In the other study of T helper/B7 isoform interaction, Lenschow et al. studied non-obese diabetic mice, a murine model for insulin-dependent diabetes mellitus which is believed to involve predominantly Th1 type effector mechanisms

(141). In apparent contradiction to the results of Kuchroo et al. (142), these investigators found that anti-B7-2 antibodies prevented the onset of disease, and anti-B7-1 antibodies resulted in more rapid onset and more severe disease (141). The degree to which these findings can be explained using the current understanding of B7/T helper cell interactions may depend on the degree to which these models are truly representative of Th1 mediated immune responses, and differences in experimental technique. Results from both these studies indicate, however, that differential B7 isoform use is important in controlling the phenotype of the immune response generated.

The mechanism through which differential signalling through the B7/CD28 costimulatory pathway may alter T helper cell phenotype is currently under investigation. A number of factors have been hypothesized to be important in controlling the type of T helper phenotype which is induced following contact with an antigen. Th2 type immune responses appear to be highly dependent on costimulation and on high initial dose of antigen, and require less costimulation in their maintenance (146), perhaps due to the requirement of CD28 costimulation in the induction of sensitivity to IL4 (147). In contrast, Th1 type immune responses appear to require much less involvement of CD28, since Th1 type responses can be generated in animals in which CD28 is either deficient or blocked (148,149). Thus B7-2 may be particularly important early in the generation of Th2 type responses to an antigen, whereas B7-1 may be critical in the maintenance of an established Th1 type response (67).

Objectives of current work.

Given the recent suggestions that differential expression of B7 isoforms may influence the development of T helper phenotype, the overall objective of this research project was to evaluate the effect of immunoregulatory cytokines on the surface expression of B7-1 and B7-2 on human APC. Specific objectives included the following:

1) To analyze the effects of immunoregulatory cytokines on B7 isoform expression on resting cultured human CD14+ monocytes.

2) To analyze the effects of immunoregulatory cytokines on B7 isoform expression on cultured human CD14+ monocytes following activation with LPS.

3) To analyze the effects of immunoregulatory cytokines on B7 isoform expression on resting cultured human CD19+ B cells.

4) To analyze the effects of immunoregulatory cytokines on B7 isoform expression on cultured human CD19+ B cells following activation with anti-IgM antibody coated sepharose beads.

MATERIALS AND METHODS:

Cell isolation and culture: Whole blood was obtained from the Canadian Red Cross, Ottawa, Canada or from healthy laboratory personnel. PBMC were isolated using standard methods (150). In brief, PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The cell layer consisting mainly of mononuclear cells was collected, washed three times in PBS and resuspended in complete IMDM supplemented with 10% FBS, 100 U/ml penicillin, and 50 μg/ml gentamicin (Sigma-Aldrich, Irvine, UK). The cells were cultured in 1 ml final volume at a concentration of 3×10^6 per ml in 24 well tissue culture plates (Corning Ltd., Corning, NY) in the presence or absence of cytokines. Human IL1α, IL1β, and GMCSF were purchased from Genzyme, Montreal, Canada. Recombinant IL4, IL5, IL6, IL10, IFNγ, TGFα and TNFα were obtained from R&D Systems, Minneapolis MN. Recombinant IL12 was generously provided by Dr. Maurice Gately, Hoffman-LaRoche, Nutley NJ. Anti-IL10 antibodies (AB-217-NA) were obtained from R&D Systems, Minneapolis MN.

The IL10 concentration used in these experiments was determined through dose response experiments (see below). Other cytokines were used in excess of concentrations demonstrated in previous surface molecule expression experiments to have a measurable effect on other markers on the cell type being studied. Cytokines were used at the following concentrations:

Cytokine	Final Concentration
IL1α	0.5 ng/ml
IL1β	50 pg/ml
IL2	50 U/ml
IL4	50 ng/ml
IL5	10 ng/ml
IL6	50 ng/ml
IL10	50 U/ml
IL12	170 U/ml
IFNγ	100 ng/ml
TNFα	40 ng/ml
TGFα	40 ng/ml
GMCSF	50 ng/ml

Isolation of B cells and Monocytes: B cells and monocytes were purified from the PBMC using anti-CD19 and anti-CD14 antibody coated immunobeads respectively (DYNAL, Lake Success NY) as described by the manufacturers. Briefly, PBMC were incubated with anti-CD19 or anti-CD14 antibody coated immunobeads at a bead to target cell ratio of 4:1 at 4°C with gentle rotation for 20 minutes. The cells/immunobeads were washed once and separated using a magnetic field. Monocytes were not detached from the immunobeads. B cells were detached using Pan-B CD19 DetachaBeads (DYNAL) as described by the manufacturer. Briefly, 10 µl DetachaBead solution were added to 1 x 10^7 B cells and gently rotated for 60 min. at room temperature. The beads were removed using a magnetic field. B cells and monocytes were analyzed for contaminating cells by flow cytometric analysis using appropriate combinations of the following antibodies: FITC conjugated anti-CD3 antibody (Becton Dickinson), PE conjugated anti-CD14 antibody (Becton Dickinson), FITC conjugated anti-CD19 antibodies (Becton Dickinson) and FITC conjugated anti-CD16 plus PE conjugated anti-CD56 antibodies (Becton Dickinson). Purified B cells contained less than 2% contaminating T cells and monocytes, and no NK cells. Purified monocytes contained less than 2% contaminating T cells and B cells, and no NK cells. Monocytes with beads attached did produced undetectable levels of TNFα and IL10, and normally upregulated TNFα and IL10 production following stimulation with LPS (Figure 1).

Cytokine ELISAs: Cytokines secreted by monocytes were measured by enzyme-linked immunosorbent assay of culture supernatants. TNFα was measured

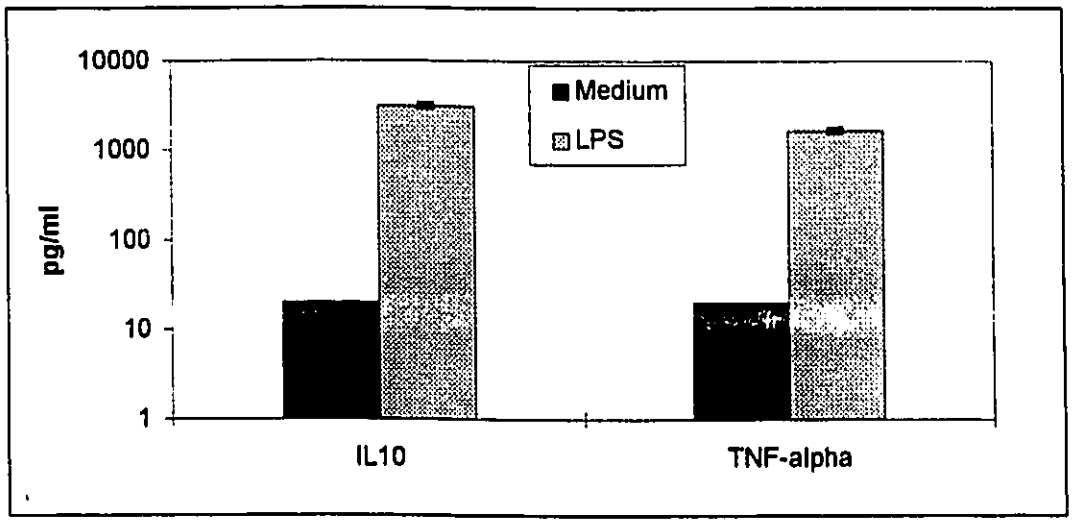


Figure 1: IL10 and TNFα Production by LPS Stimulated Monocytes. Purified monocytes (2 x 10^5 cells/ml) were cultured for 24 hrs in the presence and absence of 10 ng/ml of LPS. IL10 and TNFα were measured by ELISA following 24 hours of culture.

using a Quantikine kit (R&D Systems) essentially as described by the manufacturer. IL10 was measured by a sandwich ELISA using two different MAb that recognize two distinct epitopes as described previously (60). Briefly, plates (Nunc Immunomodules) were coated overnight at 4° C at a concentration of 3 μg/ml of purified anti-human and viral IL10 MAb, JES3-9D7 (Rat IgG1, Pharmingen, San Diego, CA) in the coating buffer (0.1M NaHCO3, pH 8.2). The plates were washed with PBS-Tween 20 and blocked with PBS-10% FBS. IL10 was detected with a second biotinylated MAb, 18562D (Rat IgG2a, Pharmingen) at a concentration of 3 μg/ml in PBS-10% FBS. Streptavidin-peroxidase (Jackson Immuno-Research) was used at a final dilution of 1:1000. The colour reaction was developed by OPD and hydrogen peroxide. Recombinant IL10 (R&D Systems) was used as a standard. The sensitivity of the IL10 ELISA was 16 pg/ml.

Cell stimulation: PBMC were cultured at a concentration of 3 x 10^6 per ml in 24 well plates (Corning) in the presence or absence of cytokines. Purified B cells (8 x 10^5 cells/ml) were stimulated with anti-IgM conjugated sepharose beads (BioRad, 10 μg/ml) and purified monocytes (2 x 10^5 cells/ml) were stimulated with LPS (Sigma, 1 μg/ml), in the presence or absence of cytokines. The cells were analyzed for the expression of B7-1 and B7-2 by flow cytometry following 24 and 48 hrs of culture.

Flow Cytometric Analysis: Cells were washed once with PBS/0.1% sodium azide and stained with 5 μl of either PE labelled anti-B7-1 (Becton-Dickinson, Lincoln Park, NJ) or PE labelled anti-B7-2 (Pharmingen) monoclonal antibodies (MAb) and 2.5 μl of FITC labelled anti-CD14 MAb (Becton-Dickinson). For

analysis of B7 isoforms on monocytes in PBMC, CD14+ cells were gated. Autofluorescence tubes and isotype matched control MAb (Becton-Dickinson) were also included. Using a Coulter EPICS XL Flow Cytometer, 5000 events were counted for purified B cells and purified monocytes, and 50,000 events were counted for PBMC.

RESULTS:

B7-1 and B7-2 expression on CD14+ monocytes in PBMC.

Freshly isolated PBMC from 9 healthy controls were analyzed for co-expression of CD14 and either B7-1 or B7-2 in the absence of stimulation by flow cytometric analysis. B7-1 was detectable on 4.8 ± 0.35% of CD14+ cells in PBMC whereas B7-2 was expressed on 85.4 ± 1.6% of CD14+ cells in PBMC. The percentage of CD14+ monocytes positive for B7-1 and B7-2 increased following culture without stimulation, reaching a peak at 24 hrs of 12.4 ± 3.2 % and 96 ± 0.8 % respectively. These results suggest that B7-2 constitutes the major B7 isoform expressed on resting and activated monocytes.

Effects of cytokines on monocyte B7-1 and B7-2 expression following culture.

To measure the effects of immunoregulatory cytokines on the expression of B7 isoforms, PBMC from five healthy individuals were cultured in the presence of either IL1α, IL1β, IL2, IL4, IL5, IL6, IL10, IL12, IFNγ, TNFα, TGFα or GMCSF followed by analysis of surface expression of B7-1 and B7-2 isoforms by flow cytometry. B7-1 and B7-2 expression on monocytes was not altered by IL1α, IL1β, IL2, IL5, IL6, IL12, GMCSF and TGFα (data not shown). The effects of the other cytokines IL4, IL10, TNFα and IFNγ were examined in more detail.

Effects of cytokines associated with Th2-type immune responses on monocyte B7 isoform expression.

Monocyte B7 isoform expression was found to be modulated by the Th2 type cytokines IL4 and IL10, and also by TNFα, which exhibits both Th1 and Th2 type characteristics depending on experimental conditions (62).

IL10: PBMC from 6 healthy individuals were cultured without stimulation in the presence or absence of IL10. IL10 downregulated the expression of B7-2 and

moderately enhanced the expression of B7-1 on CD14+ cells. Figures 2 and 3 respectively show the mean channel fluorescence (MCF) of B7-2 and B7-1 expression on CD14+ monocytes in PBMC in the presence of 50 U/ml IL10.

Histograms of B7-2 and B7-1 expression in the presence and absence of IL10 on PBMC from one representative individual are shown in Figures 4A and 4B respectively. The inhibitory effect of IL10 on B7-2 expression was dose dependent (Fig. 4C). Maximal suppression of B7-2 expression was achieved at IL10 concentrations of 25 U/ml. Kinetics of B7-2 expression on monocytes following treatment with IL10 revealed that maximum inhibition was observed after 24 hrs of culture (data not shown).

IL4: Similar effects on the expression of B7-1 and B7-2 on cultured monocytes were observed in the presence of 50 ng/ml IL4. IL4 was used at a final concentration of 50 ng/ml as this concentration is in excess of the concentration demonstrated in previous experiments to have a measurable effect on the monocyte surface molecule expression. Figures 5A and 5B illustrate the effects of IL4 on B7-2 and B7-1 expression on monocytes from 5 healthy individuals respectively.

Figures 6A and 6B show flow cytometric histograms demonstrating the effect of IL4 on B7-2 and B7-1 expression on monocytes from one representative healthy individual of the five performed. These results suggest that IL4 and IL10 act similarly and exert differential effects on the expression of B7-1 and B7-2 on cultured monocytes.

TNFα: Expression of B7-2 on cultured monocytes was also found to be downregulated by TNFα in a manner similar to the effects of IL10. TNFα was used at a final concentration of 40 ng/ml as this concentration is in excess of the concentration demonstrated in previous experiments to have a measurable effect on the monocyte surface molecule expression. Figures 7A and 7B show the effect of

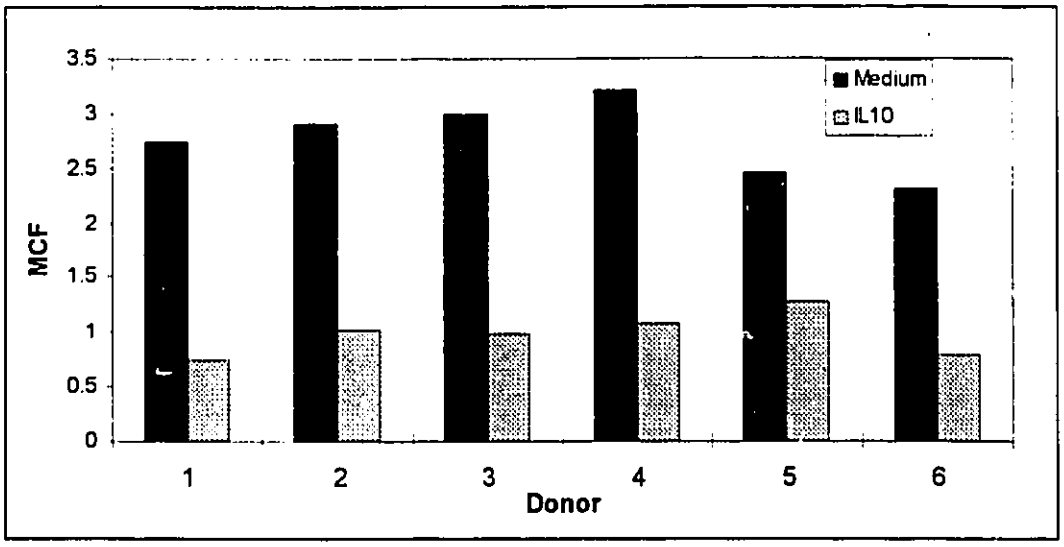


Figure 2: Effect of IL10 on B7-2 Expression by CD14+ PBMC. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 50 U/ml of recombinant human IL10 and CD14+ monocytes were analyzed for the expression of B7-2 by two colour flow cytometry.

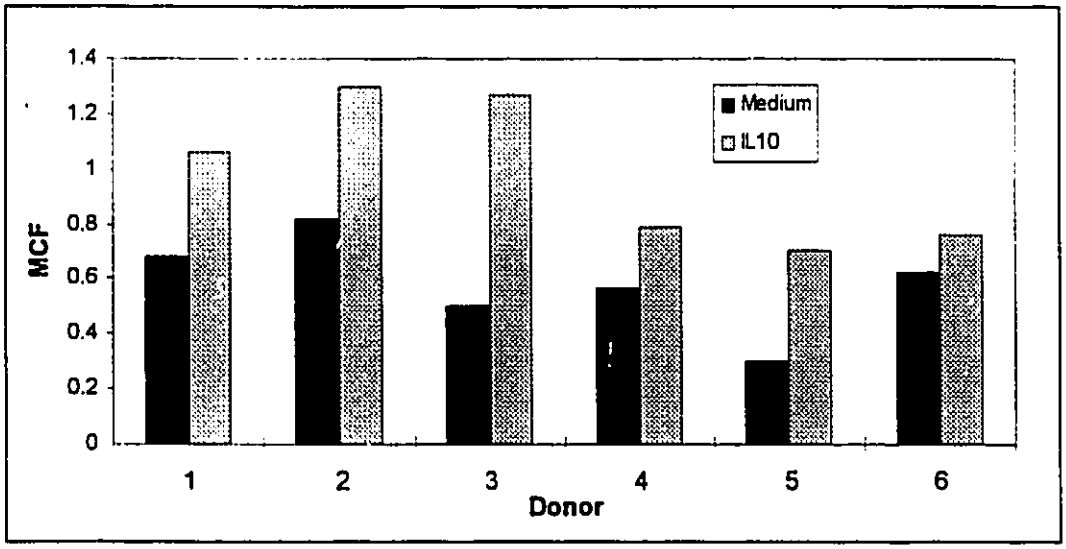
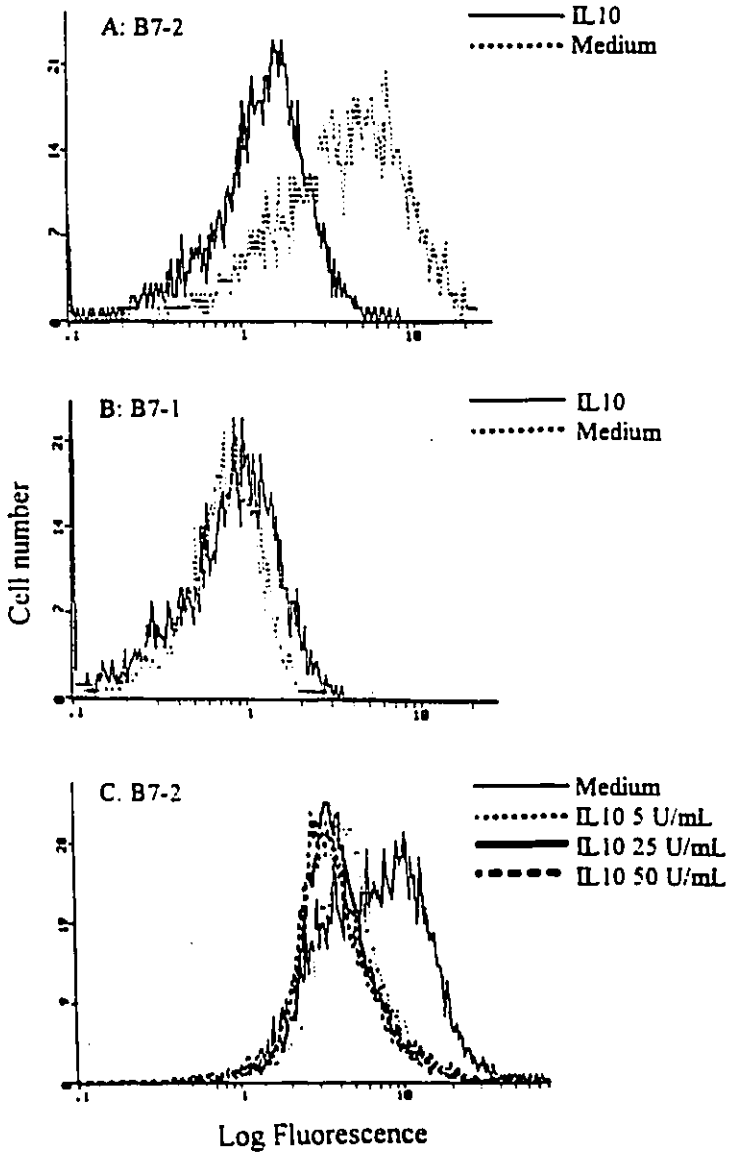


Figure 3: Effect of IL10 on B7-1 Expression by CD14+ PBMC. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 50 U/ml of recombinant human IL10 and CD14+ monocytes were analyzed for the expression of B7-1 by two colour flow cytometry.

Figure 4: Differential effects of IL10 on B7 isoform expression on CD14+ monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 50 U/ml of recombinant human IL10 and CD14+ monocytes were analyzed for expression of either B7-2 (A) or B7-1 (B) by two colour flow cytometry. Fig. 4C demonstrates the dose dependent effect of IL10 (5-50 U/ml) on B7-2 isoform expression on CD14+ resting monocytes. Histograms from one representative individual are shown.


A: B7-2
IL10
Medium
B: B7-1
IL10
Medium
Cell number
C. B7-2
Medium
IL10 5 U/mL
IL10 25 U/mL
IL10 50 U/mL
Log Fluorescence

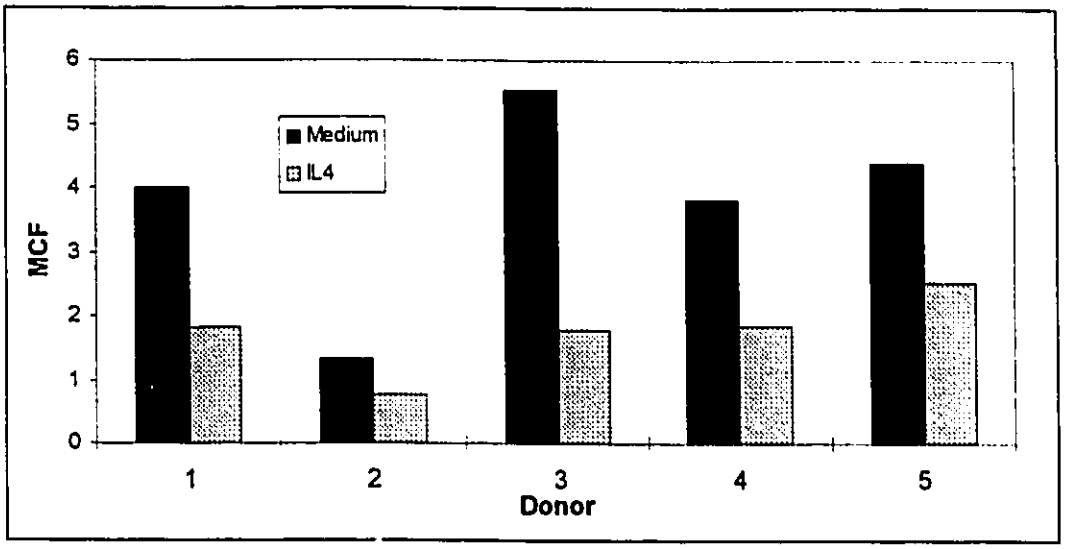


Figure 5A: Effect of IL4 on B7-2 Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 50 ng/ml of recombinant human IL4 and CD14+ monocytes were analyzed for expression of B7-2 by two colour flow cytometry.

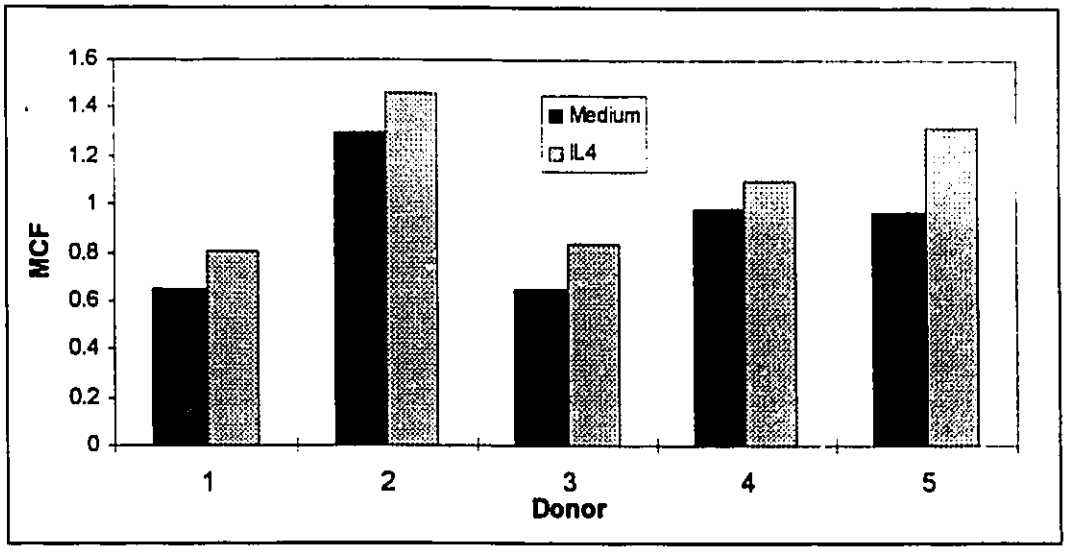
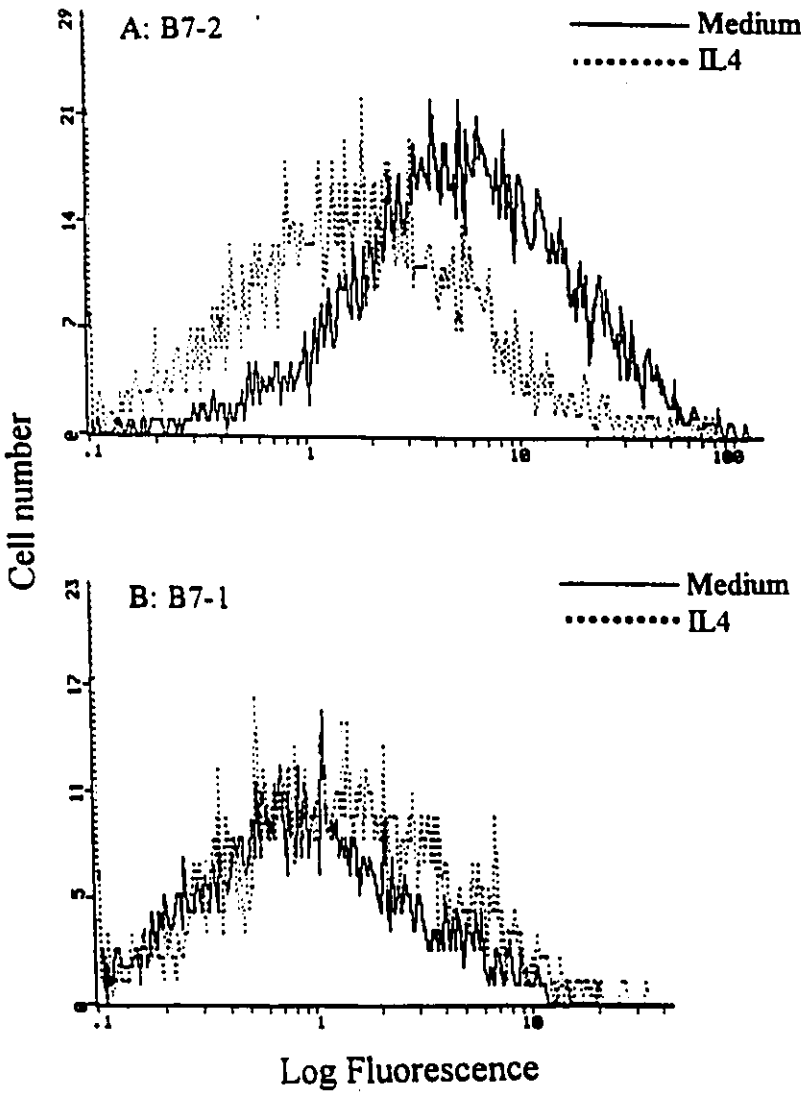


Figure 5B: Effect of IL4 on B7-1 Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 50 ng/ml of recombinant human IL4 and CD14+ monocytes were analyzed for expression of B7-1 by two colour flow cytometry.

Figure 6: Differential effects of IL4 on B7 isoform expression on CD14+ monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 50 ng/ml of recombinant human IL4 and CD14+ monocytes were analyzed for expression of either B7-2 (A) or B7-1 (B) by two colour flow cytometry. Histograms from one representative individual are shown.


A: B7-2
Medium
IL4
B: B7-1
Medium
IL4
Cell number
Log Fluorescence

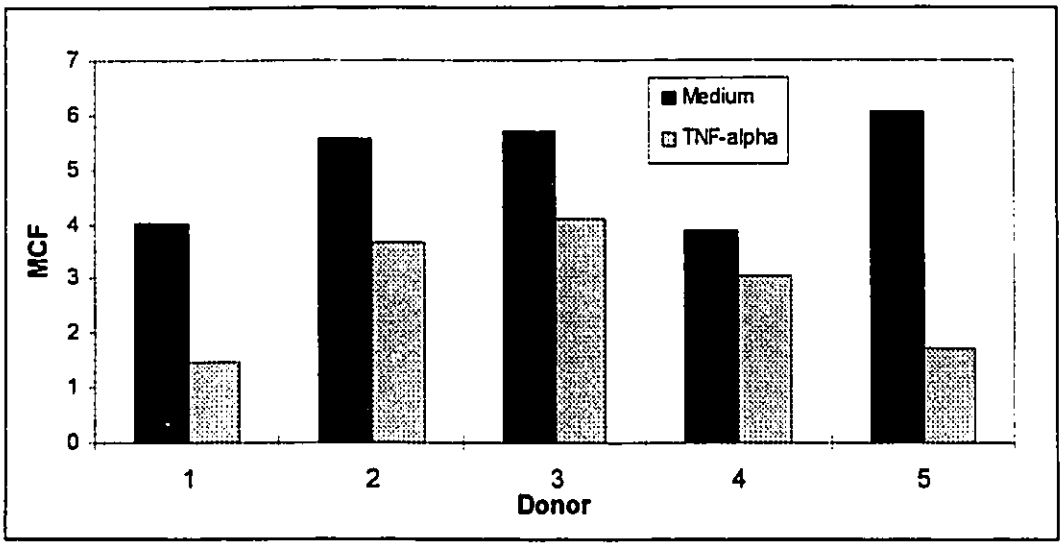


Figure 7A: Effect of TNFα on B7-2 Isoform Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 40 ng/ml of recombinant human TNFα and CD14+ monocytes were analyzed for expression of B7-2 by two colour flow cytometry.

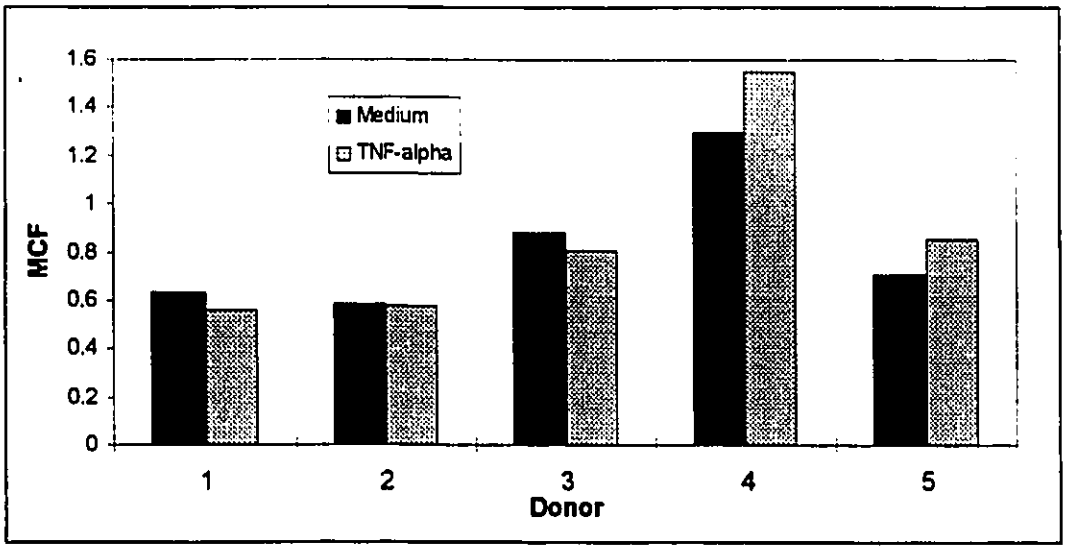


Figure 7B: Effect of TNFα on B7-1 Isoform Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 40 ng/ml of recombinant human TNFα and CD14+ monocytes were analyzed for expression of B7-1 by two colour flow cytometry.

TNFα on B7-2 and B7-1 expression respectively on monocytes from 5 healthy individuals.

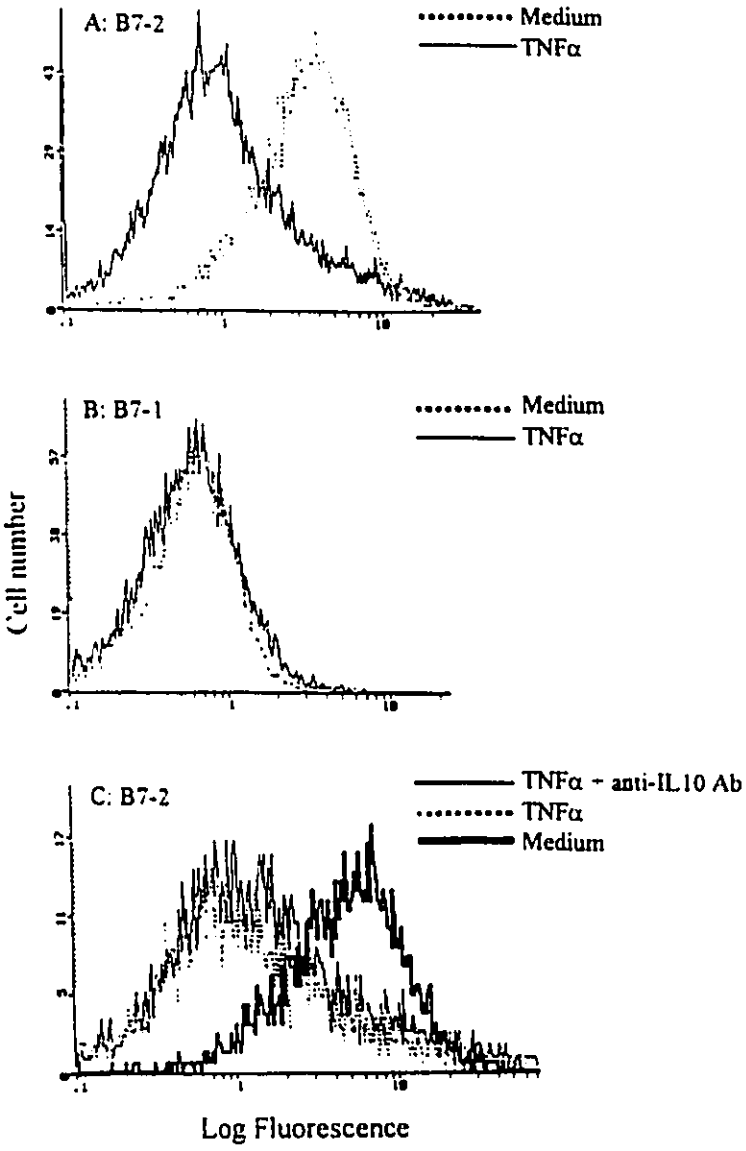
However, in contrast to IL10, variable effects of TNFα on B7-1 expression were observed. Three individuals demonstrated no difference, while 2 showed slight enhancement of B7-1 expression (Figure 7B). Histograms demonstrating B7-2 and B7-1 expression from one representative individual of the five performed are shown in Figures 8A and 8B respectively. TNFα has been shown to induce IL10 production by monocytes (151). We confirmed that IL10 production by purified monocytes is induced by TNFα (60) (Fig. 9).

To determine whether the inhibitory effects of TNFα on B7-2 expression were mediated through endogenously-produced IL10, neutralizing anti-IL10 antibodies were added to monocytes in the presence of TNFα. Anti-IL10 antibodies did not abrogate the effect of TNFα-induced B7-2 inhibition, even when high concentrations of anti-IL10 antibodies were used (20 μg/ml, Fig. 8C). These results suggest that IL10 and TNFα may modulate B7 expression by distinct and independent mechanisms.

Effects of Cytokines associated with Th1-type immune responses on Monocyte B7 isoform expression.

IFNγ: IFNγ, a Th1 type inducing cytokine (1,39), enhanced the expression of both B7-2 and B7-1 on cultured monocytes. IFNγ was used at a final concentration of 100 ng/ml as this concentration is in excess of the concentration demonstrated in previous experiments to have a measurable effect on the monocyte surface molecule expression. Figures 10A and 10B show the effects of IFNγ on B7-2 and B7-1 expression respectively on CD14+ monocytes from four donors. Figures

Figure 8: Effect of TNFα on B7 Isoform Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 40 ng/ml of recombinant human TNFα and CD14+ monocytes were analyzed for expression of either B7-2 (A) or B7-1 (B) by two colour flow cytometry. Fig. 8C demonstrates the effect of addition of anti-IL10 antibodies (20 μg/ml) in the presence of TNFα on the expression of B7-2 isoforms on CD14+ resting monocytes. Histograms from one representative individual are shown.


A: B7-2
Medium
TNFα
B: B7-1
Medium
TNFα
Cell number
C: B7-2
TNFα + anti-IL10 Ab
TNFα
Medium
Log Fluorescence

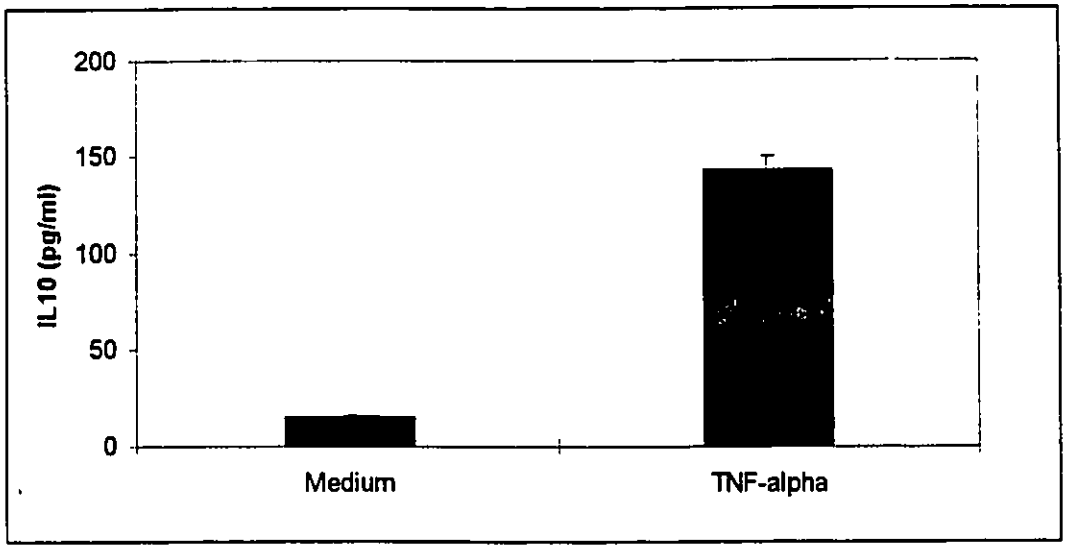


Figure 9: IL10 Production by Purified Monocytes in Presence of TNFα. Monocytes ($1x10^6$ cells/ml) purified using anti-CD14 antibody coated Immunobeads were cultured for 48 hours in the presence or absence of 40 ng/ml TNFα. IL10 production was measured by ELISA in culture supernatants.

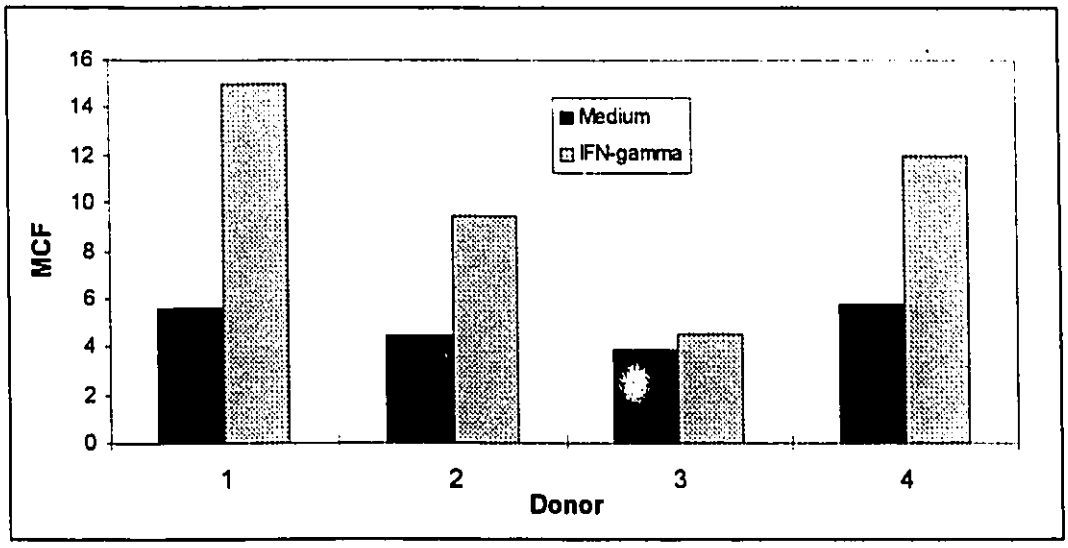


Figure 10A: Effect of IFNγ on B7-2 Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 100 ng/ml of recombinant human IFNγ and CD14+ monocytes were analyzed for expression of B7-2 by two colour flow cytometry.

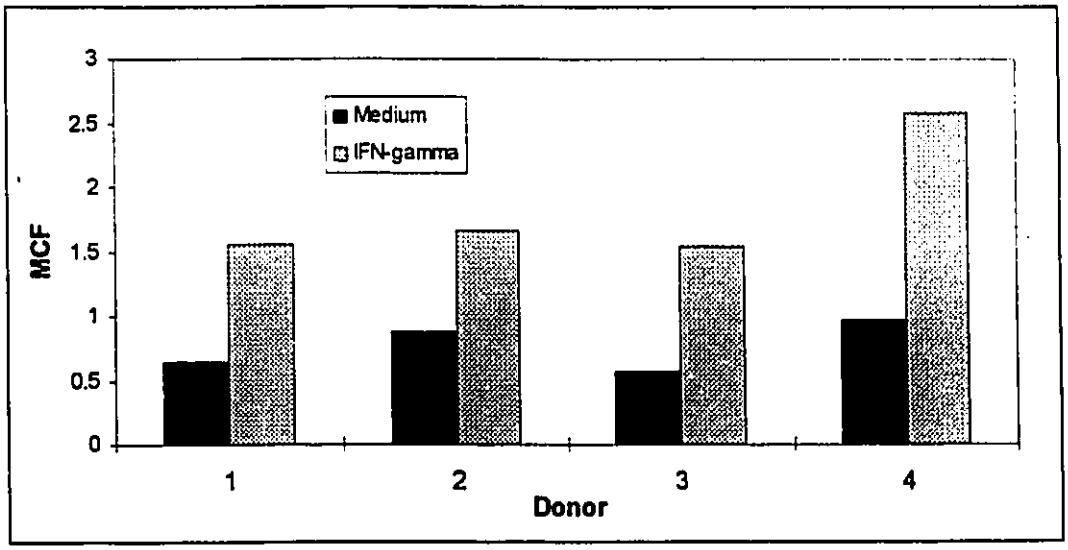


Figure 10B: Effect of IFNγ on B7-1 Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 100 ng/ml of recombinant human IFNγ and CD14+ monocytes were analyzed for expression of B7-1 by two colour flow cytometry.

11A and 11B demonstrate flow cytometric histograms from one representative individual.

IL12: IL12, a cytokine which induces Th1-type immune responses, did not alter B7 isoform expression on CD14+ monocytes in PBMC. Data from one representative donor are shown in Figures 12A and 12B. The percentage of cells positive for B7-2 and B7-1 relative to isotype control and autofluorescence tubes are plotted on the y-axis.

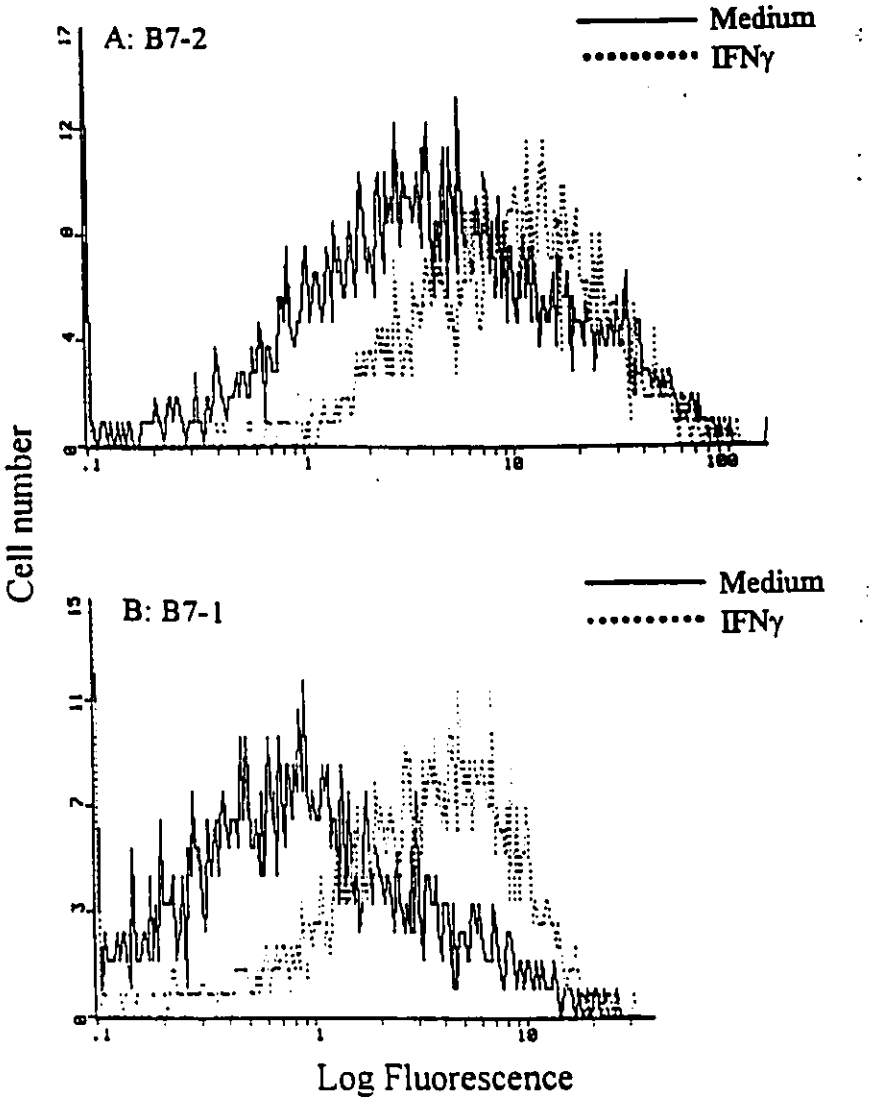
Expression of B7-1 and B7-2 on monocytes activated with LPS.

The above results show that Th1 and Th2 type cytokines mediated differential effects on the expression of B7-2 and B7-1 on resting monocytes. To further determine whether these cytokines exhibited similar effects on activated monocytes, purified monocytes were stimulated with LPS and cultured in the presence of the above mentioned cytokines for 24 hours. Purified monocytes rather than PBMC were used in order to minimize the effects on monocyte B7 isoform expression of lymphocyte-derived cytokines produced during LPS-induced activation of PBMC.

Purified monocytes from 5 healthy individuals were stimulated with LPS (10 ng/ml), and B7 isoform expression was analyzed after 24 hrs of culture. Activation of monocytes with LPS caused significant inhibition of B7-2 and moderate enhancement of B7-1 expression relative to unstimulated cells, in a similar manner to IL10. Mean results from 5 individuals are shown in Figure 13.

Histograms from one representative donor are shown in Figures 14A and 14B for B7-2 and B7-1 respectively. Monocytes were highly sensitive to the effects of LPS with respect to B7 isoform expression, with modulation evident at concentrations as low as 10 pg/ml. Monocytes stimulated with LPS were refractory

Figure 11: Effect of IFNγ on B7 Isoform Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 100 ng/ml of recombinant human IFNγ and CD14+ monocytes were analyzed for expression of either B7-2 (A) or B7-1 (B) by two colour flow cytometry. Histograms from one representative individual are shown.


A: B7-2
Medium
IFNγ
B: B7-1
Medium
IFNγ
Cell number
Log Fluorescence

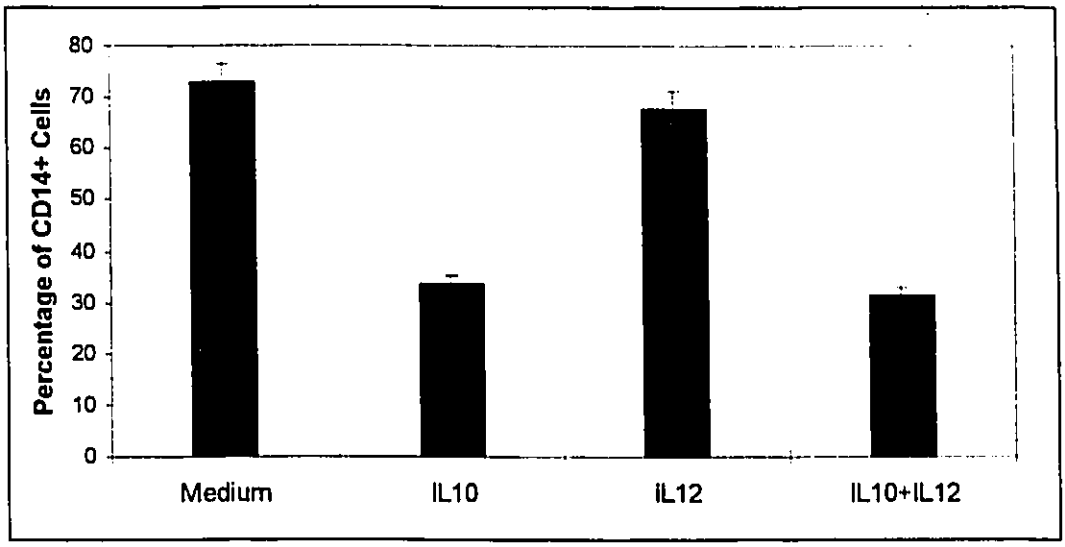


Figure 12A: Effect of IL12 on B7-2 Isoform Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 170 U/ml of recombinant human IL12 and CD14+ monocytes were analyzed for expression of B7-2 by flow cytometry.

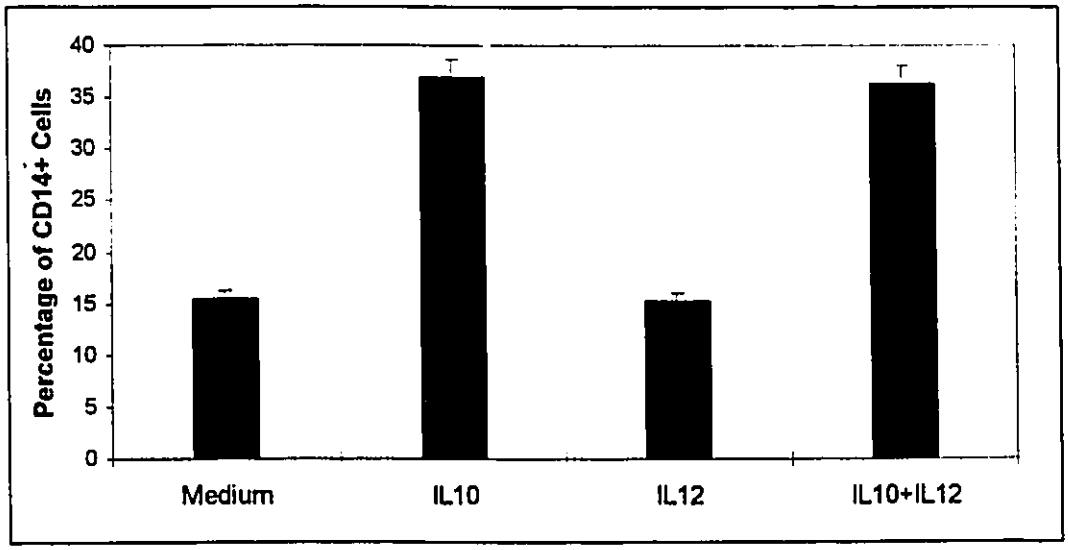


Figure 12B: Effect of IL12 on B7-1 Isoform Expression on CD14+ Monocytes. PBMC ($3x10^6$ cells/ml) were cultured for 24 hrs in the presence and absence of 170 U/ml of recombinant human IL12 and CD14+ monocytes were analyzed for expression of B7-1 by flow cytometry.

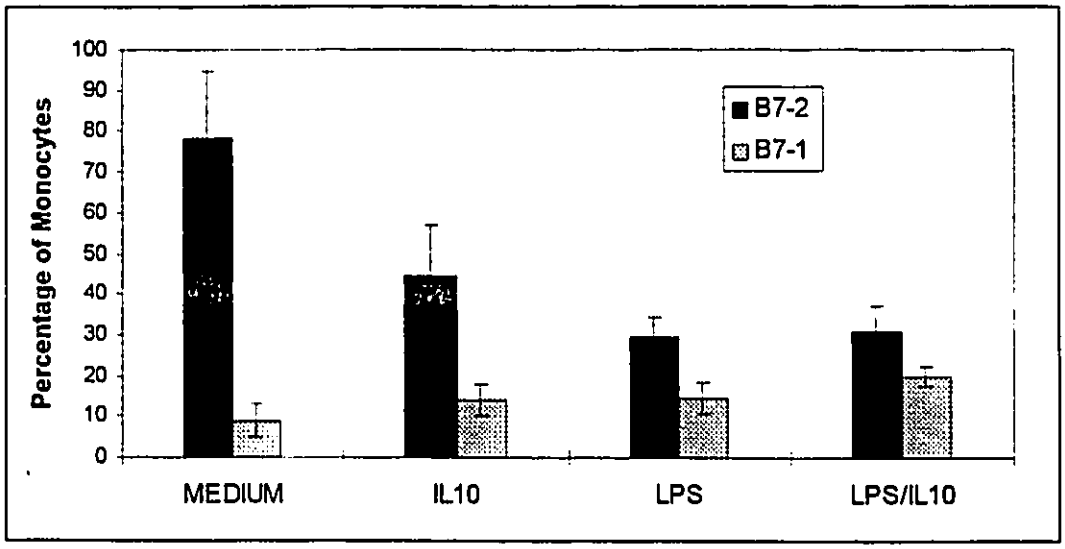
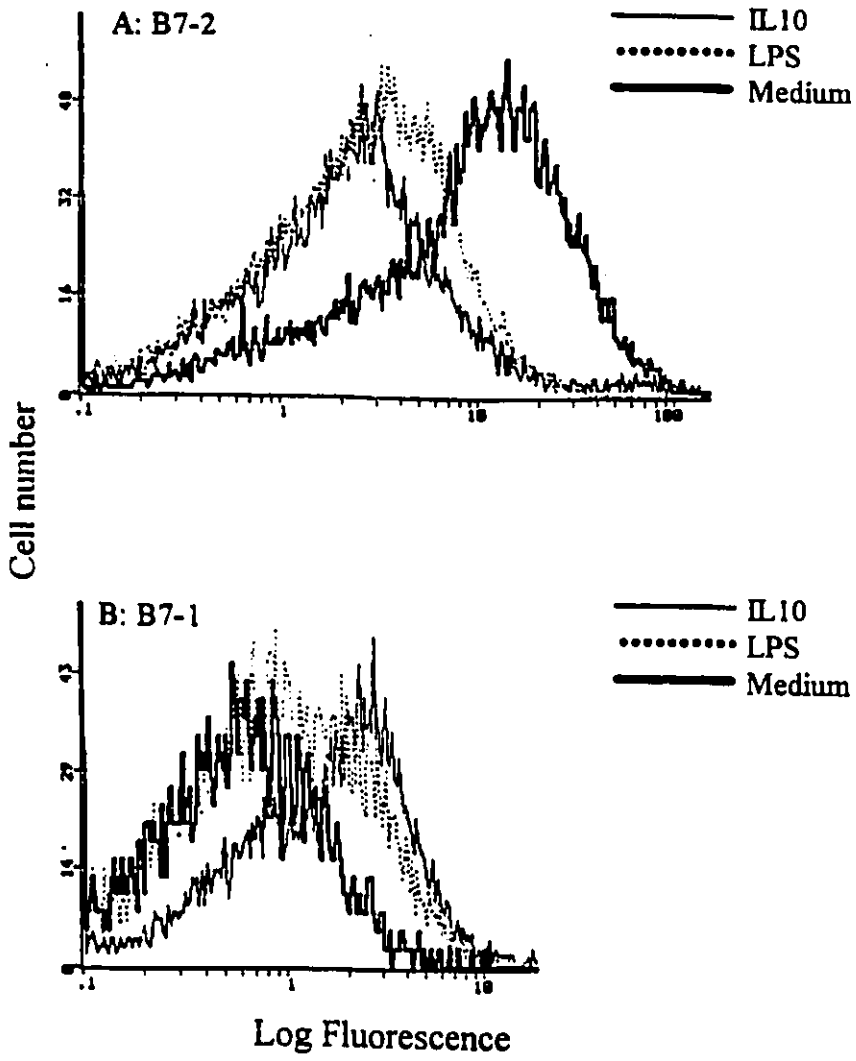


Figure 13: Effect of LPS on B7 Isoform Expression on Purified Monocytes. Purified monocytes (2 x 10^5 cells/ml) were cultured for 24 hrs in the presence and absence of 10 ng/ml of LPS and/or 50 U/ml of IL10 and B7 isoform expression was analyzed by flow cytometry.

Figure 14: Effect of LPS on B7 Isoform Expression on Purified Monocytes. Monocytes (2 x 10^5 cells/ml) purified using anti-CD14 antibody coated Immunobeads were cultured for 24 hrs in the presence and absence of 10 ng/ml of LPS or 50 U/ml of IL10 and B7-2 (A) or B7-1 (B) were analyzed by flow cytometry. Histograms from one representative individual are shown.


A: B7-2
IL10
LPS
Medium
Cell number
B: B7-1
IL10
LPS
Medium
Log Fluorescence

to the effects of the above mentioned panel of cytokines on B7 expression (data not shown). The effects of LPS on B7-2 and B7-1 expression were similar to those observed with IL10. The inhibition of B7-2 and enhancement of B7-1 expression on LPS activated monocytes may have been due to induced IL10 production (Figure 1).

Expression of B7-1 and B7-2 on resting and activated purified normal B cells.

In addition to monocytes, B lymphocytes represent another important type of circulating APC. To investigate the effect of cytokines on B7 isoform expression on B cells, normal resting and activated human B cells were analyzed for B7 expression following culture in the presence of cytokines. None of the cytokines tested, including IL1α, IL1β, IL2, IL4, IL5, IL6, IL10, IL12, IFNγ, TNFα, TGFα and GMCSF, influenced the levels of B7-1 or B7-2 expression on either resting or activated B cells (data not shown). Activation of B cells following stimulation with anti-IgM antibody coated sepharose beads did not alter B7-1 or B7-2 expression on CD19+ B cells (Figure 15).

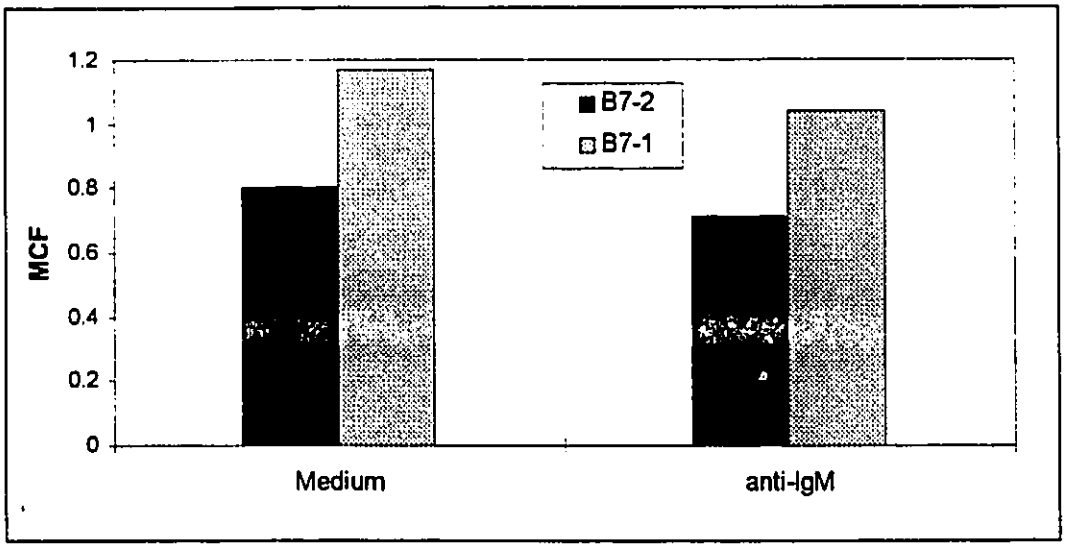


Figure 15: B7 Isoform Expression on Resting and B cells Activated Using Anti-IgM Antibody Conjugated Sepharose Beads. Purified B cells (8 x 10^5 cells/ml) were cultured for 24 hours in the presence or absence of anti-IgM antibody conjugated sepharose beads (10 μg/ml) and B7 isoform expression was measured by flow cytometry.

DISCUSSION:

Cytokine-induced alterations in surface expression of costimulatory molecules such as B7 isoforms may play critical roles in control of the immune system. The current results suggest that immunoregulatory cytokines differentially modulate the expression of B7-1 and B7-2 isoforms on unstimulated CD14+ monocytes. Cytokines which induce the development of predominantly Th2 type T cells (e.g. IL4, IL10) (39-41) inhibited the expression of B7-2 and moderately enhanced the expression of B7-1. Similar results were obtained with TNFα, a cytokine which has Th1 or Th2 type properties depending on experimental conditions (62), although B7-1 expression was not altered. IFNγ, a cytokine which induces the development of Th1 type T cells (1,6,38,39), enhanced the expression of both B7-1 and B7-2 isoforms. None of the other cytokines tested, including IL1α, IL1β, IL2, IL5, IL6, IL12, TGFα and GMCSF, modulated the expression of either B7-1 or B7-2 isoforms.

IL10 has been shown to inhibit B7-2 expression on monocytes, Langerhans cells and dendritic cells (122-125). Expression of the B7-1 isoform on Langerhans cells is downregulated by IL10 and IFNγ but is upregulated by IL1α, IL1β, IL4, GMCSF, and TNFα, whereas B7-2 expression is downregulated by IL10 but not by IFNγ (122,123). Increased expression of B7 isoforms on Langerhans cells and monocytes has been correlated with enhanced APC function (126,127). Moreover, IL10 has been shown to mediate its inhibitory effects by inhibiting the expression of B7 molecules on monocytes/macrophages (129) and may thus regulate the development of Th2 type responses by altering the expression of B7 isoforms. The

exact molecular mechanisms of induction of Th2 type T cells by IL4 and Th1 type T cells by IFNγ have not been delineated, but may be related to the ability of these cytokines to modulate B7 isoform expression on APC.

The current results demonstrate for the first time that IL4 and IL10 moderately enhance the expression of B7-1 but downregulate the expression of B7-2 on unstimulated monocytes. Furthermore, IFNγ enhances expression of both B7-1 and B7-2 isoforms. Inhibition of B7-2 and moderate enhancement of B7-1 expression observed on LPS activated monocytes may be due to the high levels of endogenously produced IL10 following stimulation with LPS. TNFα inhibited the expression of B7-2 in a manner similar to IL4 and IL10, but had no effect on the expression of B7-1. The inhibition of B7-2 on unstimulated monocytes by TNFα was speculated to have been mediated indirectly by IL10, as TNFα has been shown to induce IL10 secretion by human monocytes (151). However, addition of anti-IL10 antibodies to TNFα stimulated monocytes did not restore B7-2 expression (Fig. 3). It is possible that anti-IL10 antibodies were insufficient to neutralize endogenously produced IL10 following stimulation with TNFα. Alternatively, the concentrations of IL10 produced endogenously following stimulation with TNFα may have been too low to modulate B7-2 expression under these experimental conditions. Furthermore, addition of IL10 to purified monocytes stimulated with influenza antigen inhibited the expression of B7-2 whereas TNFα failed to do so (data not shown). These results suggest that TNFα and IL10 downregulate B7-2 expression by distinct mechanisms. However, experiments in which endogenous

IL10 production is completely blocked using antisense oligonucleotides or antisense IL10 expression vectors may aid in ruling out the possibility of IL10-mediated inhibition of B7-2 isoform expression.

Whether modulation of B7 isoform expression on monocytes can influence the T helper phenotype of an immune response is not known. Although the biological functions of individual B7 isoforms are not well understood, recent evidence suggests that they may play vital roles in the development of Th1/Th2 type immune responses (128,134,141-144). In the current studies, IL10 and IL4, potent inducers of Th2 type immunity, downregulated B7-2 and upregulated B7-1 expression on CD14+ monocytes. This is in contrast to predictions based on the findings of Kuchroo et al., who found that, in a non-obese diabetic mouse model, blockade of the B7-2 signalling pathway was associated with less severe disease, whereas blockade of the B7-1 pathway resulted in more severe disease (141). In addition, *in vitro* studies demonstrated that B7-2 cosignalling preferentially induces naive T cells to develop into Th2-like cells (134,142) and B7-1 has been associated with efficient tumour rejection (128,143). Extrapolating from these results, B7-2 would have been expected to be upregulated by IL10 and IL4 and thus skew the immune response towards a Th2 phenotype. Alternatively, cytokine induced alterations in B7 isoform expression may represent a negative feedback system of immune regulation. Thus, a predominantly Th2 type immune response would increase concentrations of IL10 and IL4 in the microenvironment, and would serve to downregulate B7-2 expression. Cytokines produced by the T cells following engagement of the TCR and the costimulatory pathways may feed back to the APC

and alter B7 isoform expression. In this case, factors other than B7 molecules are involved in induction of the initial Th2 type response, with cytokine induced changes in B7 molecules occurring later.

The mechanisms by which the initial Th2 response is generated remain to be determined. In *in vivo* models, conflicting results regarding the effects of B7 isoforms on T helper cell phenotype have been reported. Lenschow et al. found that in murine experimental allergic encephalomyelitis, B7-1 expression was associated with the development of harmful Th1 type immunity, and B7-2 with the development of protective Th2 type immunity (142). Results of future experiments may clarify these relationships. IFNγ, a potent inducer of Th1 type immunity, upregulated both B7 isoforms, and IL12 did not have the predicted opposite effects to IL10/IL4 on B7 isoform expression despite its demonstrated role as a potent inducer of Th1 type immunity (1,38,39,43,44). These findings may indicate that Th1 type immunity is induced in a different manner to Th2 type immunity, being less dependent on the B7 isoform involved in costimulation.

Among the other cytokines tested, none modulated the expression of B7 isoforms on monocytes. The observation that IL12 did not alter B7 isoform expression may be explained by the lack of functional IL12 receptors on monocytes (152), and by the fact that IL12 is produced by monocytes but exerts its biological effects mainly on CD4+ and CD8+ T cells and NK cells (63). Alternatively, IL12 may mediate its Th1-inducing effects through mechanisms distinct from B7 isoform modulation. Since IFNγ production by activated T cells has been shown to be induced by IL12 (63,153,154), IFNγ may represent a critical mediator in the

induction of Th1 type T cells through its modulation of monocyte APC function. In the current experiments, IFNγ but not IL12 modulated B7 isoform expression. Resting rather than activated T cells were utilized, indicating that IFNγ may not have been induced by IL12 in this protocol.

None of the cytokines modulated the expression of B7 isoforms on unstimulated or anti-IgM activated B cells. This is in agreement with previous reports which suggested that IL10 inhibits the APC function of monocytes but not of B cells (51). Interestingly, expression of B7-1 and B7-2 was detected on 95-100% of EBV-transformed lymphoblastoid B cells. These studies suggest that B cells, monocytes, dendritic cells and Langerhans cells may be subject to different control mechanisms with respect to their roles as APC. The specific mechanisms which control B7 isoform expression on B cells and other APC remain to be elucidated.

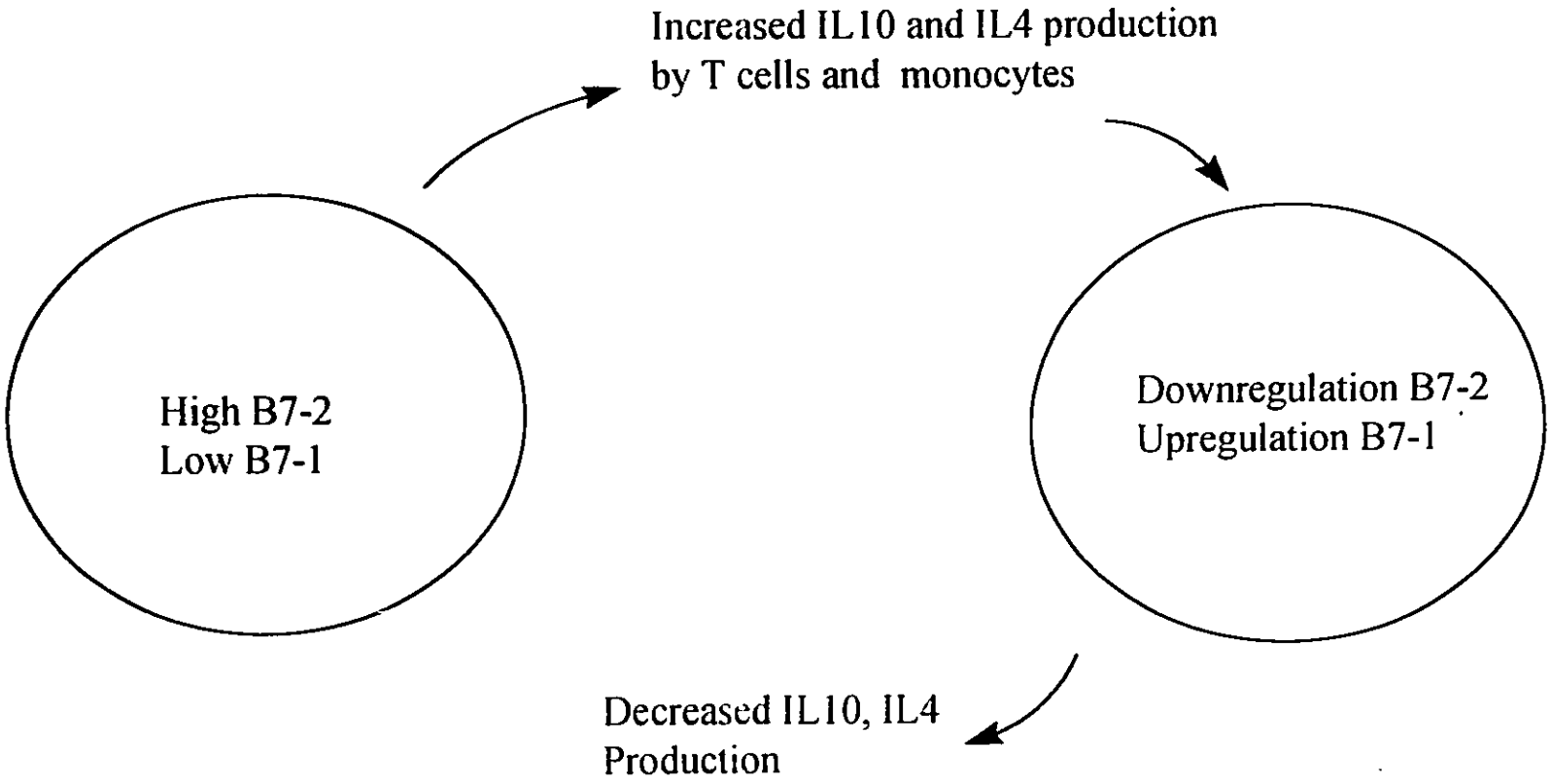
Current evidence indicates that both CD28/CTLA-4 and B7 isoforms are under complex control, with different mechanisms in effect on different cell types and at different stages of the immune response. The current findings may reflect this complexity, and the potential for applicability to other types of immune responses may be limited. However, monocytes are an important circulating APC type, and the tested cytokines have been demonstrated to be central to the immune response under other experimental conditions. Thus the results presented are significant in indicating an important potential mechanism through which IL10 and IL4 modulate B7 costimulatory molecules. If the current findings can be confirmed using *in vivo*

models, cytokine-induced alterations in B7 isoforms may theoretically mediate their effects through feedback inhibition (see Proposed Model).

A number of avenues for future research are indicated by the current data. Firstly, elucidation of the mechanisms through which the effects of immunoregulatory cytokines modulate B7 isoform expression may be important in controlling and predicting their biological effects. IL4, IL10, IFNγ and TNFα are known to modulate the expression of vital cell surface molecules such as HLA class II antigens, cytokine receptors and adhesion molecules (51,124,139,140). Whether the effect of these cytokines on the expression of B7 isoforms is mediated by other cytokines or signalling molecules is not known. Secondly, the kinetics of gene expression in conjunction with cell-surface protein expression should be studied in the context of cytokine-induced B7 isoform alterations. Thirdly, the failure of IL12 to alter APC B7 isoform expression suggests that IL12 may mediate its Th1-inducing effects through mechanisms which do not involve B7 isoforms, and therefore that different immunoregulatory cytokines may influence T helper cell phenotype in different ways. In addition, resting monocytes and monocytes which were fully activated with LPS were used in these experiments, potentially limiting applicability to *in vivo* conditions. Complete monocyte activation may have overwhelmed the effects of the exogenous cytokines on B7 isoform expression. Further experiments using suboptimal activation or alloantigenic stimulation may be necessary to more closely mimic a natural immune reaction.

The regulatory effect of cytokines on the expression of costimulatory molecules may play a vital role in the development of an immune response. *In vivo*

Proposed Model of Negative Feedback Regulation of IL10/IL4 Production via B7 Isoform Expression on Monocytes



studies involving modulations in T helper cell phenotype through selective blockade of B7 isoforms are currently being performed. Extension of these results to protocols designed to alter B7 isoforms through administration of exogenous cytokines may be attempted. However, these studies would require attempts to differentiate the effects of the added cytokines on B7 isoforms from their many other effects. A greater understanding of the relative contributions of B7-1 and B7-2 in the induction and control of the immune system may aid in the design of novel immunotherapeutic approaches to a broad range of conditions, such as HIV/AIDS, transplantation and autoimmunity.

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