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THE EXPRESSION AND SIGNAL TRANSDUCTION OF CD4, AN HIV AND INTERLEUKIN-16 RECEPTOR, IN MONOCYTIC CELLS

A Thesis Submitted to the
School of Graduate Studies,
University of Ottawa

In Partial Fulfilment of the Requirements for the Degree of
Doctor of Philosophy
Department of Biochemistry, Microbiology & Immunology
Faculty of Medicine

By

Gina M. Graziani-Bowering

ABSTRACT

The down-regulation of CD4 by cultured monocytes has been observed by our group and by other investigators. Flow cytometric analysis was performed to elucidate factors influencing this phenomenon. The addition of LPS, GM-CSF, M-CSF or IL-10 to monocyte cultures failed to inhibit the decrease in monocyte CD4 expression following overnight culture. The down-regulation was an adherence-independent phenomenon since it was not prevented by culture in Teflon vials. The type of anticoagulant into which the peripheral blood was collected did not appear to be a factor. The presence or absence of lymphocytes within the cultures was also inconsequential. The continued culture of monocytes in a whole blood environment resulted in decreased CD4 down-regulation. Experiments in which CD4 was tagged with α-CD4-PE mAb prior to cell culture revealed that the down-regulation observed was the result of CD4 internalization.

In an attempt to further understand monocyte CD4 function, we investigated the role of monocyte CD4 in signal transduction. Stimulation of Thp-1 monocytic cells with antibody to CD4 resulted in a Ca^{2+} flux, as well as in the time-dependent tyrosine phosphorylation of various proteins having molecular weights of approximately 180, 140, 120, 110, 85, 65, 55, 50 and 35 kDa. We identified the 140 and 85 kDa proteins as PLC-γ1, and the regulatory subunit of PI3-K, respectively. Using immunoprecipitation/Western immunoblotting, we were unable, however, to show any direct association between CD4 and PLC-γ1, PI3-K, or other signaling proteins. In an attempt to identify proteins capable of associating with the cytoplasmic tail of CD4, we generated a GST-$CD4_{cyt}$ fusion protein for use in far Western blots and immunoprecipitation experiments. In both types of experiments, the GST-$CD4_{cyt}$

fusion protein routinely associated with 45 and 55 kDa proteins. In the immunoprecipitation experiments, a 35 kDa protein was also observed.

The above results suggest that the expression of monocytic CD4 is regulated during the differentiation process. Furthermore, the cytoplasmic tail of monocytic CD4 is associated with various proteins which we postulate function in signal transduction, and which may also play a role in CD4 down-regulation.

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I am very grateful to my family and friends who listened patiently, celebrated my accomplishments and commiserated with me during my set-backs. This degree would not have been possible without their support.

DEDICATION

This thesis is dedicated to my parents,
Giovina and Italo,
whose hard work and many sacrifices
have made possible my successes,
and to my husband Brian,
who has made the journey less formidable.

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α-	anti
Ab	antibody/antibodies
ACD	acid citrate dextrose
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen(s)
AIDS	acquired immunodeficiency syndrome
APS	ammonium persulfate
Asn	asparagine
Asp	aspartic acid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
Ca^{2+}	calcium ion
$CaCl_2 \cdot 2H_2O$	hydrated calcium chloride
cAMP	cyclic adenosine monophosphate
CD(3,4...)	cluster of differentiation (3,4...)
cDNA	complementary deoxyribonucleic acid
CDR2	complementarity determining region 2
CDR3	complementarity determining region 3
C_H1	first constant region of immunoglobulin heavy chain
CNS	central nervous system
CO_2	carbon dioxide
Cy5	cyanin dye 5

Cys	cysteine
D1	first extracellular CD4 domain
D2	second extracellular CD4 domain
D3	third extracellular CD4 domain
D4	fourth extracellular CD4 domain
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine
ddH_20	double-distilled water
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanidine
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
dTTP	deoxythymidine
EBV	Epstein-Barr virus
ECD	energy-coupled dye
ECL	enhanced chemiluminescence.
EDTA	ethylenediaminetetra-acetic acid disodium salt
EDTA (K_3)	potassium salt of EDTA
EGTA	Ethyleneglycol-bis[beta-aminoethylether]-N,N,N',N'-tetra-acetic acid
ERK	extracellular signal-regulated kinase(s)
Ets	human homologue of the erythroblastosis virus oncogene product
FBS	fetal bovine serum
Fc	crystallizable fragment of Ig

FITC	fluorescein isothiocyanate
FL1	fluorescence 1 (peak emission at 525 nm)
FL2	fluorescence 2 (peak emission at 575 nm)
FL3	fluorescence 3 (peak emission at 620 nm)
FL4	fluorescence 4 (peak emission at 675 nm)
FS	forward scatter
× g	force of gravity
GDP	guanosine diphosphate
Gly	glycine
GM-CSF	granulocyte/macrophage-colony stimulating factor
Grb2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
GTP	guanosine triphosphate
HBS	HEPES-buffered saline
HCl	hydochloric acid
HEPES	N-2-hydroxethylpiperazine-N-2-ethane-sulphonic acid
HEK 293	human epithelial kidney 293 cells
His	histidine
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen - DR locus
hr	hour(s)
IFN-γ	interferon-gamma
Ig	immunoglobulin(s)
IL-	interleukin
$InsP_3$	inositol 1,4,5 trisphosphate
IP	immunoprecipitate/immunoprecipitation

IPTG	isopropylthio-β-D-galactoside
ITAM	immunoreceptor tyrosine-based activation motif(s)
JAK	Janus kinase
Kb	kilobase
KCl	potassium chloride
kDa	kiloDalton
LCF	lymphocyte chemoattractant factor
Leu	leucine
LOG	logarithmic
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody/antibodies
MAPK	mitogen-activated protein kinase(s)
M-CSF	macrophage-colony stimulating factor
2ME	2-mercaptoethanol
mg	milligram(s)
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute(s)
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1 beta
ml	millilitre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MuLV RTase	Murine Leukemia virus reverse transcriptase
Myb	human homologue of the Avian myeloblastosis viral oncogene product

µg	microgram(s)
µl	microliter(s)
µm	micrometer(s)
µM	micromolar
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaF	sodium fluoride
$NaHCO_3$	sodium bicarbonate
N-linked	asparagine-linked
NaN_3	sodium azide
NaOH	sodium hydroxide
$NaPO_4$	sodium phosphate
NBP1	Nef binding protein 1
nCi	nanocurie(s)
NFAT	nuclear factor of activated T cells
NF-CD4	nuclear factor for CD4
NF-κB	nuclear factor-kappa B
NHS	N-hydroxysuccinimide
NK cells	natural killer cells
nm	nanometer(s)
NP-40	nonidet P-40
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI3-K	phosphatidylinositol- kinase

PIP_2	phosphatidylinositol 4,5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC-γ1	phospholipase C-gamma one
PVDF	polyvinylidene fluoride
RANTES	regulated on activation, normal T cell expressed and secreted
RBC	red blood cell
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute Medium-1640
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SAPK	stress-activated protein kinase(s)
sCD4	soluble CD4
SDF-1	stromal cell-derived factor 1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
Ser	serine
SH2	*src* homology domains 2
SH3	*src* homology domains 3
SIV	simian immunodeficiency virus
SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
SOCS	suppressor of cytokine signaling
Src	human homologue of the v-src protein of the Rous sarcoma virus
SS	side scatter

STAT	signal transducers and activators of transcription
TAE	Tris acetate-EDTA buffer
TBST	Tris buffered saline and Tween-20
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor-beta
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-alpha
TRI	Cy5-PE conjugate
Trp	tryptophan
Tyr	tyrosine
U	unit(s)
V_H	variable region of immunoglobulin heavy chain
v/v	volume with respect to volume
vs.	versus
WBC	white blood cell
w/v	weight with respect to volume
ZAP-70	zeta-chain associated protein-70
ZEB	zinc finger, E box-binding transcription factor
Zn^{2+}	zinc ion

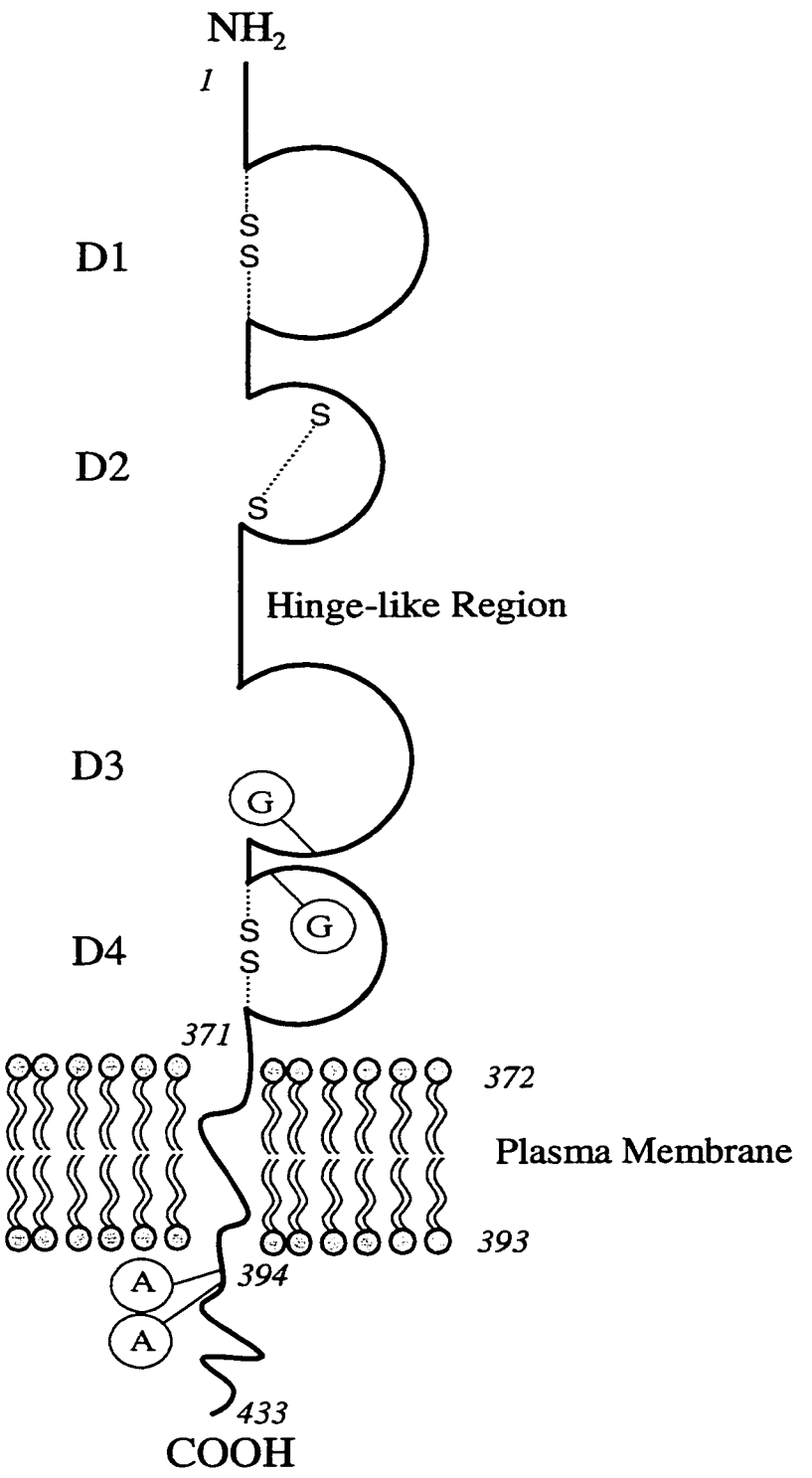
I. GENERAL INTRODUCTION

I.1. The structure of human CD4

The human CD4 molecule is a 55-60 kDa type I integral membrane glycoprotein (Foti et al. 1995) first identified on a subset of T cells which usually function as T-helper cells (Reinherz et al. 1979). The CD4 molecule has a 25 amino acid leader sequence which is cleaved, resulting in a mature protein consisting of a linear array of 4 extracellular domains (residues 1-371), a transmembrane domain (residues 372-393), and a cytoplasmic tail (residues 394-433) (Maddon et al.1985, SwissProt 1999 accession # P01730). The first extracellular domain (D1) consists of an Ig domain, whereas the remaining domains (D2-D4) consist of Ig-like domains (Brady and Barclay 1996); D2 has an unusual disulfide bridge, D3 has no disulfide bridge, and D2 and D4 are smaller than the typical Ig fold of 110 amino acids (Haseman and Capra 1989). Regardless of these variations, CD4 is classified as a member of the Ig Superfamily (Clark et al. 1987). X-ray crystallographic studies of intact sCD4 (ie. domains D1-D4) revealed a hinge-like region occurring at the junction between D2 and D3, as well as a dimerization site occurring in D4 (Wu et al. 1997). Recently, native CD4 has been found to exist as monomers, homodimers and tetramers in T cells and in cells of the monocyte/macrophage lineage (Lynch et al. 1999). Human CD4 also contains two N-linked glycosylations at Asn271 and Asn300 in domains D3 and D4, respectively, the presence of which is necessary for proper folding and expression (König et al. 1988, Tifft et al. 1992). Furthermore, Cys394 and Cys397 are acylated, the purpose of which is unclear (Crise and Rose 1992a). A schematic representation of the structure of the mature human CD4 molecule is shown in Fig.I.1.

Fig.I.1. The structure of the mature human CD4 molecule.

The mature CD4 molecule consists of a linear array of 4 extracellular domains (residues 1-371), a transmembrane domain (residues 372-393), and a cytoplasmic tail (residues 394-433). Domains D1, D2 and D4 contain disulfide bridges. N-linked glycosylations are found at Asn271 and Asn300 (denoted by circled G's), and Cys394 and Cys397 are acylated (denoted by circled A's).


NH2
1
D1
S
S
S
D2
S
Hinge-like Region
D3
G
G
D4
S
S
371
372
Plasma Membrane
393
A
394
A
433
COOH

I.2. The genetic regulation of T cell CD4

The gene for human CD4 is located on the short arm of chromosome 12 (Isobe et al. 1986). The coding portion of the gene consists of 9 exons. Exon 1 codes for the leader sequence, exons 2 and 3 for the D1 domain, exon 4 for the D2 domain, exon 5 for the D3 domain, exon 6 for the D4 domain, and exons 7 to 9 code for the transmembrane and cytoplasmic domains. The resulting CD4 mRNA transcript is 3.3 Kb. (Littman 1987).

The identification of elements regulating the expression of the CD4 gene has resulted from the study of CD4 in murine and human T cell models. To date, three enhancers, some of them specific for T-cells, have been identified for the murine CD4 gene (Sawada and Litmann 1991, Wurster et al. 1994, Adlam et al. 1997). These enhancers appear to be regulated at least in part by thymus-specific transcription factors (Sawada and Litman 1993) and by Ets transcription factors (Wurster et al. 1994). Furthermore, one of the enhancers can be converted into a silencer element by the transcriptional repressor ZEB (Brabletz et al. 1999). A murine T-cell specific promoter which binds the Myb transcription factor (Siu et al. 1992, Nakayama et al. 1993), as well as a nuclear factor designated NF-CD4 (Nakayama et al. 1993), have also been identified in the mouse, as has a transcriptional silencer (Siu et al. 1994, Sawada et al. 1994) whose regulation is extremely complex and appears to involve three different factor binding sites (Duncan et al. 1996), at least one of which can be bound by a transcriptional factor from the Notch signaling pathway (Kim and Siu 1998). In humans, two T-cell specific promoters have been identified (Salmon et al. 1993, Rushton et al. 1997), as has a T-cell specific enhancer (Blum et al. 1993) and a silencer (Donda et al. 1996).

The presence of so many positive and negative regulators of T cell CD4 gene expression points to an extremely complex regulation of CD4 expression in T cells. This process has been only partially elucidated and reveals that thymocytes and mature T cells primarily regulate CD4 expression at the level of transcription (Ellmeier et al 1999). Interestingly, the control of CD4 expression is performed differently in thymocytes *vs.* mature T cells, as exemplified by the observation that two active enhancers are required for CD4 expression by mature $CD4^-$ T cells, whereas three enhancers are required by immature $CD4^+$ $CD8^-$ thymocytes (Adlam et al. 1997).

The mechanisms by which myeloid cells regulate CD4 gene expression are unknown given the almost exclusive use of T cell models in studies performed to date. One exception, however, is the identification of a macrophage-specific enhancer within the human CD4 gene (Ellmeier et al. 1999). It is probable that other, as yet unidentified, myeloid-specific factors would also be involved in the regulation of myeloid CD4 gene expression.

The expression of CD4 in T cells and in myeloid cells is also regulated by post-transcriptional events involving protein cycling between the cell surface and the cell interior (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1998). This process will be discussed in detail later.

I.3. CD4 is expressed by many cell types

In humans, CD4 is expressed by many immunological cells. In addition to T cells, CD4 is expressed by cells of the monocyte/macrophage lineage (Moscicki et al.1983, Wood et al.1983, Levy et al.1985, Stewart et al.1986), as well as by eosinophils (Lucey et al.1989).

Dendritic cells have also been reported to express CD4, specifically, epidermal Langerhans cells (Groh et al. 1986, Wood et al. 1983, Beckstead et al. 1984), thymic dendritic cells (Landry et al. 1988), splenic dendritic cells (McIlroy et al.1995), and dendritic cells freshly isolated from human blood (O'Doherty et al.1993). CD4 expression has also been observed on some early hemopoietic progenitors (Frederickson and Basch 1989, Zauli et al.1994) and on some mature megakaryocytes (Basch et al.1996).

CD4 mRNA has been detected in normal $CD19^+$ B cells (Zhang and Henderson 1994), and in B cell lines (Malkovsky et al. 1988). CD4 expression has also been detected in B cell lines (Cutrona et al. 1999), in B cells infected with Epstein-Barr virus (Tremblay et al. 1990), in B cells activated by culture with IL-4 and CD40 ligand (Moir et al. 1999), in tonsillar B cells (Cutrona et al. 1999) and in a small population of fresh, normal, peripheral B cells (Fritsch et al. 1998).

Human herpesvirus 6 is capable of inducing *de novo* synthesis of CD4 in immune cells that do not normally express the protein. This has been observed to occur in infected $CD8^+$ T cells (Lusso et al. 1991), NK cells (Lusso et al.1993), and γ/δ T cells (Lusso et al. 1995). The ability of human herpesvirus 6 to induce CD4 expression in normally HIV nonpermissive cells makes it a viral accelerating factor in AIDS pathogenesis (Lusso and Gallo 1995).

There are also conflicting reports of CD4 expression by nonhemopoietic cells. The expression of CD4 by neurons is controversial, with some investigators observing CD4 (Funke et al. 1987, Erickson et al.1991, Omri et al.1994), while others have been unable to do so (Peudenier et al. 1991a, Peudenier et al. 1991b).

I.4. The physiological roles of CD4

I.4.1. CD4 in T cells

i. CD4 is a coreceptor for MHC class II and for CD44

In T cells, CD4 functions as an adhesion molecule/coreceptor by binding to non-polymorphic areas of MHC class II molecules on the surface of APC during antigen presentation to T cells (Doyle and Strominger 1987, Gay et al. 1987). In so doing, CD4 increases the signal transduction capacity of the TCR by a factor of approximately 100 times (Janeway 1989), an important requirement given the modest affinity of the TCR ($K_d = 10^{-5}$ to 10^{-7} M) for a peptide/MHC class II complex (Corr et al. 1994). This modest affinity ensures that the T cell response is specific for the bound peptide (König et al. 1996). CD4 also has a low affinity for MHC class II molecules. However, when CD4, the TCR, and the peptide/MHC class II complex come together, the CD4-MHC class II interaction is stabilized, which in turn increases the affinity of the TCR for the peptide/MHC class II complex. The end result is a peptide-specific T cell response which is sensitive to Ag concentrations as low as 100 to 500 peptide-occupied MHC class II molecules out of a total of more than 10^5 (Demotz et al. 1990).

MHC class II molecules are heterodimers consisting of an α-chain and a β-chain (Janeway and Travers 1997). CD4 and MHC class II interactions involve multiple regions on each protein. Residues 35-47 of the CDR2 loop in D1 of CD4 (Lamarre et al. 1989, Clayton et al. 1989, Moebius et al. 1992a), residues within D2, and residues within the D1-D2 interdomain groove interact with MHC class II (Moebius et al. 1993, Houlgatte et al. 1994). Although the D1 domain and D2 domain loops of CD4 are involved in MHC class

II binding, there does not appear to be any overlap between the CDR2 loop residues used by MHC class II and those used by HIV gp120 (Fleury et al. 1991, Autiero et al. 1995). However, the consensus appears to be that the smaller gp120 binding site in CD4 overlaps with that of the larger MHC class II binding site (Lamarre et al. 1989, Bowman et al. 1990, Moebius et al. 1992a).

CD4-binding sites have been identified in the α2 (residues 125-133; König et al. 1992), β1 (residues 41-55; König et al. 1996), and β2 domains of MHC class II. The β2 residues reported to be involved in CD4-binding differ slightly depending on the study; while residues 134-148 were found to be critical in one study (Cammarota et al. 1992), residues 137-143 were found to be critical in another (König et al. 1992). The polymorphic nature of MHC class II molecules also has consequences with respect to association with CD4 since various HLA-DR allelic forms have been found to have differential ability to interact with CD4; this influence appears to be mediated by residues 180 to 189 of the β2 chain (Fleury et al. 1995). The current model of CD4-MHC class II interaction predicts that one CD4 molecule interacts with one HLA-DR homodimer, ie. two MHC class II molecules (Gaubin et al. 1999). Furthermore, CD4 interacts with the TCR (Saizawa et al. 1987) via domains D3 and D4 (Vignali et al. 1996).

A novel role for CD4 has recently been reported with the observation that CD44, a hyaluronic acid-binding membrane protein involved in T cell activation, associates with CD4, whose associated protein tyrosine kinase $p56^{lck}$ is subsequently activated (Dianzani et al. 1999). The resulting complex is subsequently recruited to the TCR. This represents yet another example of CD4 acting as a coreceptor.

ii. CD4 plays a role in T cell signal transduction

In addition to stabilizing the TCR-Ag/MHC class II complex, CD4 is also involved in T cell signal transduction through its constitutive noncovalent association with $p56^{lck}$ (Rudd et al. 1988, Veillette et al. 1988, Barber et al. 1989), a *Src*-related nonreceptor protein tyrosine kinase which is specific for lymphocytes (Marchildon et al. 1984, Marth et al. 1985). $p56^{lck}$ consists of the following components (Veillette et al. 1991):

a) a unique (with respect to the other *Src* family members) amino terminal domain containing a myristylated glycine residue,

b) a proline-binding SH3 domain,

c) a phosphotyrosine-binding SH2 domain,

d) a highly conserved (with respect to the other *Src* family members) protein tyrosine kinase domain in the carboxyl portion of the molecule which contains an ATP binding site and a site for autophosphorylation (Tyr394), and

e) a regulatory domain consisting of a tyrosine (Tyr505) which when phosphorylated, inactivates $p56^{lck}$.

The association between CD4 and $p56^{lck}$ is mediated by two cysteine residues (Cys420 and Cys422) in the cytoplasmic tail of CD4 and by two cysteine residues (Cys20 and Cys23) in the unique amino terminal region of $p56^{lck}$ (Turner et al. 1990). Recently, the exact nature of this association was elucidated with the discovery that a Zn^{2+} ion binds to the cysteines of each protein, thus stabilizing their interaction (Huse et al. 1998, Lin et al. 1998). The identification of Zn^{2+} as a "cross-linker" or "adhesive" is a novel mode of interaction between two intracellular proteins (Huse et al. 1998, Lin et al. 1998).

CD4/p56^{lck}-mediated signal transduction plays a crucial role in initiating T cell activation (Rudd et al. 1988, Veillette and Fournel 1990). When Ag complexed with MHC class II on the surface of an antigen presenting cell binds to the TCR, a complex cascade of signaling events is generated (Qian and Weiss 1997, Zhao and Koretzky 1997, van Leewan and Samelson 1999) which include tyrosine phosphorylation, one of the earliest biochemical protein modifications induced by antigenic stimulation (June et al. 1990).

The CD4 molecule plays an active role in the association between the TCR and the Ag/MHC class II complex by stabilizing their interactions. In response to this interaction, CD4-associated p56^{lck} is activated by the dephosphorylation of its inhibitory phosphotyrosine (pTyr505), a process mediated by the common leukocyte antigen and tyrosine phosphatase CD45 (Ostergaard et al. 1989, McFarland et al. 1993). Once the inhibitory phosphate group has been removed, p56^{lck} autophosphorylates Tyr394 and becomes activated (Abraham and Veillette 1990). Activated p56^{lck} tyrosine phosphorylates residues located within the ITAM of CD3 (Iwashima et al. 1994). The SH2 domain of ZAP-70 binds to the phosphorylated tyrosine residues within the ITAM, resulting in the activation of, and recruitment by, ZAP-70, which in turn recruits and activates other signaling proteins to continue the signaling cascade (Chan et al. 1992).

Another *Src* kinase, p59^{fyn}, also participates in this process by phosphorylating the ITAM of CD3 for ZAP-70 recruitment (Samelson et al. 1990). Assuming the Ag stimulation occurs in the context of the necessary costimulatory molecules (ex. CD28) (Harris and Ronchese 1999, Watts and DeBenedette 1999), the end result of this process is the induction of the PLC-γ1 and PKC pathways (Weiss et al. 1991, Secrist et al. 1991), and of the Ras

pathway (Izquierdo et al. 1993), resulting in the transcriptional activation of the various genes required to induce cellular proliferation, differentiation and effector function (van Leeuwen and Samelson 1999).

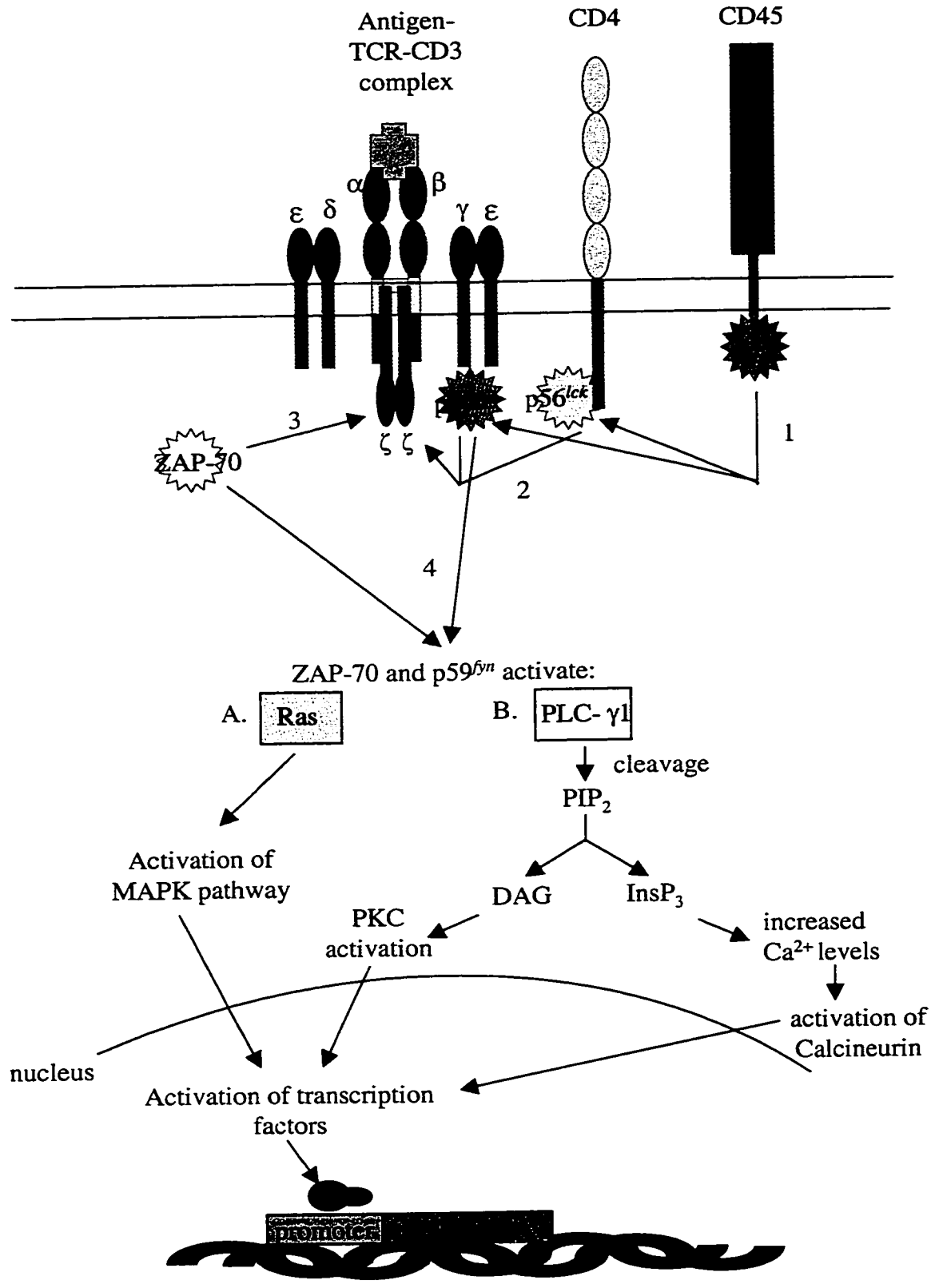
Thus, in T cells, the coreceptor function of CD4 is mediated by its association with $p56^{lck}$ and is responsible for initiating an additive cascade of tyrosine phosphorylation (Veillette 1991). A schematic depicting some of the signaling molecules involved in T cell receptor signaling is shown in Fig.I.2.

The CD4 molecule also plays an important role in thymocyte development given that the interaction between CD4 on the thymocyte and MHC class II on the thymic stroma is required for the differentiation of $CD4^{-}CD8^{+}$ double positive thymocytes into $CD4^{-}CD8^{-}$ single positive thymocytes (Kruisbeek et al. 1985). The exact mechanism by which this occurs (stochastic *vs.* instructional) is still unresolved (Ellmeier et al. 1999). In the stochastic model, either CD4 or CD8 is randomly down-regulated. Interaction of the T cell with an APC expressing MHC molecules of the class capable of interacting with the expressed coreceptor will allow the cell to survive positive selection. In the instructional model, both CD4 and CD8 are expressed. The continued expression of one coreceptor, but not the other, is dependent on the class of the MHC molecule with which the T cell interacts during Ag presentation. Regardless of the exact mechanism involved in positive selection, the role of CD4 in this process is reminiscent of the role played by CD4 on mature T cells, i.e. that of coreceptor which increases the affinity of the TCR for the MHC class II/peptide complex on the thymic stroma, and which transduces, in conjunction with the TCR complex, the signals

Fig.I.2. The role of CD4 in the initiation of TCR-mediated signal transduction.

Antigen binding to the TCR results in the recruitment of CD4-$p56^{lck}$ and CD45 to the TCR-CD3 complex and initiates a chain reaction of signal-transducing events. 1) The tyrosine phosphatase CD45 activates $p56^{lck}$ and $p59^{fyn}$. 2) Activated $p56^{lck}$ and $p59^{fyn}$ phosphorylate tyrosine residues within the ITAM of the ζ chains of CD3, resulting in (3) the recruitment of ZAP-70 to the TCR/CD3 complex; ZAP-70 binds to the phosphorylated ITAM via its SH2 domains. Once bound to the phosphorylated ITAM of the ζ chains of CD3, ZAP-70 is tyrosine phosphorylated, and thus activated, by $p56^{lck}$. 4) The activated ZAP-70 and $p56^{lck}$ subsequently activate downstream substrates including Ras and PLC-γ1, each of which participates in a different signaling pathway. A) Ras activation results in the activation of the serine/threonine kinases of the MAPK pathway, and eventually, in the activation of the MAPK which translocates into the nucleus to activate Fos, a component of the Ap-1 transcription factor. B) Activation of PLC-γ1 results in the cleavage of PIP_2 into DAG and $InsP_3$. DAG activates PKC which activates the transcription factor NF-κB. $InsP_3$ increases cytosolic Ca^{2+} levels, thus activating the phosphatase calcineurin which activates the transcription factor NFAT. The end result of these processes is the activation of cellular events (such as IL-2 production) required for cellular proliferation, differentiation and effector function.

NB. Other signaling molecules such as adaptor proteins are also involved in the above pathways.


Antigen-
TCR-CD3
complex
CD4
CD45
α
β
ε δ
γ ε
ζ ζ
p56lck
ZAP-70
3
1
2
4
ZAP-70 and p59fyn activate:
A.
Ras
B.
PLC-γ1
cleavage
PIP2
Activation of
MAPK pathway
DAG
InsP3
PKC
activation
increased
Ca2+ levels
activation of
Calcineurin
nucleus
Activation of transcription
factors
promoter

required for positive and negative selection (Ellmeier et al. 1999).

iii. CD4 is an IL-16 receptor

The CD4 molecule on T cells is also the receptor (Cruikshank et al. 1994), or part of the receptor complex (Center et al. 1995), for the chemotactic, pro-inflammatory cytokine IL-16, or lymphocyte chemoattractant factor (LCF). Unlike gp120 and MHC class II, IL-16 interacts with the D4 domain of CD4; some of the residues critical for interaction with IL-16 are common to residues involved in CD4 dimerization (Liu et al. 1999). IL-16 is produced as a 50-70 kDa, inactive precursor protein (pro-IL-16) (Baier et al. 1997, Zhang et al. 1998) which is cleaved by caspase-3 to a 17-20 kDa protein (Zhang et al. 1998). The cleavage products aggregate into a biologically active homotetramer which interacts with a CD4 homotetramer (Cruikshank et al. 1994) on the cell surface.

$CD8^+$ T cells constitutively synthesize and store biologically active IL-16 which they secrete in response to serotonin, histamine, antigen and the mitogen concanavalin A (Center et al. 1996, Mire-Sluis 1998). $CD4^+$ T cells also secrete IL-16, but only as a result of *de novo* synthesis of the biologically active protein. Mast cells, eosinophils, and airway epithelial cells of asthmatics also secrete IL-16. IL-16 secretion by these cells recruits $CD4^+$ T cells (Center and Cruikshank 1982, Cruikshank et al. 1987), monocytes (Cruikshank et al. 1987) and eosinophils (Rand et al. 1991) to sites of inflammation. The recruited cells also undergo activation. IL-2 receptor is induced in T cells, while HLA-DR is induced in both monocytes and T cells (Cruikshank et al. 1987). In eosinophils, IL-16 induces increased expression of adhesion molecules (Wan et al. 1995).

In $CD4^+$ T cells, CD4 also functions to link $p56^{lck}$ to the IL-16 signaling pathway. IL-16-mediated signal transduction is $p56^{lck}$-dependent, yet interference with the kinase activity of $p56^{lck}$ does not inhibit IL-16-induced chemotaxis, suggesting that in chemotaxis, $p56^{lck}$ functions as an adaptor that interacts with an unknown protein (Ryan et al. 1995). That $p56^{lck}$ functions as an adaptor molecule rather than as a kinase suggests that in $CD4^-$ cells that lack $p56^{lck}$, such as in the case of monocytes/macrophages (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al. 1993), some as yet unidentified, nonkinase adaptor molecule interacts with CD4 to mediate chemotaxis in response to IL-16.

Initial studies of IL-16-induced signal transduction revealed that IL-16 treatment of T cells induced increased intracellular calcium and inositol triphosphate levels (Cruikshank et al. 1991), a rapid translocation of PKC from the cytosol to the plasma membrane (Parada et al. 1996) and autophosphorylation of $p56^{lck}$ (Ryan et al. 1995).

An interesting feature of the response of $CD4^+$ T cells to IL-16 is that they enter a reversible state of unresponsiveness to stimulations through the TCR/CD3 complex if they are exposed to IL-16 prior to, or within a short period of, antigenic challenge (Cruikshank et al. 1996, Theodore et al. 1996). This unresponsiveness is at least partially mediated by IL-16 binding to the D4 domain of CD4, a domain which also contains the CD4 dimerization site (Liu et al. 1999). Binding of IL-16 to CD4 therefore prevents CD4 from oligomerizing with itself, a feature required for the formation of competent CD4/MHC class II complexes in antigen presentation (Theodore et al. 1996, Liu et al. 1999). IL-16 has also been shown to interfere with IL-2 production (Ogasawara et al. 1999). Once IL-16 is no longer bound to CD4, the cells recover and are able to respond to the antigen presented to them (Theodore

et al. 1996).

This scenario sharply contrasts the anergic state that can be induced by engagement of the CD4 molecule by antibody (Biddison et al. 1982, Bank and Chess 1985) or by HIV gp120 (Chirmule et al. 1990, Theodore et al. 1994), following which antigenic challenge of the cell induces apoptosis as a result of increased expression of Fas (Wang et al. 1994, Westendorp et al 1995). By contrast, IL-16 stimulation of cells has been shown to decrease Fas expression (Theodore et al. 1996), presumably protecting the cells from apoptosis. The reversible state of unresponsiveness to IL-16 may allow cells to be recruited to the site of inflammation in an antigen-independent fashion, following which activated cells can respond specifically to mount an appropriate immune response.

The exact mechanisms responsible for these different CD4-mediated outcomes are not well-defined, but may depend on the extent of oligomerization induced by the various CD4 ligands, and/or the different epitopes engaged by each CD4 ligand (Jabado et al. 1994, Theodore et al. 1996). That differential engagement of the CD4 molecule on the surface of T cells can lead to either positive or negative outcomes highlights the role of CD4 as an immune modulator in these cells (Theodore et al. 1996, Center et al. 1996).

IL-16 treatment of T cells has been shown to desensitize the chemokine receptor CCR5 to MIP-1β, while MIP-1β treatment interferes with IL-16-dependent CD4-mediated signaling (Vallen Mashikian et al. 1999); chemokine receptors will be discussed in greater detail later. IL-16 also inhibits HIV replication in both T cells and monocytes (Baier et al. 1995, Zhou et al. 1997, Maciaszek et al. 1997). This inhibition is not due to competition between IL-16 and HIV gp120 for binding to CD4, but occurs at the molecular level by

repressing HIV mRNA expression (Zhou et al. 1997) and HIV promoter activity (Maciaszek et al. 1997).

IL-16 has also been implicated in immune-mediated disease processes. IL-16 is thought to contribute to the pathogenesis of multiple sclerosis and asthma by virtue of its ability to recruit inflammatory cells to the CNS (Schluesener et al. 1996) and airways (Bellini et al. 1993), respectively.

Recently, a neuronal form of IL-16 capable of inducing signaling in neurons was identified (Kurschner and Yuzaki 1999), an observation which gives weight to reports by some investigators of CD4 expression by neurons (Funke et al. 1987, Erickson et al. 1991, Omri et al. 1994).

iv. CD4 may be an Ig receptor

A more obscure role for CD4 in T cells is as a receptor for Ig. Two independent groups have shown that CD4 can bind to Ig, albeit with relatively low ($K_d = 10^{-5}$ M) affinity (Lenert et al. 1990, Lederman et al. 1990). The affinity of this interaction increases when the Ig is complexed with antigen (Lenert et al. 1992), or when the Ig is aggregated (Lederman et al. 1990). This CD4/Ig interaction occurs between amino acid residues 21-49 of the D1 region of CD4 and the Fd region (V_H plus C_H1) of Ig, presumably due to protein-protein interactions between the respective Ig folds of each protein (Lenert et al. 1990). In a subsequent study, synthetic CD4 peptides were shown to enhance binding of Ig to Fc receptors in U937 monocytic cells, possibly as a result of conformational changes to the Ig molecule (Lenert et al. 1992). The implication of these findings is that CD4/Ig interactions

may play an important role in regulating the immune response. In the case of T cells, this interaction might also play a costimulatory role (Lenert et al. 1997).

I.4.2. CD4 in monocytes/macrophages

i. CD4 is expressed by many tissue-specific macrophages

In humans, CD4 is expressed by monocytes/macrophages (Moscicki et al. 1983, Wood et al. 1983, Levy et al. 1985, Stewart et al. 1986). This molecule has been identified on virtually all blood monocytes, although at lower levels than expressed by $CD4^+$ T cells (Filion et al. 1990). In human tissue macrophages, CD4 expression has been detected on placental Hofbauer cells (Goldstein et al. 1988, Lairmore et al. 1993), bone marrow stromal macrophages (Lee et al. 1988), lymph node sinus histiocytes, macrophages within germinal centres, pulmonary alveoli and connective tissue (Wood et al. 1985), as well as on macrophages in the liver (Kupffer cells) (Wood et al. 1985, Hume et al. 1987), gastrointestinal tract (Hume et al. 1987), spleen (Buckley 1991) and thymus (Ruco et al. 1989). Generally, tissue macrophages tend to be weakly $CD4^+$ (Wood et al. 1985), although CD4 levels of tissue-specific macrophages have been shown to differ from one tissue to another. High levels of CD4 have been detected on placental Hofbauer cells (Goldstein et al. 1988), whereas low level CD4 has been detected by some groups on microglial cells of the brain (Lowe et al. 1989, Porwit et al. 1989, Jordan et al. 1991, Williams et al. 1992, Wu et al. 1993, Mikovits et al. 1996, Schluesener et al. 1996). Other groups have been unable to detect microglial CD4 expression (Vazeux et al. 1987, Peudenier et al. 1991a, Peudenier et al. 1991b). The exact origin of microglia is unknown but these cells are thought to arise

from bone-marrow derived monocytes which have entered the CNS during fetal development (Theele and Streit 1993, Ling and Wong 1993).

Decreased CD4 levels have been observed on tissue culture-derived macrophages compared to blood monocytes (Crowe et al. 1987, Kazazi et al. 1989, Valentin et al. 1990, Valentin et al. 1991, Peudenier et al. 1991b, Pelchen-Matthews et al. 1998, Graziani-Bowering and Filion 2000). The variable levels of CD4 expression by different tissue-specific macrophages, as well as by tissue culture-derived macrophages, may reflect differences in their microenvironments (Kazazi et al. 1989), stage of maturation/differentiation (Kazazi et al. 1989, Valentin et al. 1991), variable sensitivities of the detection systems used by the different investigators (Vazeux et al. 1987), or a combination of these factors.

ii. CD4 plays a role in signal transduction in monocytes

$p56^{lck}$ is a lymphocyte-specific kinase (Marchildon et al. 1984, Marth et al. 1985) whose specificity is conferred by the tissue specificity of its promoters (Adler et al. 1988). As a result, monocytic CD4 has no associated $p56^{lck}$, or any other kinase activity (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al. 1993). Regardless of the lack of $p56^{lck}$, there are many examples in the literature of the involvement of monocyte CD4 in signal transduction. A complex inositol polyphosphate response and a Ca^{2+} flux are induced in monocytes upon co-cross-linking of CD4 and Fcγ receptors (Guse et al. 1992). A Ca^{2+} flux was also observed following cross-linking of CD4 in Thp-1 monocytic cells (Graziani-Bowering et al., submitted).

HIV gp120 induces a CD4-dependent down-regulation of the CCR5 chemokine receptor on the surface of human $CD4^+$ monocytes and of CCR5 and CD4 cotransfected HEK 293 cells (Wang et al. 1998).

iii. CD4 is an IL-16 receptor

IL-16 stimulation of monocytes causes up-regulation of HLA-DR (Cruikshank et al. 1987), down-regulation of CD4 and of the chemokine receptors CCR5 and CXCR4 (Hermann et al. 1999), translocation of PKC from the cytosol to the membrane (Parada et al. 1996), production of IL-1β, IL-6, IL-15 and TNF-α (Mathy et al. 2000), and activation of the SAPK (stress-activated protein kinase) and p38 pathways of the MAPK (mitogen-activated protein kinase) pathway (Krautwald 1998).

iv. CD4 may be an Ig receptor

It has been reported that CD4 can interact with Ig (Lenert et al. 1990, Lederman et al. 1990). In monocytes/macrophages, this interaction has been shown to increase the avidity of the antigen/antibody complex for the Fc receptor, which could activate monocytes when antigen levels are low, as well as result in increased uptake and processing of antigen (Lenert et al. 1992, Mehta et al. 1994). A potential disadvantage of such a process, however, would be the participation of the CD4 molecule in antibody-mediated enhancement of HIV infection (Lederman et al. 1990, Lenert et al. 1990).

iv. CD4 may be involved in the induction of IL-6

Stimulation of differentiated U937 cells with the lectin jacalin has been shown to induce IL-6 in a CD4-dependent manner; CD4-negative cells did not produce IL-6 in response to jacalin treatment (Taimi et al. 1994). IL-6 is a T and B cell growth factor which also enhances secretion of antibodies by plasma cells and which aids in the differentiation of myeloid stem cells (Kuby 1992; Janeway and Travers 1997). It is possible, therefore, that in monocytes, CD4-mediated signals are responsible for the secretion of a cytokine having immune-enhancing effects on other cells.

vi. CD4 may be involved in HIV-induced neurodegeneration

CD4 expression by microglial cells may also function in neurodegeneration given that mice whose microglial cells did not express CD4 did not suffer any LPS-induced neuronal damage, whereas transgenic mice transfected with the gene for human CD4 did suffer LPS-induced neuronal damage (Buttini et al. 1998). Furthermore, a strong correlation between the levels of CD4 expression and the degree of neurodegeneration was observed in AIDS patients who suffered from opportunistic infections but who did not have detectable HIV within the brain. Based on these observations, the authors of this study propose that CD4 acts as a pathogenic link between the immune system and the CNS in response to signals received during opportunistic infections.

I.4.3. CD4 in other cell types

Although CD4 is expressed by a wide array of cell types, its role has been elucidated

for only a few. As in the case of T cells and monocytes, CD4 mediates IL-16 responsiveness in eosinophils (Rand et al. 1991, Wan et al. 1995). In B cell lines and tonsillar B cells, cross-linking of CD4 has been shown to induce apoptosis, suggesting a regulatory role for CD4 in B cell physiology, as well as in HIV pathogenesis (Cutrona et al. 1999).

The role of CD4 on the many other cell types which express it has not been defined, but CD4 probably serves many of the same functions (signal transducer, adhesion molecule/coreceptor, IL-16 receptor/coreceptor) as for other cell types, although novel ligands and functions cannot be ruled out.

I.5. CD4 is a viral receptor

I.5.1. CD4 is a receptor for HIV and SIV

HIV-1, the etiological agent of AIDS (Barre-Sinoussi et al. 1983, Gallo et al. 1984, Levy et al. 1984) induces a profound immune deficiency characterized by a reduced $CD4^+$ T cell count (Fahey et al. 1984, Broder and Gallo 1984). This led investigators to speculate that the CD4 molecule was the HIV receptor, a theory that was soon confirmed in a series of studies in which α-CD4 mAb (Dalgleish et al. 1984, Klatzmann et al. 1984, McDougal et al. 1985) and sCD4 (Deen et al. 1988; Traunecker et al. 1988) were used to inhibit HIV infection. Furthermore, transfection of nonpermissive Raji and HeLa cells with CD4 made them susceptible to infection (Maddon et al. 1986). Finally, immunoprecipitation experiments showed a direct association between gp120 and CD4 (McDougal et al. 1986). HIV-2 and SIV also use CD4 as their receptors (Sattentau et al. 1988).

The interaction between CD4 and HIV involves the D1 and possibly D2 domains of

CD4 (Clayton et al. 1988, Landau et al. 1988), as well as the viral gp120 envelope glycoprotein (Maddon et al. 1986, Lasky et al. 1987). However, another study discounted the involvement of the D2 domain and attributed the observation by other groups that it was involved in indirect effects on D1 by mutations made in D2 (Arthos et al. 1989). The critical CD4 D1 viral binding site may span residues 42-49 (Peterson and Seed 1988), or a slightly larger stretch consisting of residues 41-55 (Arthos et al. 1989) which form a loop homologous with the CDR2 of the hypervariable region of Ig variable structures. Other studies have also identified other residues comprising another loop (CDR3) in the D1 domain that are important for HIV-binding (Ashkenazi et al. 1990, Brodsky et al. 1990). The authors propose that these other residues contribute to a discontinuous binding site for HIV and thus make an important contribution to the conformation of the HIV-binding site. However, the importance of these other residues for gp120 binding is controversial since the results are not always reproducible (Moebius et al. 1992b). Furthermore, a recent study using a rat-human CD4 chimera showed no difference in the ability of rat or human CDR3 to mediate gp120 binding, suggesting that CDR3 does not play a role in gp120 binding (Simon et al. 1997).

CD4 also plays a role in syncytia formation between infected and uninfected cells (Lifson et al. 1986, Sodroski et al. 1986). The actual CD4 domain involved in this process is controversial. Some investigators have localized this function to a membrane proximal portion of CD4 (Poulin et al. 1991), whereas others have assigned this function to the CDR3 within the D1 domain (Camerini and Seed 1990, Truneh et al. 1991, Corbeau et al. 1993). Yet a third group has determined that the D3 domain is involved in this function, as well as playing an important role in viral fusion during infection (Healey et al. 1990). However,

chimeric molecules which included the D1 and D2 domains, but not domains D3 and D4, were able to support HIV infection, suggesting that D3 and D4 are not required for HIV infection (Bedinger et al. 1988). A possible explanation for the apparent involvement of the D3 and D4 domains in the other studies is that the mAb used may have induced conformational changes in the distant D1 domains, which, while not affecting HIV binding, did not allow for the required CD4-induced conformational changes in gp120 required for post-binding events (Burkly et al. 1992). Therefore, the CD4-dependent fusion site may be localized within the D1-gp120 binding site (Sattentau 1992).

The gp120 residues critical for CD4 binding are amino acids 397-439 within the relatively conserved carboxyl portion (Lasky et al. 1987). Subsequent studies have identified other residues over a large portion of the molecule, located in conserved and variable regions, suggesting that they probably contribute to a conformation-dependent CD4 binding site (McDougal et al. 1991, James et al. 1996). The affinity of gp120 for CD4 is very high ($K_d = 10^{-9}$ M) (Lasky et al.1987, Lamarre et al.1989), although the relative affinities vary with the isolates studied due to the variant gp120 binding site for CD4 (Ivey-Hoyle et al. 1991). Interestingly, the gp120 envelope glycoprotein of HIV-2 has a slightly lower affinity for CD4 (Moore 1990).

It soon became apparent, however, that despite the importance of CD4 expression in HIV infection, CD4 expression was not sufficient. CD4-transfected murine cells could be bound by HIV, but could not be infected, suggesting that CD4 was part of a larger HIV-binding complex involving other coreceptors (Maddon et al. 1986).

An HIV coreceptor was eventually identified in the form of CXCR4, or fusin, a

seven-transmembrane G protein-coupled chemokine receptor (Feng et al. 1996) whose physiological ligand is stromal cell-derived factor-1 (SDF-1) (Bleul et al. 1996).

The discovery that $CD8^+$ T cell soluble factors capable of inhibiting HIV infection included MIP-1α, MIP-1β and RANTES (Cocchi et al. 1995), coupled with the subsequent identification of CCR5 as their chemokine receptor (Samson et al. 1996, Combadiere et al. 1996, Raport et al. 1996), led to the immediate identification by many independent groups of CCR5 as another HIV coreceptor (Deng et al. 1996, Dragic et al. 1996, Alkhatib et al. 1996, Choe et al. 1996, Doranz et al. 1996). It was subsequently shown that HIV-1 gp120 induces the formation of a complex between CD4 and chemokine receptors (Ugolini et al. 1997).

The discovery that chemokine receptors were the other required cellular factor for HIV infection also explained the mystery of HIV tropism. While the macrophage-tropic strains involved in initial infection (Schuitemaker et al. 1992) are dependent on the expression of CCR5 (Deng et al. 1996, Dragic et al. 1996, Alkhatib et al. 1996), the more cytopathic T-tropic HIV strains which emerge later in infection (Schuitemaker et al. 1992) use CXCR4 to infect cells (Feng et al. 1996). Dual-tropic viruses use both CCR5 and CXCR4 (Doranz et al. 1996).

The identification of the chemokine receptors as HIV coreceptors resulted in a better understanding of the mechanisms by which HIV binds to and fuses with its target cells (Stein et al. 1987, Maddon et al. 1988). Interactions between CD4 and gp120 induce conformational changes to both CD4 (Denisova et al. 1997, Sullivan et al. 1998, Yachou and Sekaly 1999) and gp120 (Sattentau and Moore 1991, DeVico et al. 1995, Sullivan et al.

1998). Conformational changes to gp120 increase its affinity for chemokine receptors (Wu et al. 1996, Wyatt and Sodroski 1998), an interaction which changes the association between gp120 and its noncovalently associated gp41 fusogenic domain, thus allowing gp41 to fuse with the target cell membrane (Berger et al. 1999). The end result of this process is the release of the HIV nucleocapsid into the cell, followed by the reverse transcription of viral RNA, integration of cDNA, and eventually, protein synthesis and viral assembly for the production of viral progeny (Wong-Staal 1991).

I.5.2. CD4 is a receptor for human herpesvirus 7

T cell CD4 is also a critical component of the receptor for human herpesvirus 7 (Lusso et al. 1994, Yasukawa et al. 1995, Crowley et al. 1996, Yasukawa et al. 1997), a prevalent virus which usually infects during childhood (Wyatt et al. 1991). The fact that the virus infects $CD4^+$ T cells, but does not replicate in normal monocytic cells (Crowley et al. 1996) suggests that other cellular factors are important in determining tropism. The involvement of other cell surface molecules with CD4 to form a receptor complex for human herpesvirus 7 would be reminiscent of the CD4/chemokine receptor complex used by HIV. Interestingly, human herpesvirus 7 has been detected in monocyte/macrophages from Kaposi sarcoma lesions which are coinfected with human herpesvirus 6B, while no virus could be detected in monocyte/macrophages from normal tissues (Kempf et al. 1997).

I.6. The regulation of CD4 expression

I.6.1. CD4 cycling

$CD4^+$ T cells and $CD4^-$ T cell lines differentially regulate their CD4 expression compared to monocytic cell lines, primary monocytes and monocyte-derived macrophages (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1998). In $CD4^-$ T cells and T cell lines, CD4 is constitutively endocytosed at very low rates, and the majority (approximately 90%) of CD4 is found on the cell surface at any given time, whereas in myeloid cells, the CD4 molecule shows a much greater rate of CD4 recycling, with approximately 40% of the CD4 molecules located intracellularly at any given time. The biological significance of this differential regulation of CD4 by these different cell types is unknown (Pelchen-Matthews et al. 1998).

The endocytosis of CD4 is controlled at multiple levels by the cytoplasmic tail of CD4 (Pelchen-Matthews et al. 1989, Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1992). The association of $p56^{lck}$ with lymphocytic CD4 prevents CD4 from associating with clathrin-coated pits and vesicles (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1992). This interference has been suggested to occur because $p56^{lck}$ association with Cys420 and Cys422 (Turner et al. 1990) prevents the neighbouring dileucine motif (Leu413 and L414), which plays a key role in endocytosis, from associating with clathrin adaptor proteins (Salghetti et al. 1995). In monocytic cells, CD4 is able to associate with clathrin-coated pits and vesicles because of the lack of $p56^{lck}$ association, while signals located within the cytoplasmic tail of CD4 direct CD4 to early endosomes which will re-transport the molecule back to the cell surface (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al.

1992).

Recently, it was observed that the constitutive endocytosis of CD4 by myeloid cells can be inhibited by increased cytosolic levels of cAMP (Foti et al. 1997a), a PKA-activating second messenger generated by G-protein-activated enzymes (Alberts et al. 1989). cAMP inhibits CD4 endocytosis by preventing its association with clathrin-coated pits. The authors of this study propose that this cAMP-dependent inhibition of CD4 endocytosis reflects cross-talk between CD4 and associated proteins regulated by cAMP. The exact mechanism by which cAMP interferes with the ability of CD4 to associate with clathrin-coated pits is unknown, but may involve decreased affinity of CD4 for clathrin-coated pits, or conversely, increased affinity of CD4 for non-clathrin-coated portions of the membrane. Foti et al. also propose that cAMP-mediated inhibition of CD4 endocytosis may have physiological implications by possibly prolonging the interaction between CD4 and a ligand. cAMP in myeloid cells represents a second mechanism for CD4 inhibition of endocytosis (Foti et al. 1997a), the first being the $p56^{lck}$-mediated inhibition of T cell CD4 internalization (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1992).

I.6.2. The induction of CD4 down-regulation

i. Phorbol esters down-regulate T cell CD4

In addition to constitutive CD4 endocytosis, CD4 can be down-regulated (ie. internalized without being re-expressed at the cell surface). This has been observed in $CD4^+$ T cells in response to activators of PKC such as phorbol esters (Solbach et al. 1982, Hoxie et al. 1986b, Ruegg et al. 1992, Pelchen-Matthews et al. 1993). The resulting down-

regulation is a multi-step process which begins with the phosphorylation of T cell CD4 (Acres et al. 1986) on Ser408, Ser415 and Ser431 within the cytoplasmic tail of CD4 (Shin et al. 1990), followed by its dissociation from $p56^{lck}$ (Hurley et al. 1989, Sleckman et al. 1992), a separation thought to occur because of phosphorylation-induced conformational changes in the cytoplasmic tail of CD4 (Pelchen-Matthews et al. 1993). The phosphorylated serine residues (Shin et al. 1990), together with a dileucine motif (Leu413 and L414; Pitcher et al. 1999) and 4 hydrophobic residues (Shin et al. 1991) act as a signal for association with clathrin-coated pits and subsequent diversion from the recycling pathway for degradation in lysosomes (Ruegg et al. 1992, Marsh and Pelchen-Matthews 1996, Pitcher et al. 1999). The serine phosphorylation increases the affinity of CD4 for clathrin adaptor proteins by 350 to 700-fold (Pitcher et al. 1999). CD4 gene transcription is also inhibited in these T cells, thus explaining the failure to re-express CD4 (Neudorf et al. 1991). Other stimuli which can induce T cell CD4 down-regulation by this mechanism include antigen (Acres et al. 1986, Weyand et al. 1987) and antibody cross-linking of CD4 (Cole et al. 1989, Morel et al. 1992).

ii. Gangliosides down-regulate T cell and monocyte CD4

Gangliosides, galactose-containing cerebrosides (Dorland 1988) found within cell membranes, are also capable of down-regulating CD4 in T cells (Morrison et al. 1990, Morrison et al. 1991, Repke et al. 1992, Saggioro et al. 1993, Sorice et al. 1995, Garofalo et al. 1998). For some gangliosides, the process was a PKC-independent one (Morrison et al 1990, Morrison et al. 1991, Saggioro et al. 1993). In one study, the novel PKC isoforms delta and theta were found to be involved (Garofalo et al. 1998). Regardless of the signaling

molecules involved, CD4 down-regulation in T cells occurred by internalization and degradation (Repke et al. 1992, Saggiore et al. 1993, Sorice et al. 1995, Garofalo et al. 1998) although in one study, CD4 shedding from T cells was determined to be at least partially responsible for down-regulation (Morrison et al. 1991). Ganglioside treatment of monocytic cells also induces CD4 internalization and degradation in a PKC-independent process (Sorio et al. 1993).

iii. Differentiation-inducing agents down-regulate monocyte CD4

Monocytes and monocytic cells lines can be induced to differentiate to a macrophage-like phenotype with reduced CD4 expression by a group of agents which include phorbol esters, retinoic acid, vitamin D, TNF and IFN-γ (Schlesinger et al. 1989, Rigby et al. 1990, Chorváth et al. 1990, Pimentel-Muiños et al. 1992, Hewison et al. 1992, Cassatella et al. 1992, Szabo et al. 1994). An analysis of the mechanism responsible for the failure of phorbol ester-treated HL-60 and U937 monocytic cell lines and freshly isolated monocytes to re-express CD4 following its endocytosis and degradation revealed that CD4 gene transcription was repressed (Pimentel-Muiños et al. 1992), as has been observed for phorbol ester-treated T cells (Neudorf et al. 1991). By contrast, no such CD4 gene repression was observed for monocyte-derived macrophages (Kazazi et al. 1989), or for HL-60 cells treated with TNF and IFN-γ (Cassatella et al. 1992). In the first study (Kazazi et al. 1989), CD4 continued to be found in the cytosol, leading the investigators to conclude that a post-translational process resulted in conformational changes to CD4, and ultimately, in its decreased membrane expression. In the second study, the investigator concluded that the

decreased expression of CD4 was due to decreased protein synthesis and/or transport to the cell surface (Cassatella et al. 1992). The discrepancy between the mechanisms of CD4 down-regulation observed in the various studies may be explained by the use of different cell types (cell lines *vs.* primary cells) and by the different means of inducing differentiation (phorbol esters *vs.* TNF and IFN-γ *vs. in vitro* culture).

iv. LPS down-regulates monocyte CD4

LPS treatment of monocytes also induces CD4 down-regulation (Neate et al. 1992, Herbein et al. 1995) by a mechanism which involves the induction of TNF and IL-1β (Herbein et al. 1995). A biological implication of this finding is that gram-negative bacteria may have evolved a mechanism to escape monocyte recruitment and phagocytosis since CD4 is the receptor, or part of the receptor complex, for IL-16, a chemoattractant for $CD4^+$ monocytes and T cells (Cruikshank et al. 1994, Center et al. 1995).

v. HIV infection down-regulates T cell and monocyte CD4

HIV infection of primary T cells and monocytes/macrophages, as well as cell lines, leads to CD4 down-regulation (Hoxie et al. 1986a, Stevenson et al. 1987, Mann et al. 1990, Melendez-Guerrero et al. 1990, Benson et al. 1993). It has been hypothesized that CD4 down-regulation protects the cells from becoming superinfected and also ensures that new virions are not trapped by CD4 as they bud from the cell surface (Stevenson et al. 1987). Decreased expression of CD4 by infected cells would also interfere with the immune response to virus (Lu et al. 1998). Many mechanisms have been identified for HIV-induced

down-regulation of CD4. The production of TNF-α (Karsten et al. 1996) and IFN-γ (Dhawan et al. 1995) by infected monocytes is one mechanism, as is the decreased transcription of the CD4 gene in T cells (Hoxie et al. 1986a).

Although HIV binding to T cells is not dependent on CD4-mediated signal transduction (Orloff et al. 1991) since infection can occur in the absence of the cytoplasmic tail of CD4 (Bedinger et al. 1988), HIV binding to T cells does induce phosphorylation of serine residues within the cytoplasmic tail of CD4 (Fields et al. 1988), as well as inducing dissociation of CD4 from $p56^{lck}$ (Juszczak et al. 1991), a scenario reminiscent of phorbol ester-induced CD4 down-regulation.

Three HIV proteins (gp160, Vpu and Nef) have the ability to associate with CD4 and induce its down-regulation, as determined in a number of different experimental systems employing T cells, transfected nonhemopoietic cells, and the yeast two-hybrid system.

a) gp160

Newly synthesized viral precursor envelope glycoprotein gp160 complexes with newly synthesized CD4, which is itself associated with $p56^{lck}$, resulting in the entrapment of CD4 within the endoplasmic reticulum of infected cells (Crise et al. 1990, Jabbar and Nayak 1990, Crise and Rose 1992b, Koga et al. 1994). The retention of gp160 in the endoplasmic reticulum is a consequence of its inefficient transport (Willey et al. 1988, Jabbar and Nayak 1990) which may be a result of the conformation of the gp160/CD4/$p56^{lck}$ complex (Marsh and Pelchen-Matthews 1996). The complexed CD4 subsequently becomes a target for Vpu-mediated degradation (Willey et al. 1992a). This process occurs relatively late in infection

once viral structural proteins are synthesized (Marsh and Pelchen-Matthews 1996).

b) Vpu

Vpu, a protein which functions in viral release (Klimkait et al. 1990), degrades CD4 trapped with gp160 in the endoplasmic reticulum (Buonocore and Rose 1990, Willey et al. 1992a, Willey et al. 1992b). This degradation pathway involves proteosomes of the cytosolic ubiquitination pathway (Schubert et al. 1998). It was originally reported that Vpu associates directly with the cytoplasmic tail of CD4 (Bour et al. 1995). However, subsequent analysis revealed that the interaction between the cytoplasmic domains of Vpu and CD4 are insufficient for Vpu-mediated degradation (Margottin et al. 1996). This same group subsequently reported that Vpu interacts with a novel WD (Trp-Asp) protein (having repeating [Gly-His ---- X_{23-41} --- Trp-Asp] motifs; Neer et al. 1994) and that the resulting complex is targeted to the ubiquitination pathway by a targeting factor, Skp1p (Margottin et al. 1998). The end result of the degradation of gp160-complexed CD4 is the release of gp160 and its subsequent processing to gp120 and gp41 (Willey et al. 1992a, Willey et al. 1992b).

There is also evidence that Vpu may interfere with the cellular transport of proteins from the endoplasmic reticulum to the Golgi complex (Vincent and Abdul 1995), which may be yet another mechanism for interfering with CD4 expression.

A recent study of the importance of CD4 down-regulation for HIV replication suggests that the inhibition of CD4 expression by HIV extends beyond simply preventing superinfection since the continued expression of CD4 on the cell surface results in

interactions with Vpu, thus disrupting its particle-releasing activity and resulting in a 3-5 fold decrease in virus production (Bour et al. 1999).

c) Nef

Nef is one of the first and most abundant proteins produced by HIV and SIV, and is critical for maximal viral production and pathogenesis (Lu et al. 1998). One mechanism by which Nef accomplishes this is by the down-regulation of CD4 by a variety of processes, none of which involve serine phosphorylation in T cells (Gama et al. 1991, Garcia et al. 1991). In T cells, the down-regulation of CD4 by Nef requires the CD4 cytoplasmic tail (Garcia et al. 1993), specifically, the presence of a dileucine (Leu413 and L414) motif (Aiken et al. 1994) with which Nef associates (Iafrate et al. 1997), as well as a cluster of hydrophobic amino acids in the membrane-proximal portion (Salghetti et al. 1995, Gratton et al. 1996). The down-regulation of CD4 by Nef is regulated at many levels. Nef dissociates CD4 from $p56^{lck}$ (Goldsmith et al. 1995, Kim et al. 1999), and enhances CD4 endocytosis (Rhee and Marsh 1994) by doubling the surface area of the plasma membrane containing clathrin-coated surfaces with which CD4 preferentially associates (Foti et al. 1997b). Nef also enhances CD4 intracellular sequestration (Brady et al. 1993) in early endosomes (Schwartz et al. 1995, Sanfridson et al. 1997) and lysosomes (Sanfridson et al. 1997, Kim et al. 1999), following which CD4 is degraded (Rhee and Marsh 1994).

The targeting of CD4 to this degradative pathway requires the ability of Nef to act as an adaptor between CD4 and endosomes (Piguet et al. 1999). Until recently, it was thought that Nef connected the sorting pathways in the Golgi and plasma membrane (Mangasarian

et al. 1997) by associating with adaptor protein complexes within clathrin-coated pits (Greenberg et al. 1997, Piguet et al. 1998). More recently however, Nef has been found to directly associate with NBP1 (Nef binding protein 1), the catalytic subunit of a vacuolar ATPase which is required for acidification of endosomes and lysosomes (Lu et al. 1998) and which associates with clathrin adaptor proteins (Myers and Forgac 1993). The ability of Nef to target CD4 to lysosomes is dependent on the presence of signals within Nef which mediate Nef binding to specific proteins within endosomal membranes (Piguet et al. 1999). The down-regulation of CD4 by Nef has recently been shown to be very important in preventing budding viral progeny from binding to cell surface CD4 (Ross et al. 1999).

I.6.3. Monocyte CD4 can be up-regulated by yeasts

Contrary to HIV-induced down-regulation of monocyte CD4, infection of monocytes with the yeasts *Candida albicans* and *Cryptococcus neoformans* results in increased CD4 expression (Pietrella et al. 1998). This increase requires protein synthesis and does not appear to be mediated by soluble factors. The authors conclude that the opportunistic fungi often observed in late stage AIDS may act as cofactors which can enhance viral infection and/or syncytia formation, thus contributing to disease progression.

I.7. Relevance of the current study

I.7.1. The biology of monocytes/macrophages

Cells of the monocyte/macrophage lineage have an important function in adaptive and innate immunity (Filion 1993). These cells act as scavengers of invading

microorganisms, presenters of antigens to T cells, and secretors of many immune-regulating cytokines.

The production of monocytes is a multistep process which begins with bone marrow progenitor cells (Filion 1993). These cells give rise to monoblasts which differentiate into promonocytes. Promonocytes in the bone marrow migrate into the circulation and mature into monocytes. Following approximately 30 hours, monocytes migrate into the tissue to become monocyte-derived macrophages (Meltzer et al. 1990).

Monocyte differentiation into macrophages is a complex process influenced by the microenvironment of the cell (Osusky et al. 1997a). Tissue-specific macrophages are a heterogeneous collection of cells with varying phenotypes, functions and locations (Hopper et al. 1979, Dougherty and McBride 1984, Schmitz et al. 1997). Interactions with platelets (Ammon et al. 1998), retinal epithelium cells (Osusky et al. 1997a, Osusky et al. 1997b) and astrocytes (Sievers et al. 1994), and with T cell cytokines (Tormey et al. 1997) have been shown to induce monocyte differentiation into macrophages. The cytokines M-CSF, GM-CSF, IL-6 and IL-3 have been shown to be especially important for monocyte differentiation into macrophages (Young et al. 1990, Hassan et al. 1994).

Monocyte-derived macrophages differ from monocytes both morphologically and functionally. Whereas monocytes are small, round, nonadherent cells, macrophages are larger, adherent, elongated and spindle-shaped, and have increased microfilaments and filamentous structures within the cytoplasm (Zuckerman et al. 1979, Stevenson et al. 1981, Rinehart et al. 1982, Young et al. 1990).

Cell surface antigens up-regulated in macrophages include CD71 (transferrin

receptor) (Andreesen et al. 1984, Hirata et al. 1986), HLA-DR (Becker 1984, Valentin et al. 1990, Valentin et al. 1991), integrins (Valentin et al. 1990, Young et al. 1990, Xie et al. 1998), Fc receptors (Neumann and Sorg 1980, Erickson-Miller et al. 1990, Valentin et al. 1990, Valentin et al. 1991), and mannose receptors (Schreiber et al. 1991, Rouleux-Bonnin et al. 1995). Conversely, macrophages have decreased levels of myeloperoxidase and cathepsin G (Abrink et al. 1994) and, as previously discussed, of CD4 (Crowe et al. 1987, Kazazi et al. 1989, Valentin et al. 1990, Valentin et al. 1991, Pelchen-Matthews et al. 1998, Graziani-Bowering and Filion 2000).

Macrophages also show increased capacity for ADCC (Young et al. 1990), increased phagocytic ability (Hammerstrom 1980, Neumann and Sorg 1980, Stevenson et al. 1981), increased tumour killing ability (Rinehart et al. 1982, Andreesen et al. 1983), and secrete increased amounts of lysozyme (Zuckerman et al. 1979, Stevenson et al. 1981). Macrophages also have more lysosomes and more rough endoplasmic reticulum (Rinehart et al. 1982).

I.7.2. HIV-infected monocytes/macrophages contribute to AIDS pathogenesis

Because of their expression of CD4 (Moscicki et al. 1983, Wood et al. 1983, Levy et al. 1985, Stewart et al. 1986) and of the β-chemokine receptor CCR5 which acts as an HIV coreceptor (Alkhatib et al. 1996, Choe et al. 1996, Deng et al. 1996, Doranz et al. 1996, Dragic et al. 1996), monocytes and many types of macrophages are susceptible to HIV infection (Levy et al. 1985, Gartner et al. 1986, Ho et al. 1986, Nicholson et al. 1986). However, monocytes are not as susceptible to HIV infection as are the more mature macrophage phenotype (Shen et al. 1994, Sonza et al. 1995). In certain tissues (central

nervous system, lymph nodes and lungs), the major HIV-infected cell population is the macrophage (Meltzer et al. 1990). Macrophage-tropic strains of HIV play an important role in establishing infection (Schuitemaker et al. 1992) and the persistence of HIV-infected monocytes and macrophages (Gartner et al. 1986, Ho et al. 1986) makes them viral reservoirs capable of infecting other cells (Gartner et al. 1986, Fauci et al. 1987, Folks et al. 1987, Roy et al. 1988).

HIV-infected monocytes/macrophages have impaired function and thus contribute to AIDS pathogenesis (Mosier and Sieburg 1994, Crowe 1995). HIV-infected macrophages have impaired phagocytic and killing abilities (due to decreased Fc and complement receptors), impaired antigen presentation (due to decreased MHC class I, II and B7 co-stimulatory molecules), decreased chemotactic ability, and altered cytokine profiles (increased TNF-α, TGF-β, IL-10, IL-8, IL-6, IL-1β), all of which contribute to AIDS pathogenesis (Roux-Lombard et al. 1989, Muller et al. 1990, Breen et al. 1990, Meyaard et al. 1993, Kent et al. 1994, Crowe 1995, Daftarian et al. 1995, Polyak et al. 1997). HIV infection has also been shown to interfere with monocyte differentiation into macrophages (Andreesen et al. 1990). The opportunistic infections characteristic of late-stage AIDS (*Mycobacterium tuberculosis, Mycobacterium avium, Pneumocystis carinii, Candida albicans, Toxoplasma gondii*) can be partially attributed to the inability of HIV-infected macrophages to successfully eliminate them (Siegal et al. 1986, Crowe 1995). Furthermore, HIV-infected macrophages and microglia may produce neurotoxins which contribute to AIDS-related dementia (Crowe 1995, Nottet and Gendelman 1995).

Despite our limited knowledge about the role of CD4 on cells of the

monocyte/macrophage lineage (Littman 1996, Marsh and Pelchen-Matthews 1996), the CD4 molecule is routinely used in the immunophenotyping of human monocytes/macrophages. Interestingly, murine macrophages do not express CD4, an observation which has lead some to conclude that this molecule may be dispensable for macrophage function, or at the very least, that macrophages differ from T cells in their regulation of the CD4 molecule (Crocker et al. 1987). The lack of murine CD4 expression has been attributed to species-specific differences with respect to *cis*-acting sequences in the murine CD4 gene (Ellmeier et al. 1999) since human CD4 transgenes can be expressed in a murine macrophage background (Hanna et al. 1994).

I.8. Hypotheses and statement of objectives

Our **first objective** was to characterize the regulation of monocyte CD4 during the process of cellular differentiation into tissue culture-derived macrophages. We hypothesized that the absence of CD4, the HIV co-receptor, might explain the refractoriness of overnight-cultured monocytes to infection in earlier experiments performed in our laboratory. In order to characterize monocyte CD4 regulation, we performed a comprehensive flow cytometric analysis of CD4 expression during the monocyte isolation procedure, and following cell culture under various conditions.

Whereas the signal-transducing function of T cell CD4 has been well characterized both in response to Ag, and to some extent, to HIV, very little is known with respect to the signal-transducing pathway(s) mediated by monocytic CD4. We hypothesized that despite the lack of $p56^{lck}$ association with monocyte CD4, CD4 in these cells is nonetheless an active

signaling molecule. It was our **second objective**, therefore, to determine the internal (transmembrane and cytoplasmic) sequence of monocyte CD4 for comparison to that of T cell CD4, as well as to use biochemical/immunological and functional assays to begin characterizing the pathway(s) by which monocyte CD4 transduces signal.

In meeting these objectives, it was hoped that we would gain a greater insight into the physiological role(s) of monocyte CD4 during processes such as differentiation and migration. This knowledge may eventually contribute to our understanding of the mechanisms by which HIV induces functional impairment of cells of the monocyte/ macrophage lineage, thus contributing significantly to the immune deficiency which is the hallmark of AIDS.

II. METHODS AND MATERIALS

II.1. Ethics approval for monocyte CD4 expression experiments

Ethics approval for experiments using human blood was received from the Ottawa General Hospital Research Ethics Committee.

II.2. Isolation of PBMC for monocyte CD4 expression experiments

Buffy coat or whole blood units were obtained from the Ottawa Chapter of the Red Cross. Alternatively, peripheral blood was obtained from healthy laboratory personnel following receipt of informed consent. The blood was collected into Vacutainers® (Becton Dickinson, San Jose, CA) containing either sodium heparin, EDTA (K_3) or acid citrate dextrose (ACD). PBMC were isolated by Ficoll-Paque (Pharmacia Biotech, Baie D'Urfé, PQ) by overlaying the blood (either neat or diluted 1:1 with RPMI-1640; Gibco BRL, Grand Island, NY) in a 3:2 ratio with Ficoll-Paque. The tubes were centrifuged for 30 min at RT, 400 × g. In some experiments, the plasma was collected and clarified by ultracentrifugation for 10-15 min at 4°C, 12 096 × g using the JA-20 rotor of the J2-21M centrifuge (Beckman, Toronto, ON). The autologous plasma was stored at 4°C until required for use in the Ab staining procedure. Each PBMC layer was collected and transferred into a separate 50 ml polypropylene conical tube (containing 1 ml Hepalean - optional). The PBMC were washed with RPMI-1640 for 5 min at RT, 200 × g. The washes were repeated until the platelet contamination of the PBMC was low as assessed by light microscopy; three washes were usually required. The PBMC were counted either manually using Trypan Blue (Gibco BRL), or using a Coulter Counter Z_F (Coulter Corp., Miami, FL) and used for the isolation of

monocytes. In later experiments, some of the post-Ficoll-Paque PBMC were analysed by flow cytometry, and some were cultured, as described below. Cell cultures were established as described below, depending on the experiment.

II.3. Cell cultures

II.3.1. Monocyte CD4 Expression Experiments

i. Whole blood cultures

Whole blood cultures were established in 6 ml polypropylene tubes (Sarstedt, St. Laurent, PQ) by culturing 2.5 ml whole blood in 1 ml RPMI-1640 medium containing 2 mM L-glutamine (Gibco BRL), 5 µg/ml Fungizone (Bristol-Myers Squibb Canada, Inc., Montreal, PQ), 40 µg/ml gentamycin (Gibco BRL), 10 U/ml penicillin (Gibco BRL), and 10 µg/ml streptomycin (Gibco BRL). The cultures were maintained at 37°C, 5% CO_2 until the following day.

ii. Monocyte cultures

Monocytes were isolated by adherence in flasks coated with gelatin (Bio-Rad, Mississauga, ON) and fibronectin (Becton Dickinson or Sigma, St. Louis, MO) using a modified version of a previously described procedure (Freundlich and Avdalovic 1983). Briefly, 6 to 10 ml of a 30 mg/ml sterile solution of gelatin were added to Corning T75 flasks. The flasks were incubated for 2 hr at 37°C, and residual gelatin was aspirated from the flasks. The flasks were dried for a minimum of 2 days at 65°C. On the day of the isolation, a solution consisting of 20 µg/ml fibronectin (Becton Dickinson or Sigma) and 2%

v/v Hepalean (Organon Teknika, Toronto, ON) in RPMI-1640 was prepared and added to the gelatin-coated flasks; in the original protocol, autologous plasma was used as a source of fibronectin. The flasks were incubated for at least 1 hr at RT; the residual solution was aspirated from the flasks just prior to the addition of PBMC. Following the isolation of PBMC, the cells were diluted with RPMI-1640 to a minimum concentration of 4×10^6 cells/ml. Five ml PBMC were added to gelatin/fibronectin-coated flasks containing 5 ml RPMI-1640. The PBMC were incubated at 37°C, 5% CO_2 for 1.5 hr, and the nonadherent cells were washed away by pipetting warm (37°C) RPMI-1640 against the bottom of the flasks.

The adherent cells (monocytes) were cultured in the same medium as for the whole blood cultures, but with the addition of 10% FBS (Gibco BRL); this medium will subsequently be referred to as RPMI/FBS. In some experiments, parallel monocyte cultures containing various amounts of recombinant GM-CSF (Genzyme, Cambridge, MA, Amersham, Oakville, ON, and kindly provided by Genetics Institute, Cambridge, MA) or purified GM-CSF (kindly donated by Genetics Institute), 5 or 10 U/ml recombinant M-CSF (Genzyme), 5 or 10 µg/ml LPS (Sigma), or 12 U/ml recombinant IL-10 (kindly donated by Schering Canada, Inc., Pointe Claire, PQ) were also established. The units used to characterize cytokine activity were defined by the product manufacturers. The monocyte cultures were maintained overnight and/or up to 6 days at 37°C, 5% CO_2.

iii. Post-Ficoll-Paque PBMC cultures

PBMC were cultured under various conditions. These conditions involved the culture

of 2×10^6 PBMC in uncoated Corning T25 polystyrene flasks, gelatin/fibronectin-coated Corning T25 flasks, 6 ml polypropylene tubes (Sarstedt), 30 ml Teflon vials (Savillex Corp., Minnetonka, MN), or Nunc 6 well plates (Gibco BRL). The PBMC were cultured overnight at 37°C, 5% CO_2 in 1 to 10 ml RPMI/FBS, depending on the size of the culture container.

iv. Harvesting cells for CD4 expression experiment cultures

PBMC and monocyte cultures were harvested by washing the flasks with ice cold 10mM EDTA (BDH, Inc., Toronto, ON) in RPMI-1640 and allowing the flasks to incubate on ice for 5 min intervals. The cells were collected into 50 ml polypropylene conical tubes containing 1-2 ml FBS (Gibco BRL) and were washed in PBS (Sigma), 0.1% NaN_3 (BDH) for 5 min at RT, 200 × g. The cells were resuspended in PBS, 0.1% NaN_3 in preparation for staining with mAb for flow cytometric analysis.

In experiments in which cells were cultured in both adherent environments (ie. flasks) and nonadherent environments (ie. Teflon vials), the cells were also harvested using cold EDTA/RPMI medium to control for harvesting conditions. All cells were washed with PBS, 0.1% NaN_3 and the cells were pelleted for 5 min at RT, 200 × g, resuspended in PBS, 0.1% NaN_3, blocked and stained for flow cytometric analysis.

II.3.2. Thp-1 CD4 Signal Transduction Experiments

i. Maintaining cell lines

The following cell lines were obtained from ATCC (Manassas, VA): the human monocytic cell lines U937, HL-60, and Thp-1, the human T cell lines Hut 78, Jurkat, and

J.Cam1.6 (a $p56^{lck}$-negative derivative of Jurkat cells; Goldsmith and Weiss 1987), and the human nonhemopoietic HeLa cell line (a human cervical cancer). A.201 cells, a $CD4^-$ derivative of Jurkat cells (Folks et al. 1986) were provided by Dr. Ken Dimock (University of Ottawa, Dept. of Biochemistry, Microbiology and Immunology, Ottawa, ON). The EBV-transformed human B cell line, MK, was provided by Drs. Ashok Kumar and Marko Kryworuchko (Virology Lab, Children's Hospital of Eastern Ontario, Ottawa, ON). Because of the large number of cells required in the signal transduction experiments, the $CD4^-$ Thp-1 monocytic cell line was used instead of peripheral monocytes. This cell line represents a more differentiated stage of monocytes than do U937 and HL-60 cells (Auwerx 1991).

Cells were cultured in either RPMI-1640 or OPTI-MEM®I (Gibco BRL) supplemented with 10% FBS (Gibco BRL or CanSera, Rexdale, ON) 10 U/ml penicillin and 10 μg/ml streptomycin; some cultures were also supplemented with 5 μg/ml Fungizone (Bristol Myers Squibb Canada), and 40 μg/ml gentamycin (Gibco BRL).

ii. Culture of Thp-1 cells with CD4 oligodeoxynucleotide

1×10^6 Thp-1 cells/ml were cultured for 4 days in the presence of 30 μM antisense CD4 oligodeoxynucleotide (5'-AGG GAC TCC TCC CCG GTT CAT-3'; prepared by Operon Technologies, Alameda, CA for Bio/Can Scientific) resuspended with 1X TE buffer, pH 7 (Appendix II). Briefly, Thp-1 cultures were cultured for 2 days in RPMI-1640 containing 10% FBS, 10 U/ml penicillin, 10 μg/ml streptomycin and supplemented daily with 30 μM oligodeoxynucleotide. On the third day, the cells were washed with PBS for 5 min at RT, 200 × g and starved by overnight culture in RPMI-1640 containing 2% FBS, 10

U/ml penicillin, 10 μg/ml streptomycin and 30 μM antisense oligodeoxynucleotide. On the fourth day, the cells were washed with PBS for 5 min at RT, 200 × g. The cells were cultured for 3 hr in RPMI-1640 supplemented with 30 μM antisense oligodeoxynucleotide. Separate 4 day Thp-1 cultures which were treated with TE buffer, pH 7 instead of oligodeoxynucleotide were also established as negative controls. Following the 3 hr starvation with RPMI-1640, both the oligodeoxynucleotide-positive and -negative cultures were collected and pelleted by centrifugation for 5 min at RT, 200 × g. The cells were counted using Trypan Blue and 2×10^5 cells were isolated from each culture to be used for flow cytometric analysis of surface CD4 expression. The remaining cells from each culture were divided into two aliquots and one aliquot per culture was stimulated for 3 min at 37°C with 25 μg/ml T4-4 serum (to be described in detail below); the second aliquot was not stimulated with T4-4 serum, but was incubated for 3 min at 37°C. The stimulations were stopped by the addition of cold PBS to each cell aliquot. The cells were washed for 5 min at 4°C, 200 × g, and the resulting pellets were stored at –80°C until further processing for the preparation of cell lysates.

II.4. Isolation of monocytes by negative immunomagnetic selection for CD4 sequencing

PBMC were isolated by Ficoll-Paque from a blood unit obtained from the Red Cross and the cells were counted on a Coulter Counter. The percentage of T cells present in the PBMC fraction was assumed to be approximately 65% (Sell et al. 1996). This value was used to calculate the total number of T cells present in the PBMC fraction. Since a 10:1 ratio of Dynabeads® M-450 Pan-T (CD2) beads (Dynal, Inc., Lake Success, NY) to T cells was

required, the bead volume needed to give the required number of beads was calculated based on the concentration of the Dynabeads® preparation.

This bead volume was transferred to a 15 ml conical polypropylene Falcon tube (Becton Dickinson) and allowed to sit for 1 min over a magnet. The supernatant was removed and replaced with an equal volume of PBS, 2% (v/v) FBS. The beads were resuspended and incubated over the magnet for 1 min. The supernatant was removed and replaced with an equal volume of PBS, 2% FBS.

The cells were resuspended with 25 ml PBS, 2% FBS. The washed beads were added to the cells and resuspended well. Approximately 10 ml cell/bead suspension were transferred to T75 flasks and the flasks were placed on ice and incubated for 35 min with gentle agitation. The flask contents were resuspended and the flasks were placed against a magnet. The $CD2^-$ cell fraction was collected and transferred to fresh flasks. The $CD2^-$ PBMC fraction underwent another round of negative selection, and the resulting $CD2^-$ PBMC fraction was transferred to a 50 ml conical polypropylene Falcon tube and incubated over a magnet, on ice, for 2 min. The $CD2^-$ cell fraction was transferred to a 50 ml conical polypropylene Falcon tube and the cells were pelleted by spinning for 5 min at RT, 300 × g. The supernatant was removed and the cells were resuspended with 4 ml PBS, 2% FBS. The $CD2^-$ PBMC were divided equally amongst 4 × 1.5 ml Eppendorf tubes. The tubes were incubated over a magnet, on ice, for 1 min to check for the presence of residual beads (none were observed). The cells were pooled and an aliquot was counted on a Coulter Counter.

An aliquot of cells was stained as described below for flow cytometric analysis to determine the percentage of contaminating T cells.

II.5. Staining cells for two- and three-colour flow cytometric analysis

II.5.1. Monocyte CD4 expression studies

Whole blood, PBMC and adherence-isolated monocytes were double or triple-stained to assess the relative proportions and the phenotypes of the subpopulations within each fraction before and after culture. Autofluorescent controls and the appropriate colour compensation controls were also included. Colour compensation was performed on whole blood monocytes; for each fluorochrome, the Ab that resulted in the highest intensity stain was used, ie. α-CD14-FITC, α-CD4-PE, and α-HLA-DR-TRI.

i. Whole blood

One ml whole blood from each unit of Red Cross blood or buffy coat was collected from the bag line and 500 µl Hepalean were added. Samples from Vacutainers® (heparin, EDTA (K_3) or ACD) tubes did not receive any additional heparin and were processed as described below. Whole blood volumes of 100 µl were aliquoted into 6 ml polystyrene tubes (Sarstedt) and the cells were stained with an optimum dose of mouse α-human mAb.

The staining of whole blood cultures took into account the increased volume of blood due to the harvesting medium used in the collection of the culture. We estimated that approximately 140 µl diluted whole blood would give the equivalent number of PBMC obtained in the 100 µl sample of undiluted whole blood. This increase in volume was not sufficient to compromise the effectiveness of the Q-prep procedure.

Cells were added to the Ab combinations and incubated in the dark for 10 min at RT. The combinations of Ab used in the various experiments are summarized in Table II.1.

Table II.1. Summary of the various Ab used for CD4 expression flow cytometric analysis.

	ANTIBODY	CLONE	COMPANY
AUTOFLUORESCENT CONTROL	No antibodies were added.		
ISOTYPE CONTROLS	IgG_1-PE/IgG_{2a}-FITC	2T8-2F5/7T4-1F5	Coulter
	IgG_{2b}-PE	X39	Becton Dickinson
COLOUR COMPENSATION CONTROLS	Colour compensation was performed on whole blood monocytes. For each fluorochrome, the antibody which would result in the highest intensity stain was used, ie. α-CD14-FITC, α-CD4-PE, α-HLA-DR-TRI.		
MONOCYTES	α-CD14-PE	LeuM3	Becton Dickinson
	α-CD14-PE	Mo-2	Coulter
	α-CD14-FITC	MY4	Coulter
	α-CD4-PE	T4	Coulter
	α-CD4-PE	Q4120	Sigma
	α-HLA-DR-FITC	L243	Becton Dickinson
	α-HLA-DR-TRI [a]	TU-36	Caltag Laboratories, San Francisco, CA
	Ab combinations used: Two-colour: α-CD14/ α-CD4 and α-CD14/ α-HLA-DR, or Three-colour: α-CD14/ α-CD4/α-HLA-DR		

T CELLS	α-CD3-ECD [a]	HIT3A	Coulter
	α-CD3-FITC	Leu4	Becton Dickinson
	α-CD3-TRI [b]	S4.1	Caltag Laboratories
	α-CD3-Quantum Red [c]	UCHT-1	Sigma
	α-CD4-PE: various clones as described for "MONOCYTES"		
	Ab combinations used: α-CD3/ α-CD4		
B CELLS	α-CD19-ECD	HD237	Coulter
	α-CD19-FITC	SJ25-C1	Sigma
	α-CD19-FITC	FMC63	Serotec, Mississauga, ON
	α-CD20-PE	Leu16	Becton Dickinson
	α-HLA-DR: various clones as described for "MONOCYTES"		
	Ab combinations used: α-CD19/α-HLA-DR and/or α-CD20/ α-HLA-DR		
CD4-TAGGING EXPERIMENTS	goat α–mouse Ig-FITC	Not applicable	Biosource International, Camarillo, CA

[a] ECD is a Texas Red-PE tandem dye
TRI [b] and Quantum Red [c] are Cy5-PE tandem dyes

Isotype controls were also included initially, but were eventually discontinued once it was determined that isotype binding was negligible.

Red blood cells were lysed and the white blood cells stabilized using the 35 sec cycle on the Q-Prep System (Coulter). Cells were washed (optional) for 5 min at RT, 200 × g with PBS and resuspended with PBS to a final volume of 0.5 ml.

In experiments using Ab conjugated to Cy5-PE tandem dyes, a blocking step was performed prior to staining with mAb (Dr. L.G. Filion; unpublished results) to block monocyte receptors specific for the Cy5 component of the conjugate (Stewart and Stewart 1994). Five µl BSA-Cy5 were added per 100 µl whole blood prior to the addition of the tandem dye. The BSA-Cy5 reagent was prepared by conjugating BSA (Sigma) to Fluorolink™ Cy5™ Reactive Dye (Amersham Life Science, Inc., Pittsburgh, PA) as per the manufacturer's instructions (described in Appendix II).

ii. Post-Ficoll-Paque PBMC and adherence-isolated monocytes

Cells were resuspended in PBS, 0.1% NaN_3 to a concentration of 5×10^5 cells/ml and blocked with an equal volume of autologous plasma or with 10 µg/ml gamimmune (an Ig preparation from the Canadian Red Cross Society, Ottawa, ON), and, when required, with 5 µl BSA-Cy5, for 10 min at RT. Two hundred µl cells were transferred to 6 ml polystyrene tubes to which mAb had been added. The cells were incubated in the dark for 10 min at RT, washed in PBS, 0.1% NaN_3 for 5 min at RT, 200 × g, and resuspended to a final volume of 0.5 ml with PBS, 0.1% NaN_3.

iii. CD4 Tagging experiments

PBMC were placed on ice and 2×10^6 PBMC were resuspended with sterile PBS to a volume of 1 ml and blocked with 1 ml of 10 μg/ml of gamimmune. The cells were incubated in the dark for 10 min with 25 μl α-CD4-PE mAb and washed in sterile PBS for 5 min at 4°C, 300 × g. The pellet was resuspended and the cells were cultured overnight in RPMI/FBS in Nunc 6-well plates. Untagged PBMC cultures were established as controls.

Untagged and CD4-tagged PBMC were harvested with EDTA/RPMI and washed as previously described. The cells were blocked with gamimmune (10 μg/ml), aliquoted and stained. Untagged PBMC were stained with either α-CD4-PE mAb or with goat α-mouse Ig-FITC Ab. CD4-tagged PBMC were stained with goat α-mouse Ig-FITC Ab.

II.5.2. CD4 Signal Transduction Studies

i. Cell lines for sequencing experiments

Adherent HeLa cells were trypsinized by incubating the cells for 3 min at 37°C with trypsin, followed by two washes with PBS for 5 min at RT, 150 × g. Hut78 and U937 cells were collected into PBS ,0.1% NaN_3 and pelleted for 5 min at RT, 150 × g.

HeLa, Hut78 and U937 cells were resuspended in PBS,0.1% NaN_3, counted using Trypan Blue and stained for flow cytometric analysis as previously described. The following panel of mouse α-human mAb was used:

Tube 1. Autofluorescent control (no Ab added)
Tube 2. 2.5 μl IgG_1-FITC isotype control (Becton Dickinson)
Tube 3. 10 μl α-HLA-ABC-FITC (clone B-H9; Serotec)
Tube 4. 2.5 μl α-CD4-PE (clone T4; Coulter)

ii. $CD2^-$ PBMC isolated by negative immunomagnetic selection for CD4 sequencing experiments

An aliquot of $CD2^-$ PBMC isolated by negative immunomagnetic selection was stained for flow cytometric analysis using the mAb panel summarized in Table II.2.

iii. Antisense CD4 oligodeoxynucleotide experiments

1×10^5 Thp-1 cells collected from oligodeoxynucleotide-positive and -negative cultures were blocked and stained with α-CD4-PE mAb (clone Q4120; Sigma) as previously described. For each culture condition, an autofluorescent control consisting of 1×10^5 blocked, unstained Thp-1 cells was also included.

II.6. Flow cytometric analysis of cells

II.6.1. Monocyte CD4 expression studies

Cell surface marker expression was determined in initial experiments using the Profile II Flow Cytometer (Coulter), and later, using the EPICS XL Flow Cytometer (Coulter), both of which were equipped with an argon ion laser emitting at 488 nm. In order to compare results obtained at different time points within an experiment, channel targeting was performed at the beginning of each analysis session using Standard Brite Beads (Coulter). Forward *vs.* side scatter histograms were established to visualize the cells and to gate out dead cells and debris. In the case of whole blood and PBMC samples, gates were established around the lymphocytes and monocytes and these cell subpopulations were analysed separately. A minimum of 2 000 events was recorded. In the case of the Profile

Table II.2. Summary of the mAb panel used for the flow cytometric analysis of monocyte CD4 expression and T cell contamination of $CD2^-$ PBMC isolated by negative immunomagnetic selection.

Tube # and Purpose	mAb combination
1. Autofluorescent control	none
2. T cell determination	α-CD3-FITC/α-CD4-PE
3. Monocyte determination	α-CD14-FITC/α-CD4-PE
4. Monocyte determination	α-HLA-DR-FITC/α-CD14-PE
5. B cell determination	α-HLA-DR-FITC/α-CD20-PE
6. NK cell determination	α-CD16-FITC/α-CD56-PE

The mAb were obtained from the following companies: α-CD3-FITC (clone Leu4): Becton Dickinson; α-CD4-PE (clone T4): Coulter; α-CD14-FITC (clone MY4): Coulter; α-CD14-PE (clone LeuM3): Becton Dickinson; α-HLA-DR-FITC (clone L243): Becton Dickinson; α-CD20–PE (clone Leu16): Becton Dickinson; α-CD16-FITC (clone B-E16): Serotec, and α-CD56-PE (clone NKH-1): Coulter.

II Flow Cytometer, data was acquired using the running software, and was reanalysed using the EPICS XL Version 1.5 Software (Coulter). In the case of the EPICS XL Flow Cytometer, data was both acquired and reanalysed using the Epics XL Version 1.5 software. Figures were generated with the WinMDI 2.8 program (Trotter 1999).

II.6.2. Monocyte CD4 sequencing experiments

Monocytes within the $CD2^-$ PBMC fraction which was isolated by negative immunomagnetic selection, and the cell lines used as positive and negative controls were analysed using the EPICS XL Flow Cytometer.

II.6.3. Thp-1 CD4 oligodeoxynucleotide experiments

CD4 surface expression of Thp-1 cells cultured in the presence or absence of oligodeoxynucleotide was assessed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) which was equipped with an argon ion laser emitting at 488 nm. Data was acquired and analysed using CellQuest software Version 3.2.1fl (Becton Dickinson). To determine the level of CD4 expression for each condition, the mean channel number of the autofluorescent control was subtracted from the mean channel number of the α-CD4-PE mAb-stained cells to obtain the corrected mean channel number of CD4 expression. The percentage reduction of CD4 expression was calculated using the following equation:

$$[1 - (CMC^+/CMC^-)] \times 100$$

where CMC^+ and CMC^- = corrected mean channel numbers of CD4 expression for oligodeoxynucleotide-positive and -negative cultures, respectively.

II.7. Statistical analysis for monocyte CD4 expression experiments

To determine the degree of monocyte CD4 or HLA-DR expression in whole blood, PBMC or adherence-isolated monocyte fractions for any particular time point or culture condition, paired T tests were performed using the mean channel number of Ab-stained cells, after correction for autofluorescence. Paired T tests were also performed to compare CD4 or HLA-DR expression between time points or conditions. The word "significant" is used to indicate a difference at $P < 0.05$.

II.8. Sequencing the transmembrane and cytoplasmic tail domains of monocyte CD4

II.8.1. RNA extraction

2×10^6 $CD2^-$ PBMC isolated by negative immunomagnetic selection were transferred to 1.5 ml Eppendorfs to which 1 ml TRI-reagent (Molecular Research Center, Cincinnati, OH) was added. The cells were resuspended well and incubated for 5 min at RT. The lysates were stored at -80°C until the RNA extraction was continued. Frozen homogenates were thawed at RT. Lysates were extracted once with 200 µl chloroform (BDH) and the nucleic acids were precipitated for 10 min at RT with 2.5 volumes isopropanol (BDH). RNA was pelleted by centrifugation for 10 min at 4°C, 12 000 × g. The RNA pellets were washed with 1 ml DEPC (BDH)-treated 75% ethanol (Commercial Alcohols, Inc., Brampton, ON) by centrifuging for 5 min at 4°C, 7 500 ×g and air dried for 10 min. The RNA pellets were resuspended in 20 µl DEPC-treated ddH_20 and heated for 15 min at 60°C. The purity of the isolated RNA was checked using the DUB8 UV/visible spectrophotometer (Beckman Instruments Canada, Inc., Mississauga, ON). RNA integrity was assessed by running an

aliquot on a 0.8% agarose gel. Briefly, 2 µl RNA or 0.24-9.5 Kb RNA ladder (Gibco BRL) were diluted with 8 µl 1X TAE (Appendix II) and heated for 15 min at 65°C, then quenched on ice for 5 min. Prior to loading, 2 µl 6X RNA loading buffer (Appendix II) were added to the samples. The samples were loaded into 0.8% agarose (Bio-Rad) gels prepared in 1X TAE with or without (0.5 µg/µl) ethidium bromide (Gibco BRL), and electrophoresed at 50-75 volts. Gels not containing ethidium bromide were stained for 5 min in a 0.5 µg/ml ethidium bromide bath, and destained for 5 to 10 min in ddH_20, or by running the gel for 5 min at 50-75 volts. Gels were visualized on a UV source (Fotodyne, Inc., New Berlin, WI) and photographed using the Mitsubishi Videocopy Processor (Bio/Can Scientific, Mississauga, ON). The images were saved using the Gel Print 2000i (Biophotonics Corp., Bio/Can Scientific).

II.8.2. Reverse transcription

A master mix containing 5 mM $MgCl_2$, 1X PCR Buffer II, 1 mM each dATP, dCTP, dGTP, and dTTP, 1 U/µl RNase Inhibitor, 2.5 U/µl MuLV RTase, and 2.5 µM each random hexamers and oligo $d(T)_{16}$ was prepared; all components were from the Gene Amp RNA PCR Kit (Perkin Elmer, Branchburg, NJ). Two µl RNA or 2 µl ddH_20 (negative control) were added to each Thin-walled reaction tube (Perkin Elmer) for a final volume of 20 µl. The reaction tubes were vortexed and overlaid with mineral oil. Using the Perkin Elmer DNA Thermal Cycler, the reactions were incubated at 22°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min.

II.8.3. PCR

Master mixes containing 1.5 mM $MgCl_2$, 1X Buffer, 0.04 U/µl AmpliTaq DNA polymerase, 0.2 mM each dATP, dCTP, dGTP, and dTTP, and 0.4 mM either 3' or 5' CD4 primers, or 3' or 5' β-actin primers, were prepared; all components, with the exception of the primers, were from the Gene Amp RNA PCR Kit. The primer sequences were as follows: 5' CD4 primer: 5'-GTG AAC CTG GTG GTG ATG AGA GC-3'; 3' CD4 primer: 5'-GGG GCT ACA TGT CTT CTG AAA CCG GTG-3'; 5' β-actin primer: 5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3' ; 3' β-actin primer: 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'. Primers were originally obtained from Clontech Laboratories, Inc. (Palo Alto, CA); CD4 primers were later prepared by the University of Ottawa Biotechnology Research Institute. Five to 10 µl cDNA (from reverse transcription reaction), 5 µl cDNA provided as a positive control for the primers, or 5 µl ddH_20 (negative control) were added to each Thin-walled reaction tube for a total reaction volume of 50 µl. Reactions in which the AmpliTaq DNA polymerase was replaced with ddH_20 were also established as additional negative controls. The reaction tubes were vortexed, overlaid with mineral oil (Perkin Elmer), and amplified with the Perkin Elmer DNA Thermal Cycler as follows: 95°C for 2 min, followed by 30 to 35 cycles of 95°C for 45 sec, 60°C for 45 sec, 72°C for 2 min, followed by 72°C for 7 min.

II.8.4. Assessing RT-PCR products

Products were checked on 1.8-2% agarose gels prepared in 1X TAE buffer (Appendix II). Five µl RT-PCR product were resuspended with 1X TAE and 6X DNA Loading Buffer

(Appendix II). A 1:50 dilution of 100 bp DNA ladder (Gibco BRL) prepared in 1X TAE was denatured by heating at 65°C for 5 min in a block heater, and quenched on ice for 5 min. Two µl 6X DNA Loading Buffer were added to the ladder dilution prior to loading into the gel. The samples were electrophoresed as described.

II.8.5. Automated sequencing of CD4 cDNA

Automated sequencing of CD4 cDNA from 3 different RT-PCR preparations obtained for each $CD4^+$ cell line/type ($CD2^-$ PBMC, Hut 78 and U937 cells) was performed by the University of Ottawa Biotechnology Research Institute. The same CD4 primers used for PCR were used for sequencing.

II.9. Antibodies, positive control lysates and sCD4 used in Western immunoblotting and immunoprecipitation experiments

PE-conjugated α-CD4 murine mAb clone Q4120, rabbit α-mouse Ab and rabbit α-goat Ab were obtained from Sigma. Murine α-phosphotyrosine mAb clone 4G10, α-$p56^{lck}$ murine mAb (clone 3A5), murine α-PI3-K (p85) mAb and Jurkat positive control cell lysate were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Murine α-GST mAb, rabbit polyclonal Ab to PLC-γ1, rabbit polyclonal Ab to ERK-1, rabbit polyclonal Ab to ERK-2, and goat polyclonal Ab to actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit serum to Shp-1 was generated in-house as previously described (Kon-Kozlowski et al. 1996). sCD4 and T4-4 rabbit serum to sCD4 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH

(Rockville, MD), courtesy of Dr. R. Sweet, SmithKline Beecham Pharmaceuticals (Deen et al. 1988).

II.10. CD4 stimulation of Thp-1 cells

Thp-1 cells were cultured at 37°C, 5% CO_2 in either OptiMem®I or RPMI-1640 supplemented with 10% FBS, 10 U/ml penicillin and 10 μg/ml streptomycin. Prior to stimulation with T4-4 rabbit serum to sCD4, the cells were cultured overnight in medium containing 2% FBS and washed the next morning for 5 min at RT, 200 × g with PBS prior to being further starved for 3 to 4 hr by culture in serum-free medium. Following this starvation, the cells were collected by centrifugation for 5 min at RT, 200 × g, counted using Trypan Blue and aliquoted into 1 ml volumes containing 10 × 10^6 cells. Cells were stimulated by incubation with 25 μg/ml T4-4 serum for 3 min at 37°C. As a negative control, starved but unstimulated Thp-1 cells were also incubated at 37 °C for 3 min. In some experiments, the following negative control stimulations were also included: 25 μg/ml rabbit serum to Shp-1, or 25 μg/ml T4-4 serum which had been pre-adsorbed by incubation with either unbound sCD4 or BSA (ICN Biomedicals, Montreal, PQ), or sCD4 or BSA immobilized on NHS-activated Sepharose 4 Fast Flow Beads (Pharmacia Biotech) as per the manufacturer's instructions (Appendix II). Following stimulation, the cells were washed with cold PBS for 5 min at RT, 200 × g and the pellets were stored at –80°C until further processing for preparation of cell lysates.

II.11. Preparation of cell lysates

Cell pellets prepared from various cell lines were lysed by the addition of lysis buffer (Appendix II). The resuspended pellets were incubated for 30 to 60 min on ice, and the lysates were centrifuged for 20 min at 4°C, 9 850 × g to separate insoluble material from the lysates. Protein concentration was determined using the Bio-Rad Protein Colorimetric Assay, as per the manufacturer's directions.

II.12. Immunoprecipitation

Immunoprecipitation reactions consisted of 1.5 to 2 mg Thp-1 total protein lysate, 50 µl of a 50% Protein A or Protein G-Sepharose 6MB Bead slurry (Pharmacia Biotech) and the recommended amount of Ab. Lysis buffer was added to bring the immunoprecipitation reactions to a final volume of 1 ml, and the reactions were incubated by inversion for 2 hr at 4°C. The immune complexes were collected by centrifugation for 5 min at 4°C, 9 850 × g, and washed three times for 5 min at 4°C, 9 850 × g with 1 ml lysis buffer. The final pellets were resuspended with 25 µl Laemmli Sample Buffer (Bio-Rad).

II.13. Western immunoblotting

Immune complexes and prestained protein SDS-PAGE standards (Bio-Rad) were eletrophoresed through SDS polyacrylamide gels prepared according to Laemmli (Laemmli 1970). The resolving gels consisted of 8 to 10% (w/v) acrylamide (Bio-Rad) in 375 mM Tris-HCl, pH 8.1 (Gibco BRL), 0.1% (w/v) SDS (Bio-Rad), 0.15% (w/v) APS (Bio-Rad) and 0.2% (v/v) TEMED (Sigma). The stacking gels consisted of 5% acrylamide in 127 mM Tris-

HCl, pH 6.8, 0.1% SDS, 0.2% APS, and 0.2% TEMED. The electrophoresed proteins were electroblotted onto Immobilon-P PVDF Transfer membranes [Millipore (Canada) Ltd., Nepean, ON] and the membranes were blocked with 5% (w/v) BSA in 1X TBST (Appendix II). Blocked membranes were incubated with primary Ab diluted in 5% BSA in 1X TBST, washed with 1X TBST, and, unless otherwise stated, incubated with approximately 100 nCi/ml I^{125}-Protein A (NEN Life Science Products, Inc. Boston, MA) diluted in 5% BSA. The membranes were washed with 1X TBST, and exposed to BioMax X-ray film (Kodak, New Haven, CT) at –80°C. In experiments in which the Ab used to immunoblot had low affinity for the Protein A component of I^{125}-Protein A, a secondary Ab which acted as a linker between the primary Ab and the ^{125}I-Protein A was included. Film was developed using the Kodak M35A X-omat Processor.

In other experiments, chemiluminescent detection was performed instead of radioactive detection. In these experiments, membranes were incubated with horseradish peroxidase-conjugated secondary Ab or Protein G (Bio-Rad) instead of ^{125}I-Protein A. The membranes were subsequently incubated for 1 to 2 min with ECL solution prepared by mixing equal volumes of luminol reagent with oxidizing reagent; Renaissance® Western Blot Chemiluminescence Reagent (Plus) Kit (NEN Life Sciences Products, Inc.). The membranes were inserted into hybridization bags (Gibco BRL) and exposed to film at RT for varying lengths of time prior to developing.

When required, blots were stripped by incubation for 30 min in a 50°C H_2O bath with stripping solution (Appendix II). Following stripping, the membranes were washed extensively with 1X TBST and exposed to film to ensure efficient stripping prior to

subsequent reblotting with other Ab.

II.14. Preparation of GST-$CD4_{cyt}$ fusion protein

GST-$CD4_{cyt}$ fusion protein was generated by subcloning PCR-amplified human CD4 cytoplasmic tail (residues 396 to 433) into pGEX-2T (Pharmacia Biotech). The steps undertaken to produce this fusion protein are described below.

II.14.1. Preparation of $CD4_{cyt}$ tail insert

i. PCR

A master mix containing 1X PCR buffer with Mg (Boehringer Mannheim Canada, Laval, PQ), 1 mM dNTP mix (Invitrogen, Carlsbad, CA), 0.02 µg/µl 5' CD4+BamHI primer and 0.02 µg/µl 3' CD4+EcoRI primer were prepared. The primer sequences were as follows: 5' primer: 5'-AAA AAA GGA TCC AGG TGC CGG CAC CGA AGG-3', and 3' primer: 5'-AAA AAA GAA TTC TCA AAT GGG GCT ACA TGT-3'; primers were synthesized by the Molecular Genetics Laboratory of the Children's Hospital of Eastern Ontario, Ottawa, ON. One µl CD4 cDNA from U937 (cDNA leftover from RT-PCR products generated for sequencing monocyte CD4) were added to each reaction tube. The reactions were incubated for 5 min at 95°C on a block heater. One µl Taq DNA polymerase (Boehringer Mannheim Canada) was added to each reaction for a final concentration of 0.02 U/µl in a 50 µl reaction volume. The reaction tubes were spun momentarily to mix and overlaid with mineral oil. The reactions were amplified with the Perkin Elmer DNA Thermal Cycler as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min,

followed by 72°C for 7 min. The PCR product was checked on a 1.8% agarose (Gibco BRL) gel prepared in 1X TAE buffer and containing 0.5 µg/µl ethidium bromide. Five µl PCR product or 2 µl 100 bp ladder (Pharmacia Biotech) were mixed with 5 µl DNA Loading Buffer prior to loading.

ii. Purification of $CD4_{cyt}$ PCR product and restriction enzyme digestion

The PCR product consisting of the cytoplasmic tail of CD4 with 5' BamHI and 3' EcoRI extensions was purified using the Qiaex II Agarose Gel Extraction Kit (Qiagen, Mississauga, ON) as described in Appendix II. A digest reaction consisting of 34 µl purified PCR product, 2 µl each 10 U/µl BamHI and EcoRI restriction enzymes (Boehringer Mannheim) and 5 µl 10X SuRE/Cut Buffer B (Boehringer Mannheim) was established in a total volume of 50 µl. The reactions were incubated overnight at 37°C. Following the overnight digestion, separate preparations consisting of either 5 µl of the digest reaction or 5 µl of undigested DNA were mixed with 5 µl DNA loading buffer and electrophoresed on 1.8% agarose gels to confirm digestion. Following confirmation of the successful digestion of the PCR product, the digested PCR product was isolated using the Qiaquick PCR Purification Kit (Qiagen), as described in Appendix II.

iii. GST vector DNA preparation

Fifty-nine µl pGEX-2T cloning vector DNA (Pharmacia Biotech) was digested with 2 µl each 10 U/µl BamHI and EcoRI and 2 µl 10X SuRE/Cut Buffer B in a total reaction volume of 68 µl. The digest was confirmed on a 1% agarose gel. The digested GST vector

DNA was purified by electrophoresis in a 1.2% low melting point agarose gel (Gibco BRL). The GST vector DNA was excised from the gel and purified by phenol:chloroform extraction (as described in Appendix II) and checked on a 1% agarose gel.

iv. Ligation of the digested $CD4_{cyt}$ PCR product with the digested GST vector DNA

A ligation reaction consisting of 8 µl digested, purified PCR product, 1 µl digested, purified GST-vector, and 1X ligation buffer (Boehringer Mannheim) was prepared in 1X TE buffer. The reaction was heated for 16 sec in a 50-55°C water bath. T4 DNA ligase (Boehringer Mannheim) was added to a final concentration of 0.2 U/µl in a 20 µl reaction volume. The reaction was incubated overnight at 16°C in a Perkin Elmer DNA Thermal Cycler.

v. Transformation of Epicurian Coli XL1-Blue Competent cells with GST-$CD4_{cyt}$ DNA

2ME was added to prechilled 15 ml Falcon 2059 polypropylene tubes (Becton Dickinson) such that the final concentration would be 25 mM. Epicurian Coli XL1-Blue Competent cells (Stratagene, La Jolla, CA; genotype listed in Appendix II) were added to the tubes; 100 µl of cells were used for the transformation using the ligation reaction, and 50 µl of cells were used to establish the positive and negative controls, respectively. The reactions were incubated for 10 min on ice; the tubes were swirled every 2 minutes. DNA was added to the tubes as follows: 10 µl ligation reaction to the 100 µl cells and 0.5 µl pUC18 DNA (Stratagene) to the positive control tube containing 50 µl cells; no DNA was

added to the negative control tube. The reactions were incubated on ice for 30 min, heat pulsed for 45 sec in a 42°C water bath and incubated on ice for 2 min. Using sterile technique, SOC medium (Gibco BRL; Appendix II) was added to the tubes as follows: 900 µl SOC medium to the tube containing the ligation reaction, and 450 µl SOC medium to the positive and negative control tubes, respectively. The reactions were incubated for 1 hr at 37°C, 225 rpm in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ). Cells were plated onto 2YT plates containing agar (Appendix II) and 50 µg/ml ampicillin (Sigma). The plates were incubated overnight at 37°C.

The following day, ampicillin-resistant colonies were selected from the plates inoculated with cells transformed with the ligation reaction. These colonies were used to inoculate cultures to be used for maxipreps and minipreps and to streak back-up plates.

vi. Induction of expression of GST-CD4$_{cyt}$ fusion protein

Working in 14 ml Falcon 2059 polypropylene tubes, XL1-Blue cultures transformed with GST-CD4$_{cyt}$ were established in 2 ml 2YT broth (Appendix II) supplemented with 50 µg/ml ampicillin. The cultures were incubated overnight at 37°C, 225 rpm. The next morning, the overnight cultures were subcultured by transferring 1 ml culture into 50 ml fresh 2YT broth supplemented with 50 ug/ml ampicillin. The cultures were incubated for 1 hr at 37°C, 225 rpm. Following the 1 hr incubation, IPTG (Gibco BRL) was added to each culture to a final concentration of 1 mM. The cultures were incubated for 3 hr at 37°C, 225 rpm. Following induction, the cultures were collected into 14 ml Falcon 2059 polypropylene tubes and centrifuged for 15 min at RT, 1 315 × g using the Adams Compact II Centrifuge

(Becton Dickinson). The supernatants were removed and the bacterial pellets were frozen at -80°C.

vii. Isolation of GST and GST-CD4$_{cyt}$ proteins

One ml RIPA lysis buffer (Appendix II) was added to each frozen bacterial pellet, which was subsequently vortexed. Working on ice, the lysates were sonicated for 3 to 4 cycles of pulse (30 sec) and rest (10 sec) using the Microson Ultrasonic Cell Disruptor (Heat Systems Ultrasonics, Inc., Farmingdale, NY). The lysates were transferred to 1.5 ml Eppendorfs and centrifuged for 20 min at 4°C, 9 850 × g to separate insoluble material from the lysates. The supernatants were transferred to fresh 1.5 ml Eppendorfs containing 50 µl of a 50% Glutathione Sepharose 4B Bead slurry (Pharmacia Biotech). The reactions were incubated by inversion for 2 hr at 4°C. The immune complexes were collected by centrifugation for 5 min at 4°C, 9 850 × g and washed three times for 5 min at 4°C, 9 850 × g with 1 ml RIPA buffer. The final pellets were resuspended with an equal volume of lysis buffer to give a 50% bead slurry.

To assess the relative concentrations of fusion protein complexed to Glutathione Sepharose 4B Beads, aliquots were electrophoresed through 12% SDS polyacrylamide gels; known quantities of a BSA standard (Pierce, Rockford, IL) were also included as references. The gels were stained with Coomassie Brilliant Blue Solution (Appendix II) for 1 hr at RT on a shaker, then destained with destaining solution (Appendix II) for several hours at RT on a shaker. The concentration of fusion protein complexed to Glutathione Sepharose 4B Beads was determined by comparing its intensity with that of the known BSA standards,

adjusting for the difference in protein size, and factoring in the volume of fusion protein-bead complex electrophoresed. A sample calculation is shown in Appendix II.

viii. DNA extraction (maxiprep)

DNA was extracted from Epicurian Coli XL1-Blue Competent cells transformed with GST-$CD4_{cyt}$ DNA using the EndoFree Plasmid Maxi Kit, as per the manufacturer's instructions.

ix. Screening of maxiprep plasmid DNA for the presence of the GST-$CD4_{cyt}$ insert

A digest reaction consisting of 10 µl maxiprep DNA , 2 µl each 10 U/µl BamHI and EcoRI restriction enzymes and 1.5 µl 10X SuRE/Cut Buffer B was established in a total volume of 15.5 µl. The reaction was incubated overnight at 37°C. Following the overnight digestion, an aliquot of the reaction, as well as an aliquot of the undigested maxiprep vector, were run on agarose gels to confirm digestion.

x. Automated sequencing of maxiprep DNA

Automated sequencing of GST-$CD4_{cyt}$ maxiprep DNA was performed by the University of Ottawa Biotechnology Research Institute to ensure that the GST-$CD4_{cyt}$ construct did not contain any mutations. The following primers were used: 5'GST-long: 5'-GGT GGT GGC GAC CAT CCT-3'; 5'GST-short: CCA AAA TCG GAT CTG GTT-3'; and 3' GST-short: 5'-CAG ATC GTC AGT CAG TC-3'.

xi. Transforming C600hflA and BL21 E. coli cells with GST-CD4$_{cyt}$ or with GST and comparison of fusion protein expression levels with XL1-Blue cells

In a prechilled 15 ml Falcon 2059 polypropylene tube, 200 µl competent C600hflA (Clontech Laboratories, Inc.) or competent BL21 cells (a kind gift from Dr. Louise Larose, Mount Sinai Hospital, Toronto, ON) were mixed with 1-2 µl isolated, digested and sequenced GST-CD4$_{cyt}$ maxiprep DNA or a lab stock of GST maxiprep DNA; the procedure for making C600hflA and BL21 *E. coli* cells competent is described in Appendix II, as are the genotypes of the cells. A negative control consisting of cells, but no DNA, was also established. The cells were incubated on ice for 1 hr, then heat-shocked for 2 min at 42°C in a water bath. The cells were incubated on ice for 2 min. 800 µl SOC medium were added to the cells and the cells were incubated for 1 hr at 37°C, 225 rpm. The transformed cells were plated onto 2YT agar plates containing 50 µg/ml ampicillin and cultured overnight at 37°C. The following day, colonies of transformed cells were used to inoculate 2YT cultures supplemented with 50 µg/ml ampicillin. Following overnight culture, the cells were induced as previously described. The relative amounts of GST-CD4$_{cyt}$ and GST proteins expressed by the transformed C600hflA and BL21 *E. coli* strains were compared to the levels produced by XL1-Blue cells.

II.15. Far Western blots

GST and GST-CD4$_{cyt}$ proteins immobilized onto Glutathione Sepharose 4B Beads were eluted by incubating the complexes for 10 min at RT with a half volume of glutathione elution buffer (Appendix II). The complexes were centrifuged for 5 min at 4°C, 9 850 × g

and the supernatants containing the eluted fusion proteins were collected for use in far Western blots. In this technique, proteins (GST and GST-$CD4_{cyt}$ in these experiments) rather than antibodies are used for blotting membranes. To determine the concentration of eluted GST and GST-$CD4_{cyt}$, aliquots of the proteins and known quantities of BSA were electrophoresed and the gels stained with Coomassie Brilliant Blue solution as previously described.

Unstimulated and T4-4-stimulated Thp-1 cell lysates were electrophoresed in duplicate, and the proteins were transferred to membranes. Blocked membranes were incubated with 1 μg/ml eluted GST or GST-$CD4_{cyt}$ protein. Bound GST or GST-$CD4_{cyt}$ were detected by incubation of the membranes with murine α-GST mAb, followed by rabbit α-mouse Ab and ^{125}I-Protein A.

II.16. GST-$CD4_{cyt}$ immunoprecipitation experiments

For each immunoprecipitation reaction, 3.5 to 5 mg Thp-1 total protein lysate were resuspended to a 1 ml volume with lysis buffer. The lysates were precleared with 30 μg GST protein (immobilized to Glutathione Sepharose 4B Beads) by inversion for 1 hr at 4°C. Following preclearing, the lysates were centrifuged for 5 min at 4°C, 9 850 × g, and the precleared supernatants were transferred to fresh Eppendorf tubes for a second round of preclearing with GST protein. The lysates were centrifuged for 5 min at 4°C, 9 850 × g and the precleared supernatants were incubated by inversion at 4°C for 2 to 18 hr with 30 μg GST or GST-$CD4_{cyt}$ proteins (immobilized to Glutathione Sepharose 4B Beads). The immune complexes were pelleted by centrifugation for 5 min at 4°C, 9 850 × g. The complexes were

washed three times with 1 ml lysis buffer for 5 min at 4°C, 9 850 × g. The final pellets were resuspended with 25 µl Laemmli Sample Buffer. The complexes were electrophoresed and the gels were stained for 1 hr with Coomassie Brilliant Blue solution as previously described.

II.17. Calcium flux experiments

A 1mM Fluo-3/AM (Molecular Probes, Eugene, OR) solution in 1 mM DMSO (Sigma) and 3.75% w/v Pluronic F-127 (Sigma) was prepared and used to load Thp-1 cells (Brousseau et al. 1998). Thp-1 cells were washed with PBS for 5 min at RT, 200 × g. The cells were resuspended with Buffer A (RPMI-1640 containing 20 mM HEPES, pH 7) and the cells were counted using Trypan Blue. 5×10^6 viable cells were washed twice for 10 min at RT, 400 × g with Buffer A. The cells were resuspended with Buffer A to a final volume of 5 ml, and Fluo3/AM was added to a final concentration of 1 µM. The cells were vortexed gently, then incubated in the dark for 45 min at 37°C, 50 rpm in a Gyrotory Water Bath Shaker (New Brunswick Scientific Co., Inc., Edison, NJ). Five ml Buffer B (Buffer A containing 5% v/v heat inactivated FBS, pH 7.4) were added to the cells and the cells were incubated for 15 min at 37°C, 50 rpm. Thirty ml Buffer B were added to the cells and the cells were washed for 10 min at RT, 400 × g. The cells were resuspended with 10 ml Buffer B to give a final cell concentration of 5×10^5 cells/ml. The cells were aliquoted into 1ml volumes.

A FACScan flow cytometer with CellQuest software Version 3.2.1fl. was used to perform cell analysis of Ca^{2+} flux in response to various stimuli. A protocol was established to include 3 histograms: FS *vs.* SS, time *vs.* FL1, and count *vs.* FL1. An automatic stop was

included at 512 sec.

At the beginning of each acquisition session, a cell sample was used to establish the FS *vs.* SS gate, a flow rate of approximately 200 cells/sec, and an FL1 mean fluorescence intensity for the entire gated cell population of between 100 and 200 mean fluorescence units on a linear scale. In order to maintain the temperature of the cells at 37°C during the data acquisition, each tube was wrapped with a hot pack which had been prewarmed to 37°C. A 10% bleach solution was used to wash the tubes of the flow cytometer after each sample. A second cell sample was used to establish baseline FL1 mean channel fluorescence intensity (count *vs.* FL1 histogram) in the absence of any stimuli, for a total of 512 sec. For all subsequent samples to which various stimuli were added, baseline FL1 signal was recorded for 2 min 5 sec, data acquisition was paused, the stimulus was added quickly and the cells vortexed, and data acquisition was resumed and continued for a total of 512 sec. Positive and negative controls in the form of 20 μM calcium ionophore A23187 (Sigma) and 5 mM EGTA (Sigma) respectively, were also included for each experiment.

III. MONOCYTE CD4 EXPRESSION

III.1. Introduction

The CD4 molecule is a 55-60 kDa membrane glycoprotein first identified on a subset of T cells (Reinherz et al. 1979) which usually function as T-helper cells (White et al. 1978, Reinherz et al. 1979). In humans, CD4 is also expressed by other cell types, including eosinophils (Lucey et al. 1989), as well as by cells of the dendritic and monocyte/ macrophage lineages (Moscicki et al. 1983, Wood et al. 1983, Levy et al. 1985, Stewart et al. 1986); CD4 has been identified on virtually all blood monocytes, although at lower levels than expressed by $CD4^+$ T cells (Filion et al. 1990).

Decreased CD4 levels have been observed on tissue-culture derived-macrophages compared to blood monocytes (Crowe et al. 1987, Kazazi et al. 1989, Valentin et al. 1990, Valentin et al. 1991, Peudenier et al. 1991b, Pelchen-Matthews et al. 1998, Graziani-Bowering and Filion 2000). The variable levels of CD4 expression by different tissue-specific macrophages, as well as by tissue culture-derived macrophages, may reflect differences in their microenvironments (Kazazi et al. 1989), stage of maturation/ differentiation (Kazazi et al. 1989, Valentin et al. 1991), variable sensitivities of the detection systems used by the different investigators (Vazeux et al. 1987), or a combination of these factors.

The *in vivo* role(s) of CD4 on human monocytes/macrophages is not well-defined (Littman 1996, Marsh and Pelchen-Matthews 1996). IL-16 is a chemoattractant for human $CD4^+$ monocytes (Cruikshank et al. 1987) and $CD4^+$ eosinophils (Rand et al. 1991), as well as for rat and human $CD4^+$ T cells (Center and Cruikshank 1982, Cruikshank et al. 1987).

Since IL-16 uses CD4 as a receptor (Cruikshank et al. 1994) or coreceptor (Center et al. 1995) on T cells, CD4 probably serves the same function on $CD4^-$ monocytes and eosinophils (Center et al. 1995).

We wished to characterize the regulation of monocyte CD4 during the process of cellular differentiation into tissue culture-derived macrophages. We hypothesized that the absence of CD4, the HIV coreceptor, might explain the refractoriness of overnight-cultured monocytes to infection in earlier experiments (Filion et al. unpublished observations). In order to characterize the regulation of monocyte CD4 expression, we performed a comprehensive flow cytometric analysis of CD4 expression during monocyte isolation and culture under various conditions.

III.2. Results

III.2.1. Monocyte experiments

i. Culturing monocytes results in CD4 down-regulation

The level of CD4 expression of monocytes isolated from PBMC by adherence to gelatin/fibronectin was examined at various time points following their isolation. Monocytes cultured overnight in RPMI/FBS expressed significantly reduced levels of CD4 (Table III.1), ranging from undetectable (Fig.III.1; n=18), to low level expression (n=13). Following 6 days in culture in RPMI/FBS, monocyte CD4 expression was variable (n=14), ranging from undetectable, to significant expression by either the entire population, or by a subpopulation (Table III.1).

The overnight culture of monocytes resulted in increased HLA-DR levels compared

Table III.1. Monocyte CD4 expression levels before and after overnight and/or 6 days in culture in RPMI/FBS.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN OVERNIGHT MONOCYTE OR PBMC CULTURES		MONOCYTE CD4 EXPRESSION IN 6 DAY MONOCYTE CULTURES	
ISOLATION #	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]
1	0.265	6.38	0.232 [c]	0.228 [c]	N.D. [d]	N.D. [d]
2	0.216	4.07	0.266 [c]	0.405 [c]	N.D. [d]	N.D. [d]
3	0.193	3.60	0.223 [c]	0.367 [c]	N.D. [d]	N.D. [d]
4	0.239	2.54	N.D. [d]	N.D. [d]	0.869	4.64 (63.1%) [e]
5	0.190	2.90	0.313 [c]	0.424 [c]	0.998	1.57
6	0.159	2.29	0.198 [c]	0.213 [c]	0.330	0.603
7	0.232	4.07	N.D. [d]	N.D. [d]	0.727	5.82 (83.6%) [e]
8	0.296	4.49	N.D. [d]	N.D. [d]	0.774	1.24
9	0.266	2.79	N.D. [d]	N.D. [d]	0.771	2.57
10	0.216	2.97	0.529 [c]	0.691 [c]	3.95	5.48
11	0.181	2.76	0.296 [c]	0.308 [c]	2.17	2.46
12	0.218	3.08	0.271 [c]	0.313 [c]	2.16	3.74
13	0.204	2.54	0.228 [c]	0.267 [c]	1.53	2.17
14	0.223	2.75	0.192 [c]	0.197 [c]	2.15	5.56
15	0.235	3.02	0.206 [c]	0.222 [c]	1.49	2.07
16	0.235	3.10	0.301 [c]	0.204 [c]	1.44	1.33
17	0.310	2.67	0.358 [c]	0.324 [c]	1.22	1.27
18	0.276	5.33	0.324 [f]	0.200 [f]	N.D. [d]	N.D. [d]

[a] mean channel number of autofluorescence (FL2 LOG)
[b] mean channel number of α-CD4-PE signal
[c] monocyte culture
[d] N.D.: not done
[e] increased CD4 expression was observed for a subpopulation of monocytes

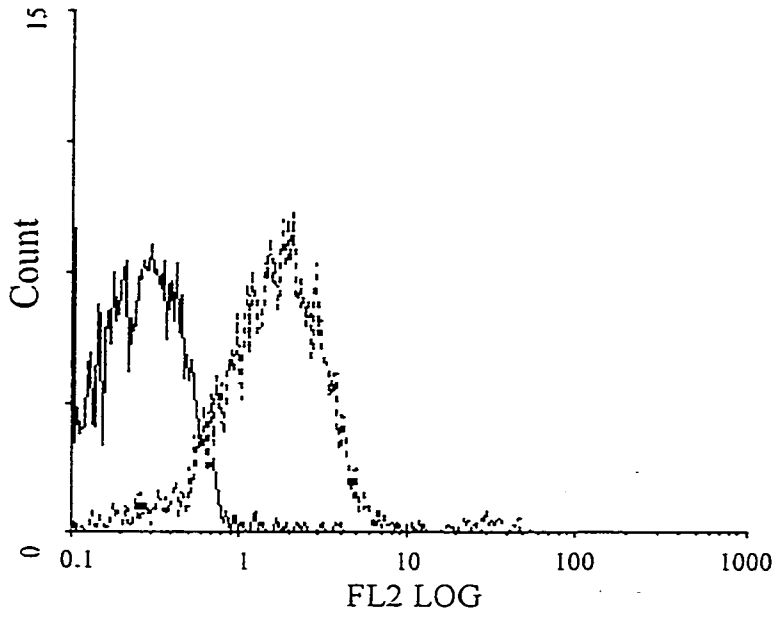
	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN OVERNIGHT MONOCYTE OR PBMC CULTURES		MONOCYTE CD4 EXPRESSION IN 6 DAY MONOCYTE CULTURES	
ISOLATION #	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]
19	0.640	8.02	0.551 [f]	0.846 [f]	N.D. [d]	N.D. [d]
20	0.224	4.79	0.705 [f]	0.692 [f]	N.D. [d]	N.D. [d]
21	0.291	5.54	0.269 [f]	0.225 [f]	N.D. [d]	N.D. [d]
22	0.385	6.61	0.642 [f]	0.486 [f]	N.D. [d]	N.D. [d]
23	0.590	9.15	0.457 [f]	0.777 [f]	N.D. [d]	N.D. [d]
24	0.560	3.74	0.365 [f]	0.485 [f]	N.D. [d]	N.D. [d]
25	0.269	3.09	0.253 [f]	0.688 [f]	N.D. [d]	N.D. [d]
26	0.190 [g]	4.02 [g]	0.492 [f]	1.02 [f]	N.D. [d]	N.D. [d]
27	0.125 [g]	3.87 [g]	0.269 [f]	0.244 [f]	N.D. [d]	N.D. [d]
28	0.302	6.88	0.256 [f]	0.426 [f]	N.D. [d]	N.D. [d]
29	0.427	4.63	0.256 [f]	0.269 [f]	N.D. [d]	N.D. [d]
30	0.315	4.51	0.234 [f]	0.362 [f]	N.D. [d]	N.D. [d]
31	0.284	4.49	0.249 [f]	0.264 [f]	N.D. [d]	N.D. [d]
32	0.298	5.09	0.201 [f]	0.645 [f]	N.D. [d]	N.D. [d]
33	0.315 [h]	7.45 [h]	0.427 [f,h]	1.32 [f,h]	N.D. [d]	N.D. [d]
34	0.459 [h]	2.05 [h]	0.173 [f,h]	0.820 [f,h]	N.D. [d]	N.D. [d]
35	0.336 [h]	5.68 [h]	0.148 [f,h]	0.883 [f,h]	N.D. [d]	N.D. [d]
mean [i] ±S.E.M.		4.04 ±0.29 [j]		0.159 ±0.047 [j,k]		1.43 ±0.44 [j,l]

[f] PBMC culture
[g] whole blood data not available due to Q-Prep malfunction; data from post-Ficoll-Paque PBMC
[h] data from citrate condition of anticoagulant experiments
[i] corrected for autofluorescence
[j] statistically significant CD4 expression relative to autofluorescence; $P < 0.050$
[k] statistically significant reduction in CD4 expression relative to whole blood; $P < 0.050$
[l] statistically significant increase in CD4 expression relative to overnight culture; $P < 0.050$

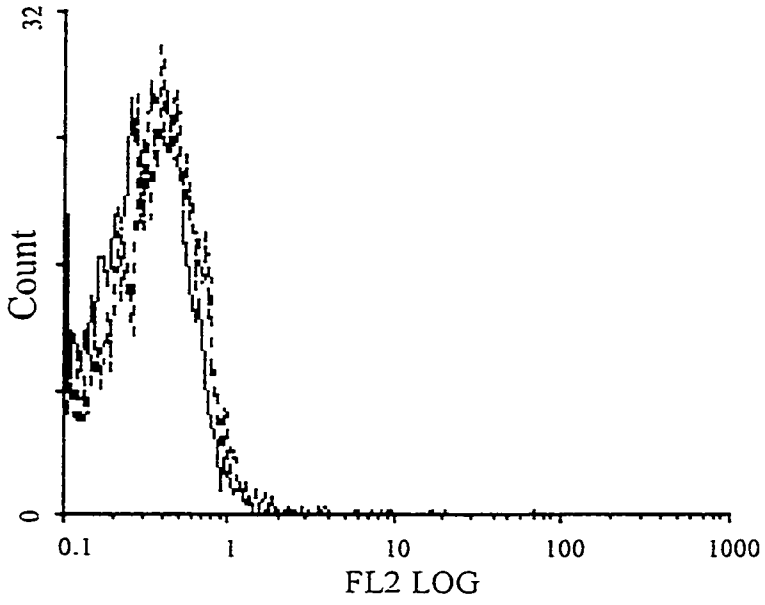
FIG.III.1. Comparison of monocyte CD4 expression before and after overnight culturing.

CD4 expression by monocytes was assessed in a) whole blood, and b) following monocyte isolation and overnight culture in RPMI/FBS medium; monocytes were electronically gated based on their forward *vs.* side scatter characteristics. The results of the flow cytometric analysis of a representative experiment in which monocyte CD4 was completely down-regulated (n=18) are shown. Autofluorescence (——); α-CD4-PE (-------).

a)


15
Count
0
0.1
1
10
100
1000
FL2 LOG

b)


32
Count
0
0.1
1
10
100
1000
FL2 LOG

to whole blood monocytes (Table III.2); HLA-DR was measured to assess monocyte activation/differentiation. HLA-DR was further up-regulated following 6 day culturing (Table III.2), although the increase did not reach statistical significance; this lack of statistical significance is most likely due to the wide range of expression, from a low of 9.01 mean channel units, to a high of 414.7 mean channel units. However, comparison of HLA-DR levels between time points within each isolation reveals that HLA-DR levels did increase with time.

ii. Growth factors/external stimuli do not modulate monocyte CD4 expression following overnight culture

Experiments were performed to determine if CD4 expression of gelatin/fibronectin-purified monocytes could be modulated by growth/differentiation factors such as GM-CSF (Perno et al. 1990) and M-CSF (Kalter et al. 1991), by IL-10, a suppressor of monocyte/macrophage cytokine production (Weissman et al. 1994), and by LPS, a potent monocyte/macrophage activator (Wolff 1973, Pabst and Johnston 1980, Aderem et al. 1986). The addition of 100 U/ml GM-CSF (n = 4), 10 U/ml M-CSF (n =3), 5 μg/ml LPS (n=3), or 12 U/ml IL-10 (n = 3) to monocyte cultures did not prevent CD4 down-regulation following overnight culture (Table III.3). HLA-DR levels were up-regulated with respect to whole blood levels following overnight culture in RPMI/FBS (Table III.2). Monocytes cultured overnight in the presence of IL-10 showed lower levels of HLA-DR expression compared to monocytes cultured in RPMI/FBS (Table III.4); no statistical analysis was performed, however, because of the bimodal distribution of HLA-DR expression observed

Table III.2. Monocyte HLA-DR expression levels before and after overnight and/or 6 days in culture in RPMI/FBS.

	BASELINE MONOCYTE HLA-DR EXPRESSION IN WHOLE BLOOD		MONOCYTE HLA-DR EXPRESSION IN OVERNIGHT MONOCYTE OR PBMC CULTURES		MONOCYTE HLA-DR EXPRESSION IN 6 DAY MONOCYTE CULTURES	
ISOLATION #	*mc# Auto* [a]	*mc# HLA-DR* [b]	*mc# Auto* [a]	*mc# HLA-DR* [b]	*mc# Auto* [a]	*mc# HLA-DR* [b]
1	0.235	18.3	0.611 [c]	86.4 [c]	N.D. [d]	N.D. [d]
2	0.618	28.4	0.289 [c]	43.6 [c]	N.D. [d]	N.D. [d]
3	0.489	30.4	0.191 [c]	51.7 [c]	N.D. [d]	N.D. [d]
4	0.232	26.0	N.D. [d]	N.D. [d]	4.58	414.7
5	0.622	4.85	0.566 [c]	14.9 [c]	2.82	37.7
6	0.290	8.17	0.389 [c]	13.7 [c]	2.85	14.9
7	0.329	15.5	N.D. [d]	N.D. [d]	5.10	169.3
8	0.255	28.0	N.D. [d]	N.D. [d]	3.59	129.4
9	0.377	11.8	N.D. [d]	N.D. [d]	3.46	55.9
10	0.976	12.8	1.49 [c]	49.7 [c]	13.4	270.7
11	0.265	12.6	0.369 [c]	17.7 [c]	1.33	38.7
12	0.354	4.66	0.340 [c]	20.0 [c]	2.03	29.9
13	0.304	4.35	0.295 [c]	8.46 [c]	1.41	32.7
14	0.358	6.18	0.637 [c]	21.4 [c]	2.08	33.7
15	0.340	4.60	0.338 [c]	8.87 [c]	1.37	12.2
16	0.359	8.38	0.514 [c]	11.3 [c]	1.48	9.01

[a] mean channel number of autofluorescence (FL1 LOG or FL4 LOG)
[b] mean channel number of α-HLA-DR-FITC or α-HLA-DR-TRI staining
[c] monocyte culture
[d] N.D.: not done
[e] PBMC culture
[f] whole blood data not available due to Q-Prep malfunction; data from post-Ficoll-Paque PBMC
[g] corrected for autofluorescence

	BASELINE MONOCYTE HLA-DR EXPRESSION IN WHOLE BLOOD		MONOCYTE HLA-DR EXPRESSION IN OVERNIGHT MONOCYTE OR PBMC CULTURES		MONOCYTE HLA-DR EXPRESSION IN 6 DAY MONOCYTE CULTURES	
ISOLATION #	*mc# Auto* [a]	*mc# HLA-DR* [b]	*mc# Auto* [a]	*mc# HLA-DR* [b]	*mc# Auto* [a]	*mc# HLA-DR* [b]
17	0.480	7.13	0.458 [c]	14.6 [c]	1.56 [c]	36.6 [c]
18	0.299	13.3	0.378 [e]	30.9 [e]	N.D. [d]	N.D. [d]
19	0.439	15.8	0.353 [e]	40.0 [e]	N.D. [d]	N.D. [d]
20	0.244	11.0	0.681 [e]	30.0 [e]	N.D. [d]	N.D. [d]
21	0.599	15.1	0.594 [e]	48.5 [e]	N.D. [d]	N.D. [d]
22	0.702	18.9	0.895 [e]	36.1 [e]	N.D. [d]	N.D. [d]
23	0.630	11.9	0.548 [e]	33.6 [e]	N.D. [d]	N.D. [d]
24	0.488	11.6	0.583 [e]	45.5 [e]	N.D. [d]	N.D. [d]
25	0.599	6.65	0.505 [e]	27.4 [e]	N.D. [d]	N.D. [d]
26	0.205 [f]	26.8 [f]	0.532 [e]	191.7 [e]	N.D. [d]	N.D. [d]
27	0.141 [f]	7.97 [f]	0.275 [e]	56.5 [e]	N.D. [d]	N.D. [d]
28	0.216	14.0	0.239 [e]	81.0 [e]	N.D. [d]	N.D. [d]
29	0.289	5.96	0.447 [e]	6.97 [e]	N.D. [d]	N.D. [d]
30	0.447	5.46	0.366 [e]	9.54 [e]	N.D. [d]	N.D. [d]
31	0.486	3.17	0.348 [e]	2.54 [e]	N.D. [d]	N.D. [d]
32	0.428	4.01	0.305 [e]	6.56 [e]	N.D. [d]	N.D. [d]
mean [g] ±S.E.M.		10.33 ± 1.13 [h]		35.56 ± 7.08 [h,i]		88.45 ± 31.2 [h,j]

[h] statistically significant HLA-DR expression relative to autofluorescence; $P < 0.050$

[i] statistically significant increase in HLA-DR expression relative to whole blood monocytes; $P < 0.050$

[j] increase in HLA-DR expression by day-6cultured monocytes relative to overnight cultured monocytes does not reach statistical significance; $P > 0.050$

Table III.3. Monocyte CD4 expression levels before and after overnight culture in RPMI/FBS, with or without various monocyte/macrophage growth factors, suppressors, and activators.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN OVERNIGHT MONOCYTE CULTURES									
			RPMI/FBS		+ GM-CSF (100 U/ml)		+ M-CSF (10 U/ml)		+ IL-10 (12 U/ml)		+ LPS (5 µg/ml)	
ISOLATION #	*mcfl Auto* [a]	*mcfl CD4* [b]	*mcfl Auto* [a]	*mcfl CD4* [b]	*mcfl Auto* [a]	*mcfl CD4* [b]	*mcfl Auto* [a]	*mcfl CD4* [b]	*mcfl Auto* [a]	*mcfl CD4* [b]	*mcfl Auto* [a]	*mcfl CD4* [b]
1	0.265	6.38	0.232	0.228	0.247	0.235	0.231	0.212	N.D. [c]	N.D. [c]	0.231	0.277
2	0.216	4.07	0.266	0.405	0.286	0.497	0.366	0.574	N.D. [c]	N.D. [c]	0.369	0.373
3	0.193	3.60	0.223	0.367	0.243	0.344	0.233	0.349	N.D. [c]	N.D. [c]	0.248	0.296
12	0.218	3.08	0.271	0.313	0.362	0.397	N.D. [c]	N.D. [c]	0.503	0.525	N.D. [c]	N.D. [c]
14	0.223	2.75	0.192	0.197	N.D. [c]	N.D. [c]	N.D. [c]	N.D. [c]	0.224	0.317	N.D. [c]	N.D. [c]
16	0.235	3.10	0.301	0.204	N.D. [c]	N.D. [c]	N.D. [c]	N.D. [c]	0.358	0.190	N.D. [c]	N.D. [c]
mean [d] ±S.E.M.		3.61 ±0.58 [e]		0.038 ±0.031 [f]		0.084 ±0.048 [g]		0.102 ±0.066 [g]		-0.018 ±0.078 [g]		0.033 ±0.014 [g]

[a] mean channel number of autofluorescence (FL2 LOG)
[b] mean channel number of α-CD4-PE signal
[c] N.D.: not done
[d] corrected for autofluorescence
[e] statistically significant CD4 expression relative to autofluorescence; $P < 0.050$
[f] statistically significant reduction in CD4 expression relative to whole blood monocytes; $P < 0.050$
[g] no statistically significant difference in CD4 expression between monocytes cultured in RPMI/FBS and cultures supplemented with various factors

Table III.4. Monocyte HLA-DR expression following overnight culture with IL-10.

	MONOCYTE HLA-DR EXPRESSION IN OVERNIGHT MONOCYTE CULTURES			
	RPMI/FBS		+ IL-10	
ISOLATION #	*mc# Auto* [a]	*mc# HLA-DR* [b]	*mc# Auto* [a]	*mc# HLA-DR* [b]
12	0.340	20.0	0.600	0.646 (25.0%) [c,d] 10.5 (75.0%) [c,e]
14	0.637	21.4	3.68	3.50
16	0.514	10.8	0.575	0.908 (20.8%) [c,d] 6.14 (79.2%) [c,e]
mean [f] ± S.E.M.		16.9 ± 3.3 [g]		0.066 ±0.148 [d] 5.10 ± 2.92 [e]

[a] mean channel number of autofluorescence (FL1 LOG)
[b] mean channel number of α-HLA-DR-FITC signal
[c] two populations were observed with respect to HLA-DR expression; low[d] expression and high[e] expression
[f] corrected for autofluorescence
[g] statistically significant HLA-DR expression relative to autofluorescence; $P < 0.050$

for 2 of the 3 cultures. Lymphocyte contamination levels ($CD3^+$ T cells and $CD19^+$ or $CD20^+$ B cells) were determined for all overnight monocyte culture conditions and ranged from 0.2% to 12.8%.

The culture of purified monocytes for 6 days in the presence of LPS (n=4), M-CSF (n=7) or GM-CSF (n=23; various concentrations ranging from 0.1 to 1 000 U/ml) did not affect CD4 expression relative to monocytes cultured in RPMI/FBS; the results of a representative experiment are shown in Table III.5. However, the culture of purified monocytes for 6 days in the presence of 12 U/ml IL-10 (n=3) resulted in increased monocyte CD4 re-expression compared to monocytes cultured in RPMI/FBS (n = 3); Table III.6 and Fig.III.2. This CD4 re-expression did not quite reach statistical significance, although we expect that significance would have been reached had more experiments been performed.

Lymphocyte contamination levels ($CD3^+$ T cells and $CD19^+$ and $CD20^+$ B cells) were determined for all day-6 monocyte culture conditions and ranged from 0.2% to 16.8%.

III.2.2. The down-regulation of monocyte CD4 is adherence-independent

The role of culture-mediated adherence was assessed by determining monocyte CD4 levels following isolation of PBMC by Ficoll-Paque. In 5 of 18 PBMC fractions assessed, down-regulation of monocyte CD4 could be observed at this time point, prior to the establishment of PBMC cultures; the results of an experiment in which such a down-regulation was observed are shown in Fig.III.3.

Further experiments were performed to examine the effect of adherent and nonadherent culture conditions on monocyte CD4 down-regulation. The effect of adherence

Table III.5. Monocyte CD4 expression levels (of a representative experiment) before and after 6 days of culture in RPMI/FBS, with or without various monocyte/macrophage growth factors and activators.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN DAY-6 MONOCYTE CULTURES							
			RPMI/FBS		+ GM-CSF (400 U/ml)		+ M-CSF (10 U/ml)		+ LPS (5 µg/ml)	
ISOLATION #	*mcll Auto* [a]	*mcll CD4* [b]	*mcll Auto* [a]	*mcll CD4* [b]	*mcll Auto* [a]	*mcll CD4* [b]	*mcll Auto* [a]	*mcll CD4* [b]	*mcll Auto* [a]	*mcll CD4* [b]
8	0.296	4.49	0.774	1.24	0.852	1.26	0.685	0.935	0.573	0.792

[a] mean channel number of autofluorescence (FL2 LOG)
[b] mean channel number of α-CD4-PE signal

Table III.6. Monocyte CD4 expression following 6 days in culture with IL-10.

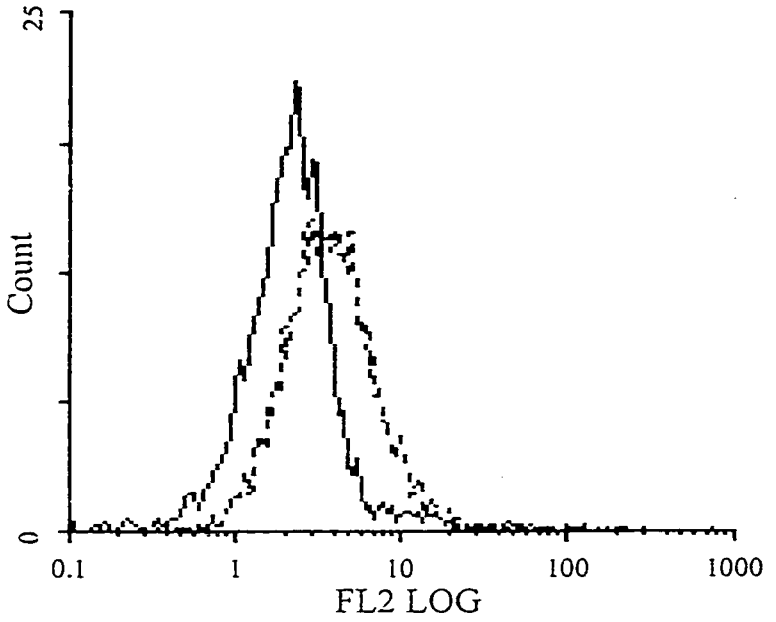
	MONOCYTE CD4 EXPRESSION IN OVERNIGHT MONOCYTE CULTURES			
	RPMI/FBS		+ IL-10	
ISOLATION #	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]
12	2.16	3.74	2.10	5.85
14	2.15	5.56	3.50	10.7
16	1.44	1.33	2.52	4.17
mean [c] ± S.E.M.		1.63 ± 1.02		4.20 ± 1.62 [d]

[a] mean channel number of autofluorescence (FL2 LOG)
[b] mean channel number of α-CD4-PE signal
[c] corrected for autofluorescence
[d] increase in CD4 expression does not quite reach statistical significance relative to RPMI/FBS culture; P value between 0.060 and 0.050

Fig.III.2. Effect of IL-10 on monocyte CD4 re-expression following 6 days in culture.

The results of the flow cytometric analysis of a representative experiment in which monocytes were cultured for 6 days in a) the absence of IL-10, or b) the presence of IL-10; monocytes were electronically gated based on their forward *vs.* side scatter characteristics. Autofluorescence (——); α-CD4-PE (-------). (n=3)

a)


25
Count
0
0.1
1
10
100
1000
FL2 LOG

b)

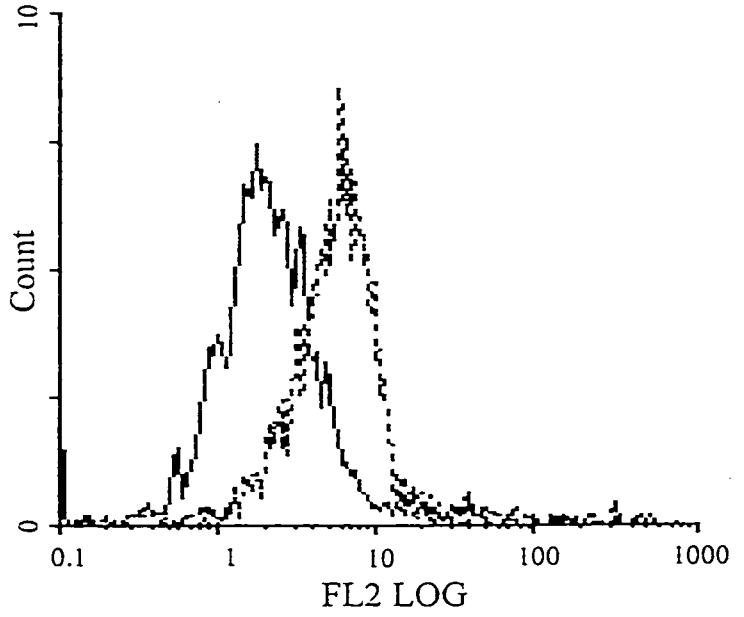
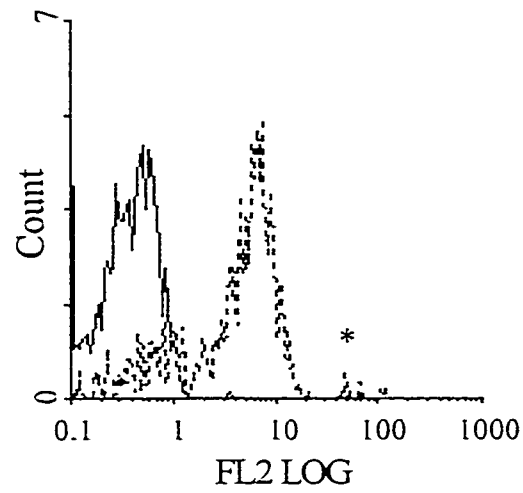

10
Count
0
0.1
1
10
100
1000
FL2 LOG

Fig.III.3. Assessment of CD4 expression by monocytes following isolation of PBMC by Ficoll-Paque.

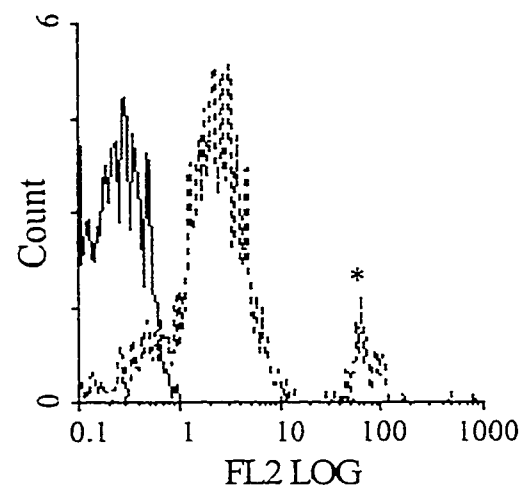
CD4 expression by monocytes was assessed in whole blood and following isolation of PBMC by Ficoll-Paque; monocytes were electronically gated based on their forward *vs.* side scatter characteristics. The results of the flow cytometric analysis of a representative experiment in which monocyte CD4 expression was down-regulated following the Ficoll-Paque procedure are shown. a) Autofluorescence (——) and α-CD4-PE (-------) in whole blood monocytes. b) Autofluorescence (——) and α-CD4-PE (-------) in monocytes following isolation of PBMC by Ficoll-Paque. c) Comparison of CD4 expression by whole blood monocytes (——) to CD4 expression by monocytes in PBMC following isolation by Ficoll-Paque (-------).

* Contaminating $CD4^+$ T cells in monocyte gate as confirmed by the $CD3^-$ status of this population.

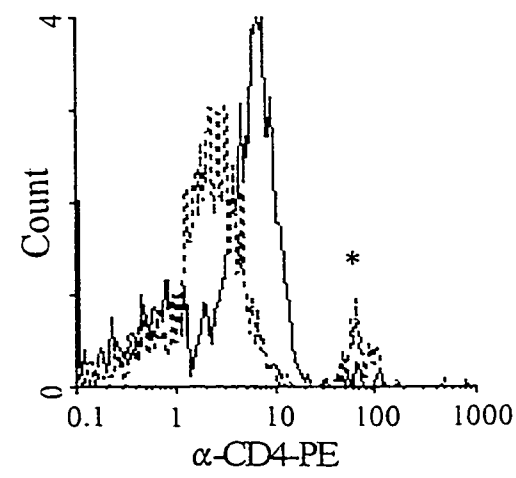
a)


7
Count
0
*
0.1
1
10
100
1000
FL2 LOG

b)


6
Count
0
*
0.1
1
10
100
1000
FL2 LOG

c)


4
Count
0
*
0.1
1
10
100
1000
α-CD4-PE

mediated by gelatin/fibronectin-coated polystyrene surfaces (n=6), and by uncoated polystyrene surfaces (n=6) was examined; PBMC were used rather than purified monocytes since we had established that the presence or absence of lymphocytes had no effect on monocyte CD4 expression (Table III.1). The effect of nonadherence was also examined by culturing PBMC in Teflon vials (Van Der Meer et al. 1981) (n=3). Following overnight culture, monocyte CD4 expression was significantly and comparably down-regulated for all culture conditions (Table III.7).

III.2.3. Whole blood may contain some CD4-stabilizing factor(s)

The effect of Ficoll-Paque isolation of PBMC was examined for its possible involvement in monocyte CD4 down-regulation. Monocyte CD4 expression before and after overnight culturing was assessed for PBMC isolated by Ficoll-Paque, and for whole blood cultures containing cells which had not undergone Ficoll-Paque isolation.

Following overnight culturing of PBMC, the level of monocyte CD4 expression was significantly down-regulated in all 4 experiments (Table III.8), consistent with previous observations. In 3 of 4 experiments, monocytes cultured overnight in a whole blood environment also showed down-regulation of CD4 levels (Table III.8, isolations 30–32). In isolation 30, the down-regulation of monocyte CD4 was obvious only by overlaying the α-CD4-PE histogram for baseline monocyte CD4 expression in whole blood, with the α-CD4-PE histogram of monocyte CD4 expression following overnight culture in whole blood. The level of monocyte CD4 expression in these three whole blood cultures was slightly higher than monocyte CD4 levels observed for overnight PBMC cultures. Monocytes in one of the

Table III.7. Monocyte CD4 expression levels before and after overnight culture in adherent and nonadherent environments.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN OVERNIGHT PBMC CULTURES					
			Gelatin/Fibronectin-Coated Polystyrene Flasks		Uncoated Polystyrene Flasks		Teflon Vials	
ISOLATION #	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]
18	0.276	5.33	0.324	0.200	0.440	0.253	N.D. [c]	N.D. [c]
20	0.224	4.79	0.705	0.692	0.566	0.714	N.D. [c]	N.D. [c]
21	0.291	5.54	0.269	0.225	0.310	0.289	N.D. [c]	N.D. [c]
26	0.190 [d]	4.02 [d]	0.492	1.02	0.352	1.32	0.368	1.21
27	0.125 [d]	3.87 [d]	0.269	0.244	0.220	0.220	0.220	0.279
28	0.302	6.88	0.256	0.426	0.198	0.389	0.237	0.420
mean [e] ± S.E.M.		4.84 ± 0.43 [f]		0.082 ± 0.098 [g]		0.183 ± 0.166 [g]		0.361 ± 0.243 [h]

[a] mean channel number of autofluoresence (FL2 LOG)
[b] mean channel number of α-CD4-PE signal
[c] N.D.: not done
[d] whole blood data not available due to Q-Prep malfunction; data from post-Ficoll-Paque PBMC
[e] corrected for autofluorescence
[f] statistically significant CD4 expression relative to autofluorescence; $P < 0.050$
[g] statistically significant decrease in CD4 expression relative to whole blood; $P < 0.050$
[h] although the reduction in CD4 expression in Teflon cultured monocytes compared to whole blood monocytes did not quite reach statistical significance (P value between 0.060 and 0.050), no statistically significant difference in CD4 expression was observed between the 3 types of overnight cultures ($P > 0.050$)

Table III.8. Monocyte CD4 expression levels (as a function of Ficoll-Paque isolation[a]) before and after overnight culture in a whole blood or RPMI/FBS environment.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN OVERNIGHT CULTURES			
			Whole Blood Cultures		PBMC Cultures in RPMI/FBS	
ISOLATION #	*mc# Auto* [b]	*mc# CD4* [c]	*mc# Auto* [b]	*mc# CD4* [c]	*mc# Auto* [b]	*mc# CD4* [c]
29	0.427	4.63	0.253	4.93	0.256	0.269
30	0.315	4.51	0.266	4.02	0.234	0.362
31	0.284	4.49	0.233	1.82	0.249	0.264
32	0.298	5.09	0.275	1.98	0.201	0.645
mean [d] ± S.E.M.		4.35 ± 0.15 [e]		2.93 ± 0.77 [e]		0.150 ± 0.102 [f,g]

[a] For each experiment, monocytes were cultured with and without prior isolation of PBMC by Ficoll-Paque.
[b] mean channel number of autofluorescence (FL2 LOG)
[c] mean channel number of α-CD4-PE signal
[d] corrected for autofluorescence
[e] statistically significant CD4 expression relative to autofluorescence; $P < 0.050$
[f] statistically significant reduction in CD4 expression relative to whole blood monocytes; $P < 0.050$
[g] statistically significant reduction in CD4 expression relative to monocytes in whole blood cultures; $P < 0.050$

whole blood cultures continued to express CD4 levels comparable to those observed in peripheral blood (Table III.8, isolation 29). Overall, CD4 expression by monocytes cultured overnight in a whole blood environment was significantly higher compared to monocytes cultured overnight in RPMI/FBS.

III.2.4. The down-regulation of monocyte CD4 is not dependent on the nature of the anticoagulant used

The effect of various anticoagulants on monocyte CD4 expression levels was assessed (n=3). CD4 expression of monocytes was assessed for PBMC cultures established from peripheral blood collected in Vacutainer® tubes containing sodium heparin, acid citrate dextrose (ACD) or EDTA (K_3). Following overnight culturing, monocyte CD4 levels were equally down-regulated, regardless of the anticoagulant used (Table III.9).

III.2.5. Monocyte CD4 is down-regulated by internalization

The fate of monocyte CD4 was determined in tagging experiments (n=3) in which PBMC cultures were stained with α-CD4-PE mAb prior to overnight culture; untagged PBMC cultures were established as controls.

Untagged and α-CD4-PE-tagged PBMC cultures were harvested following overnight culture. The untagged PBMC cultures were subsequently stained with α-CD4-PE mAb, whereas the tagged PBMC cultures were not. Flow cytometric analysis of the α-CD4-PE tagged cultures revealed a PE signal for the monocyte (Table III.10A and Fig.III.4e) and

Table III.9. Monocyte CD4 expression levels (as a function of anticoagulant [a]), before and after overnight culture in RPMI/FBS.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD						MONOCYTE CD4 EXPRESSION IN OVERNIGHT PBMC CULTURES					
	Citrate		EDTA		Heparin		Citrate		EDTA		Heparin	
ISOLATION #	*mcll Auto* [b]	*mcll CD4* [c]	*mcll Auto* [b]	*mcll CD4* [c]	*mcll Auto* [b]	*mcll CD4* [c]	*mcll Auto* [b]	*mcll CD4* [c]	*mcll Auto* [b]	*mcll CD4* [c]	*mcll Auto* [b]	*mcll CD4* [c]
33	0.315	7.45	0.445	3.70	0.456	6.51	0.427	1.32	0.353	0.956	0.334	0.943
34	0.459	2.05	0.408	4.24	0.371	2.61	0.173	0.820	0.140	0.922	0.174	0.954
35	0.336	5.68	0.353	5.79	0.359	3.95	0.148	0.883	0.143	0.721	0.147	0.726
mean [d] ± S.E.M.		4.69 ± 1.63 [e]		4.17 ± 0.65 [e]		3.96 ± 1.12 [e]		0.758 ± 0.072 [f]		0.654 ± 0.064 [f]		0.656 ± 0.063 [f]

[a] For each isolation, whole blood was collected in 3 different anticoagulants and assessed for CD4 expression. PBMC were subsequently isolated from each whole blood/anticoagulant combination, and the overnight PBMC cultures were assessed for monocyte CD4 expression.

[b] mean channel number of autofluorescence (FL2 LOG)

[c] mean channel number of α-CD4-PE signal

[d] corrected for autofluorescence

[e] no statistically significant difference in CD4 expression between any of the whole blood conditions; $P > 0.050$

[f] no statistically significant difference in CD4 expression between any of the overnight cultures; $P > 0.050$

Table III.10A. Monocyte CD4 tagging experiments: α-CD4-PE signal.

	BASELINE MONOCYTE CD4 EXPRESSION IN WHOLE BLOOD		MONOCYTE CD4 EXPRESSION IN OVERNIGHT PBMC CULTURES		
			Untagged Cultures		CD4-Tagged Cultures
ISOLATION #	*mc# Auto* [a]	*mc# CD4* [b]	*mc# Auto* [a]	*mc# CD4* [b]	*mc# CD4* [c]
19	0.640	8.02	0.551	0.846	1.68
24	0.560	3.74	0.365	0.485	2.47
25	0.269	3.09	0.253	0.688	4.52
mean [d] ± S.E.M.		4.46 ± 1.46		0.283 ± 0.091	2.89 ± 0.85 [e]

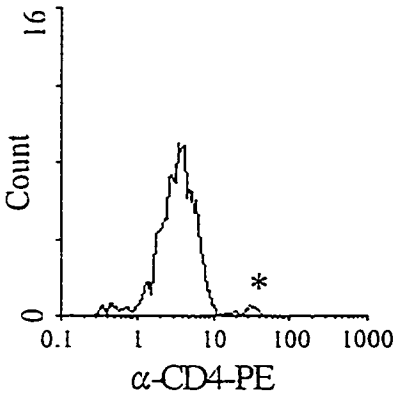
[a] mean channel number of autofluorescence (FL2 LOG)
[b] mean channel number of α-CD4-PE signal
[c] mean channel number of α-CD4-PE signal (mAb added prior to overnight culturing)
[d] corrected for autofluorescence (except for CD4-tagged culture)
[e] difference between α-CD4-PE signal for untagged and tagged cultures does not reach statistical difference; $P > 0.050$

Fig.III.4. Tagging of monocyte CD4.

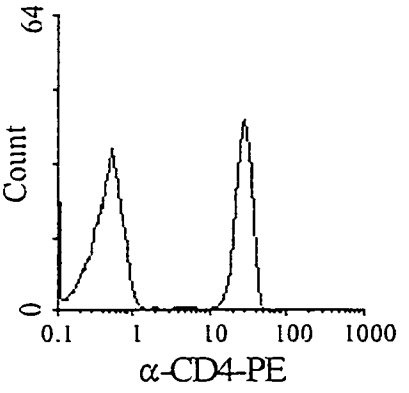
The level of CD4 expression of untagged and CD4-tagged PBMC following overnight culture was assessed; monocytes and lymphocytes were electronically gated based on their respective forward *vs.* side scatter characteristics. The results of a representative experiment are shown: a) baseline CD4 expression by monocytes in whole blood; b) baseline CD4 expression by $CD4^+$ T cells in whole blood; c) CD4 expression of untagged monocytes following overnight culture, with subsequent staining for CD4; d) CD4 expression of untagged lymphocytes following overnight culture with subsequent staining for CD4; e) CD4-tagged monocytes following overnight culture (no additional α-CD4-PE mAb was added to the cells after overnight culturing); f) CD4-tagged lymphocytes following overnight culture (no additional α-CD4-PE mAb was added to the cells after overnight culturing). (n=3)

* Contaminating $CD4^+$ T cells in monocyte gate as confirmed by the $CD3^-$ status of this population.

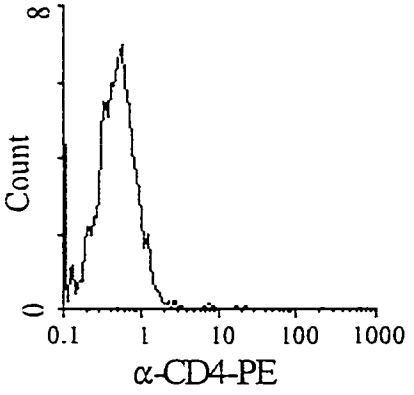
a) whole blood monocytes



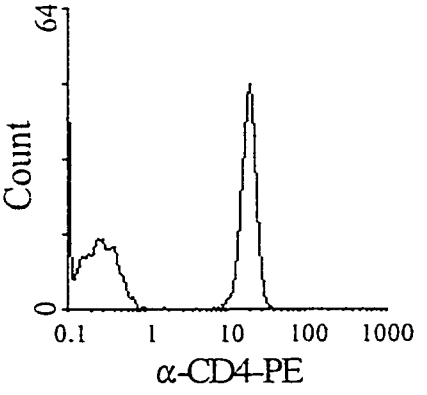
b) whole blood lymphocytes



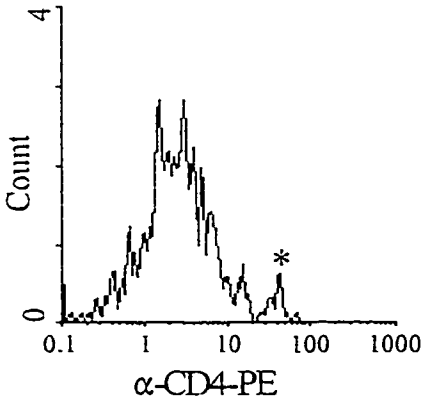
c) untagged monocytes



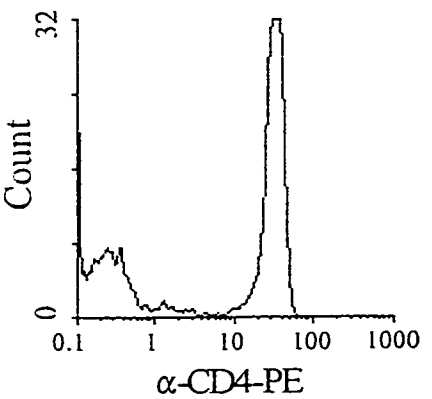
d) untagged lymphocytes



e) CD4-tagged monocytes



f) CD4-tagged lymphocytes



lymphocyte populations (Fig.III.4f). The untagged PBMC cultures, which were stained with α-CD4-PE mAb upon harvesting, showed staining of $CD4^-$ lymphocytes (Fig.III.4d), consistent with the levels observed for whole blood lymphocytes (Fig.III.4b), but negligible staining of the untagged monocytes (Table III.10A and Fig.III.4c).

Following overnight culture, the location of the CD4/α-CD4-PE mAb complex of the monocyte and lymphocyte subpopulations was determined using a goat α-mouse Ig-FITC antibody. The untagged cultures were used to control for nonspecific binding by the goat-α mouse Ig-FITC antibody. Negligible binding by the goat α-mouse Ig-FITC antibody was observed for the untagged and tagged monocytes (Table III.10B and Fig.III.5a and c, respectively), as well as for the untagged lymphocytes (Fig.III.5b), as determined by comparison to the FITC autofluorescence signal for each of these conditions. However, the goat α-mouse Ig-FITC antibody did bind the tagged lymphocytes (Fig.III.5d).

The conclusion that CD4 on tagged monocytes had been internalized was based on the observations that a PE signal could be observed for these cells, suggesting the continued presence of the α-CD4-PE mAb, while the goat α-mouse-FITC Ab failed to bind the tagged monocytes, but could bind the tagged lymphocytes. To confirm that the PE signal observed for the tagged monocytes was in fact due to the continued presence of the α-CD4-PE mAb, and not due to a general increase in autofluorescence in response to overnight culturing in the presence of antibody, experiments were performed (n=2) in which PBMC were cultured overnight with either α-CD4-PE mAb, or with unconjugated α-CD4 mAb; a control consisting of untagged PBMC was also included. Following overnight culture, the monocytes from each PBMC culture were compared with respect to their FL2 LOG signals.

Table III.10B. Monocyte CD4 tagging experiments: use of goat α-mouse Ig-FITC Ab to localize CD4/α-CD4-PE mAb complex of tagged monocytes.

	MONOCYTE CD4 EXPRESSION IN OVERNIGHT PBMC CULTURES			
	Untagged Cultures		CD4-Tagged Cultures	
ISOLATION #	*mc# Auto* [a]	*mc# GaM-FITC* [b]	*mc# Auto* [a]	*mc# GaM-FITC* [b]
19	0.357	0.367	0.372	0.367
24	0.510	0.664	0.654	0.898
25	0.505	0.561	0.763	0.717
mean [c] ± S.E.M.		0.073 ± 0.042 [d]		0.064 ± 0.091 [d, e]

[a] mean channel number of autofluorescence (FL1 LOG)
[b] mean channel number of Goat α-Mouse Ig-FITC signal
[c] corrected for autofluorescence
[d] no statistically significant binding of Goat α-Mouse Ab to monocytes; $P > 0.050$
[e] no statistically significant difference in level of Goat α-Mouse Ab between untagged and tagged cultures; $P > 0.050$

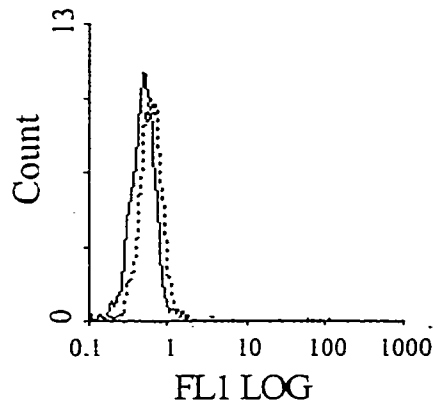
Fig.III.5. Localization of the CD4/α-CD4-PE mAb complex with goat α-mouse Ig-FITC Ab.

The experiment was performed as outlined in Fig.III.4. Goat α-mouse Ig-FITC Ab was used to stain cultures harvested following overnight culture to assess the presence of the CD4/α-CD4-PE mAb complex on the cell surface; monocytes and lymphocytes were electronically gated based on their respective forward *vs.* side scatter characteristics. The goat α-mouse Ig-FITC signal was assessed relative to autofluorescence for each of the following cell populations: a) untagged monocytes; b) untagged lymphocytes; c) CD4-tagged monocytes; d) CD4-tagged lymphocytes. Autofluorescence (——); goat α-mouse Ig-FITC (-------).

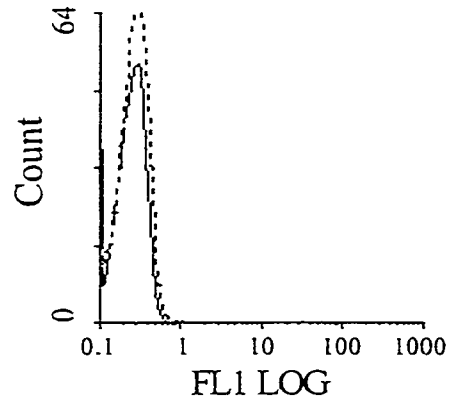
The degree of binding of the goat α-mouse Ig-FITC Ab was also compared for e) the untagged and CD4-tagged monocytes, and f) the untagged and CD4-tagged lymphocytes; untagged (——); tagged (-------). (n=3)

* This goat α-mouse Ig-FITC signal is most likely due to the presence of contaminating $CD4^+$ T cells in the monocyte gate.

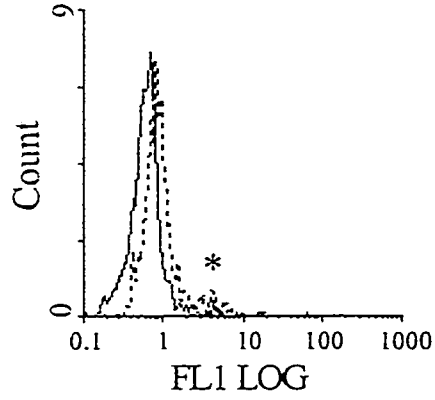
a) untagged monocytes



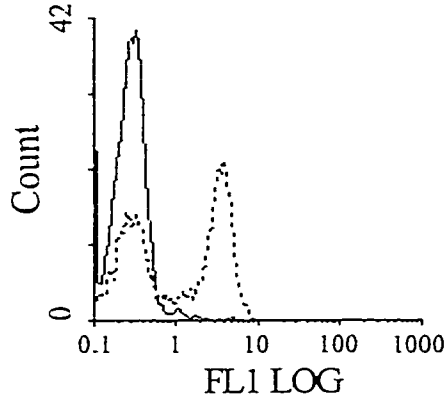
b) untagged lymphocytes



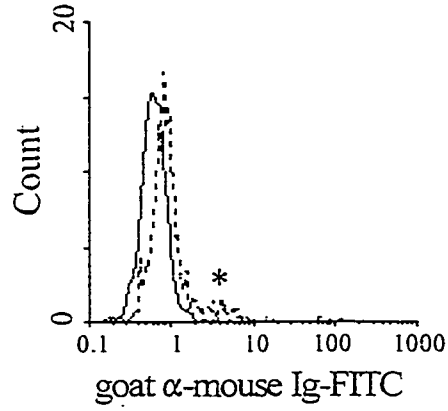
c) CD4-tagged monocytes



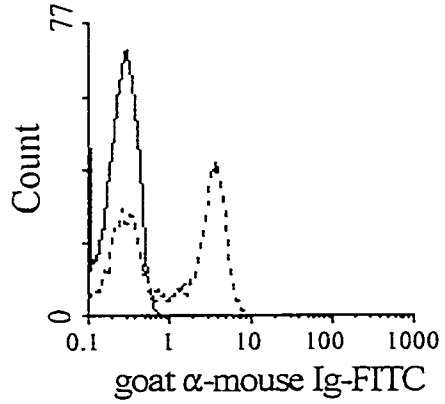
d) CD4-tagged lymphocytes



e) untagged *vs.* tagged monocytes



f) untagged *vs.* tagged lymphocytes



The monocytes cultured with unconjugated α-CD4 mAb did not show any increase in FL2 LOG signal compared to the autofluorescent control of the untagged monocytes, whereas the monocytes tagged with α-CD4-PE mAb did show an increase in FL2 LOG signal (Fig. III.6). These observations establish that culturing monocytes with α-CD4 mAb does not increase autofluorescence in FL2 LOG, thus confirming that the increased FL2 LOG signal observed for the α-CD4-PE mAb was in fact due to the presence of the mAb following the internalization of the CD4/α-CD4-PE mAb complex.

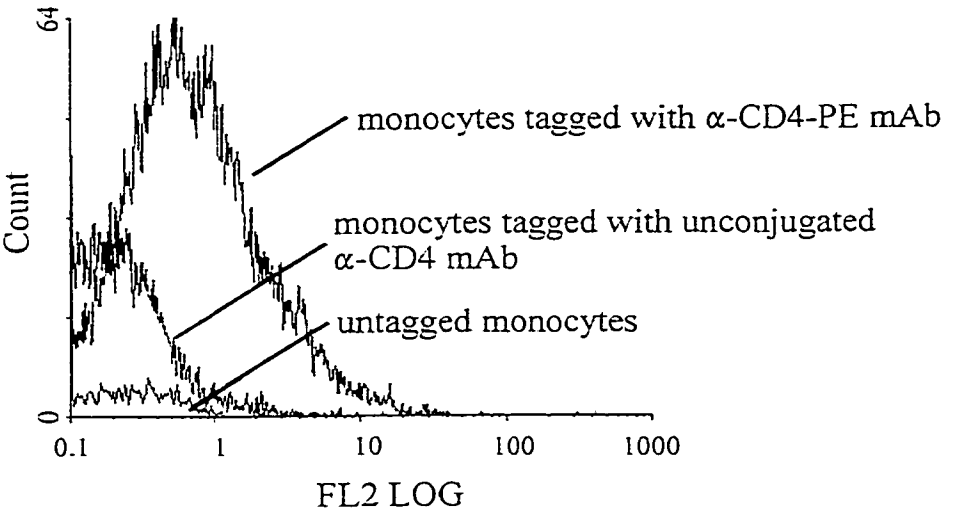
III.3. Summary of results

The overnight culture of monocytes resulted in the down-regulation of CD4 as the cells differentiated into macrophages. The addition of LPS, GM-CSF, M-CSF or IL-10 to monocyte cultures failed to inhibit the decrease in monocyte CD4 expression following overnight culture. The down-regulation was an adherence-independent phenomenon since it was not prevented by culture in Teflon vials. The type of anticoagulant into which the peripheral blood was collected did not appear to be a factor. The presence or absence of lymphocytes within the cultures was also inconsequential. The continued culture of monocytes in a whole blood environment resulted in decreased CD4 down-regulation. Finally, we were able to determine by means of tagging monocyte CD4 with an α-CD4-PE mAb that the down-regulation observed was the result of the internalization of the molecule.

In order to characterize the function of monocyte CD4, we next examined whether monocyte CD4 is capable of transducing signals, and if so, by what pathway(s).

Fig.III.6. Assessment of the effect on FL2 LOG signal of overnight culture of monocytes tagged with unconjugated α-CD4 mAb.

Untagged PBMC, or PBMC tagged with either unconjugated α-CD4 mAb or with α-CD4-PE mAb were cultured overnight. The following day, the cells were harvested and the FL2 LOG signal for monocytes cultured in each condition was assessed; monocytes were electronically gated based on their forward *vs.* side scatter characteristics.


64
Count
0
0.1
1
10
100
1000
FL2 LOG
monocytes tagged with α-CD4-PE mAb
monocytes tagged with unconjugated α-CD4 mAb
untagged monocytes

IV. MONOCYTE CD4 SIGNAL TRANSDUCTION

IV.1. Introduction

In $CD4^+$ T cells, CD4 plays a crucial role in the signal transduction response to antigen presented to them in the context of MHC class II molecules (Qian and Weiss 1997, Zhao and Koretzky 1997, van Leewan and Samelson 1999). This signal is mediated by virtue of the association of CD4 with the *Src*-related protein tyrosine kinase $p56^{lck}$ which functions to tyrosine-phosphorylate proteins associated with the T cell antigen receptor complex (Rudd et al. 1988, Veillette et al. 1988). In conjunction with signals mediated by CD3, the TCR, and their respective associated signaling proteins, the CD4-$p56^{lck}$ complex functions to induce the T cell proliferation, differentiation, and cytokine synthesis and release required to mount an effective immune response to the triggering antigen (van Leeuwen and Samelson 1999). Furthermore, in HIV-infected T cells, gp120-induced apoptosis is mediated by the CD4-$p56^{lck}$ complex; the responsible pathway has yet to be determined (Corbeil et al 1996). Such a process may contribute to the depletion of $CD4^+$ T cells, a hallmark of AIDS pathogenesis (Corbeil et al 1996).

Unlike T cell CD4, monocytic CD4 has no associated kinase activity (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al. 1993). Some investigators have, however, observed CD4-mediated signaling in monocyte/macrophages. For example, a complex inositol polyphosphate response and a Ca^{2+} flux were induced in monocytes in which CD4 and Fcγ receptors were co-cross-linked (Guse et al. 1992). IL-16 stimulation of various $CD4^+$ monocytic cell lines induces a translocation of PKC from the cytosol to the membrane (Parada et al. 1996), as well as activation of the SAPK and p38 signaling

pathways (Krautwald 1998). Finally, HIV gp120 has been shown to induce a CD4-dependent down- regulation of the CCR5 chemokine receptor in monocytes (Wang et al. 1998).

In an attempt to further define the function of monocyte CD4, we investigated the role of monocyte CD4 in signal transduction. Specifically, we wished to determine the internal (transmembrane and cytoplasmic) sequence of monocyte CD4 for comparison to that of T cell CD4, as well as to use biochemical/immunological and functional assays to begin characterizing the pathway(s) by which monocyte CD4 transduces signal.

IV.2. Results

IV.2.1. Sequencing the transmembrane and cytoplasmic domains of monocyte CD4

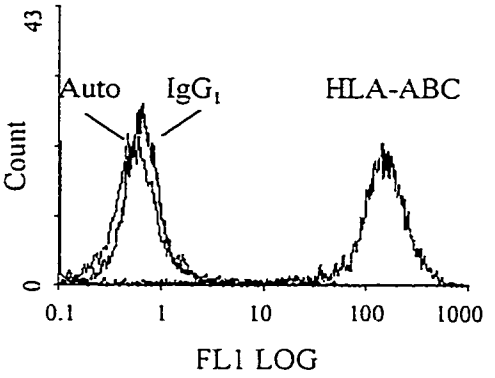
i. Flow cytometric analysis of HeLa, U937 and Hut78 cell lines for CD4 expression

A small degree of nonspecific binding was observed for U937, Hut 78 and HeLa cells stained with the IgG_1-FITC isotype control (Fig.IV.1 a, c and e, respectively). All cells stained brightly with the α-HLA-ABC-FITC mAb as determined by comparison to autofluorescence and staining by the isotype control (Fig.IV.1 a, c and e, respectively). The U937 monocytic cell line and Hut 78 T cell line both showed a high level of staining with the α-CD4-PE mAb (Fig.IV.1b and d, respectively) as determined by comparison to autofluorescence (Fig.IV.1 b and d, respectively) and staining by the isotype control (Fig. IV.1 a and c, respectively). In contrast, the degree of staining of HeLa cells with the α-CD4-PE mAb (Fig.IV.1f) was comparable to that observed with the isotype control (Fig. IV.1e).

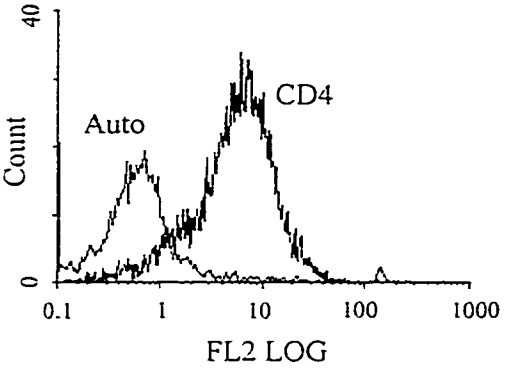
Fig. IV.1. Flow cytometric analysis of various cell lines to assess their expression of CD4.

The monocytic cell line U937 (a, b), the T cell line Hut 78 (c, d), and the nonhemopoietic HeLa cell line (e, f) were stained with an IgG_1-FITC isotype murine Ab control (a, c and e), murine α-HLA-ABC-FITC mAb (a, c and e), and murine α-CD4-PE mAb (b, d and f).

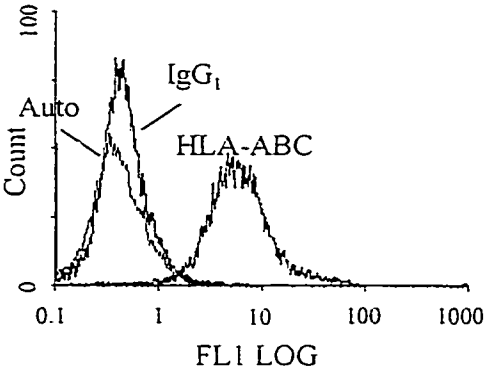
a) U937



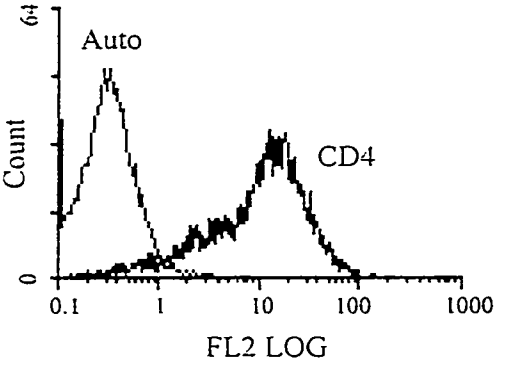
b) U937



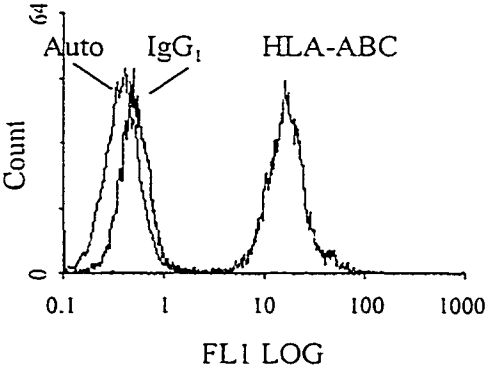
c) Hut 78



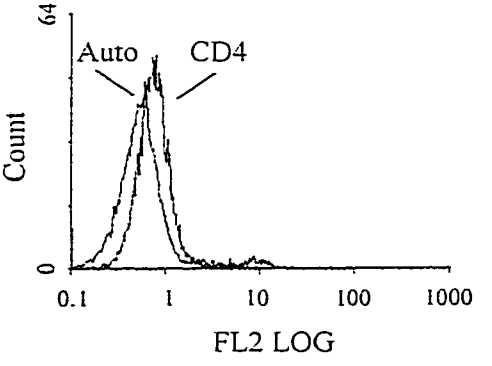
d) Hut 78



e) HeLa



f) HeLa



ii. Flow cytometric analysis of CD2⁻ PBMC for CD4 expression by monocytes, and for lack of T cells

Failure of our adherence-based technique for the isolation of monocytes (discussed further in Appendix I) necessitated another approach for the isolation of monocytes relatively free of contaminating $CD4^+$ T cells. To this end, we used a negative selection technique in which T cells were removed from a PBMC fraction using Dynal magnetic beads coated with mAb specific for CD2, a pan T cell marker (Lee et al. 1999). This approach resulted in the successful removal of most T cells; flow cytometric analysis of the PBMC fraction following treatment with the Dynal beads revealed that only 1.0 % of the remaining cells expressed CD3 (Table IV.1 and Fig.IV.2a), another pan T cell marker (Lee et al. 1999). Monocytes accounted for 43.8 to 57.8% of the isolated cells (Table IV.1 and Fig. IV.2b), depending on the combination of markers (CD14/CD4 *vs.* HLA-DR/CD14, respectively) used to define this population; 91.3% of the $CD14^+$ monocytes expressed CD4 . The presence of other cell types (such as NK cells) besides the monocytes of interest was inconsequential as these other cell types do not express CD4, and therefore, would not interfere in our sequencing work.

iii. CD4 and β-actin RT-PCR

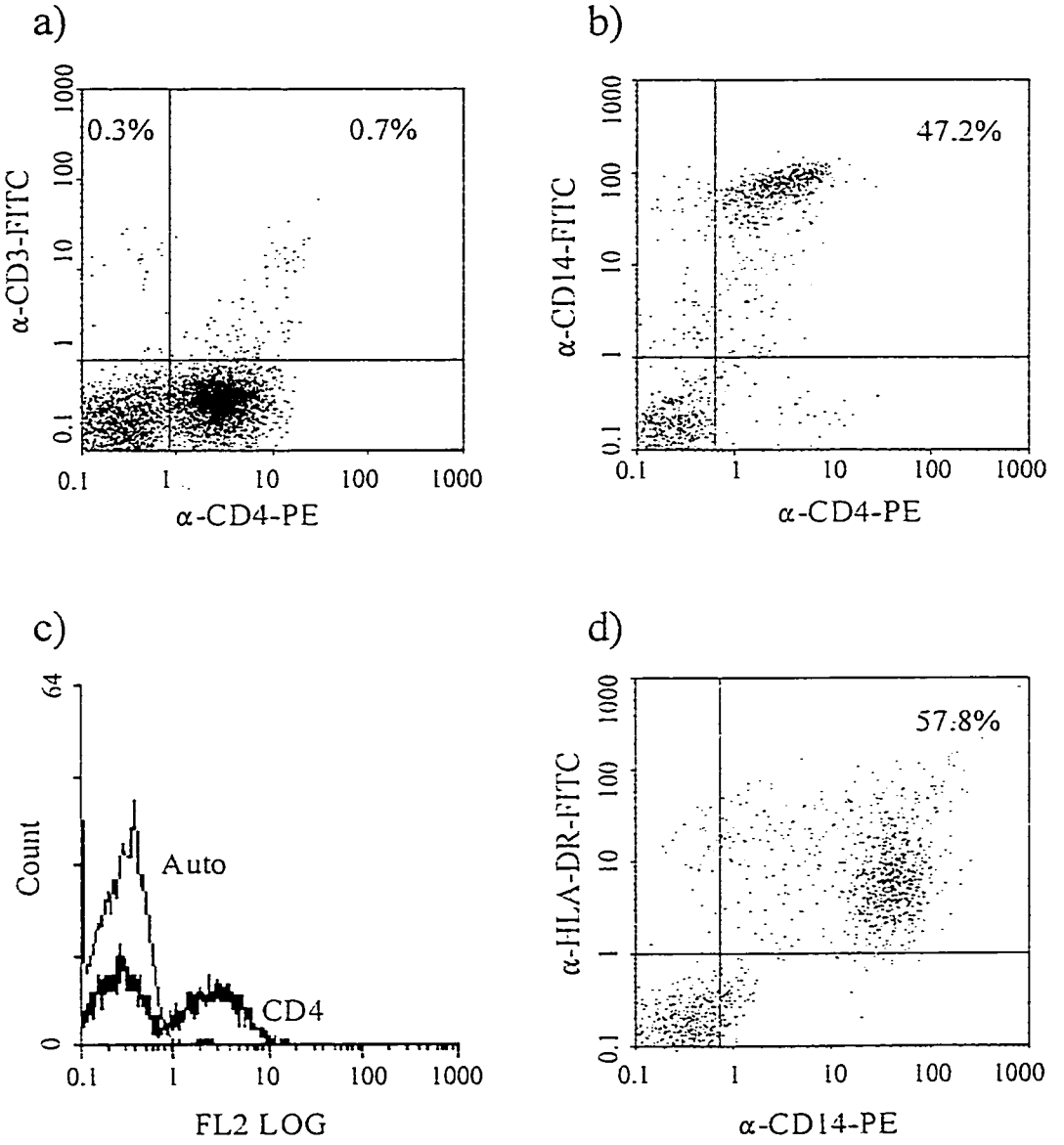
RNA from HeLa, U937, Hut78 and $CD2^-$ PBMC was subjected to RT-PCR using primers specific for β-actin and using primers flanking the transmembrane and cytoplasmic tail sequence of CD4. U937 cells were included as a monocytic control free of contaminating T cells, whereas Hut78 cells, a T cell line, were included as another control since the published sequence for human CD4 was obtained from human T cells (Maddon et al. 1985).

Table IV.1. Results of the flow cytometric analysis of PBMC following negative selection for CD2 expression.

Cell population	mAb combination	%
Monocytes	α-CD14-FITC/α-CD4-PE α-HLA-DR-FITC/α-CD14-PE	43.8 57.8
Total T cells	α-CD3-FITC	1.0
$CD4^+$ T cells	α-CD3-FITC/α-CD4-PE	0.7
B cells	α-HLA-DR-FITC/α-CD20–PE	3.3
NK cells	α-CD16-FITC/α-CD56-PE	10.6

Fig.IV.2. Flow cytometric analysis of $CD2^-$ PBMC.

Flow cytometric analysis of $CD2^-$ PBMC was performed using various murine mAb combinations: a) α-CD3-FITC/α-CD4-PE staining; b) α-CD14-FITC/α-CD4-PE staining; c) Comparison of autofluorescence (FL2 LOG) and α-CD4-PE staining; d) α-HLA-DR-FITC/α-CD14-PE staining.


a)
0.3%
0.7%
α-CD3-FITC
α-CD4-PE
0.1
1
10
100
1000
b)
47.2%
α-CD14-FITC
α-CD4-PE
c)
64
0
Count
Auto
CD4
FL2 LOG
d)
57.8%
α-HLA-DR-FITC
α-CD14-PE

Whereas β-actin could be amplified for all 4 cell types (Fig.IV.3b), the 438 bp CD4 fragment could only be amplified for the U937, Hut 78 and $CD2^-$ PBMC fraction (Fig.IV.3a), as expected.

iv. Sequencing CD4

The purified PCR product was subjected to sequencing. The sequence obtained for the Hut 78 T cell line, U937 monocytic cell line and monocytes present in the $CD2^-$ PBMC fraction were identical and differed with respect to the published T cell sequence (Maddon et al. 1985) at a single nucleotide, resulting in a silent mutation: the published nucleotide sequence for amino acid 424 (cysteine) is TGC, whereas our sequence was TGT.

IV.2.2. Detection of CD4 in Western blots

T4-4 rabbit serum against sCD4 was tested to ensure that it could be used successfully in Western blots to detect CD4 (Dr. Ray Sweet, personal communication). sCD4, as well as CD4 expression in the U937 and Thp-1 monocytic cell lines were detected by Western blot analysis, whereas no CD4 was detected in the nonhemopoietic HeLa cell line, as expected (Fig. IV.4; n=2).

IV.2.3. Confirmation that monocytic cells do not express $p56^{lck}$

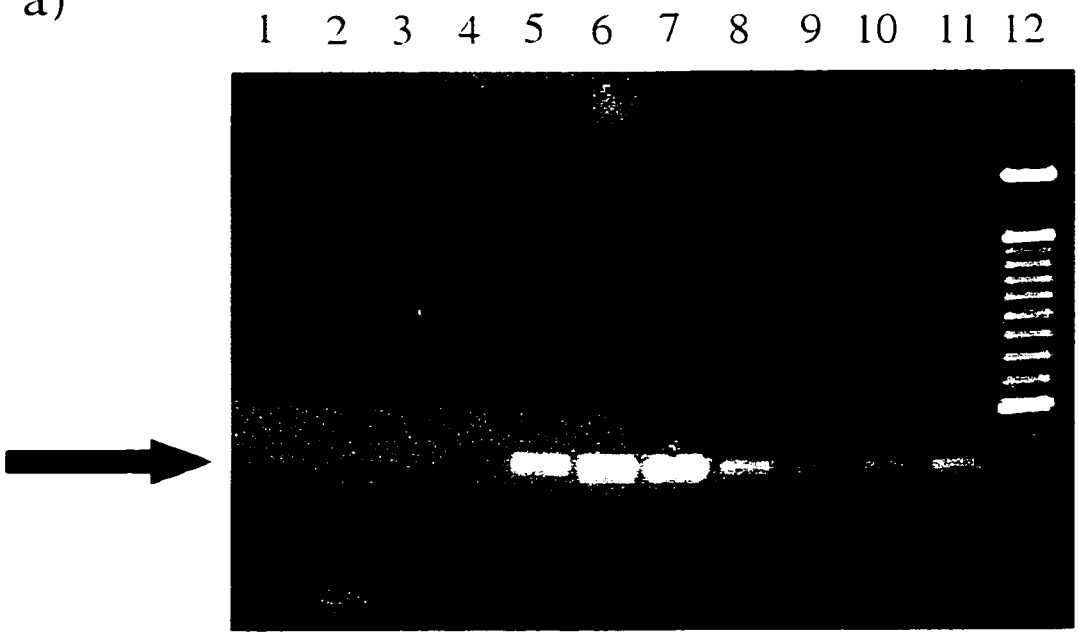
To confirm that human monocytic cells do not express $p56^{lck}$, cell lysates from various cell lines were subjected to Western blot analysis using a murine α-$p56^{lck}$ mAb (n=2). $p56^{lck}$ was successfully detected for Jurkats and for A.201 cells, a $CD4^-$ Jurkat derivative

Fig. IV.3. RT-PCR of CD4 and β-actin from HeLa, U937, Hut78 and $CD2^-$ PBMC.

a) Total cellular RNA was reverse transcribed using random hexamers and oligo $d(T)_{16}$. An aliquot was PCR amplified using CD4-specific primers. Products were resolved by electrophoresis through a 1.8% agarose gel. Lane 1: H_20 template negative control for PCR reaction (i.e. no cDNA); Lane 2: HeLa cDNA; Lane 3: H_20 template negative control for the reverse transcription reaction (i.e. no RNA); Lane 4: H_20 enzyme control for PCR reaction (i.e. no Taq polymerase); Lanes 5 and 6: U937 cDNA; Lanes 7 and 8: Hut 78 cDNA; Lanes 9 and 10: $CD2^-$ PBMC cDNA; Lane 11: CD4 positive control cDNA; Lane 12: 100 bp ladder. The position of CD4 cDNA is indicated by an arrow.

b) Total cellular RNA was reverse transcribed using random hexamers and oligo $d(T)_{16}$. An aliquot was PCR amplified using β-actin-specific primers. Products were resolved by electrophoresis through a 1.8% agarose gel. Lane1: U937 cDNA; Lane 2: Hut 78 cDNA; Lane 3: HeLa cDNA; lane 4: $CD2^-$ PBMC cDNA; Lane 6: β-actin positive control cDNA; Lane 8: 100 bp ladder. The position of β-actin cDNA is indicated by an arrow.

a)



b)

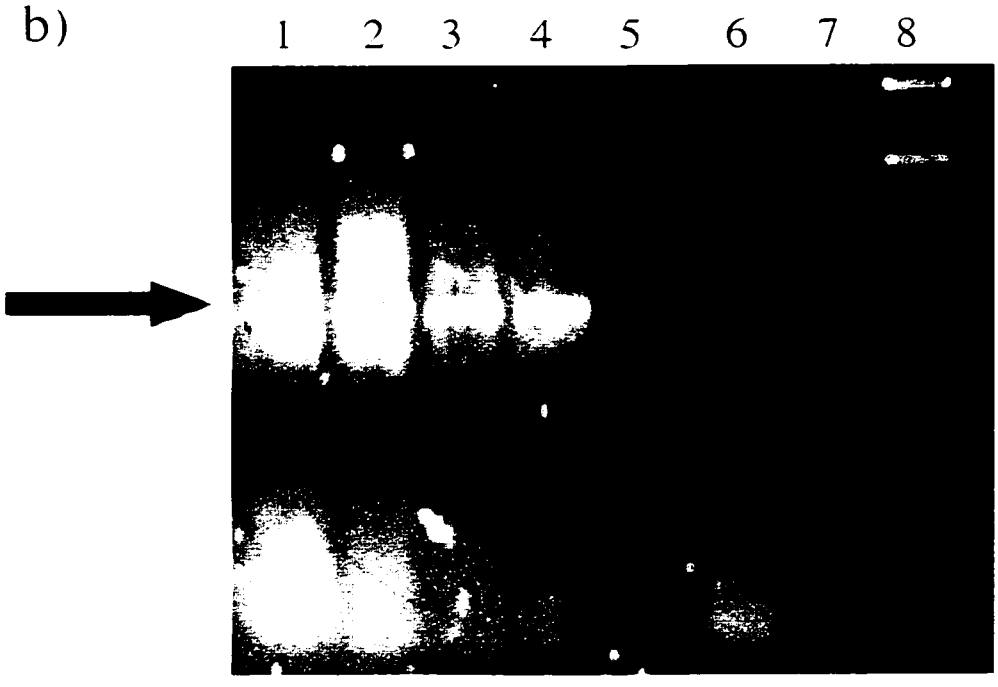
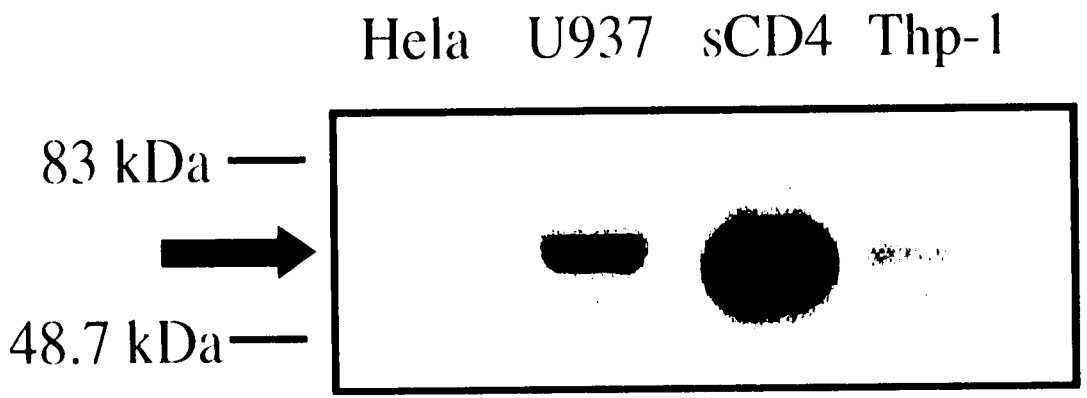


Fig. IV.4. Successful detection of CD4 by Western blot analysis using T4-4 rabbit serum to sCD4.

100 µg total protein lysate from U937, Thp-1 and HeLa cells, and 0.1 µg sCD4 were electrophoresed and analysed by immunoblotting. The blocked membrane was incubated with 10 µg/ml T4-4 rabbit serum to sCD4 and detected using ^{125}I-Protein A. The position of CD4 is indicated by an arrow. Mobilities of the molecular mass (MW) standards are shown on the left. (n=2)


Hela
U937
sCD4
Thp-1
83 kDa
48.7 kDa

(Fig.IV.5). $p56^{lck}$ was also detected, although in reduced quantities, in the EBV-transformed B cell line, MK. No $p56^{lck}$ could be detected in any of the Thp-1, U937 or HL-60 monocytic cell lines, in the J.Cam1.6 $p56^{lck}$-negative Jurkat derivative, or in the nonhemopoietic HeLa cell line.

IV.2.4. Stimulation of Thp-1 cells through the CD4 receptor results in a Ca^{2+} flux

To determine if CD4 expressed on the surface of the human monocytic cell line Thp-1 was capable of transducing extracellular signals, we stimulated Thp-1 cells with T4-4 rabbit serum to sCD4 and measured the CD4-mediated changes in intracellular Ca^{2+} levels (n=3). Because of the large numbers of cells required in this and subsequent experiments, the $CD4^+$ Thp-1 monocytic cell line was used instead of peripheral monocytes. This cell line represents a more differentiated stage of monocytes than do U937 and HL-60 cells (Auwerx 1991). Stimulation of Thp-1 cells with T4-4 induced a Ca^{2+} flux (Fig.IV.6b) compared to the intracellular baseline Ca^{2+} levels observed for unstimulated cells (Fig.IV.6a). No Ca^{2+} flux was observed, however, upon the addition of T4-4 serum which had been pre-adsorbed with a sCD4-NHS-activated Sepharose bead complex (Fig.IV.6c), or upon the addition of an irrelevant rabbit serum generated against Shp-1, an intracellular tyrosine phosphatase (Fig.IV.6d). Positive and negative technical controls consisting of the calcium ionophore A23187, and the metal ion chelator EGTA, respectively, were also included (Fig.IV.6e).

IV.2.5. Stimulation of Thp-1 cells through the CD4 receptor results in the time-dependent induction of protein tyrosine phosphorylation

α-phosphotyrosine immunoblotting analysis of unstimulated and T4-4 stimulated

Fig. IV.5. Monocytic cells do not express the protein tyrosine kinase p56^{lck}.

100 µg total protein lysate from various cell lines were electrophoresed and analysed by immunoblotting. The blocked membrane was incubated with 0.5 µg/ml murine α-p56^{lck} mAb, followed by a 1:3 000 dilution of Protein G-horseradish peroxidase for detection by ECL. The position of p56^{lck} is indicated by an arrow. Mobilities of the molecular mass (MW) standards are shown on the left. (n=2)

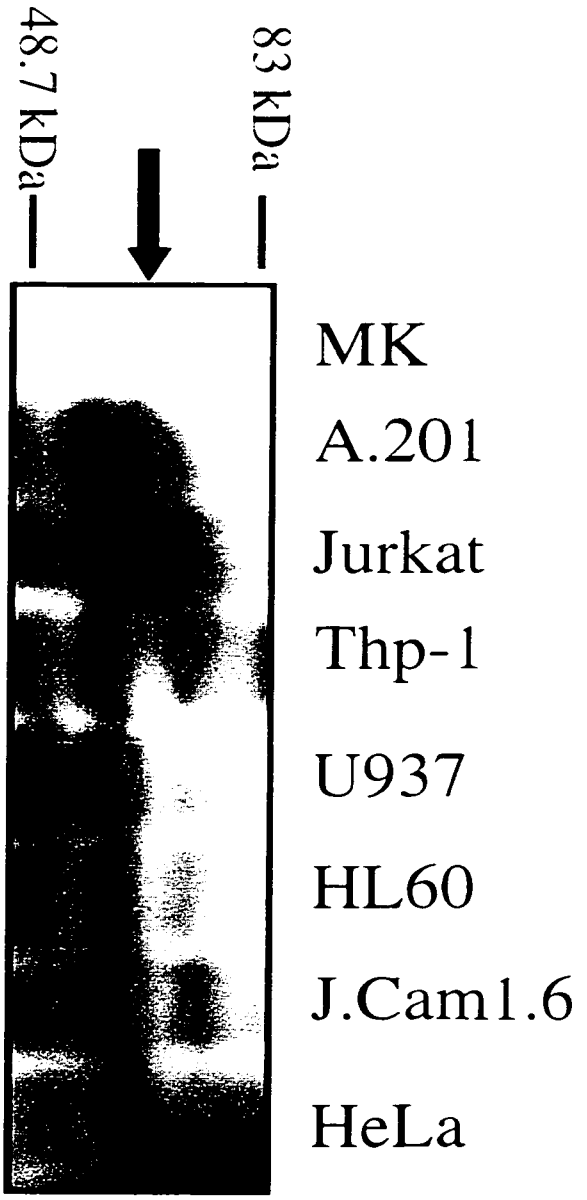
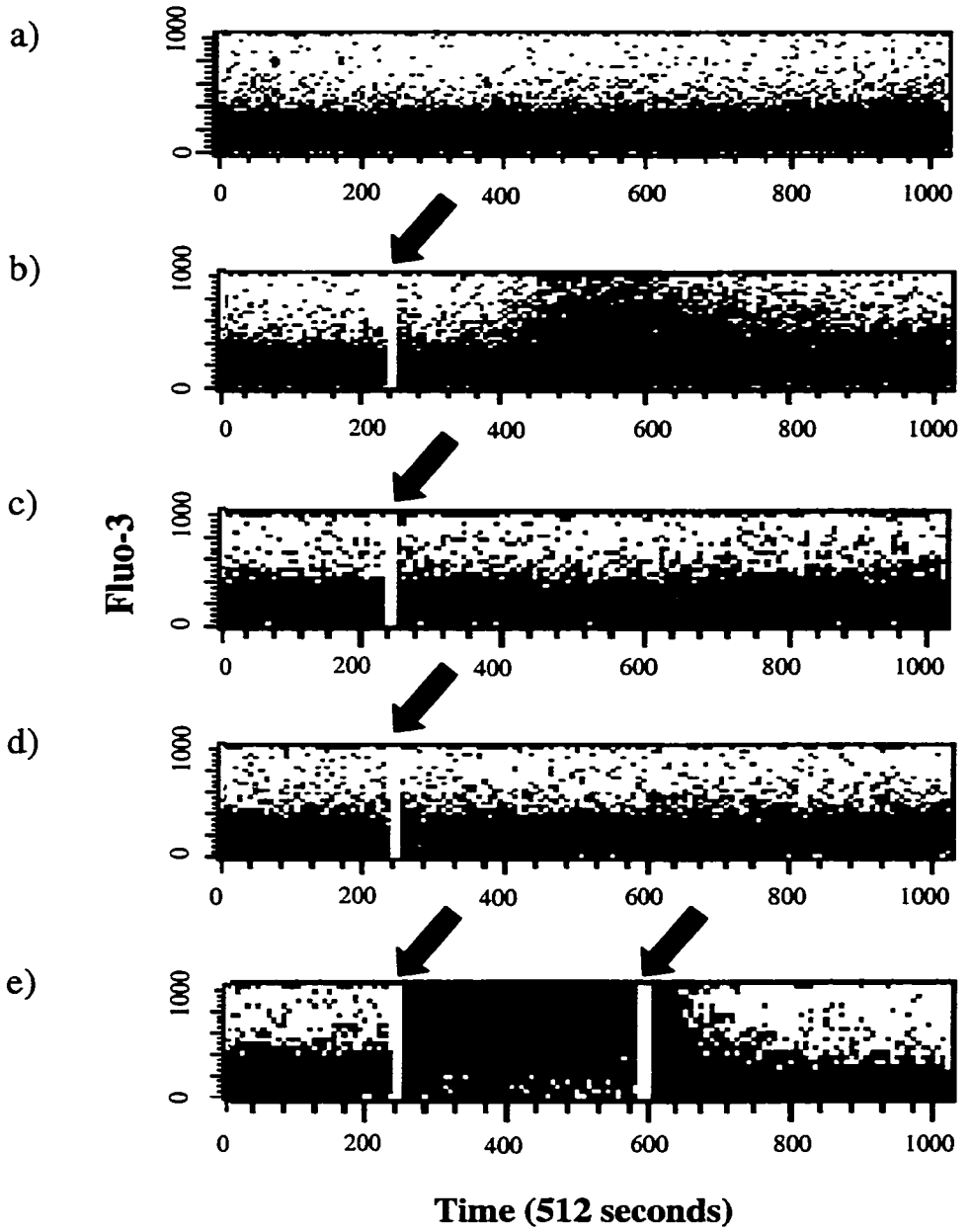

48.7 kDa
83 kDa
MK
A.201
Jurkat
Thp-1
U937
HL60
J.Cam1.6
HeLa

Fig. IV.6 Stimulation of Thp-1 monocytic cells with T4-4 rabbit serum to sCD4 induces Ca^{2+} flux.

Thp-1 cells loaded with Fluo3-AM were stimulated with various stimuli (as indicated by the arrows) and the resulting Ca^{2+} flux was measured by flow cytometric analysis.
a) Baseline Ca^{2+} levels in unstimulated cells; b) Stimulation with T4-4 rabbit serum to sCD4; c) Stimulation with T4-4 rabbit serum pre-adsorbed with sCD4 (conjugated to NHS-activated Sepharose beads); d) Stimulation with rabbit serum to Shp-1; e) Stimulation with the Ca^{2+} ionophore A23187 (first arrow) followed by the addition of the metal ion chelator EGTA (second arrow). (n=3)


a)
b)
c)
d)
e)
Fluo-3
1000
0
0
200
400
600
800
1000
Time (512 seconds)

Thp-1 lysates revealed a time-dependent tyrosine phosphorylation of various proteins; the phosphorylation peaked at 1 to 3 min, and declined gradually thereafter. A representative experiment (n=3) is shown in Fig.IV.7 - upper panel. To ensure that equal amounts of protein lysate were loaded in each lane, the membrane was stripped and blotted with T4-4 serum (Fig. IV.7. - lower panel).

IV.2.6. The tyrosine phosphorylation induced by T4-4 serum to sCD4 is mediated by CD4

To control for the possibility that the use of T4-4, a polyclonal rabbit serum to CD4, may have induced some non-CD4-specific signaling, we performed experiments in which a polyclonal rabbit serum to Shp-1, a protein not expressed on the Thp-1 cell surface, was used to stimulate the cells. The use of rabbit serum to Shp-1 (used at the same concentration as T4-4 serum) induced less tyrosine phosphorylation compared to the T4-4 stimulation, although the level of tyrosine phosphorylation was enhanced compared to the unstimulated control as shown in Fig. IV.8a - upper panel (n=4).

To further confirm that at least part of the induction of tyrosine phosphorylation observed was actually mediated by T4-4 binding to CD4, we attempted to adsorb the CD4-specific reactivity of the T4-4 serum. T4-4 serum that had been pre-adsorbed with sCD4 prior to use in the stimulation reaction induced less tyrosine phosphorylation compared to the levels induced by the unadsorbed T4-4 serum (Fig. IV.8a - upper panel). The decreased tyrosine phosphorylation observed for stimulations performed with Shp-1 serum and with T4-4 serum adsorbed with sCD4 was not due to decreased amounts of protein lysate loaded

Fig. IV.7 Stimulation of Thp-1 monocytic cells with T4-4 rabbit serum to sCD4 results in the time-dependent tyrosine phosphorylation of various proteins.

Upper panel: 10×10^6 serum-starved Thp-1 cells were stimulated for various times at 37°C with 25 µg/ml T4-4 rabbit serum to sCD4. 100 µg total protein lysate were electrophoresed and transferred. The blocked membranes were incubated with 1 µg/ml α-phosphotyrosine mAb, followed by a 1:2 000 dilution of Rabbit α-Mouse Ab, and detected using I^{125}-Protein A. Induced tyrosine-phosphorylated proteins (pp) having molecular weights of 180, 140, 120, 110, 85, 65, 55, 50 and 35 kDa are indicated by *. *Lower panel:* The blot was stripped, incubated with 10 µg/ml T4-4 serum and detected using I^{125}-Protein A; the position of CD4 is indicated by an arrow. Mobilities of the molecular mass (MW) standards are shown on the left. (n=3)

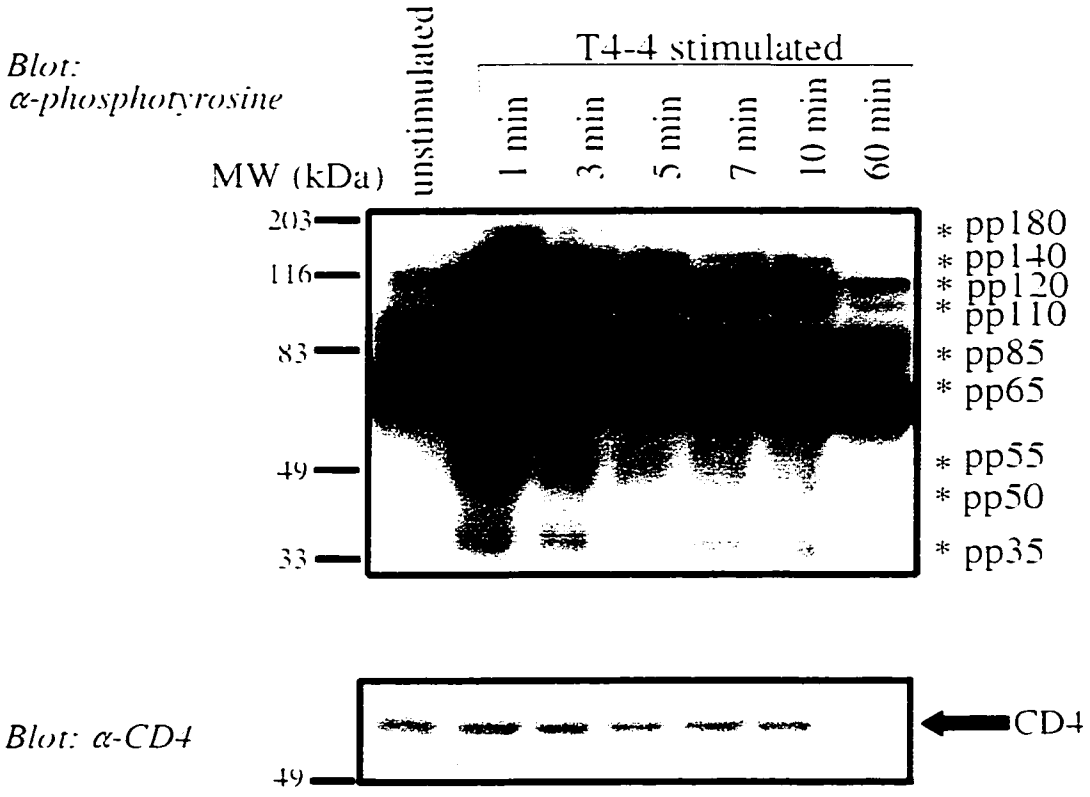

Blot:
α-phosphotyrosine
unstimulated
T4-4 stimulated
1 min
3 min
5 min
7 min
10 min
60 min
MW (kDa)
203
116
83
49
33
* pp180
* pp140
* pp120
* pp110
* pp85
* pp65
* pp55
* pp50
* pp35
Blot: α-CD4
49
CD4

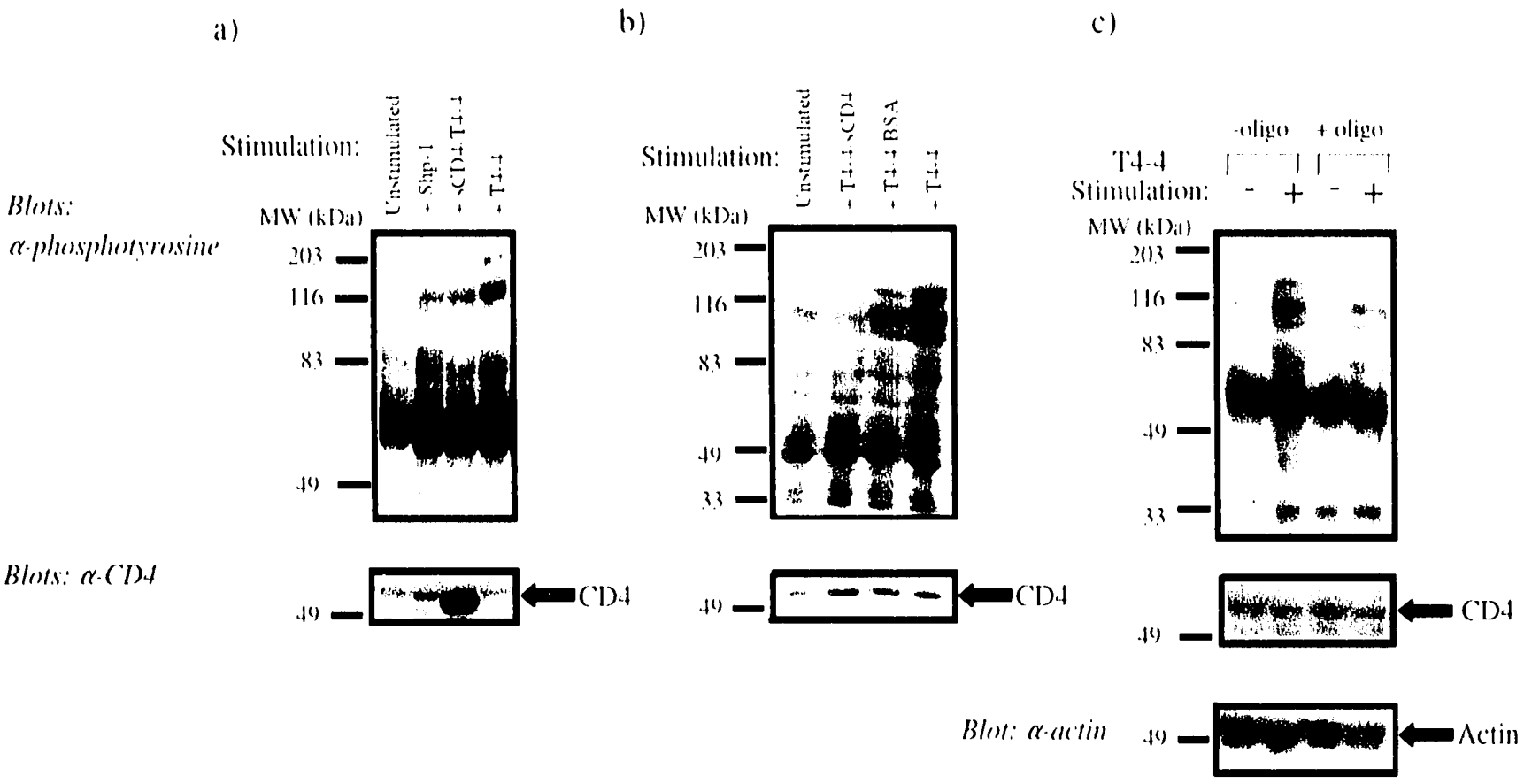
Fig. IV.8. The tyrosine phosphorylation induced by stimulation of Thp-1 monocytic cells with T4-4 rabbit serum to sCD4 is mediated by CD4.

a) 10×10^6 serum-starved Thp-1 cells were stimulated for 3 min at 37°C with various stimulants. 100 µg total protein lysate were electrophoresed and analysed by immunoblotting. *Upper panel:* The blocked membrane was incubated with 1 µg/ml α-phosphotyrosine mAb, followed by a 1:2 000 dilution of Rabbit α-Mouse Ab, and detected using I^{125}-Protein A. *Lower panel:* The blot was stripped and incubated with 10 µg/ml T4-4 serum and detected using I^{125}-Protein A; the position of CD4 is indicated by an arrow. (n=3)

b) 10×10^6 serum-starved Thp-1 cells were stimulated for 3 min at 37°C with various stimulants. 100 µg total protein lysate were electrophoresed and analysed by immunoblotting. *Upper panel:* The blocked membrane was incubated with 1 µg/ml α-phosphotyrosine mAb followed by a 1:2 000 dilution of Rabbit α-Mouse Ab, and detected using I^{125}-Protein A. *Lower panel:* The blot was stripped and incubated with 10 µg/ml T4-4 serum and detected using I^{125}-Protein A; the position of CD4 is indicated by an arrow. (n=2)

c) 1×10^6 Thp-1 cells/ml were cultured for 4 days in the presence or absence of 30 µM CD4 antisense oligodeoxynucleotide, then serum-starved and stimulated with T4-4 rabbit serum. 100 µg total protein lysate were electrophoresed and analysed by immunoblotting. *Upper panel:* The blocked membranes were incubated with 1 µg/ml α-phosphotyrosine mAb followed by a 1: 2 000 dilution of Rabbit α-Mouse Ab, and detected using I^{125}-Protein A. *Middle panel:* The blot was stripped, incubated with 10 µg/ml T4-4 serum and detected using I^{125}-Protein A; the position of CD4 is indicated by an arrow. *Lower panel:* The blot was stripped and incubated with a 1:200 dilution of goat polyclonal Ab to actin, followed by a 1:500 dilution of Rabbit α-Goat Ab, and detected using I^{125}-Protein A; the position of actin is indicated by an arrow. (n=1)

Mobilities of the molecular mass (MW) standards are shown on the left.


a)
b)
c)
Stimulation:
Unstimulated
+ Shp-1
+ sCD4 T4-4
+ T4-4
Blots:
α-phosphotyrosine
MW (kDa)
203
116
83
49
Blots: α-CD4
CD4
Stimulation:
Unstimulated
+ T4-4 sCD4
+ T4-4 BSA
+ T4-4
MW (kDa)
203
116
83
49
33
CD4
-oligo
+ oligo
T4-4
Stimulation:
- + - +
MW (kDa)
203
116
83
49
33
CD4
Blot: α-actin
Actin

in the wells, as indicated by the levels of CD4 detected in this blot (Fig.IV.8a - lower panel; n=2); the prominent, slightly lower molecular weight band observed in lane 3 is the sCD4 used to adsorb the T4-4 serum prior to stimulation.

As a second strategy to confirm the specificity of the T4-4 stimulation, we attempted to deplete the CD4-specific reactivity of the T4-4 serum by preincubation with sCD4 coupled to NHS-activated Sepharose Beads. Following the incubation, the T4-4 serum was recovered and used for subsequent Thp-1 stimulation. A negative control in the form of T4-4 serum incubated with the irrelevant protein BSA (coupled to NHS-activated Sepharose Beads) was also included. The T4-4 serum adsorbed with the sCD4-NHS-activated Sepharose bead complex induced lower levels of tyrosine phosphorylation compared to the use of T4-4 alone (Fig.IV.8b - upper panel); the level of tyrosine phosphorylation for the sCD4-adsorbed T4-4 stimulation was comparable to that observed for the unstimulated control. The stimulation of Thp-1 cells with T4-4 serum incubated with BSA (coupled to NHS-activated Sepharose Beads) resulted in greater levels of tyrosine phosphorylation compared to stimulation with T4-4 serum pre-adsorbed with the sCD4/bead complex, and was comparable to the levels observed for stimulation with T4-4 alone. The differences in tyrosine phosphorylation levels observed for the various stimuli were not due to different amounts of protein lysate loaded per lane since comparable levels of CD4 were detected following the stripping and reblotting of the membrane with T4-4 serum (Fig.IV.8b - lower panel; n=2).

A third strategy used to confirm that the induction of tyrosine phosphorylation observed was actually mediated by T4-4 binding to CD4 involved the use of CD4 antisense oligodeoxynucleotide to down-regulate CD4 surface expression, and thus, CD4-mediated

tyrosine phosphorylation (n=1). Thp-1 cells were cultured for 4 days in the presence of 30 μM CD4 antisense oligodeoxynucleotide. The cells were subsequently starved, followed by stimulation with T4-4 serum. Thp-1 cells cultured in the presence of oligodeoxynucleotide and subsequently stimulated with T4-4 serum showed decreased levels of tyrosine phosphorylation compared to Thp-1 cells that had not been cultured in the presence of oligodeoxynucleotide, but which had undergone stimulation with T4-4 serum (Fig. IV.8c - upper panel). To confirm that CD4 surface levels had been reduced by culturing the cells in the presence of oligodeoxynucleotide, cell aliquots were collected prior to stimulation from each culture condition and the cells were surface stained with α-CD4-PE mAb and analysed by flow cytometry. The addition of 30 μM oligodeoxynucleotide to the Thp-1 culture resulted in a 10.6% decrease in CD4 expression compared to the oligodeoxynucleotide-negative culture. To confirm that total (surface and cytoplasmic) CD4 expression had been reduced, the α-phosphotyrosine blot (Fig.IV.8c - upper panel) was reprobed with T4-4 serum (Fig.IV.8c - middle panel). To control for the amount of protein lysate loaded into each lane, the blot was stripped and reprobed with goat polyclonal antibody to actin (Fig.IV.8c - lower panel).

IV.2.7. T4-4 stimulation of Thp-1 cells does not activate the ERK pathway

Many different signaling pathways have been characterized. Central among these are the ERK (extracellular signal-regulated kinase) serine/threonine kinases of the MAPK pathway; the ERK branch mediates signals in response to mitogens and growth factors (Kyriakis 1999). To determine if the ERK branch of the MAPK pathway was activated by

T4-4 stimulation of Thp-1 cells, unstimulated and T4-4 stimulated lysates were immunoblotted with Ab to p44 ERK-1 and p42 ERK-2; an aliquot of a commercial Jurkat lysate preparation was also included as a positive control for ERK-1 and ERK-2 expression (n=1). ERK activation occurs in response to tyrosine and threonine phosphorylation (Kyriakis 1999) which should reduce the mobility of the activated ERK proteins compared to inactivated ERK proteins. However, no difference was observed in the relative mobility of the ERKs in stimulated Thp-1 lysates compared to unstimulated lysates (Fig.IV.9a). To further confirm that T4-4 stimulation had not induced tyrosine phosphorylation of ERK-1 and/or ERK-2, the blots were stripped and reprobed with murine α-phosphotyrosine mAb. Although the T4-4 stimulated lysates showed increased tyrosine phosphorylation compared to the unstimulated lysates, no increased tyrosine phosphorylation of 42 or 44 kDa proteins could be detected in the stimulated lysates compared to the unstimulated lysates (Fig.IV.9b).

IV.2.8. PLC-γ1 and PI3-K undergo tyrosine phosphorylation in response to T4-4 stimulation

T4-4 stimulation of Thp-1 cells routinely induced the tyrosine phosphorylation of proteins of specific molecular weights. Among the higher molecular weight species was a protein of approximately 140 kDa (Fig.IV.7 and Fig.IV.10 - upper panel). Thp-1 cells stimulated with T4-4 serum underwent a Ca^{2+} flux (Fig.IV.6), a response mediated by phosphoinositol-specific phospholipase C (Katan 1998). The molecular weight of PLC-γ1 is approximately 148 kDa (Burgess et al. 1990) which corresponds to the approximately 140 kDa tyrosine-phosphorylated protein observed following T4-4 stimulation. Furthermore, it

Fig. IV.9. T4-4 stimulation of Thp-1 cells does not activate the ERK pathway.

a) 10×10^6 serum-starved Thp-1 cells were stimulated for 3 min at 37°C with 25 µg/ml T4-4 rabbit serum to sCD4. 100 µg total protein lysate and 20 µg Jurkat positive control cell lysate were electrophoresed and analysed by immunoblotting. a) The blocked membranes were incubated with a 1:300 dilution of rabbit polyclonal Ab to either Erk-1 (left) or Erk-2 (right) and detected using I^{125}-Protein A. b) The membranes were stripped, blocked, and incubated with 1 µg/ml α-phosphotyrosine mAb, followed by a 1:2 000 dilution of Rabbit α-Mouse Ab, and detected using I^{125}-Protein A. The positions of ERK-1 and ERK-2 are indicated by arrows. Mobilities of the molecular mass (MW) standards are shown on the left. (n=1)

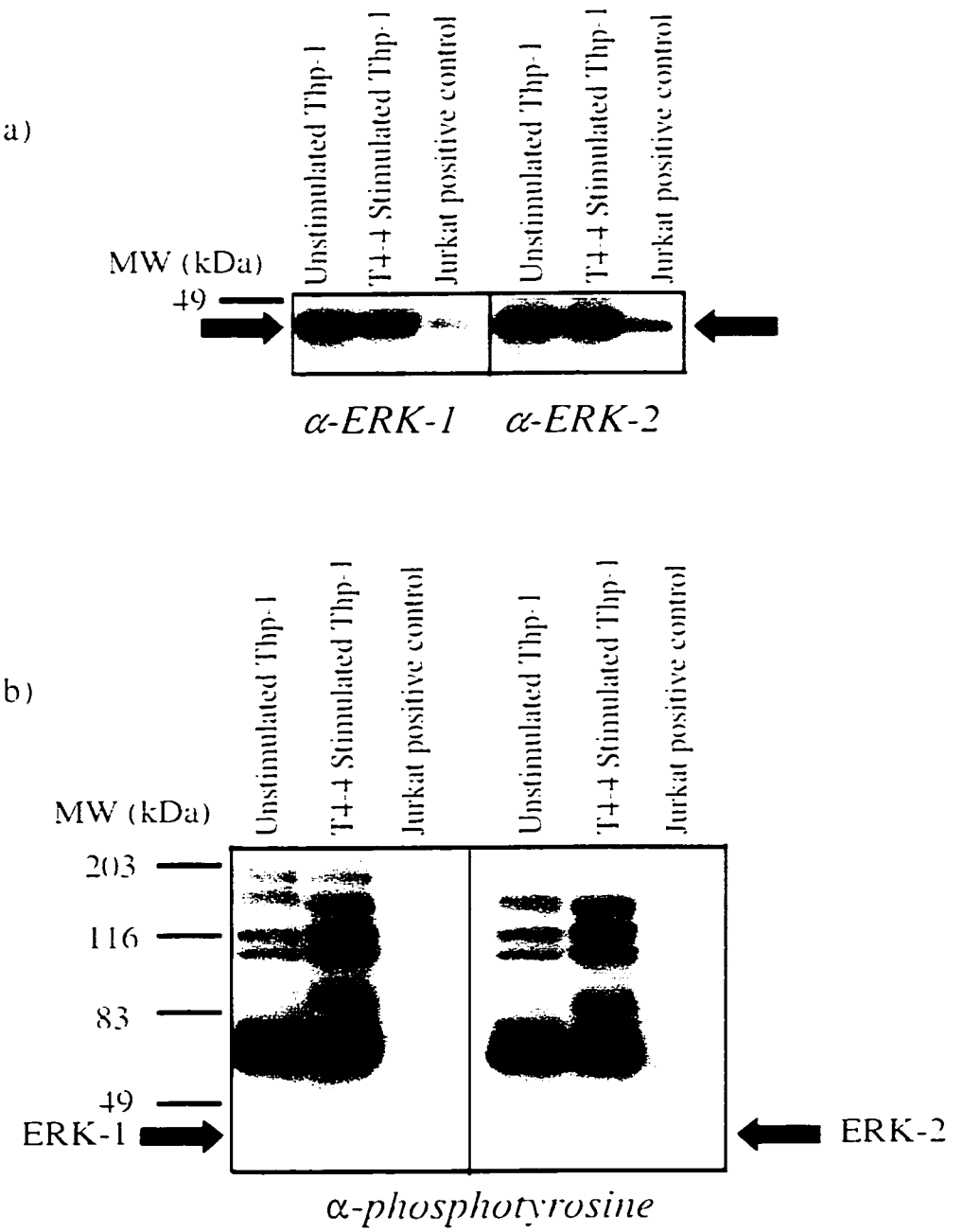

a)
Unstimulated Thp-1
T4-4 Stimulated Thp-1
Jurkat positive control
Unstimulated Thp-1
T4-4 Stimulated Thp-1
Jurkat positive control
MW (kDa)
49
α-ERK-1
α-ERK-2
b)
Unstimulated Thp-1
T4-4 Stimulated Thp-1
Jurkat positive control
Unstimulated Thp-1
T4-4 Stimulated Thp-1
Jurkat positive control
MW (kDa)
203
116
83
49
ERK-1
ERK-2
α-phosphotyrosine

Fig. IV.10. T4-4 stimulation of Thp-1 cells induces tyrosine phosphorylation of PLC-γ1 and PI3-K.

Tyrosine-phosphorylated proteins were immunoprecipitated from 2 mg of unstimulated and T4-4 stimulated Thp-1 total protein lysates using 10 μg α-phosphotyrosine mAb. The immunoprecipitation reactions, as well as 100 μg lysate controls, were electrophoresed and analysed by immunoblotting. The same membrane was subjected to four rounds of immunoblotting:

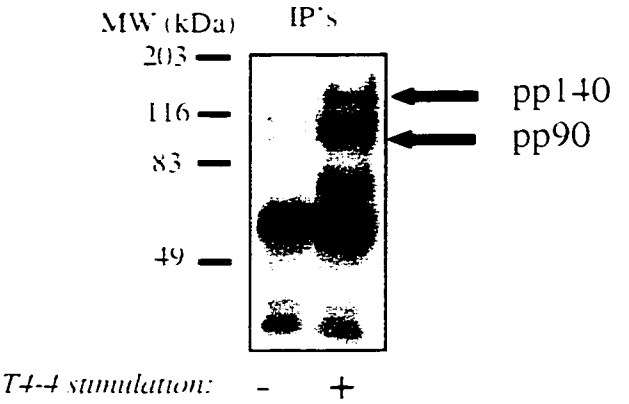
Upper panel: The blot was incubated with 1 μg/ml α-phosphotyrosine mAb followed by a 1:2000 dilution of Rabbit α-Mouse Ab and detected using I^{125}-Protein A.

Middle panel: The blot was incubated with 1 μg/ml rabbit polyclonal Ab to PLC-γ1 and detected using I^{125}-Protein A, followed by incubation with a 1:1000 dilution of murine mAb to PI3-K (p85 subunit) and with a 1:2000 dilution of Rabbit α-Mouse Ab and detected using I^{125}-Protein A. The positions of PLC-γ1 and PI3-K are indicated by arrows.

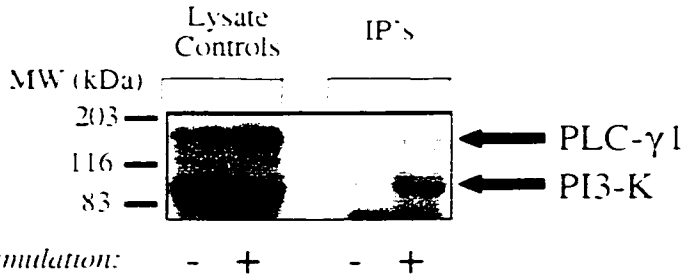
Lower panel: The blot was stripped, blocked and incubated with 10 μg/ml T4-4 rabbit serum and detected using I^{125}-Protein A. The position of CD4 is indicated by an arrow.

Mobilities of the molecular mass (MW) standards are shown on the left. (n=4)

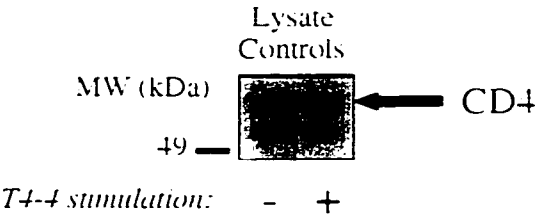
IP: α-phosphotyrosine
Blot: α-phosphotyrosine



IP: α-phosphotyrosine
Blot: α-PLC-γ1
α-PI3-K



Blot: α-CD4



has been reported that PI3-K forms a complex with CD4 and $p56^{lck}$ in human T cells (Prasad et al. 1993). We speculated, therefore, that the approximately 85-90 kDa Thp-1 protein observed to undergo tyrosine phosphorylation in response to T4-4 stimulation (Fig.IV.7 and Fig.IV.10 - upper panel) might be the regulatory subunit of PI3-K (p85). To determine if PLC-γ1 and PI3-K were two of the proteins that became tyrosine phosphorylated in response to T4-4 stimulation, we performed α-phosphotyrosine immunoprecipitations of unstimulated and T4-4 stimulated Thp-1 cells (n=4). Following electrophoresis and transfer of the immune complexes, the membranes were blotted with rabbit antibody to PLC-γ1. The level of PLC-γ1 immunoprecipitated and detected upon T4-4 stimulation was increased compared to the unstimulated condition (Fig.IV.10 - middle panel). Given that PLC-γ1 (p148) and the regulatory subunit (p85) of PI3-K have significantly different molecular weights, and given the absence of any background in the 85 kDa range following blotting with antibody to PLC-γ1, the membrane was reblotted, without stripping, for PI3-K (p85). The level of immunoprecipitated PI3-K was consistently increased upon T4-4 stimulation, compared to the unstimulated condition (Fig.IV.10 - middle panel). The membranes were subsequently stripped and blotted with T4-4 to control for the relative amounts of proteins present in the various lysate preparations (Fig.IV.10 - lower panel): slightly decreased levels of CD4 were present in the T4-4 stimulated lysate compared to the unstimulated lysate, suggesting that the protein concentration of this lysate may have been slightly lower. These results suggest that the α-phosphotyrosine immunoprecipitation performed with the T4-4 stimulated lysate contained relatively lower amounts of protein, and yet, the highest levels of phosphorylated PLC-γ1 and PI3-K were nonetheless observed in these lysates. To confirm that the enhanced

detection of PLC-γ1 and PI3-K was in fact due to successful immunoprecipitation of tyrosine phosphorylated proteins, the membrane was reprobed with α-phosphotyrosine mAb (Fig.IV.10 - upper panel). In the α-phosphotyrosine blot, the highest amount of immunoprecipitated tyrosine-phosphorylated protein was observed for the immunoprecipitation reaction performed with the T4-4 stimulated lysate, as expected.

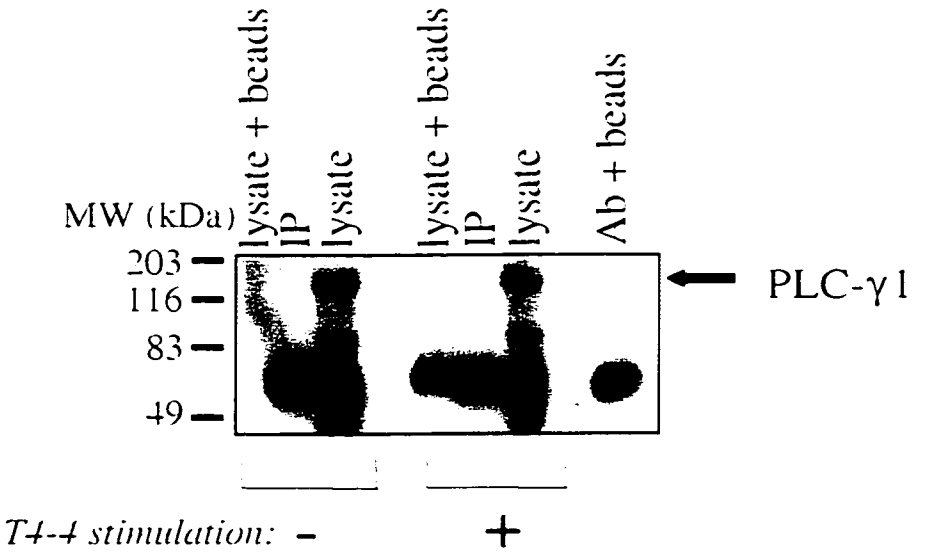
IV.2.9. CD4 does not appear to directly interact with either tyrosine phosphorylated PLC-γ1 or PI3-K

T4-4 stimulation of Thp-1 cells was shown to induce the tyrosine phosphorylation of PLC-γ1 and PI3-K. To determine if either PI3-K or PLC-γ1 were directly associated with CD4, CD4 in unstimulated and T4-4 stimulated Thp-1 lysates was immunoprecipitated using T4-4 serum. To control for nonspecific interactions between T4-4 serum and immunoprecipitated proteins, negative controls consisting of lysate and Protein A-Sepharose beads but no T4-4 serum, and T4-4 serum and Protein A-Sepharose beads but no lysate, were also included. The electrophoresed immune complexes were subsequently blotted for the presence of PI3-K, PLC-γ1, and a number of other known signaling molecules. A representative experiment in which the T4-4 immunoprecipitation reaction was screened for the presence of PLC-γ1 is shown in Fig.IV.11 - upper panel. Despite the successful detection of PLC-γ1 in the lysate controls, and despite the prolonged exposure of the blots (up to 1 week), no PLC-γ1 was detected in the CD4 immunoprecipitation reactions. The membrane had been originally probed with T4-4 serum to confirm that CD4 had been successfully immunoprecipitated (Fig.IV.11 - lower panel). Attempts to detect PI3-K in CD4

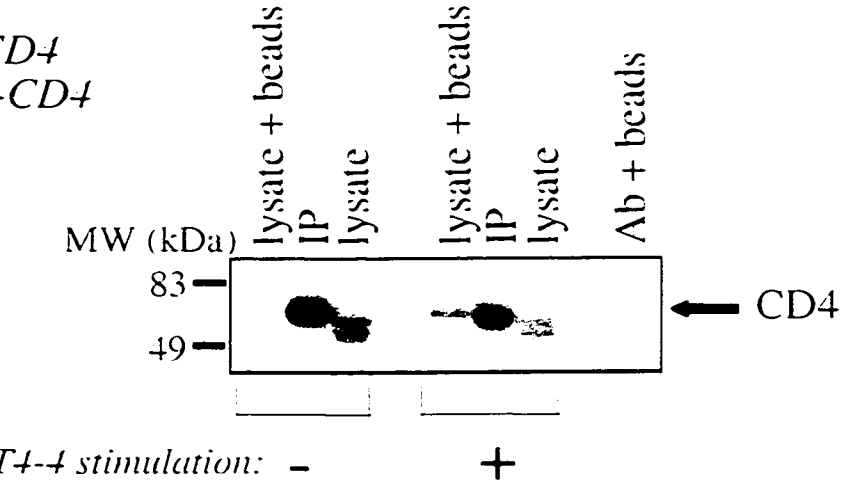
Fig. IV.11. CD4 and PLC-γ1 are not directly associated.

CD4 was immunoprecipitated from 2 mg of unstimulated or T4-4 stimulated Thp-1 total protein lysate using 5 μg T4-4 rabbit serum. The immunoprecipitation reactions, as well as 100 μg lysate controls, were electrophoresed and transferred. The blocked membrane was incubated with 10 μg/ml T4-4 rabbit serum (lower panel), and with 1 μg/ml rabbit polyclonal Ab to PLC-γ1 (upper panel); both rounds of immunoblotting were detected using I^{125}-Protein A. The positions of PLC-γ1 and CD4 are indicated by arrows. Mobilities of the molecular mass (MW) standards are shown on the left. (n=1)

IP: α-CD4
Blot: α-PLC-γ1



IP: α-CD4
Blot: α-CD4



immunoprecipitation reactions were also unsuccessful. In addition, we performed similar experiments to determine if CD4 directly associated with a number of other known signaling molecules. These molecules, partially chosen based on the molecular weights of Thp-1 proteins shown to undergo tyrosine phosphorylation in response to T4-4 stimulation, included kinases (HCK, PKC), G proteins (G_α, G_β and Rap-1), adaptor molecules (NCK, SLP-76), and VAV, an exclusively hemopoietic GDP/GTP exchange factor. None of these molecules could be detected in the CD4 immunoprecipitation reactions.

IV.2.10. GST-$CD4_{cyt}$ constitutively associates with 35, 45 and 55 kDa proteins

The failure to observe direct association between CD4 and any of the candidate signaling molecules for which we screened necessitated a different strategy to determine which Thp-1 proteins interact with the cytoplasmic tail of CD4. To this end, we produced a GST-$CD4_{cyt}$ fusion protein (Fig.IV.12a) for use in far Western blot analysis and in immunoprecipitation experiments. Production of the GST-$CD4_{cyt}$ fusion protein began by preparing a 141 bp insert (Fig.IV.12b) consisting of DNA coding for the cytoplasmic tail of CD4 in addition to extensions coding for BamHI and EcoRI restriction enzyme sites; these extensions would be required for the ligation of the CD4 construct into the pGEX-2T vector.

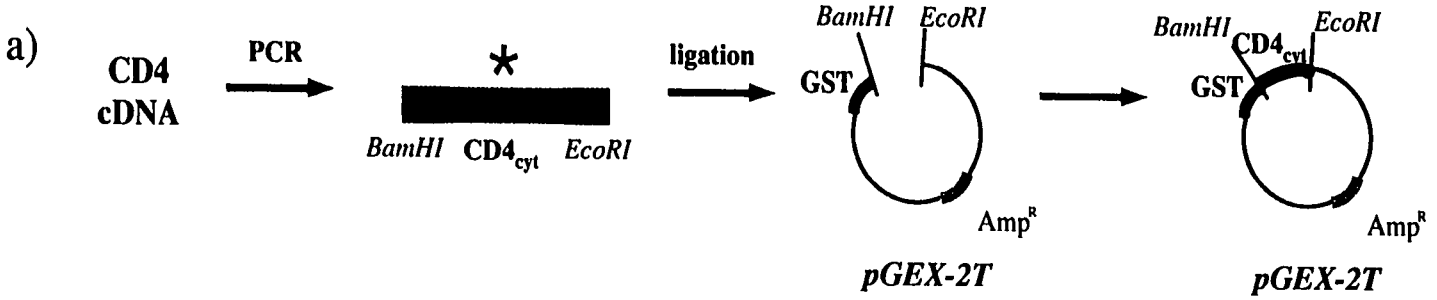
Upon the successful production of the fusion protein in the XL-1 Blue strain of *E. coli*, 2 other *E. coli* strains, C600hflA and BL21 cells were also assessed for their relative expression levels of GST and GST-$CD4_{cyt}$; the BL21 cells were found to express the highest levels of fusion protein as quantified by Coomassie Brilliant Blue staining of electrophoresed fusion protein aliquots (Fig.IV.13). Purified GST-$CD4_{cyt}$ fusion protein and GST control

Fig. IV.12a. Schematic of the production of the GST-$CD4_{cyt}$ construct.

GST-$CD4_{cyt}$ fusion protein was generated by subcloning PCR-amplified human CD4 cytoplasmic tail (residues 396 to 433) into the pGEX-2T expression plasmid. Briefly, $CD4_{cyt}$ cDNA was PCR amplified using primers specific for the cytoplasmic tail of CD4; the primers also coded for BamHI and EcoRI extensions* (see Fig.IV.12b). Following BamHI and EcoRI restriction enzyme digestion of the resulting PCR product and of the pGEX-2T vector, the digested and purified PCR product and vector were ligated, and the resulting construct was used to transform various *E. coli* strains. The cells were induced using IPTG and the resulting GST-$CD4_{cyt}$ fusion protein was purified using Glutathione Sepharose 4B Beads; the resulting complex was used in immunoprecipitation experiments. Alternatively, the fusion protein was eluted from the beads for use in far Western blots.

Fig. IV.12b. Production of the $CD4_{cyt}$ construct by PCR.

CD4 cDNA from U937 cells was PCR amplified using primers specific for the cytoplasmic tail of CD4, and containing extensions coding for BamHI and EcoRI restriction sites (5' and 3' primers, respectively). The resulting PCR product was resolved by electrophoresis through a 1.8% agarose gel. Lane 1: 100 bp ladder; Lane 2: 141 bp construct* consisting of DNA coding for the cytoplasmic tail of CD4 in addition to BamHI and EcoRI restriction sites; the position of the construct is indicated by an arrow.


a)
CD4
cDNA
PCR
*
BamHI CD4cyt EcoRI
ligation
BamHI EcoRI
GST
Amp^R
pGEX-2T
BamHI CD4cyt EcoRI
GST
Amp^R
pGEX-2T

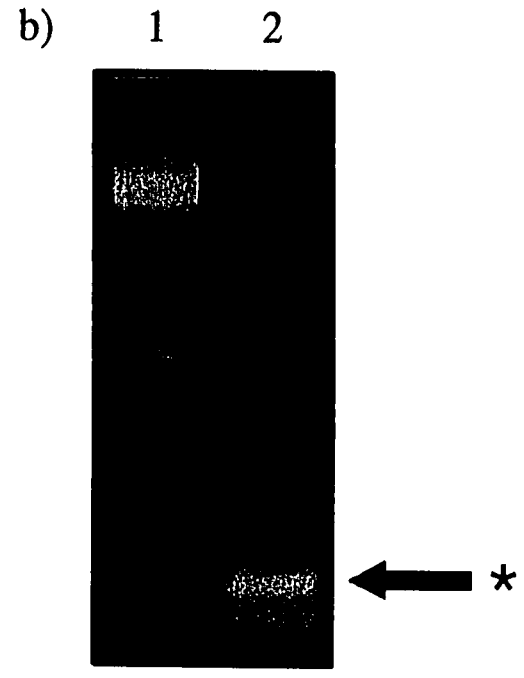
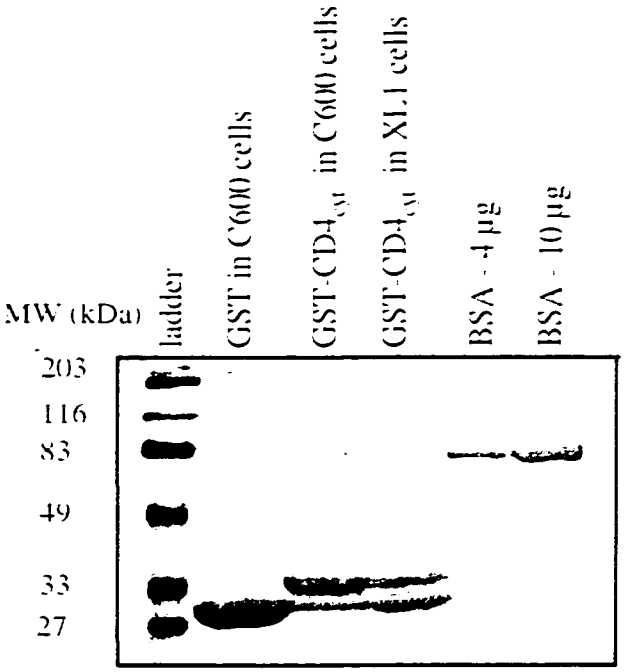

b)
1
2
*

Fig.IV.13. Relative expression of GST-CD4$_{cyt}$ by various *E. coli* strains.

Epicurian Coli XL-1 Blue (a), C600 (a) and BL21 (b) strains of *E. coli* were compared for their ability to express GST-CD4$_{cyt}$ fusion protein. Bacterial cells were transformed with pGEX-2T cloning vector containing the GST-CD4$_{cyt}$ construct and induced to express fusion protein. GST-CD4$_{cyt}$ was isolated and electrophoresed on 12% agarose gels which were stained with Coomassie Brilliant Blue.

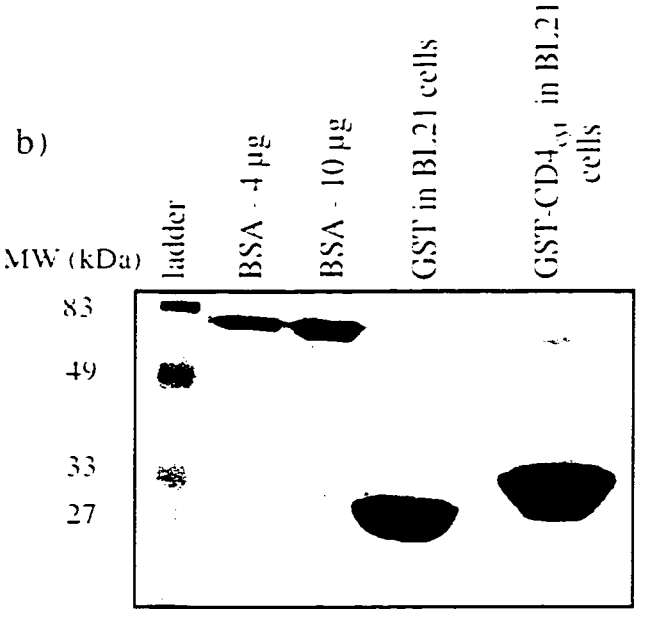
a)



GST-$CD4_{cyt}$:
C600 cells: 4.8 µg protein/ml culture
XL1 cells: 2.4 µg protein/ml culture

GST: 10.8 µg protein/ml culture

b)



GST-$CD4_{cyt}$: 7.2 µg protein/ml culture

GST: 6.25 µg protein/ml culture

proteins were subsequently used in far Western blot analysis and in immunoprecipitation experiments.

Far Western blot analysis was performed in which Thp-1 proteins from unstimulated and T4-4 stimulated lysates were electrophoresed and transferred and assessed for their abilities to bind the CD4 cytoplasmic tail component of the GST-$CD4_{cyt}$ fusion protein (n=3). In these experiments the GST-$CD4_{cyt}$ fusion protein was capable of binding proteins having molecular weights of approximately 140, 110, 90, 85, 55, and 45 kDa; a representative experiment is shown in Fig.IV.14. These molecular weights are comparable to the molecular weights of proteins observed to undergo increased tyrosine phosphorylation in response to T4-4 stimulation (Fig.IV.7). In contrast, the GST control protein bound weakly, if at all, to the proteins bound by GST-$CD4_{cyt}$. Furthermore, the banding patterns obtained for the GST-$CD4_{cyt}$ blotted membranes were the same for both unstimulated and T4-4 stimulated lysates, suggesting that the CD4 portion of the GST-$CD4_{cyt}$ fusion protein bound to these proteins constitutively.

The GST-$CD4_{cyt}$ fusion protein construct was also used in a series of immunoprecipitation experiments in which unstimulated and T4-4 stimulated Thp-1 lysates were incubated with fusion protein to determine if any Thp-1 proteins are capable of binding the CD4 cytoplasmic tail component of the GST-$CD4_{cyt}$ fusion protein. In these experiments 45 and 55 kDa proteins were routinely observed (n=8 and 7, respectively) in the GST-$CD4_{cyt}$ immunoprecipitation reactions; these proteins were not observed in the GST immunoprecipitation reactions (Fig.IV.15). In some experiments (n=3), an enhanced 35 kDa protein was also observed for the GST-$CD4_{cyt}$ immunoprecipitation reaction (Fig.IV.15). The

Fig. IV.14. GST-CD4$_{cyt}$ fusion protein binds to numerous Thp-1 proteins as determined by far Western blot analysis.

100 μg unstimulated and T4-4 stimulated Thp-1 protein lysates were electrophoresed in duplicate and analysed by immunoblotting. The blocked membranes were incubated with 1 μg/ml eluted GST or GST-CD4$_{cyt}$ protein and detected with a 1:200 dilution of murine α-GST mAb, followed by a 1:2 000 dilution of Rabbit α-Mouse Ab, and detected using I^{125}-Protein A. GST-CD4$_{cyt}$-binding Thp-1 proteins having molecular weights of 140, 110, 90, and 85 kDa are indicated by *, and 55 and 45 kDa proteins are indicated by arrows. Mobilities of the molecular mass (MW) standards are shown on the left. (n=3)

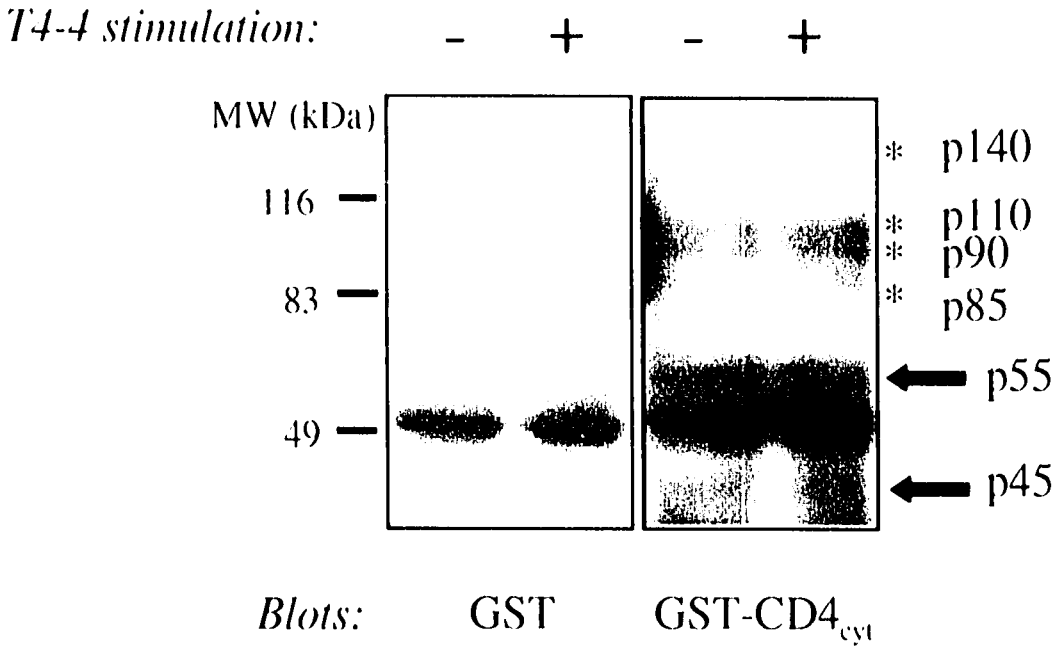
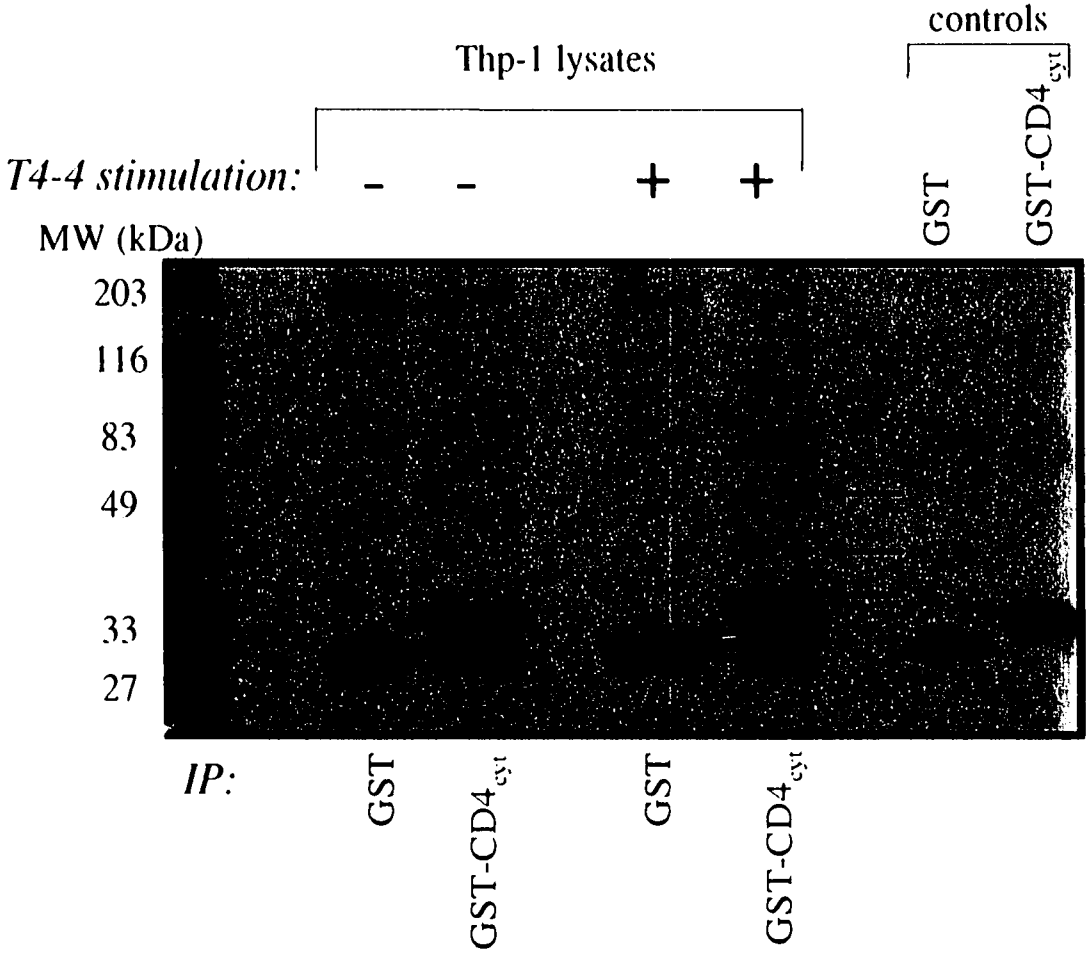

T4-4 stimulation:
-
+
-
+
MW (kDa)
116
83
49
p140
p110
p90
p85
p55
p45
Blots:
GST
GST-CD4cyt

Fig. IV.15. GST-CD4$_{cyt}$ fusion protein immunoprecipitates three proteins from Thp-1 total protein lysate, irrespective of CD4 stimulation.

Unstimulated and T4-4 stimulated lysates (3.5 mg) were precleared twice with 30 μg GST control protein. The precleared lysates were subjected to an overnight immunoprecipitation with 30 μg of either GST or GST-CD4$_{cyt}$ protein. The Thp-1 proteins directly associated with the GST or GST-CD4$_{cyt}$ proteins were visualized by separating the immune complexes by electrophoresis and staining the gel with Coomassie Brilliant Blue. Electrophoresed GST and GST-CD4$_{cyt}$ proteins (in the absence of Thp-1 lysates) were included as controls. GST-CD4$_{cyt}$-binding Thp-1 proteins having molecular weights of 35, 45 and 55 kDa are indicated by arrows. Mobilities of the molecular mass (MW) standards are shown on the left. (n=9)


controls
Thp-1 lysates
T4-4 stimulation:
- - + +
GST
GST-CD4cyt
MW (kDa)
203
116
83
49
33
27
IP:
GST
GST-CD4cyt
GST
GST-CD4cyt

association between GST-$CD4_{cyt}$ and the 35, 45 and 55 kDa proteins occurred irrespective of whether the lysates were from unstimulated or T4-4 stimulated cells; similar observations were made for the 45 and 55 kDa proteins in the far Western blot experiments. Equal amounts of GST and GST-$CD4_{cyt}$ proteins were used for the immunoprecipitation reactions as indicated by the similar intensities of these bands (at 30 and 33 kDa, respectively) (Fig.IV.15).

IV.3. Summary of results

We have shown that stimulation of Thp-1 monocytic cells with antibody to CD4 results in a Ca^{2+} flux, as well as in the time-dependent tyrosine phosphorylation of various proteins having molecular weights of approximately 180, 140, 120, 110, 85, 65, 55, 50 and 35 kDa. We have identified the 140 and 85 kDa proteins as PLC-γ1, and the regulatory subunit of PI3-K, respectively. Using immunoprecipitation/Western immunoblotting, we were unable, however, to show any direct association between CD4 and PLC-γ1, PI3-K, or other signaling proteins. In an attempt to identify proteins capable of associating with the cytoplasmic tail of CD4, we generated a GST-$CD4_{cyt}$ fusion protein for use in far Western blots and immunoprecipitation experiments. In both types of experiments, the GST-$CD4_{cyt}$ fusion protein routinely associated with 45 and 55 kDa proteins. In the immunoprecipitation experiments, an additional protein of 35 kDa was also observed.

V. DISCUSSION

The CD4 molecule expressed by monocytes/macrophages has been poorly studied, and thus, is poorly understood (Littman 1996, Marsh and Pelchen-Matthews 1996). This project was undertaken in order to gain greater insight into the physiological role, as well as the regulation of, monocytic CD4.

V.1. Monocyte CD4 expression

Our first objective was to characterize the regulation of monocyte CD4 during the process of cellular differentiation into tissue culture-derived macrophages. We hypothesized that the absence of CD4, the HIV coreceptor, might explain the refractoriness of overnight-cultured monocytes to infection in earlier experiments performed in our laboratory. In order to characterize monocyte CD4 regulation, we performed a comprehensive flow cytometric analysis of CD4 expression during the monocyte isolation procedure and following cell culture under various conditions.

The down-regulation of CD4 by cultured monocytes has been reported by others (Crowe et al. 1987, Kazazi et al. 1989, Valentin et al. 1990, Valentin et al. 1991, Peudenier et al. 1991b, Pelchen-Matthews et al. 1998). One proposed mechanism for culture-induced CD4 down-regulation involves a post-translational process which results in conformational changes to CD4, and ultimately, in its decreased membrane expression (Kazazi et al. 1989). Other investigators have failed to observe down-regulation of monocyte CD4 upon culture (Collman et al. 1990). Our study, which includes 35 isolations, represents a comprehensive analysis of CD4 expression on monocytes from healthy donors, under most experimental

conditions employed in the laboratory (Graziani-Bowering and Filion 2000).

Monocyte CD4 levels were assessed in whole blood within hours of their isolation from the donor, in post-Ficoll-Paque PBMC fractions, and following overnight and 6 day culture. Down-regulation was immediately observed following the isolation of PBMC by Ficoll-Paque in 5 of 18 experiments. Following overnight culture in various conditions, monocyte CD4 was consistently down-regulated, either completely or to negligible levels. Various monocyte/macrophage growth factors, activators or suppressors were tested for their ability to maintain monocyte CD4 following overnight culture, or to enhance re-expression following 6 days in culture. The literature contains reports of enhanced HIV infection and replication in monocyte cultures supplemented with GM-CSF (Perno et al. 1990, Goletti et al. 1996) or M-CSF (Bergamini et al. 1994, Kalter et al. 1991). We hypothesized that enhanced infection and replication of HIV in supplemented monocyte cultures might be the result of maintenance of the CD4 molecule on the surface of cultured monocytes. The addition of M-CSF to macrophage cultures has been reported to increase CD4 expression (Bergamini et al. 1994), although the quantities used were in excess of those used in our experiments. In our experiments, the addition of either GM-CSF or M-CSF to our cultures failed to inhibit monocyte CD4 down-regulation, nor did it enhance re-expression in day 6 cultures.

LPS, a potent activator of monocytes/macrophages (Wolff 1973, Pabst and Johnston 1980, Aderem et al. 1986) has been shown to down-regulate monocyte CD4 expression (Herbein et al. 1995, Neate et al. 1992); this down-regulation is attributed to the induction of TNF and IL-1β (Herbein et al. 1995). CD4 on monocytes, T cells and eosinophils acts as

a receptor for IL-16, a chemoattractant factor which recruits these immune cells to a site of inflammation (Cruikshank et al. 1994, Center et al. 1995). LPS-induced down-regulation of monocyte CD4 may, therefore, have evolved as a mechanism by which gram-negative bacteria evade monocytic recruitment and phagocytosis. However, as for the GM-CSF and M-CSF-treated cultures examined in our experiments, LPS-treated monocyte cultures showed CD4 down-regulation comparable to the levels observed for untreated overnight cultures.

IL-10, an immunosuppressive cytokine able to suppress T cell and macrophage cytokine production (Weissman et al. 1994) and to inhibit signaling through cytokine receptors (Ito et al. 1999, Donnelly et al. 1999) is expressed by T cells, B cells and macrophages (Montaner et al. 1994). IL-10 also inhibits the maturation of cultured monocytes (Chang et al. 1996) and impairs antigen presentation by macrophages (Muller et al. 1998). In HIV infection, elevated IL-10 levels (Weissman et al. 1994, Barcellini et al. 1996) play an important role in the pathogenesis of HIV/AIDS by causing increased CCR5 expression, and thus, more efficient infection of macrophages (Sozzani et al. 1998), resulting in increased viral replication in T cells and macrophages (Weissman et al. 1995). In our experiments, overnight culture of monocytes in the presence of IL-10 had no effect on monocyte CD4, but did result in decreased HLA-DR expression. Similar observations with respect to HLA-DR have been made by other investigators (de Waal Malefyt et al. 1991). Interestingly, the culture of monocytes for 6 days in the presence of IL-10 resulted in increased expression of CD4 compared to control cultures not supplemented with IL-10. The enhanced expression of CD4 by IL-10 may be due to the ability of IL-10 to suppress

cytokines, including inflammatory cytokines (de Waal Malefyt 1998), as well as the ability of IL-10 to inhibit signaling through cytokine receptors (Ito et al. 1999, Donnelly et al. 1999). The inflammatory cytokines IL-1β, TNF-α and IFN-γ have been implicated in various monocyte/macrophage models of CD4 down-regulation (Cassatella et al. 1992, Dhawan et al. 1995, Herbein et al. 1995, Karsten et al. 1996). It is possible, therefore, that in our monocyte model, CD4 is down-regulated in response to various inflammatory cytokines produced by the monocytes in response to their *in vitro* environment, and that the continued presence of IL-10, a cytokine usually induced late after activation (de Waal Malefyt 1998), may interfere with this inflammatory cytokine response, thus partially inhibiting the repression of CD4 expression in day 6 cultures. The ability of IL-10 to modulate CD4 expression in day 6, but not in overnight cultures may reflect the requirement for up-regulation of the IL-10 receptor, and/ or the requirement for the synthesis of SOCS (suppressor of cytokine signaling) proteins which interfere with the JAK/STAT pathway of cytokine signaling (Ito et al. 1999, Donnelly et al. 1999).

We also addressed the issue of whether adherence could be the cause of monocyte CD4 down-regulation following overnight culturing. The expression by monocytes of high-affinity receptors for fibronectin (Bevilacqua et al. 1981) is the basis of the isolation procedure used in the initial experiments (Freundlich and Avdalociv 1983). To test the hypothesis that monocyte CD4 down-regulation is induced by adherence, which may or may not be mediated by the fibronectin-receptor, we established parallel PBMC cultures in adherent flasks (uncoated flasks and gelatin-fibronectin-coated flasks), as well as in Teflon vials to inhibit the adherence of monocytes (Van Der Meer et al. 1981). Following overnight

culturing of the PBMC in each of these environments, CD4 expression was comparably down-regulated, indicating that this is not an adherence-mediated phenomenon. Our observation that the culture of monocytes in Teflon vials, a nonadherent environment, does not inhibit CD4 down-regulation is supported by the findings of other investigators. Crowe et al. (1987) have shown minimal levels of CD4 expression in monocytes cultured for 6 days on Teflon surfaces. Interestingly, the observation that the culture of monocytes on Teflon surfaces does not inhibit CD4 down-regulation is not a universal one: another group of investigators have shown that monocytes cultured on Teflon maintained CD4 expression, whereas adherent monocytes did not (Kazazi et al. 1989). The reasons for the discrepancies among the results of the various groups is unknown, but may be related to a number of factors including source of blood, manner in which monocytes were isolated and cultured, donor-specific factors, and/or differences in flow cytometric technique. We are confident nonetheless that at least in our experiments, the down-regulation of CD4 by cultured monocytes is an adherence-independent phenomenon. This conclusion is further supported by the results of our whole blood and anticoagulant experiments (discussed below) in which monocytes cultured as part of a PBMC fraction in nonadherent polypropylene tubes routinely showed down-regulation of CD4 expression following overnight culturing.

We also investigated the possible role of anticoagulants on the down-regulation of monocyte CD4; heparin, for instance, has been shown to interact with the CD4 molecule (Lederman et al. 1989). However, regardless of whether blood was collected in citrate, EDTA or heparin, comparable monocyte CD4 down-regulation was observed following overnight culture of PBMC.

Finally, we examined the possible involvement of Ficoll-Paque isolation of PBMC on monocyte CD4 down-regulation. In 3 of 4 experiments in which PBMC did not undergo Ficoll-Paque isolation but were cultured instead in a whole blood environment, monocytes in the whole blood cultures did show decreased CD4 expression. However, the CD4 levels in these cultures were greater than those of monocytes cultured in parallel cultures containing Ficoll-Paque-isolated PBMC. In the remaining isolation, monocytes in the whole blood culture did not show loss of CD4. These results suggest that whole blood may contain some factor which stabilizes monocyte CD4 expression and/or inhibits monocyte differentiation into macrophages. The isolation of PBMC or pure monocytes may lead to the removal of this factor, whereas the addition of FBS-free medium to whole blood may dilute this factor, perhaps explaining why monocytes in the whole blood cultures expressed slightly higher levels of CD4 than did monocytes in PBMC fractions cultured in medium. This hypothesis could also explain why in one experiment, monocytes cultured in a whole blood environment continued to express CD4, whereas monocytes in the parallel PBMC culture did not. It is possible that this particular donor had higher levels of this putative factor in his blood, and that the addition of FBS-free medium to establish the whole blood culture was insufficient to dilute out the factor enough to destabilize monocyte CD4 expression. The putative factor is probably not IL-16, a known natural ligand of CD4 (Cruikshank et al. 1987, Cruikshank et al. 1994, Center et al. 1995), since IL-16 has been shown to down-regulate CD4 expression in monocyte-derived macrophages (Hermann et al. 1999).

Differential regulation of CD4 on monocytes and T cells was observed in overnight PBMC cultures; while monocyte CD4 was routinely down-regulated, T cell CD4 levels were

fairly consistent, most likely due to the association of T cell CD4 with $p56^{lck}$, which keeps CD4 anchored on the cell surface (Pelchen-Matthews et al. 1992). Because of the lack of $p56^{lck}$ expression by monocytes (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al.1993), monocyte CD4 is not anchored to the cell membrane, thus explaining the ability of monocytic cell lines and monocytes to constitutively endocytose and recycle their CD4 molecules (Pelchen-Matthews et al. 1992, Pelchen-Matthews et al. 1998).

We have shown, albeit indirectly, that internalization is the process by which monocyte CD4 was down-regulated in our experiments. Monocytes and lymphocytes from cultures tagged with α-CD4-PE mAb prior to culture continued to display a PE signal following overnight culture and harvesting. The addition of a goat α-mouse Ig-FITC antibody to these harvested cells revealed binding to the tagged lymphocytes, but negligible binding to the tagged monocytes, indicating that the CD4/α-CD4-PE mAb complex remained on the cell surface of the tagged lymphocytes, but was almost completely internalized in the case of the tagged monocytes. Attempts to stain PBMC from the untagged overnight culture revealed that the majority of monocyte CD4 had been down-regulated. We conclude that the internalization of monocyte CD4 observed in these experiments reflects the down-regulation we routinely observe following overnight culturing, and is not an artifact of our tagging procedure. Other processes, such as protein shedding from the cell surface, may also be involved in CD4 down-regulation, although we did not investigate such mechanisms.

Analysis of monocytes cultured for 6 days showed variable CD4 expression levels, ranging from undetectable to high. We speculate that these variations may be at least partially attributed to donor variability in response to numerous factors, including sensitivity to

mediators/factors involved in the collection of blood or the manipulation of the cells (Neate et al. 1992), or possible differences in the cytokine response of cells from the various donors to their *in vitro* environment.

The down-regulation of monocyte CD4 observed in our *in vitro* experiments may reflect naturally occurring *in vivo* processes which occur as monocytes migrate from the circulation to certain tissues. Generally, tissue macrophages tend to be weakly $CD4^-$ (Wood et al. 1985). However, certain tissue-specific macrophages tend to show extremely low level CD4 expression, perhaps explaining the conflicting reports of microglial CD4 expression by some (Lowe et al. 1989, Porwit et al. 1989, Jordan et al. 1991, Williams et al. 1992, Wu et al. 1993, Mikovits et al. 1996, Schluesener et al. 1996) and the failure by others to make the same observation (Vazeux et al. 1987, Peudenier et al. 1991a, Peudenier et al. 1991b). Cytokines (such as IL-10), adhesion factors, and/or other cell types in the microenvironment may play important roles in the regulation of monocyte CD4 expression *in vivo*.

This study was initiated in response to the failure to induce productive HIV infection following overnight culture of monocytes. We had speculated that this failure was due to the absence of the CD4 molecule on the surface of the cells. Although we did show that CD4 expression was decreased in these cultures, CD4 could still be detected in some cultures. Although CD4 is the main cellular coreceptor for HIV (Klatzmann et al. 1984, Dalgleish et al. 1984, McDougal et al. 1985) in conjunction with chemokine receptors (Feng et al. 1996 Deng et al. 1996, Dragic et al. 1996, Alkhatib et al. 1996, Choe et al. 1996, Doranz et al. 1996), attempts to correlate CD4 levels with HIV infectability of monocytes have been unsuccessful (Crowe et al. 1987, Sonza et al. 1995). Furthermore, Ab-coated virus may be

involved in Fc receptor-mediated HIV infection of monocytes (Homsey et al. 1989), a mechanism which could compensate for CD4 down-regulation. The failure to productively infect overnight-cultured monocytes with HIV may not have been due to CD4-dependent limitations at the level of viral entry. Macrophage susceptibility to HIV infection has been shown to depend on the levels of expression of the CCR5 coreceptor, the levels of which increase as the cells differentiate (Tuttle et al. 1998).

HIV replication of infected cells requires the cellular transcription factor NF-κB (Roulston et al. 1995). In T cells, this requirement is met in activated, replicating cells (McDougal et al. 1985). In contrast, monocytes undergo little replication, but do differentiate into macrophages, a process which is also driven by increased production of NF-κB (Griffin et al. 1989, Lewin et al. 1997).

We conclude, therefore, that the inability to productively infect overnight-cultured monocytes was not due to insufficient levels of CD4 expression, but rather to insufficient maturation of the cells, and thus inadequate CCR5 expression and/or inadequate NF-κB production. This conclusion is consistent with current protocols in which monocytes are allowed to differentiate into tissue-culture-derived macrophages for several days in order to maximize HIV infection (Rich et al. 1992, Coligan et al. 1993, Sonza et al. 1996).

At this time, we conclude that the observed down-regulation of monocyte CD4 is probably due to the differentiation of blood monocytes into tissue-culture-derived macrophages (Kazazi et al. 1989, Valentin et al. 1991), rather than to some artifact of the isolation procedure. Despite its regular use in the immunophenotyping of monocytes, the physiological role of CD4 in this cell population has been poorly studied and is poorly

understood (Littman 1996, Marsh and Pelchen-Matthews 1996). Further work is therefore required in order to gain a better understanding of how monocyte CD4 is regulated, as well as whether its physiological role extends beyond that of the IL-16 receptor (Cruikshank et al. 1987, Cruikshank et al. 1994, Center et al. 1995).

V.2. Monocyte CD4 signal transduction

In an attempt to further understand monocyte CD4 function, we investigated the role of monocyte CD4 in signal transduction. Whereas the role of CD4-mediated signal transduction has been well established for $CD4^+$ T cells, less is known about the role of this molecule in monocytes. It was our goal, therefore, to perform a comprehensive study of CD4-mediated signal transduction in monocytic cells using biochemical/immunological and functional assays.

Species heterogeneity with respect to CD4 expression by macrophages has been observed; while human and rat macrophages express CD4, murine macrophages do not, despite the expression of CD4 by a subset of murine T cells (Crocker et al. 1987). The lack of CD4 expression by murine macrophages has been attributed to the production of a truncated CD4 transcript (Moore et al. 1992). Since there were no literature reports for the protein sequence of the cytoplasmic domain of monocytic CD4, we examined whether this portion of the CD4 molecule in human monocytes differed from that found in human T cells. The partial CD4 amino acid sequence (including the juxtamembrane, transmembrane and cytoplasmic domains except for the last amino acid) obtained for the Hut 78 T cell line, U937 monocytic cell line, and monocytes present in a $CD2^-$ PBMC fraction were identical; they

differed with respect to the published T cell sequence (Maddon et al. 1985) at a single nucleotide, resulting in a silent mutation. The published nucleotide sequence for amino acid 424 (cysteine) is TGC, whereas our sequence was TGT.

Despite the failure to detect any direct interaction between CD4 and $p56^{lck}$ or other kinases (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al.1993), a finding which we confirmed with respect to $p56^{lck}$ expression, there is evidence that CD4 is not an inert remnant originating from the pluripotent hemopoietic stem cell precursor shared by both lymphoid and myeloid cells. Rather, CD4 has been shown to be an active signaling molecule capable of responding to various stimuli. For example, a complex inositol polyphosphate response and a Ca^{2+} flux are induced in monocytes upon co-cross-linking of CD4 and Fcγ receptors (Guse et al. 1992). IL-16 stimulation of various $CD4^+$ monocytic cell lines induces a translocation of PKC from the cytosol to the membrane (Parada et al. 1996), as well as activation of the SAPK and p38 MAPK signaling pathways (Krautwald 1998). Finally, HIV gp120 induces a CD4-dependent down-regulation of the CCR5 chemokine receptor on the surface of human $CD4^+$ monocytes and of CCR5 and CD4 co-transfected HEK 293 cells (Wang et al. 1998). This effect was not observed for cells which either did not express CD4, or which expressed CD4 whose cytoplasmic tail was missing. Furthermore, the effects of gp120 could be mimicked by mAb to CD4. The exact mechanism by which CD4 and the chemokine receptor cross-talk occurs has yet to be determined.

The observations made in some of these studies cited must, however, be interpreted cautiously with respect to the exact role played by CD4 in the observed responses. In one study, CD4-mediated signal transduction required a coreceptor consisting of the Fcγ receptor

(Guse et al.1992). In the case of studies performed with the chemokine IL-16, it has been shown that while CD4 is required for IL-16 to mediate its effects (Cruikshank et al. 1994), it is unknown whether CD4 is sufficient; CD4 may complex with other receptors to form a larger receptor complex for IL-16 (Center et al. 1995).

We propose that a chemokine receptor may form part of this complex since IL-16 has been shown to induce the down-regulation of CCR5 and CXCR4 on monocyte-derived macrophages (Hermann et al. 1999), while causing the desensitization of CCR5 to MIP-1β in T cells (Vallen Mashikian et al. 1999). Such a CD4-chemokine receptor complex is required for HIV infection of monocytes/macrophages and $CD4^+$ T cells (Feng et al. 1996 Deng et al. 1996, Dragic et al. 1996, Alkhatib et al. 1996, Choe et al. 1996, Doranz et al. 1996). While the participation of known or suspected coreceptors acting in conjunction with monocyte CD4 is similar to the role of CD4 as a coreceptor in T cell receptor signaling, the involvement of these other signaling proteins makes it difficult to dissect out the exact role of CD4 in each of the monocyte signaling studies.

We have established that monocyte CD4 is capable of transducing signals independently of other surface receptors. Thp-1 monocytic cells loaded with Fluo-3/AM and stimulated with T4-4 serum underwent a dramatic Ca^{2+} flux; this response was not observed when T4-4 was adsorbed with sCD4 prior to stimulation, nor did a Ca^{2+} flux occur when the cells were stimulated with an equal amount of an irrelevant rabbit serum to Shp-1. These results establish that the response observed upon T4-4 stimulation is mediated by CD4 and not by Fc receptors. In Guse's study, the use of the α-CD4 mAb MAX.16H5 was insufficient to cause Ca^{2+} flux; this could be achieved only by co-cross-linking CD4 and Fcγ

receptors (Guse et al. 1992). The discrepancy between these studies may be attributed to differences in the relative affinities for CD4 of the mAb and the T4-4 serum used in each study, or to differences in the ability of various CD4 epitopes to mediate signal transduction; this latter scenario has been observed for the CD4 molecule expressed by the Jurkat T cell line (Baldari et al. 1995). It is possible, therefore, that the particular epitope against which the α-CD4 MAX.16H5 mAb was generated is not capable of mediating signal transduction in monocytes, whereas one (or more) of the other epitopes with which polyclonal T4-4 reacts is a more effective mediator of signal transduction.

In addition to a Ca^{2+} flux, we also observed a time-dependent tyrosine phosphorylation of many proteins in response to CD4-mediated stimulation. The CD4-specificity of our phosphotyrosine response was again established by using T4-4 pre-adsorbed with sCD4, which resulted in a decreased level of tyrosine phosphorylation; this dramatic decrease did not occur if BSA was used to pre-adsorb the T4-4 serum. Furthermore, the use of the irrelevant rabbit serum to Shp-1 did not induce as much tyrosine phosphorylation as was observed for the T4-4 stimulation. Finally, the use of CD4 antisense oligodeoxynucleotide to down-regulate CD4 surface expression also resulted in decreased levels of phosphotyrosine in response to T4-4 stimulation.

To elucidate the mechanism by which tyrosine phosphorylation was induced by T4-4 stimulation, we attempted to identify the proteins that underwent tyrosine phosphorylation. It has been reported that PI3-K forms a complex with CD4 and $p56^{lck}$ in human T cells (Prasad et al. 1993). We hypothesized that PI3-K might form a similar complex with monocyte CD4 and some other, as yet unidentified protein(s), and that PI3-K might,

therefore, be one of the phosphoproteins observed in our blots. The tyrosine phosphorylation of PI3-K was confirmed based on our ability to detect it among the immunoprecipitated proteins which were tyrosine phosphorylated in response to T4-4 stimulation.

Based on the observations that T4-4 stimulation resulted in a CD4-specific Ca^{2+} flux, we proposed that another of the tyrosine-phosphorylated proteins observed in the phosphotyrosine blots might be PLC-γ1. The detection of tyrosine phosphorylated PLC-γ1 was not as strong as the detection of tyrosine phosphorylated PI3-K. This may reflect technical factors (such as the larger size of PLC-γ1) which may have influenced the efficiency of the α-phosphotyrosine immunoprecipitation reactions.

Despite our findings that PLC-γ1 and PI3-K were tyrosine phosphorylated in response to CD4 stimulation, we were unable to show any direct interaction between either of these signaling molecules with immunoprecipitated CD4. These findings were not unexpected, however, since the SH2 domains of these and other signaling proteins recognize tyrosine-phosphorylated residues (Moran et al. 1990), and the cytoplasmic tail of CD4 does not contain any tyrosine residues (Maddon et al. 1985). Attempts to immunoprecipitate CD4 and detect a number of other known signaling molecules were also unsuccessful. The use of antibodies to perform immunoprecipitations yielded no information regarding the identity of CD4-associated molecules. The odds were low that we would successfully identify a CD4-associated protein by screening for molecules chosen as potential candidates on the basis of the molecular weights of the observed tyrosine phosphorylated proteins. Technical limitations of using antibodies to immunoprecipitate proteins were also a concern; in early experiments we attempted to immunoprecipitate various signaling molecules and

immunoblot these reactions for the presence of CD4. Unfortunately, the heavy chain of the immunoprecipitating antibody was often detected in the antibody and Sepharose bead controls. Given that the heavy chain and the CD4 molecule are 55 and 56 kDa respectively, it was impossible to determine if the signal observed at this molecular weight was CD4 which came down with the immunoprecipitated signaling molecule, or the heavy chain of the immunoprecipitating antibody. An additional limitation of the traditional immunoprecipitation/immunoblotting technique was that we could probe for only characterized proteins for which commercial antibodies exist; this approach would prove futile in the event that monocyte CD4 associates with proteins which have yet to be characterized.

As an alternative strategy to identify proteins linking CD4 to the PLC-γ1 and PI3-K signaling pathways, we generated a GST-$CD4_{cyt}$ fusion protein for use in far Western blots and immunoprecipitation experiments. In both types of experiments, 45 and 55 kDa proteins were found to associate with the GST-$CD4_{cyt}$ fusion protein, but not with the GST control protein. In the immunoprecipitation experiments, a 35 kDa protein was also observed. These associations occurred regardless of whether the cells had been stimulated with T4-4. Such a constitutive association of CD4 with these proteins is reminiscent of the constitutive association of CD4 and $p56^{lck}$ in T cells (Rudd et al. 1988, Veillette et al. 1988). The identities of the 35, 45 and 55 kDa proteins are currently unknown.

Based on our observations, we propose that in Thp-1 monocytic cells, the cytoplasmic tail of CD4 is constitutively associated with 35, 45 and/or 55 kDa proteins. Upon stimulation with T4-4 serum, a signal is transduced by CD4, resulting in the tyrosine

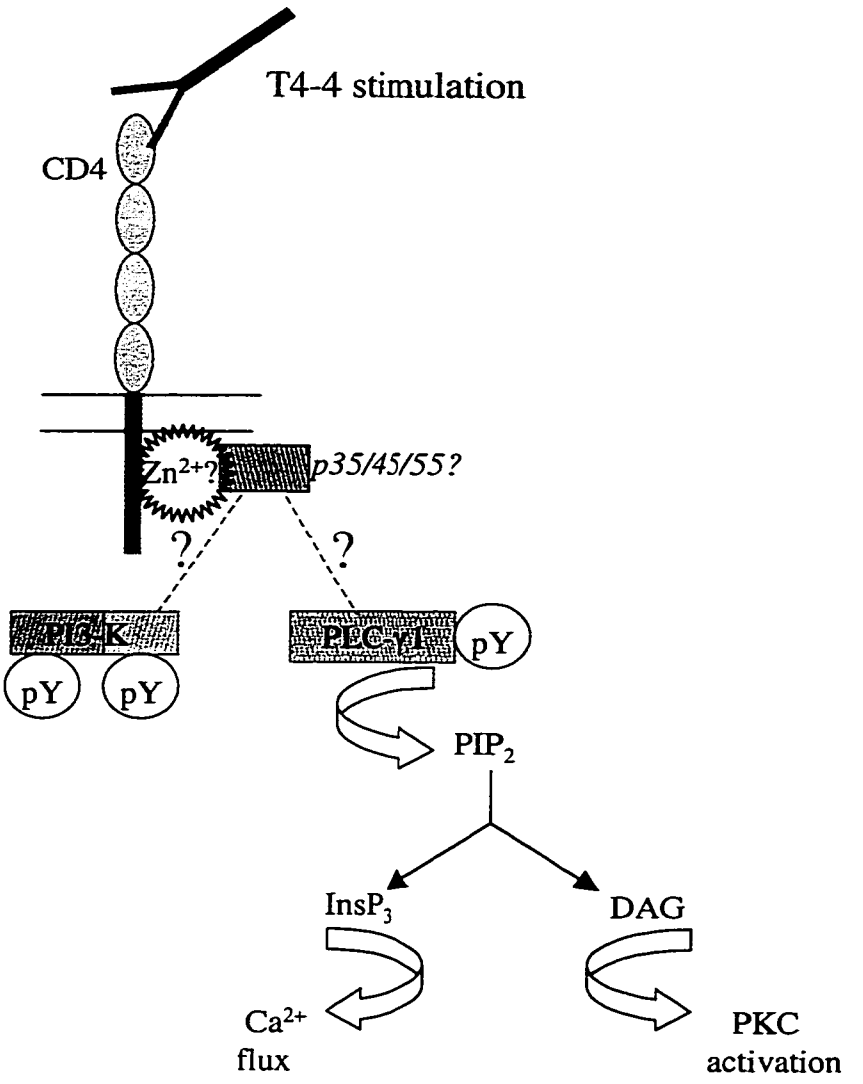
phosphorylation, and therefore, activation, of PLC-γ1 and PI3-K; we presume that the p35, p45 and/or p55 molecules function as linkers/adaptors between CD4 and these signaling molecules. The activated PLC-γ1 will in turn cleave PIP_2, thus activating PKC, and inducing a Ca^{2+} flux (Fig.V.1).

Our study has provided insight into the initial events which occur upon engagement of the CD4 receptor in monocytic cells. The ultimate outcome of this engagement has yet to be determined. Many different signaling pathways have been characterized. Central among these are the serine/threonine kinases which participate in the MAPK pathways. The MAPK pathways can be divided into the ERK pathway, the c-JUN/SAPK pathway, and the p38 MAPK pathway (Kyriakis 1999). The ERK pathway mediates signals in response to mitogens and growth factors, whereas the c-JUN/SAPK and p38 MAP kinase pathways are activated by stress factors such as inflammatory cytokines (IL-1β, TNF-α), ionizing radiation, osmotic shock, heat shock, oxidative stress, and chemical and physiological stresses.

IL-16 stimulation of $CD4^+$ macrophages does not activate the ERK pathway, but does activate p46 and p54 of the SAPK pathway, as well as the p38 pathway of the MAPK pathways (Krautwald 1998). Similarly, in our study, we were unable to detect any activation of ERK-1 and ERK-2, and we found proteins of 45 and 55 kDa to be constitutively associated with our GST-$CD4_{cyt}$ fusion protein. The identities of these 45 and 55 kDa proteins are unknown, but we do not expect them to correspond to the p46 and p54 proteins of the SAPK pathway. These proteins are found downstream in the SAPK pathway, whereas we expect our p45 and p55 molecules to be upstream in their respective pathway(s), given their ability to interact with the cytoplasmic tail of CD4, a membrane glycoprotein.

Fig.V.1. Proposed model of CD4 signaling in Thp-1 monocytic cells.

Based on our observations, we propose that in Thp-1 monocytic cells, the cytoplasmic tail of CD4 is constitutively associated with 35, 45 and/or 55 kDa proteins. Upon stimulation with T4-4 serum, a signal is transduced by CD4, resulting in the tyrosine phosphorylation (pY), and therefore, activation, of PLC-γ1 and PI3-K; we presume that p35, p45 and/or p55 molecules function as linkers/adaptors between CD4 and these signaling molecules. The activated PLC-γ1 will in turn cleave PIP_2, thus activating PKC and inducing a Ca^{2+} flux. The interactions between CD4 and its associated proteins may be mediated by a Zn^{2+} ion.


T4-4 stimulation
CD4
Zn2+?
p35/45/55?
?
?
PI3-K
PLC-γ1
pY
pY
pY
pY
PIP2
InsP3
DAG
Ca2+
flux
PKC
activation

Furthermore, p46 and p54 of the SAPK pathway are kinases, and no CD4-associated kinase activity has been detected in monocytes (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al. 1993). Further studies will be required to determine the identities of the p35, p45 and p55 proteins which were found to associate with our GST-$CD4_{cyt}$ fusion protein, as well as to determine if T4-4 stimulation of Thp-1 cells results in the activation of the SAPK and p38 pathways.

V.3. Concluding remarks

In an attempt to elucidate the regulation and function of monocyte CD4 at the cellular and molecular levels, we performed a systematic study of the down-regulation of monocyte CD4 during the course of monocyte differentiation into tissue culture-derived macrophages, as well as beginning the elucidation of the CD4-mediated signal transduction pathway in Thp-1 cells, a monocytic cell line.

Expression of the CD4 protein by both T cells and/or monocytes/macrophages can be modulated by a number of factors including HIV infection (Hoxie et al. 1986a, Stevenson et al. 1987, Mann et al. 1990, Melendez-Guerrero et al. 1990, Benson et al. 1993), phorbol ester treatment (Solbach et al. 1982, Hoxie et al. 1986b, Ruegg et al. 1992, Pelchen-Matthews et al. 1993), antigenic stimulation (Acres et al. 1986, Weyand et al. 1987) and antibody cross-linking of CD4 (Cole et al. 1989, Morel et al. 1992). In many of these cases, such as for HIV infection and phorbol ester treatment, the cytoplasmic tail of CD4 is phosphorylated on its serine residues by PKC (Acres et al. 1986, Fields et al. 1988, Shin et al. 1990), thus enhancing its endocytosis (Pitcher et al. 1999).

However, not all CD4 down-regulation is PKC-dependent, as has been shown by ganglioside-induced down-regulation of monocytic CD4 (Sorio et al. 1993), and by HIV gp160 (Crise et al. 1990, Jabbar and Nayak 1990, Crise and Rose 1992b, Koga et al. 1994), Vpu (Buonocore and Rose 1990, Willey et al. 1992a, Willey et al. 1992b) and Nef (Rhee and Marsh 1994, Goldsmith et al. 1995, Kim et al. 1999) -induced down-regulation.

We and others (Crowe et al. 1987, Kazazi et al. 1989, Valentin et al. 1990, Valentin et al. 1991, Peudenier et al. 1991b, Pelchen-Matthews et al. 1998, Graziani-Bowering and Filion 2000) have observed CD4 down-regulation in response to monocyte differentiation into tissue culture-derived macrophages. Unlike most of the other stimuli which induce CD4 down-regulation (HIV infection, mAb cross-linking), the down-regulation observed following differentiation may occur independently of an extracellular ligand binding to CD4, as occurs in response to the PKC-activating phorbol esters. Alternatively, CD4 down-regulation in monocyte-derived macrophages may be induced in response to some unidentified ligand synthesized by monocytes as they differentiate into macrophages, or in response to a tissue-specific ligand.

In addition to the removal of CD4 expressed on the cell surface, CD4 down-regulation must also involve the prevention of new CD4 from being expressed. This has been observed to occur at the level of transcription in phorbol ester-treated HL-60 and U937 monocytic cell lines and in freshly isolated monocytes (Pimentel-Muiños et al. 1992). Conversely, a post-translational process was observed for CD4 down-regulation in the case of monocyte-derived macrophages (Kazazi et al. 1989), and for HL-60 cells treated with TNF and IFN-γ (Cassatella et al. 1992).

The mechanism responsible for the inhibition of CD4 synthesis in our system was not determined. Although our original experimental design did include transcriptional studies, such studies require very pure populations of monocytes since the CD4 mRNA contributed by contaminating T cells would be indistinguishable from monocytic CD4 mRNA. The failure of our adherence-based monocyte isolation technique during our study prevented us from pursuing these experiments (we would eventually, however, develop an adherence-independent method for the isolation of monocytes; Graziani-Bowering et al. 1997; Appendix I). It is possible, however, that CD4 transcription would be reduced in our model of monocyte differentiation as this mechanism is the primary mechanism by which thymocytes and mature T cells regulate CD4 expression (Ellmeier et al 1999). However, other mechanisms may also be involved.

The role of CD4 on monocytes is not well defined (Littman 1996, Marsh and Pelchen-Matthews 1996). The best described physiological function to date is that of IL-16 receptor (Cruikshank et al. 1994), although it is unknown if CD4 acts independently, or complexes with other receptors (Center et al. 1995). That CD4 complexed with various chemokine receptors is necessary for HIV infection of cells (Feng et al. 1996, Deng et al. 1996, Dragic et al. 1996, Alkhatib et al. 1996, Choe et al. 1996, Doranz et al. 1996) establishes a precedence for the ability of CD4 to associate with chemokine receptors, which we suspect may also occur in the case of IL-16 binding. Evidence for such a complex is provided by the ability of IL-16 to induce the down-regulation of CCR5 and CXCR4 on monocyte-derived macrophages (Hermann et al. 1999), while causing the desensitization of CCR5 to MIP-1β in T cells (Vallen Mashikian et al. 1999).

We have identified 3 proteins, p35, p45 and p55 as having the ability to directly associate with the cytoplasmic tail of CD4. The identities of these proteins are unknown. Our existing knowledge of CD4 in T cells and in monocytes/macrophages does, however, allow us to speculate on the nature of these proteins. These proteins may include proteins which associate with the cytoskeleton, a scenario reminiscent of $p56^{lck}$ in T cells (Louie et al. 1988). Alternatively, one of these proteins may mediate the association between CD4 and the clathrin-mediated receptor endocytosis pathway (Fig.V.2), as has been observed for the Nef protein of HIV (Piguet et al. 1998). We speculate that the constitutive association of monocyte CD4 with such a protein might provide a mechanism by which CD4 is constitutively endocytosed in myeloid cells (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1998).

Differentiation of monocytes in response to isolation from whole blood and subsequent culture may involve activation of PKC and subsequent phosphorylation of the serine residues of the cytoplasmic tail of CD4 (Fig.V.3a), resulting in enhanced endocytosis (Pitcher et al. 1999), as well as shuttling of the protein to lysosomes instead of the usual recycling from endosomes back to the cell surface (Ruegg et al. 1992, Marsh and Pelchen-Matthews 1996, Pitcher et al. 1999). Alternatively, a PKC-independent pathway (Fig.V.3b) may be involved in which proteins, analogous to the Nef protein of HIV(Piguet et al. 1998) interact with CD4, or possibly, with a CD4-containing complex, to mediate its increased association with clathrin-coated pits and subsequent internalization and degradation. Such proteins may be NF-κB-dependent, and thus be induced during the differentiation process, possibly explaining the time-requirement for CD4 down-regulation.

Fig.V.2. Proposed model of constitutive endocytosis of CD4 in monocytic cells.

The association of the cytoplasmic tail of monocyte CD4 with p35, p45 and/or p55 may increase the affinity of CD4 for clathrin-coated pits, thus contributing to the constitutive endocytosis of CD4 in myeloid cells. Following internalization, CD4 sequestered in early endosomes is recycled back to the cell surface.

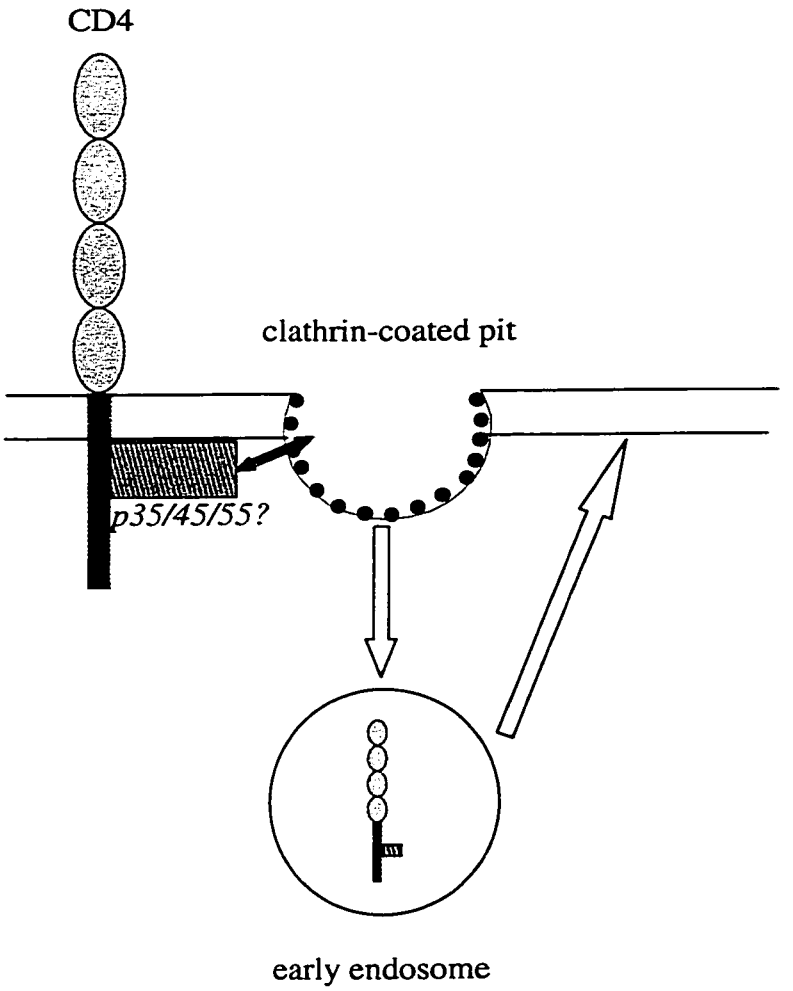
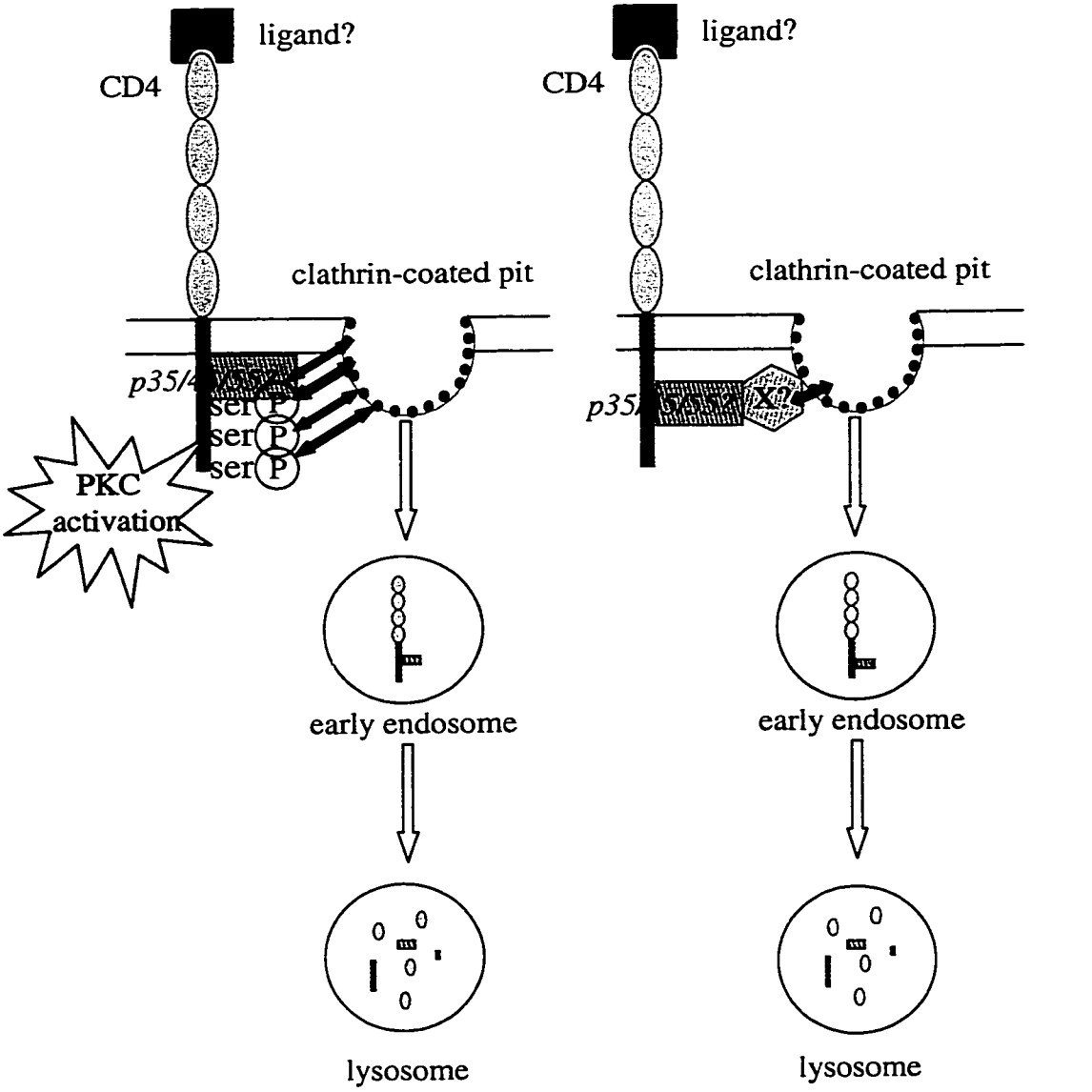

CD4
clathrin-coated pit
p35/45/55?
early endosome

Fig.V.3. Proposed models of CD4 down-regulation during monocytic differentiation.

a) Serine phosphorylation-dependent model. During the cellular differentiation of monocytes into macrophages, PKC is activated, possibly in response to a ligand binding to CD4. The subsequent phosphorylation of serine residues within the cytoplasmic tail of CD4 further enhances the affinity of CD4 for clathrin-coated pits and redirects CD4 from the recycling pathway to the degradative pathway.

b)Serine phosphorylation-independent model. During the cellular differentiation of monocyte into macrophages, a novel protein interacts with the complex formed between CD4 and p35, p45 and/or p55. This protein functions to further enhance the affinity of CD4 for clathrin-coated pits and redirects CD4 from the recycling pathway to the degradative pathway.


a) Serine phosphorylation-dependent
b) Serine phosphorylation-independent
ligand?
CD4
clathrin-coated pit
PKC
activation
early endosome
lysosome
ligand?
CD4
clathrin-coated pit
early endosome
lysosome

As previously discussed, the p35, p45 and/or p55 proteins may function as adaptor proteins in signal transduction (Fig.V.1). Adaptor proteins are small proteins which lack intrinsic enzymatic activity and which may sometimes consist almost entirely of SH2 and SH3 domains (McCarty 1998), as in the case of Grb2 (Lowenstein et al. 1992). Adaptor proteins such as the 23 kDa Grb2 (Lowenstein et al. 1992), the 47 kDa NCK (Lehmann et al. 1990), and the various isoforms of SHC (46-66 kDa; Pelicci et al. 1992) are within the size range of our CD4-associated proteins, although our experiments failed to show a direct association between NCK and CD4. Nonetheless, our CD4-associated proteins may include other adaptor proteins. The possibility that adaptor proteins may associate with CD4 could explain how despite the lack of CD4 intrinsic and associated kinase activity (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al.1993), antibody to CD4 can nonetheless induce tyrosine phosphorylation and a Ca^{2+} flux as was observed in our study. Furthermore, $p56^{lck}$ complexed with CD4 in T cells has been shown to function as an adaptor protein in the case of IL-16-induced chemotaxis (Ryan et al. 1995), as well as in the gp120-induced apoptosis of HIV-infected T cells (Corbeil et al. 1996), despite the intrinsic kinase activity of $p56^{lck}$ (Marchildon et al. 1984, Marth et al. 1985).

The nature of the association between CD4 and these proteins is also unknown. The recent discovery that the association between CD4 and $p56^{lck}$ is mediated by a Zn^{2+} ion (Huse et al. 1998, Lin et al. 1998) may provide a clue as to how CD4 associates with other proteins which have traditional binding motifs unable to mediate an interaction with CD4. An adaptor molecule containing SH2 and/or SH3 domains could bind CD4 via a metal ion and in turn associate with other signaling proteins (Fig.V.1). This association could explain

how CD4 can mediate tyrosine phosphorylation without itself being directly associated with a kinase (Pelchen-Matthews et al. 1991, Vinante et al. 1992, Sorio et al.1993).

The fact that CD4 down-regulation occurs in response to differentiation may reflect a maturation-stage dependent usage of this protein. It is possible that circulating monocytes require CD4 in order to migrate into tissues, in response to IL-16, for instance, and once committed to a tissue, down-regulate the no longer required receptor. We have observed, however, that monocyte CD4 can be slightly re-expressed following 6 days in culture. This may reflect a different function of CD4 at this stage of maturation, such as the possible involvement of CD4 in the production of IL-6, as is observed in the case of jacalin-treated differentiated U937 cells (Taimi et al. 1994). The decreased amounts of CD4 at this stage of development might reflect a different affinity for a different ligand, such as MHC class II molecules which monocytes would probably not encounter in the circulation. However, the interaction of macrophage CD4 with MHC class II molecules is merely speculative at this time (Foti et al. 1995). CD4 has also been shown to interact with Ig (Lederman et al. 1990, Lenert et al. 1990, Lenert et al. 1992, Lenert et al. 1997), an association which in the case of macrophages, could increase the avidity of the antigen/antibody complex for the Fc receptor, thus activating macrophages when antigen levels are low, and/or resulting in increased uptake and processing of antigen (Lenert et al. 1992).

Further studies will be required to identify the p35, p45 and p55 proteins immunoprecipitated by the GST-$CD4_{cyt}$ fusion protein. It would also be of interest to determine if the cytoplasmic tail of CD4 in overnight-cultured monocytes (isolated by the OptiPrep technique - Appendix I) undergoes serine phosphorylation. To date, such studies

have been done only in response to differentiation-inducing agents, such as the nonphysiological phorbol ester treatment. A limiting factor in these types of experiments, however, would be the difficulty in obtaining sufficient numbers of monocytes from individual donors.

We conclude that monocyte CD4 down-regulation occurs in response to cellular differentiation, a process which appears to be more dependent on the actual removal of monocytes from their whole blood environment rather than any of the experimental factors examined. We propose that the *in vitro* culture of monocytes may, at some level, mimic the process of monocyte migration from the circulation to tissues where the cells differentiate into tissue-specific macrophages (Meltzer et al. 1990).

Furthermore, monocyte CD4 is capable of transducing signal, and, as in the case of T cells, appears to do so by associating intracellularly with other proteins. The identities of these proteins, as well as the nature of their association with CD4, are unknown, but may involve Zn^{2+} ion-mediated associations, as has been observed for T cell CD4 and $p56^{lck}$ (Huse et al. 1998, Lin et al. 1998). It is also possible that the CD4-associated proteins may also function as adaptors which link CD4 to the endocytic pathway, thus explaining the constitutive endocytosis of CD4 in myeloid cells (Pelchen-Matthews et al. 1991, Pelchen-Matthews et al. 1998), as well as providing a mechanism for the down-regulation we observed as monocytes differentiate into macrophages.

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VII. APPENDIX I

THE DEVELOPMENT OF A QUICK, EASY AND INEXPENSIVE METHOD FOR THE ISOLATION OF HUMAN PERIPHERAL BLOOD MONOCYTES

VII.1. Introduction

The study of monocytes and tissue culture-derived macrophages is complicated by the difficulty of isolating monocytes from peripheral blood. Monocytes account for approximately 10-20% of PBMC (Jones et al. 1989) and attempts to isolate these cells often result in a high percentage of contaminating lymphocytes. Traditional monocyte isolation techniques include exploiting the ability of these cells to adhere to glass and plastic (Coligan et al. 1994a). However, monocyte isolation by adherence is a labourious and time-consuming technique requiring several hours for the isolation of PBMC, the adherence step, and subsequent washes to remove nonadherent lymphocytes. Furthermore, this technique results not only in low yield (approximately 10% recovery in our isolations), but also in the activation of the monocytes (Coligan et al. 1994a). Although we had used an adherence-based technique for the isolation of monocytes in the CD4 expression studies (Chapter III), changes made by the Red Cross to their citrate anticoagulant formulation resulted in increased adherence of lymphocytes to our gelatin-fibronectin-coated flask surfaces, thus precluding the use of this method in subsequent studies. We were, therefore, forced to examine nonadherence based techniques for the isolation of monocytes.

Other methods used to isolate monocytes require the use of expensive and technically-demanding instruments such as elutriators (counter flow centrifuges) and cell sorters (Coligan et al. 1994a). While the use of these instruments can result in the isolation

of pure fractions of monocytes, the techniques are time-consuming, and in the case of cell sorting, expensive antibodies are required (Coligan et al. 1994a). The latter technique has the added disadvantage that the procedure also activates monocytes; this is avoided in elutriation (Coligan et al. 1994a).

The use of magnetic beads conjugated to monoclonal antibodies is another expensive procedure for the isolation of monocytes. This approach has been used for both the positive (Holz et al. 1991, Mattsson et al. 1993) and negative selection (Aman et al. 1996, Betjes et al. 1993) of monocytes/macrophages. Large quantities of beads are required when isolating monocytes by negative selection given that these cells constitute only a fraction of the total PBMC population (Jones et al. 1989). Positive selection of monocytes using magnetic beads is a more cost-effective approach, however, the mAb-bead complexes often cannot be removed from the isolated monocytes, thus interfering with the analysis of the cell surface markers to which the magnetic bead-conjugated mAb have bound. Furthermore, antibodies against CD14, the molecule most commonly used to identify monocytes, react with only 70-85% of monocytes, depending on the particular epitope against which the α-CD14 mAb is directed (Griffin et al. 1981, Todd III et al. 1981, Dimitriu-Bona et al. 1983). Attempts to isolate monocytes by magnetic beads or cell sorting based on the expression of CD14 would, therefore, lead to the loss of the $CD14^-$ monocyte subpopulation. Since there is no single marker that is both specific for monocytes and is expressed by all monocytes, it is imperative that attempts to isolate monocytes not be based solely on the expression of a single marker. A final disadvantage of the use of magnetic beads to isolate monocytes is that this procedure, like cell sorting and adherence, results in monocyte activation.

Other monocyte isolation techniques include density-gradient centrifugation, which isolates cells on the basis of their size and density. Various gradient-density media, such as sucrose and Percoll, have been used to isolate monocytes, although each of these gradient-density media has its disadvantages (Ford et al. 1994). Specifically, at the concentrations required for cell fractionation, sucrose solutions are hyperosmotic and very viscous, leading to osmotic stress to the cells of interest and slow sedimentation rates, respectively. Percoll, a colloidal silica gradient-density medium, has lower osmolality and viscosity than sucrose, however, relatively high g-forces are required to generate the gradients needed to isolate cells; monocyte isolation requires 21 000 × g (Coligan et al. 1994a). Percoll gradients have the added disadvantage that their constituent colloidal silica particles can be ingested by monocytes (Wakefield et al. 1982), leading to their activation (Coligan et al. 1994a). Furthermore, repeated attempts in our laboratory to isolate monocytes using Percoll resulted in unacceptable levels of lymphocyte contamination.

A density-gradient medium recently introduced by Nycomed Pharma AS, Diagnostic Division, Oslo, Norway (Ford et al. 1994) has proven to be a valuable tool in our attempts to isolate monocytes quickly, easily and inexpensively. We report the successful adaptation of the OptiPrep monocyte isolation technique from a small-scale system to a larger-scale system (Graziani-Bowering et al. 1997). This technique is a convenient, fast (less than 2 hours), relatively simple, and inexpensive alternative to traditional monocyte isolation techniques.

VII.2. Methods and Materials

VII.2.1. Blood collection

Peripheral blood was obtained from healthy lab personnel following receipt of informed consent. For each donor, the blood was collected into a minimum of six 10 ml EDTA (K_3) Vacutainer® tubes (Becton Dickinson) for a minimum blood volume of 50 ml.

VII.2.2. Preparation of reagents

Isolation solutions:

a) HEPES-buffered saline (HBS): 0.8% (w/v) NaCl (BDH), 10 mM HEPES-NaOH, pH 7.4; the HEPES-NaOH solution consisted of a 40 mM stock solution of HEPES (BDH) whose pH had been adjusted to 7.4 with a 1 M NaOH solution (Fisher Scientific, Nepean, ON). Prior to use as the top saline layer, the necessary volume of HBS was syringe-filtered using a 0.2 μm Sterile Acrodisc (Gelman Sciences, Montreal, PQ).

b) Solution A: 1 mM EDTA (BDH) in HBS. Solution A was filter-sterilized using a bottle top 0.2 μm filter (Corning Costar).

c) Solution B (made fresh for each isolation): 0.5% (w/v) BSA (Sigma) in Solution A. Dissolution of the powder in the buffer was allowed to occur at RT for 30 min without stirring to prevent foaming. Once dissolved, Solution B was syringe-filtered using a 0.2 μm Sterile Acrodisc (Gelman Sciences).

d) 1.078 g/ml solution: 1 part OptiPrep and 3 parts Solution B.

e) 1.068 g/ml solution: 1 part OptiPrep and 4 parts Solution B.

The Cy5 component of TRI-colour conjugated mAb binds to specific receptors on the

surface of monocytes (Stewart and Stewart 1994). Fluorolink™ Cy5™ Reactive Dye, which was conjugated to BSA as per the manufacturer's instructions, was used to block this binding as described in section II.5.1.i.

VII.2.3. Preparation of buffy coat

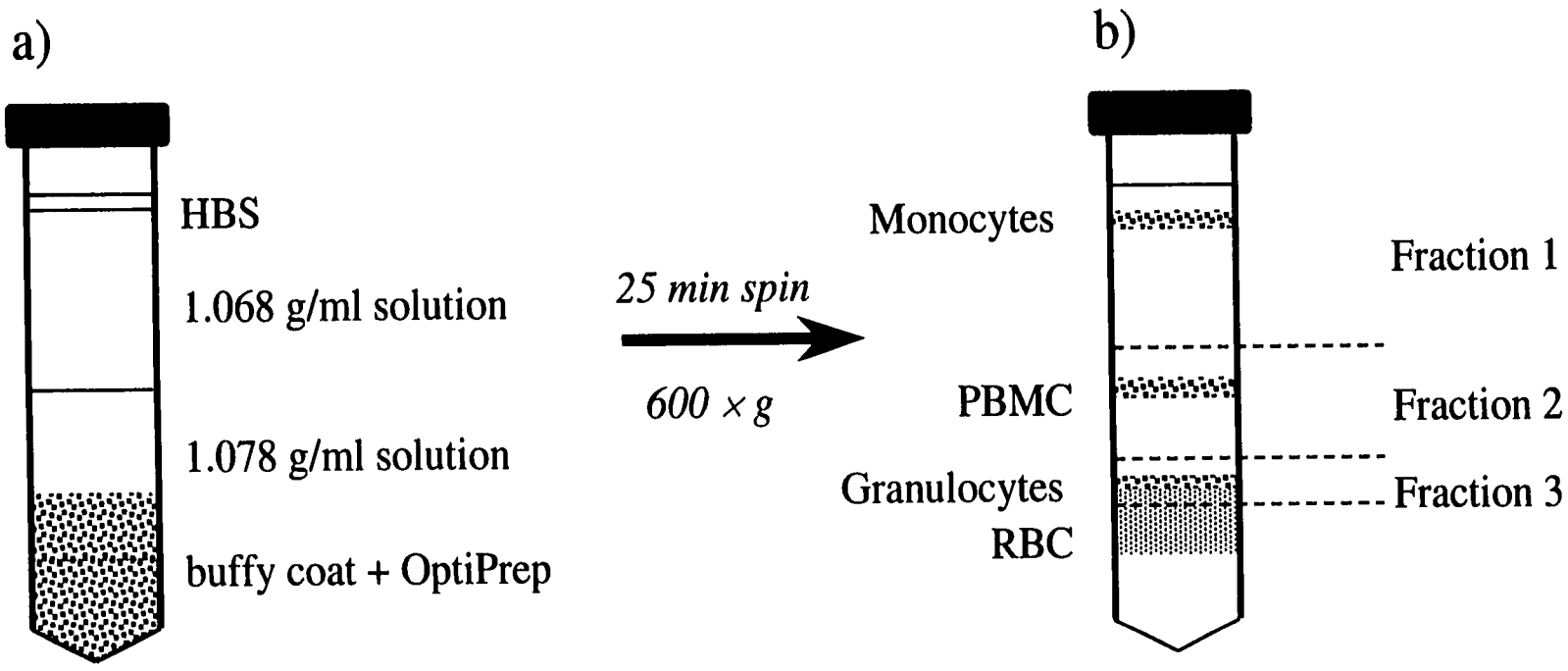
Upon collection of the peripheral blood into EDTA Vacutainers®, the blood was pooled into a 50 ml OPTICUL™ Falcon polypropylene conical tube (Becton Dickinson) and centrifuged for 20 min at RT, 550 × g in an IEC Centra-7R centrifuge with a swinging bucket rotor (International Equipment Co., USA). One ml of the resulting plasma was collected and saved for later use in the antibody-staining procedure, and the remaining plasma was aspirated. Ten ml buffy coat were collected and transferred into a fresh 50 ml polypropylene conical tube, and 500 µl buffy coat were saved for later use in the antibody-staining procedure.

VII.2.4. Monocyte isolation

The OptiPrep solution (generously donated by Nycomed Pharma AS, Diagnostic Division, Oslo, Norway) was mixed well before 4 ml were added to, and mixed with, 10 ml buffy coat. Using a disposable 9 inch glass pipette (Becton Dickinson), the buffy coat/OptiPrep mixture was overlaid with 7.5 ml of the 1.078 g/ml lymphocyte-specific density layer. This layer was overlaid with 20 ml of the 1.068 g/ml solution and 0.5 ml HBS (Fig. VII.1a). Care was taken to prevent mixing of the layers. The buffy coat/OptiPrep mixture was centrifuged for 25 min at RT, 600 × g in an IEC Centra-7R centrifuge with a

Fig.VII.1. Schematic representation (not to scale) of the up-scaled OptiPrep monocyte isolation procedure.

a) The set-up of the buffy coat/OptiPrep mixture and the various density layers. b) The banding patterns of the cells following a 25 min spin at 600 × g, and the corresponding fractions collected for flow cytometric analysis.


a)
HBS
1.068 g/ml solution
1.078 g/ml solution
buffy coat + OptiPrep
25 min spin
600 × g
b)
Monocytes
PBMC
Granulocytes
RBC
Fraction 1
Fraction 2
Fraction 3

swinging bucket rotor; no brake was applied during deceleration. Fractions of the resulting phases were collected as follows (Fig.VII.1b): *first fraction:* the first 18 - 20 ml, with care not to collect any of the WBC band observed at the interface of the 1.078 g/ml and 1.068 g/ml layers; *second fraction:* the next 14 ml (approximately), including the WBC band, but not including any cells at the interface of the 1.078 g/ml and the buffy coat/OptiPrep layers; *third fraction:* the next 6 ml (approximately), including cells at the interface of the 1.078 g/ml phase and the buffy coat/OptiPrep layer. The second and third fractions were collected only for the first 4 isolations in order to determine the relative content of monocytes of the various fractions.

Each fraction was washed with 5 ml PBS (Sigma), 0.1% NaN_3 (BDH) for 5 min at RT, 200 × g. For all isolations, Fraction 1 was aspirated of its supernatant and the cells were resuspended with PBS, 0.1% NaN_3 to a final volume of 1 ml. Fraction 2 was processed as per Fraction 1. For Fraction 3, the supernatant was aspirated and approximately 1 ml cells was collected from the interface of the layer and the PBS, 0.1% NaN_3 supernatant.

VII.2.5. Preparation of cells for flow cytometric analysis

Flow cytometric analysis was performed on whole blood to ensure that each donor's blood contained normal percentages and phenotypes of each leukocyte population. The staining procedure was performed as described in Section II.5. The combination of mAb used is summarized in Table VII.1; each mAb was used at 2.5 µl per test.

Cells from each fraction were also analysed by flow cytometry. The cells were stained using the same combination of mAb used for the whole blood and buffy coat samples.

Table VII.1. Summary of the mAb panel used for the flow cytometric analysis of cells isolated by the up-scaled OptiPrep technique.

Tube # and Purpose	mAb Combination
1. Autofluorescent control	none
2. TRI colour compensation [a]	α-CD45-TRI
3. PE colour compensation [a]	α-CD4-PE
4. FITC colour compensation [a]	α-CD14-FITC
5. Monocyte determination	α-CD45-TRI/α-CD4-PE/α-CD14-FITC
6. Monocyte and granulocyte determination	α-CD45-TRI/α-CD4-PE/α-CD15-FITC
7. T cell determination	α-CD45-TRI/α-CD4-PE/α-CD3-FITC
8. B cell and granulocyte determination	α-CD45-TRI/α-CD20–PE/α-CD15-FITC
9. Assessment of monocyte activation [b]	α-CD45-TRI/α-CD4-PE/α-HLA-DR-FITC

[a] included for whole blood samples only
[b] included only for some experiments

The monoclonal antibodies were obtained from the following companies: α-CD45-TRI (clone BRA-55; Sigma), α-CD4-PE (clone Q4120; Sigma), α-CD14-FITC (clone MY4; Coulter Corp., Hialeah, FL), α-CD3-FITC (clone UCHT-1; Sigma), α-CD20-PE (clone Leu16; Becton Dickinson Immunocytometry Systems, San Jose, CA), α-CD15-FITC (Caltag Laboratories, San Francisco, CA), and α-HLA-DR-FITC (Becton Dickinson Immunocytometry Systems).

The cells were incubated with the mAb for 10 min at RT in the dark. The whole blood, buffy coat and Fraction 3 samples underwent automated RBC lysis using the Multi Q-Prep System (Coulter). Fraction 1 and 2 samples, which contained few RBC, were not subjected to the Q-Prep procedure, and following the 10 min incubation of these samples with mAb, 500 µl PBS, 0.1% NaN_3 were added to each of the tubes from these fractions.

VII.2.6. Viability staining of samples

Two viability assays were included for isolations 5 and 6. These consisted of a standard propidium iodide stain for the assessment of cell membrane integrity, and a commercial live/dead assay. The latter assay used the probes calcein-AM and ethidium homodimer.

The propidium iodide (Sigma) assay was performed using the standard protocol (Coligan et al. 1994b). The cells were analysed by flow cytometry, using autofluorescence as a negative control.

For the live/dead assay, a 4 µM working solution of ethidium homodimer (Molecular Probes, Inc., Eugene, OR) in PBS was prepared from a 12 mM stock solution prepared in

DMSO (Sigma). A 10 nM working solution of calcein-AM (Molecular Probes) in PBS was prepared from a 1 mM stock solution of calcein-AM prepared in DMSO (prepared fresh for each experiment). One hundred and fifty µl working solution of each stain were added to 100 µl cells from Fraction 1, and the cells were incubated in the dark for 45 min at RT. Just prior to flow cytometric analysis of the cells, an additional 25 µl each stain was added to the sample. Colour compensation using cells stained with only calcein-AM was performed prior to analysis of the cells stained with both calcein-AM and ethidium homodimer. Previous standardization of this commercial assay by others in our laboratory had shown that there was no overlap of the ethidium homodimer signal (FL2 LOG) into the calcein-AM signal (FL1 LOG), but some overlap of the calcein-AM signal into the ethidium homodimer signal did occur; colour compensation was, therefore, required for only the calcein-AM stain. For each assay, the autofluorescence tube used as the negative control for mAb staining was also used as a negative control for viability staining.

VII.2.7. Addition of Flow-Count Fluorospheres™ for absolute count determination

Flow-Count Fluorospheres™ (Coulter) were used to perform automated cell counts of the buffy coat and of Fraction 1 in order to determine the number of isolated monocytes and the percentage monocyte yield.

Aliquots of Flow-Count Fluorospheres™ were brought to RT, vortexed for 12 sec, and 100 µl were added to tubes 5 and 6 (refer to Table VII.1) of the buffy coat and Fraction 1 samples which had been brought to RT. The tubes were vortexed for 5 sec upon the addition of the Flow-Count Fluorospheres™, and again prior to the analysis of the samples.

VII.2.8. Flow cytometric analysis of cells

Analysis was performed using the EPICS XL MCL Flow Cytometer (Coulter). This instrument was equipped with an argon laser emitting at 488 nm. Controls included autofluorescence and colour compensation as outlined in section VII.5. A minimum of 1000 events was counted in the monocyte gate for each cell sample analysed.

A cell analysis protocol was established to assess the purity of monocytes in Fraction 1, as well as the degree of contaminating lymphocytes and granulocytes. A FS *vs.* SS histogram was established to visualize the cells, and a CD45 *vs.* SS histogram was used to differentiate cells from debris (Horvatinovich et al. 1996). $CD45^-$ bright events were analysed for their staining by the mAb listed in section VII.2.5 and Table VII.1. in order to determine the purity of the isolated cells.

Flow Count Fluorospheres™ were used for the determination of the number of monocytes within a sample. This determination required the use of a separate protocol which was established according to the manufacturer's instructions. Briefly, a FS *vs.* SS histogram was established to visualize the cells. Two more histograms were also established; an α-CD4-PE *vs.* α-CD14-FITC or α-CD15-FITC histogram for the identification of monocytes, and a FS *vs.* FL3 LOG histogram for the identification of the Flow Count Fluorospheres™, around which a rectilinear gate (the CAL region) was established.

Monocyte concentrations within a sample were obtained by entering the known concentration of the Flow Count Fluorospheres™ into the CAL option of the SYSTEM II™ acquisition and analysis software (Coulter) of the flow cytometer. Once a minimum of 1000 Flow Count Fluorospheres™ events was collected in the CAL region, a monocyte

concentration (X cells/μl) was automatically calculated for the gated monocytes within a sample. These values were used to determine monocyte yield as described in section VII.2.9.

A third protocol was used to assess the viability of the isolated cells using the standard propidium iodide stain, and the commercial calcein-AM/ethidium homodimer live/dead assay. A FS *vs.* SS histogram was established to visualize the cells and a gate was drawn around the cells to separate them from debris. The cells could be distinguished from debris only in this manner since the viability stains fluoresced in the same range as the fluorochrome-conjugated mAb, thus precluding their use as a means of identifying cells or cell populations (ie. monocytes from contaminating lymphocytes).

VII.2.9. Calculations for the determination of monocyte yield

Using the monocyte concentration (X cells/μl) determined by using the Flow Count Fluorospheres™ (as described in VII.2.8), the total number of monocytes within the buffy coat and Fraction 1, as well as the percent monocyte yield in Fraction 1 following the OptiPrep monocyte isolation were determined using the following equations:

Equation #1: Determining the total number of monocytes in 10 ml buffy coat:

X cells/μl × 10 000 μl × 2 *

* where 2 = dilution factor since only 50 μl buffy coat were used to determine the cell concentration, but the automated count assumes 100 μl of sample are used

Equation #2: Determining the total number of monocytes in Fraction 1 (resuspended to 1 ml):

X cells/μl × 1000 μl

Equation #3: Determining the percent monocyte yield:

$$\frac{\text{total number of monocytes isolated in Fraction 1}}{\text{total number of monocytes in buffy coat}} \times 100$$

VII.3. Results

VII.3.1. Assessment of monocyte purity and yield

The results of experiments in which monocytes were isolated using an up-scaled adaptation of the OptiPrep system are reported. The assessment of monocyte purity and yield of these isolations is summarized in Table VII.2.

Histograms based on the flow cytometric analysis of isolation 5A, a representative experiment, are shown in Fig.VII.2. In the first isolation, 88.6% of isolated cells were monocytes, as determined using mAb against CD14 and CD4. The mean monocyte purity of the subsequent isolations (n = 6) in which monocytes were defined as $CD15^{+}$dim $CD4^{+}$dim cells, was 91.7% and ranged from 87.9% to 96.4% (Table VII.2). The mean yield of all isolations was 26.1%, with the individual yields ranging from 10.8% to 41.4%. These values were relative to the number of monocytes in the buffy coat prior to the OptiPrep isolation. The mean number of isolated monocytes per experiment was 3.6×10^{6} monocytes for the first 4 isolations in which 14 ml of buffy coat/OptiPrep mixture were used per isolation, and 2.0×10^{6} monocytes for the last 3 isolations in which half of this volume was used. The degree of contamination by nonmonocytic cells in each isolation is summarized in Table VII.3.

Table VII.2. Assessment of monocyte purity and yield of Fraction 1 in up-scaled OptiPrep isolations, as determined by flow cytometric analysis.

ISOLATION #	% $CD45^+$ BRIGHT EVENTS IN FRACTION 1	% MONOCYTE PURITY [a] (% OF $CD15^+$ DIM $CD4^+$ DIM CELLS)	% $CD14^+$ $CD4^+$ DIM MONOCYTES [a]	NUMBER OF MONOCYTES ISOLATED ($\times 10^6$)	% MONOCYTE YIELD [b]
1	97.1	N.D.	88.6	5.5	29.3
2	95.8	96.4	84.2	0.9	10.8
3	98.1	93.2	82.7	4.4	41.4
4	99.7	91.2	76.6	3.7	15.3
5A [c]	99.0	91.7	76.2	1.6	21.6
5B [c,d]	99.0	90.0	74.7	2.2	29.7
6A [c,d]	97.4	87.9	71.7	2.2	34.4

mean = 91.7	mean = 79.2	mean (#1-4) = 3.6 [e]	mean = 26.1
		mean (#5-6) = 2.0 [f]	

[a] The percentages reported are based on the percentage of $CD45^{+}$ bright events reported in column 2.
[b] The % Monocyte Yield is relative to the number of monocytes within the buffy coat.
[c] These isolations were performed with 7 ml of buffy coat/OptiPrep mixture instead of the usual 14 ml.
[d] These isolations were performed with the 1.074 g/ml lymphocyte-trapping layer instead of the 1.078 g/ml solution.
[e] The mean (#1-4) represents the mean number of monocytes obtained in isolations #1-4.
[f] The mean (#5-6) represents the mean number of monocytes obtained in isolations #5 and 6, which were performed with 7 ml of the buffy coat/OptiPrep mixture.

Fig.VII.2. Flow cytometric assessment of monocyte purity of isolation 5A.

a) The forward *vs.* side scatter profile of cells isolated in Fraction 1; b) The assessment of cellularity of the isolated cells; c) The assessment of monocyte purity as defined by $CD15^{+}$dim $CD4^{-}$dim cells; d) The assessment of T cell contamination as defined by $CD3^{+}$ cells; e) The assessment of B cell contamination as defined by $CD20^{+}$ bright cells; f) The assessment of granulocyte contamination as defined by $CD15^{+}$ bright cells.

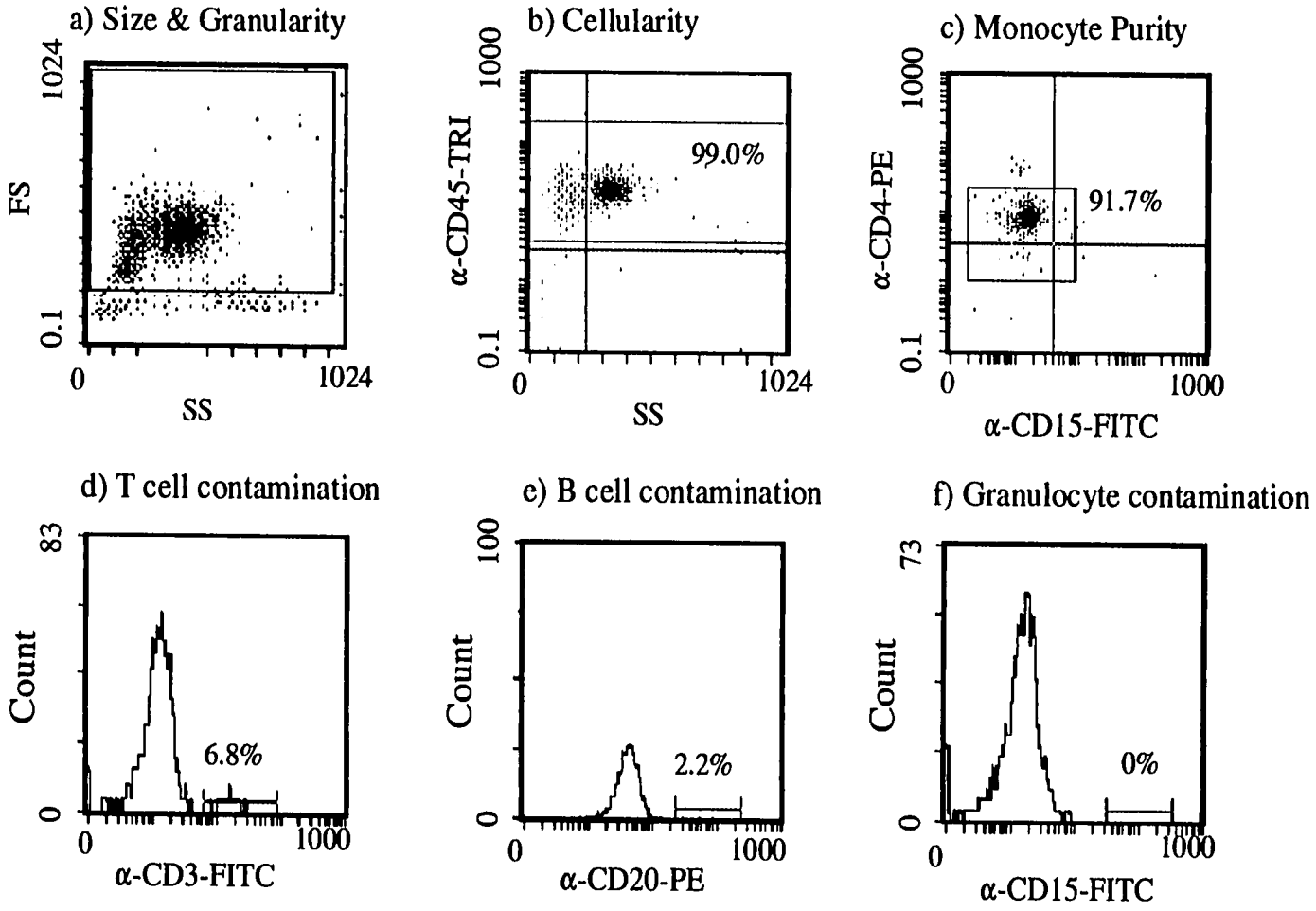

a) Size & Granularity
1024
FS
0.1
0
1024
SS
b) Cellularity
1000
α-CD45-TRI
0.1
99.0%
0
1024
SS
c) Monocyte Purity
1000
α-CD4-PE
0.1
91.7%
0
1000
α-CD15-FITC
d) T cell contamination
83
Count
0
6.8%
0
1000
α-CD3-FITC
e) B cell contamination
100
Count
0
2.2%
0
1000
α-CD20-PE
f) Granulocyte contamination
73
Count
0
0%
0
1000
α-CD15-FITC

Table VII.3. Assessment of nonmonocytic cell contamination of Fraction 1 in up-scaled OptiPrep isolations, as determined by flow cytometric analysis.

ISOLATION #	% CONTAMINATING T CELLS [a] (CD3$^+$ CELLS)	% CONTAMINATING B CELLS [a] (CD20$^+$ BRIGHT CELLS)	% CONTAMINATING GRANULOCYTES [a] (CD15$^+$ BRIGHT CELLS)
1	6.5	2.5	0 [b]
2	2.7	1.2	0 [b]
3	9.1	5.0	0.4
4	9.4	1.8	0 [b]
5A [c]	6.8	2.2	0 [b]
5B [c]	9.8	2.0	1.5
6A [c]	12.6	2.8	0 [b]

[a] The percentages reported are based on the percentage of CD45$^+$ bright events reported in column 2 of Table VII.2.

[b] No distinct CD15$^+$ bright peak was observed.

[c] These isolations were performed with 7 ml of buffy coat/OptiPrep mixture instead of the usual 14 ml.

The other fractions were also assessed for their monocyte content by flow cytometric analysis, the results of which are summarized in Table VII.4. The second fraction included the lymphocyte-trapping layer (1.078 g/ml for isolations 1-4, or 1.074 g/ml layer for isolations 5B and 6A), as well as a small volume from the interface of the lymphocyte-trapping layer and the 1.068 g/ml layers. For the isolations using the 1.078 g/ml lymphocyte-trapping layer, a distinct WBC band was visible at this interface following centrifugation. Flow cytometric analysis of the second fractions revealed that they contained mostly lymphocytes, whereas the third fraction of each isolation, at the interface of the lymphocyte-trapping layer and the buffy coat/OptiPrep mixture, consisted predominantly of granulocytes (Table VII.4).

VII.3.2. Assessment of monocyte activation

The activation of monocytes by the OptiPrep solution was assessed by performing semi-quantitative flow cytometric analysis of HLA-DR expression which is up-regulated in activated monocytes (Beller 1984, Gonwa et al. 1986). HLA-DR levels of isolated monocytes were compared to baseline levels established for buffy coat monocytes. In the 2 experiments examined, the levels of monocyte HLA-DR expression before and after contact with OptiPrep were comparable (Table VII.5).

VII.3.3. Assessment of monocyte viability

Viability assays were performed to determine if the OptiPrep solution was toxic to the cells. The viability of isolated monocytes was assessed using a standard propidium

Table VII.4. Flow cytometric analysis of Fractions 2 and 3 in up-scaled OptiPrep monocyte isolations.

	FRACTION 2		FRACTION 3	
ISOLATION #	% OF MAJOR CELL POPULATION [a]	% MONOCYTES [a,b]	% OF MAJOR CELL POPULATION [a]	% MONOCYTES [a,b]
1	47.2% $CD3^+$ T cells	31.2 [c] ($CD14^+$ $CD4^+$ dim cells)	87.1% $CD15^+$ bright granulocytes	1.8 [c] ($CD14^+$ $CD4^+$ dim cells)
2	52.3% $CD3^+$ T cells	22.5	57.0% $CD15^+$ bright granulocytes	2.4 (based on FS *vs.* SS profile)
3	66.8% $CD3^+$ T cells	8.1 [c] ($CD14^+$ $CD4^+$ dim cells)	66.5% $CD15^+$ bright granulocytes	1.3 [c] ($CD14^+$ $CD4^+$ dim cells)
4	55.0% $CD3^+$ T cells	22.1	75.5% $CD15^+$ bright granulocytes	0 [d]

[a] The percentages reported are based on the percentage of $CD45^+$ bright events reported in column 2 of Table VII.2.

[b] Monocytes were defined as $CD15^+$ dim $CD4^+$ dim unless stated otherwise:

[c] no $CD15^+$ $CD4^+$ data was available

[d] no distinct $CD15^+$ dim $CD4^+$ dim population was observed

Table VII.5. Assessment of cell viability and activation of monocytes obtained in up-scaled OptiPrep isolations, as determined by flow cytometric analysis.

	LIVE/DEAD ASSAY (CALCEIN-AM AND ETHIDIUM HOMODIMER STAINING)			PROPIDIUM IODIDE ASSAY	MONOCYTE HLA-DR EXPRESSION	
					BUFFY COAT	FRACTION 1
ISOLATION #	% VIABLE	% DYING	% DEAD	% POSITIVE	MC# [a]	MC# [a]
5A	95.8	4.2	0	6.1	13.0	15.9
5B	98.2	1.8	0	5.1	13.0	11.6
6A	98.4	1.4	0	1.6	11.0	10.3

[a] The mc# represents the mean channel number for staining of monocytes with an α-HLA-DR-FITC mAb.

iodide assay and a commercial live/dead assay (Table VII.5). In 2 experiments, 1.6 to 6.1% of isolated monocytes stained with propidium iodide, a marker for compromised cell membranes. In these same experiments, 1.4 to 4.2% of isolated monocytes stained with both calcein and ethidium homodimer, indicating a dying state. The remaining cells were alive based on their staining only with calcein. The flow cytometric analysis of isolation 5A is shown in Fig.VII.3. In this isolation, 95.1% of the cells were calcein$^+$ and ethidium homodimer$^-$ and were, therefore, alive (Fig.VII.3a). Two populations of dying cells were observed on the basis of their staining with ethidium homodimer and their continued ability to hydrolyse calcein-AM into calcein. One population (2.9%) showed early signs of death, whereas the second population of cells (1.2%) had progressed further towards death. The standard propidium iodide stain for membrane integrity indicated that 6.1% of the cells had compromised membranes (Fig.VII.3b). This value of 6.1% was consistent with the value of 4.1% ethidium homodimer$^-$ cells observed in the live/dead assay.

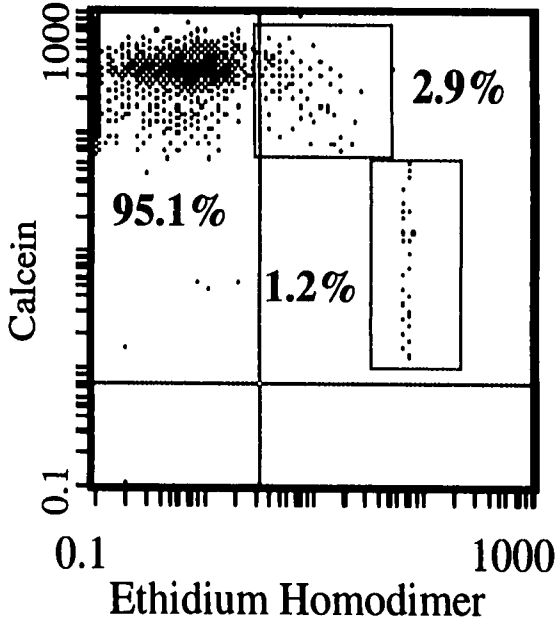
VII.4. Discussion

OptiPrep, a new density-gradient medium, consists of a sterile, endotoxin-negative solution of 60% Iodixanol in water, and has a density of 1.32 g/ml (Graham et al. 1994). Iodixanol has been used for the isolation of murine liver organelles (Graham et al. 1994), caveolae membranes (Smart et al. 1995) and plasma lipoproteins (Graham et al. 1996), as well as for the subfractionation of hepatocyte endoplasmic reticulum (Cartwright et al. 1997). We have used OptiPrep, the commercial preparation of Iodixanol, for the isolation of human monocytes from peripheral blood. The efficacy of the monocyte isolation method relies on the generally lower median density and larger size of monocytes compared to lymphocytes

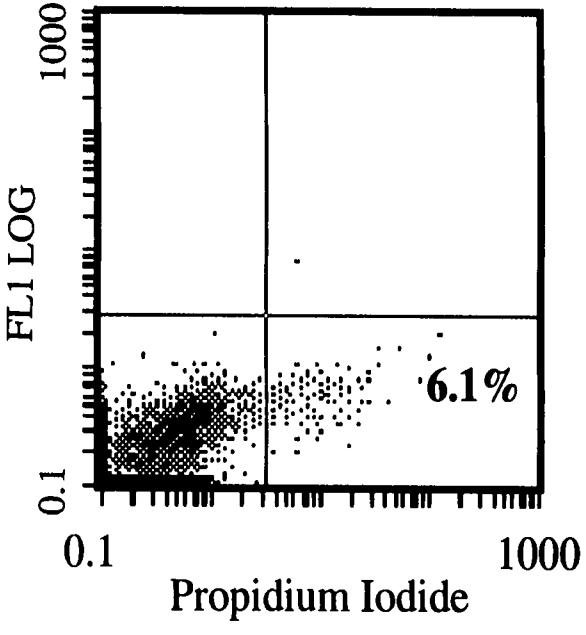
Fig.VII.3. Flow cytometric assessment of cell viability in isolation 5A.

a) A live/dead assay was used to distinguish metabolically active cells from dead cells. In this isolation, 95.1% of the cells were alive based on their ability to hydrolyse calcein-AM into calcein. A total of 4.1% of the cells were dying, based on their ability to hydrolyse calcein-AM and stain with ethidium homodimer. No dead cells were present, given the absence of cells stained only by ethidium homodimer. b) A standard propidium iodide stain for membrane integrity was performed and indicated that 6.1% of the cells had compromised membranes. This value is consistent with the 4.1% ethidium homodimer-stained cells observed in the live/dead assay.

a) Live/Dead Assay



b) Membrane Integrity Assay



(1.064 *vs.* 1.073 g/ml, and 15-20 *vs.* 6-20 μm, respectively) (Centrifugation techniques III: Isolation of blood cells (1995), p. 16; Nycomed Pharma literature). Thus, in this system in which a buffy coat fraction is layered beneath a double-density barrier of 1.078 and 1.068 g/ml, at least some of the monocyte population should float to the surface of the 1.068 g/ml layer more rapidly than most of the lymphocytes. The isolation of monocytes by this procedure requires a centrifuge with a swinging bucket rotor capable of achieving 600 × g, a relatively low g-force in comparison to the 21 000 × g required for Percoll isolation of monocytes (Coligan et al. 1994a).

The adaptation of the OptiPrep isolation procedure to a larger volume than originally recommended by the manufacturer led us to the conditions outlined in Materials and Methods. These conditions resulted in the successful isolation of monocytes.

Monocytes are routinely identified on the basis of CD14 expression (Goyert et al. 1989). However, antibodies against CD14 react with only 70-85% of monocytes, depending on the particular epitope against which the α-CD14 mAb is directed (Griffin et al. 1981, Todd III et al. 1981, Dimitriu-Bona et al. 1983). Previous work in our laboratory using Coulter's α-CD14 mAb against the Mo2 epitope showed a range of 58.7% to 85.8% of cells in the monocyte gate as being $CD14^+$ (n = 5 patients sampled 3 times; Dr. L.G. Filion; unpublished results). A more accurate marker is CD15 which is expressed on most monocytes, as well as on all granulocytes, although these latter cells express much higher levels (Skubitz et al. 1989). The combination of α-CD15 and α-CD4 mAb is, to date, the best means of identifying monocytes exclusively (Dr. L.G. Filion; unpublished results). In the first experiment however, no α-CD15 mAb was included in the staining panel and

monocytes were identified and quantified by their dual expression of CD14 and CD4. The value of 88.6% monocyte purity in this isolation is nonetheless quite accurate given that the sum of the contaminating nonmonocytic cells is 9.0% (Table VII.3), thus allowing us to account for almost 100% of all isolated cells. All subsequent isolations included the use of an α-CD15 mAb in conjunction with an α-CD4 mAb; the average purity of these isolations was 91.7% (n=6). The inadequacy of CD14 staining for the identification of monocytes is illustrated by the significantly lower average purity of 79.2% (n = 7; Table VII.2) obtained when these cells were identified on the basis of CD14/CD4 expression.

The average yield of the first 3 up-scaled isolations was 27.2%, which is typically higher than yields obtained by some traditional monocyte isolation techniques such as adherence. Flow cytometric analysis of Fractions 2 and 3 (Table VII.4) revealed that the majority of the monocytes remained in the second fraction as seen by the significant number of monocytes in Fraction 2, and the almost complete absence of monocytes in Fraction 3.

There are many possible reasons for the failure of many of the monocytes to float to the top of the 1.068 g/ml layer. The rational behind this isolation technique is that monocytes, being less dense and larger than lymphocytes, have a higher flotation rate than lymphocytes. However, both cell types demonstrate considerable heterogeneity of both of these parameters and undoubtedly, smaller monocytes and larger lymphocytes have very similar flotation properties. Donor-specific factors may also be involved since the actual density and size range of monocytes (and lymphocytes) may vary from individual to individual. The manipulation of the cells during the isolation procedure might also lead to changes in the size and density of the monocytes. Furthermore, because particles concentrate

at density interfaces, the majority cell types (granulocytes and/or lymphocytes) may effectively trap the minority cell type (monocytes) at each of the density interfaces, especially in cases where the buffy coat contained many cells.

Several approaches were undertaken to increase monocyte yield. These approaches included increasing centrifugation time (to give monocytes longer to float to the 1.068 g/ml layer), using only half of the buffy coat/OptiPrep volume (to decrease cell load), reducing the volume of the 1.078 g/ml layer from 10 ml to 7.5 ml (to decrease the distance through with the monocytes had to float to reach the 1.068 g/ml layer), changing the density of the lymphocyte-trapping solution from 1.078 g/ml to 1.074 g/ml (to reduce the density difference between the lymphocyte and monocyte density-specific layers, thus reducing the concentration of cells at the interface of these layers), and subjecting the second fraction of cells to another round of isolation.

Increasing centrifugation time did not, however, increase monocyte yield, and resulted in increased lymphocyte contamination. A 35 min centrifugation resulted in 22.4% lymphocyte contamination (isolation 6B), compared to only 14.4% lymphocyte contamination for a parallel 25 min spin (isolation 6A; Table VII.2).

The use of 7 ml of buffy coat/OptiPrep instead of 14 ml had no effect on monocyte yield, but did not adversely affect monocyte purity (isolations 5A, 5B and 6A; Table VII.2).

The reduction in volume of the 1.078 g/ml layer did not increase monocyte yield (isolations 4 and 5A; Table VII.2), but since this change did not affect monocyte purity, and since it minimized the amount of reagents required, this modification was incorporated into the protocol.

Reducing the density of the lymphocyte-trapping solution from 1.078 g/ml to 1.074 g/ml gave variable results with respect to monocyte yield and purity. In isolations 5B and 6A, monocyte purities of 90.0% and 87.9% $CD15^+$ $CD4^+$ cells and yields of 29.7% and 34.4% were obtained, respectively (Table VII.2). However, isolations from other donors in subsequent experiments were less successful. In one experiment, monocyte purity was high at 90.8% $CD15^+$ $CD4^+$ cells, but yield was very low at 2.7%. In another experiment, monocyte purity was only 81.4% $CD15^+$ $CD4^+$ cells and the number of monocytes isolated was very low at only 0.18×10^6 $CD15^+$ $CD4^+$ cells. Although no percent yield could be calculated for this experiment because of a malfunctioning Q-prep which resulted in the loss of the buffy coat data required for the calculation of percent yield, the number of cells obtained was approximately one log lower than the number of cells obtained in the successful experiments listed in Table VII.2.

Finally, an attempt was made to isolate monocytes trapped in the second fraction by subjecting this fraction to a second round of isolation. This approach was unsuccessful, however; $CD15^+$ $CD4^+$ monocytes accounted for only 23.5% of the re-isolated cells.

The final standardized protocol is as outlined in Materials and Methods. This protocol can be used for the successful isolation of monocytes from 14 or 7 ml of buffy coat/OptiPrep mixture.

Most monocyte isolation techniques result in cell activation. This is especially true when monocytes are isolated by adherence, magnetic beads, and flow cytometric sorting, but less so when monocytes are isolated by elutriation (Coligan et al. 1994a). An increase in HLA-DR expression of monocytes is usually indicative of activation (Beller 1984, Gonwa

et al. 1986). Monocytes isolated by the OptiPrep method were assessed for HLA-DR expression by flow cytometric analysis. HLA-DR expression levels by monocytes in the buffy coat fraction were used as baseline levels, and the level of expression was determined following the isolation of monocytes in Fraction 1. In all 3 isolations examined, no significant increase in HLA-DR expression was observed following the isolation of the monocytes, as indicated by the comparable mean channel values observed (Table VII.5). Given that Iodixanol, the main compound of OptiPrep, is known not to interact with, or bind to, cells (Biological Separations Issue no. 1, 1995; Nycomed Pharma news bulletin), these results were as expected.

Originally used in humans as an X-ray contrast medium, Iodixanol has undergone rigorous clinical testing (Ford et al. 1994). OptiPrep has also been assessed for possible cytopathic effects on cells. As expected, no measurable cytotoxicity was found for OptiPrep-isolated bull spermatozoa, nor for MOLT-4 T cells cultured in the presence of OptiPrep (Ford et al. 1994). The viability of monocytes isolated using the OptiPrep system was assessed using a live/dead assay, as well as with a propidium iodide assay for cell membrane integrity. The live/dead assay allows for the determination of the metabolic state of cells: viable, dying, or dead. The propidium iodide assay was also performed since it is an established technique for the determination of compromised cell membrane integrity (Coligan et al. 1994b).

The two assays were each performed on cells from 3 isolations (Table VII.5). In all cases, more than 95% of the cells were alive, based on their staining for calcein but not for ethidium homodimer. A small (< 5%) population of dying cells (calcein$^-$ and ethidium

homodimer$^+$) were also observed for each isolation. No dead cells (calcein$^-$ and ethidium homodimer$^+$) were observed for any of the isolations. The standard propidium iodide assay for cell membrane integrity gave comparable values to those obtained with ethidium homodimer (Table VII.5). Both assays clearly show that the conditions used for the isolation of monocytes by the OptiPrep technique are not toxic.

Despite the retention of the majority of the monocytes within the second fraction of each isolation, and despite the somewhat wide range of monocyte yield, we have successfully adapted a previously established small-scale monocyte isolation technique to a more useful large-scale procedure. The high degree of monocyte purity obtained by this technique was at least equal to, and often exceeded, the purity obtained by other techniques. Furthermore, this procedure precludes the requirement for expensive and/or technically-demanding instruments such as elutriators, cell sorters and ultracentrifuges. The cost of the OptiPrep solution required per isolation is less than $10 (Canadian), much less than the cost of the large quantities of monoclonal antibodies required for cell sorting or for magnetic bead isolations. These techniques have the added disadvantage that the α-CD14 mAb (in the case of cell sorting) or mAb-bead conjugates (in magnetic bead isolations) used to isolate monocytes often cannot be removed, thus leading to continued cell activation and/or interference with the analysis of monocyte CD14. Furthermore, because not all monocytes express CD14 (Griffin et al. 1981, Todd III et al. 1981, Dimitriu-Bona et al. 1983), the use of this marker in the isolation of these cells would result in the loss of the CD14$^-$ monocyte subpopulation, leading to further decreases in monocyte recovery.

The OptiPrep monocyte isolation technique also has advantages over standard

adherence assays for the isolation of monocytes. The OptiPrep technique can be completed in less than 1.5 hours, which is significantly less than the standard adherence procedure which requires the isolation of PBMC by Ficoll-Paque, subsequent washes, the adherence step (a minimum of 1 hour), and the subsequent washes to remove nonadherent lymphocytes (Coligan et al. 1994a).

A disadvantage of the OptiPrep system is the requirement for EDTA as the anticoagulant into which the blood is collected. The exact reason for the incompatibility of other anticoagulants with the OptiPrep system is unknown. One possible explanation in the case of heparin is that this anticoagulant has been known to cause white blood cells to clump (Brown et al. 1993); cell clumping could interfere with the flotation of the cells to the appropriate density layer.

Regardless of the requirement for EDTA, we conclude that our up-scaled OptiPrep monocyte isolation system is a convenient, fast, relatively simple, and inexpensive alternative to traditional monocyte isolation techniques. Furthermore, the resulting monocyte purity and yield obtained by this up-scaled OptiPrep system at least equalled, and often exceeded, those obtained by these other techniques. In the event that an isolation should contain an unacceptable degree of lymphocyte contamination, magnetic beads could be used to remove the unwanted cells. The monocytes obtained by this technique are viable and have not been activated.

Not only does our work represent the first report in the literature of the use of the OptiPrep system for the isolation of monocytes, but we are also the first to up-scale the original small-scale procedure for the purpose of increasing monocyte yield. Also, our work

represents the first time that the purity of OptiPrep-isolated monocytes has been assessed by flow cytometric analysis, an automated, highly quantitative technique, instead of the less accurate nonspecific esterase staining. Finally, this technique replaced the adherence-based technique previously used in our laboratory, allowing for the continued study of monocytes by other students.

VIII. APPENDIX II

A. SOLUTIONS

Coomassie Brilliant Blue Solution

50% Omnisolve distilled methanol (BDH)
0.05% Coomassie Brilliant Blue G-250 (Bio-Rad)
10% glacial acetic acid (BDH)

Destaining solution

5% Omnisolve distilled methanol (BDH)
7% glacial acetic acid (BDH)

6X DNA Loading Buffer

60 mM Tris, pH 8 (Fisher Scientific)
6 mM EDTA, pH 8 (BDH)
30% glycerol (BDH)
A dab of bromophenol blue (Sigma) and xylene cyanol FF (Sigma) powders were added to colour the solution.
The solution was vortexed and syringe-filtered.

Glutathione elution buffer

10 mM reduced glutathione (Sigma)
50 mM Tris-HCl, pH 8 (Sigma)

Lysis buffer

20 mM Tris-HCl, pH 8 (Sigma)
150 mM NaCl (BDH)
1% (v/v) NP-40 (Sigma)
10 % (v/v) glycerol (BDH)
10 mM NaF (Sigma)
25 µg/ml aprotinin (Sigma)
25 µg/ml leupeptin (Sigma)
1 mM sodium orthovanadate (Sigma)

RIPA lysis buffer

0.1% SDS (Sigma)
1% sodium deoxycholate (Sigma)
1% Triton X-100 (Fisher Scientific)
150 mM NaCl (BDH)
2 mM EDTA (Sigma)
24 mM Tris-HCl, pH 8.3 (Sigma)
25 µg/ml aprotinin

25 µg/ml leupeptin
1 mM sodium orthovanadate

6X RNA loading buffer
50% glycerol
1mM EDTA
0.25% bromophenol blue
0.25% xylene cyanol FF
The solution was prepared in DEPC-treated ddH_20.

SOC medium (Gibco BRL)
2% w/v tryptone
0.5% w/v yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose

Stripping solution
0.7 mM DTT (Sigma)
62.5 mM Tris-HCl, pH 6.7 (Sigma)
100 mM 2ME (Sigma)
2% SDS (Bio-Rad)

1X TAE
40 mM Tris-acetate (Sigma)
1 mM EDTA (Sigma)

1X TBST
10 mM Tris-HCl, pH 8 (Sigma)
150 mM NaCl (BDH)
0.05% Tween-20 (Sigma)

1X TE
10 mM Tris-HCl, pH 8 (Sigma)
1 mM EDTA, pH 8 (Sigma)

2YT agar
3.1% w/v 2YT medium (Gibco BRL)
1.5% w/v Bacto-Agar (Difco Laboratories, Detroit, MI)
The solution was sterilized by autoclaving.

2YT broth

3.1% w/v 2YT medium* (Gibco BRL)
The solution was sterilized by autoclaving.

*2YT medium = SELECT Peptone 140 (an enzymatic digest of casein) + SELECT Yeast Extract + NaCl

B. TECHNIQUES

I. Conjugating BSA to Fluorolink™ Cy5™ Reactive Dye

200 mM Na_2CO_3 (Sigma) and 317 mM $NaHCO_3$ (Sigma) solutions were used to prepare a buffer consisting of 16% v/v Na_2CO_3 and 34% v/v $NaHCO_3$. A 1 mg/ml solution of BSA in buffer was prepared and allowed to sit at RT for 15 min to dissolve. One ml BSA solution was added to 1 mg Cy5 and incubated for 1 hour at RT, with inversion every 10 min. Unconjugated dye was removed by dialysis. Dialysis tubing (Fisher Scientific, Nepean, ON) with a molecular weight cut-off of 10 000 was used, and the dialysis was performed overnight at 4°C using PBS, 0.1% NaN_3 as the separation solution. The final concentration of the BSA-Cy5 conjugate was 1 mg/ml.

II. Immobilizing proteins onto NHS-activated Sepharose 4 Fast Flow Beads

One ml NHS-activated Sepharose 4 Fast Flow Beads (Pharmacia Biotech) was washed three times for 5 min at 4°C, 200 × g with 10-12 ml cold 1mM HCl. Following the third wash, the supernatant was removed and an equal volume of *1X coupling buffer* [0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3] was added to the beads to give a 50% slurry.

One hundred to 200 µl of the peptide to be cross-linked to the washed NHS-activated

Sepharose beads were added to a 2 ml screwcap tube; if the peptide was lyophilized, it was dissolved with *phosphate buffer* (0.01 M $NaPO_4$, pH 7.3; Sigma). Three hundred µl *1X coupling buffer* was added to the peptide and the mixture was centrifuged for 5 min at 4°C, 9 850 × g. One ml of the 50% NHS-activated Sepharose bead slurry was added to the peptide/buffer mixture and the reaction was inverted for 2 hr at RT. Five hundred µl *blocking buffer* (0.5 M ethanolamine, pH 8.3; Sigma) was added to the reaction and the reaction was inverted for 1 hour at RT. The reaction was centrifuged for 5 min at RT, 200 × g. The supernatant was removed and the reaction was washed with 1 ml 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 (high pH wash) for 5 min at RT, 200 × g. The supernatant was removed and the reaction was washed with 1 ml 0.1 M sodium acetate (Sigma), 0.5 M NaCl, pH 4.0 (low pH wash) for 5 min at RT, 200 × g. The high pH, low pH washes were repeated for a total of 6 wash cycles. The final pellet was resuspended with an equal volume of *phosphate buffer* to give a 50% bead slurry.

III. Isolation of PCR product using the Qiaex II Agarose Gel Extraction Kit

The PCR product consisting of the cytoplasmic tail of CD4 with 5' BamHI and 3' EcoRI extensions was purified using the Qiaex II agarose gel extraction kit. The desired DNA bands were excised from the agarose gel, with care to minimize the excess agarose. Each gel slice was transferred into separate 1.5 ml Eppendorfs and weighed. To each Eppendorf, 3 volumes (µl) Buffer QX1 were added for every 1 volume (mg) of agarose. The Qiaex II beads were resuspended by vortexing for 30 sec. Twenty µl Qiaex II beads were added to each sample. The samples were incubated for 10 min in a 50°C water bath, with

vortexing every 2 min. Using Litmus paper, the pH of each sample was checked to ensure a pH of $\leq$ 7.5. The samples were centrifuged for 30 sec at RT, 10 000 × g. The supernatants were removed and replaced with 500 µl Buffer QX1. The samples were vortexed, then centrifuged for 30 sec at RT, 10 000 × g. All traces of supernatant were removed. Five hundred µl Buffer PE were added to each pellet; Buffer PE was prepared by adding 96-100% ethanol to the PE concentrate, according to directions on the bottle. The pellets were vortexed, then centrifuged for 30 sec at RT, 10 000 × g. All residual supernatant was removed, and the pellets were washed again with Buffer PE. Following the second wash, the supernatants were removed and the pellets were air dried at RT for approximately 20 min, or until they became white. The DNA was eluted from the pellets by the addition of 25 µl 10 mM Tris-HCl, pH 8. The samples were vortexed and allowed to sit at RT for 5 min. The samples were centrifuged for 30 sec at RT, 10 000 × g. The DNA-containing supernatants were transferred to fresh Eppendorfs. A second elution step was performed on the pellets, and the resulting eluates were pooled with those from the first elution step. An aliquot of the isolated DNA was checked on an agarose gel.

IV. PCR purification of digested PCR product using the Qiaquick PCR Purification Kit

Following the confirmation of the successful digestion of the PCR product, the digested PCR product was isolated using the Qiaquick PCR Purification Kit (Qiagen). To each digested volume, 5 volumes of Buffer PB were added. The samples was added to Qiaquick columns inserted into 2 ml collection tubes. The samples were centrifuged for 45

sec at RT, 10 000 × g. The flow-through from the collection tubes was discarded. The columns were re-inserted into the collection tubes, and the digested DNA was washed with 750 µl Buffer PE (wash buffer) which was prepared by adding 96-100% ethanol to the PE concentrate, according to directions on the bottle. The samples were centrifuged for 45 sec at RT, 9 850 × g. The flow-through was discarded from the collection tubes and the columns were re-inserted back into the collection tubes and centrifuged for 1 min at RT, 9 850 × g. The residual flow-through was discarded and the columns were transferred to 1.5 ml Eppendorfs. The DNA was eluted by the addition of 30 µl Buffer EB (elution buffer) to the *center* of the columns. The samples were incubated for 1 min at RT, and centrifuge for 1 min at RT, 9 850 × g. Aliquots of the eluted DNA collected into the Eppendorfs were checked on an agarose gel.

V. Phenol:chloroform extraction of DNA

DNA bands were excised from agarose gels and the slices were transferred to 1.5 ml Eppendorf tubes to which 300 µl 20 mM TE were added. The DNA/TE mixtures were incubated for 20 min in a 50°C water bath; the tubes were shaken periodically. One volume of equilibrated phenol (Gibco BRL) was added to the tubes and the tubes were vortexed. The mixtures were centrifuged for 5 min at RT, 10 000 × g. The DNA-containing aqueous phases was transferred to fresh 1.5 ml Eppendorfs to which an equal volume of 25:24:1 (v/v/v) phenol/chloroform (BDH)/isoamyl alcohol (Sigma) was added. The tubes were vortexed, then centrifuged for 2 min at RT, 10 000 × g. The DNA-containing aqueous phases was transferred to fresh 1.5 ml Eppendorfs for a second round of extraction with

25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol. The DNA-containing aqueous phases were subjected to ethanol precipitation by the addition of a 1/10 volume of cold 7.5 M ammonium acetate (Sigma) and 2 volumes of cold 95% ethanol (RDL Alcohols, a division of Rieder Distillery, Grimbsy, ON). The DNA was precipitated on dry ice at -80 °C for at least 30 min. The tubes were centrifuged for 15 min at RT, 10 000 × g. The supernatants were removed and 100 µl of cold 70% ethanol were added. The DNA was precipitated on dry ice at -80 °C for 15 min. The tubes were centrifuged for 15 min at RT, 10 000 × g. The ethanol was removed and the tubes were covered with parafilm which was punctured with small holes. The DNA pellets were dried in the Speedvac Plus SC110A (Savant) for 5 min on low temperature. The DNA pellets were resuspended with 20 µl 10 mM Tris-HCl, ddH_20 or TE. Aliquots of DNA was electrophoresed on a 1.2% agarose gel to assess quality and quantity.

VI. Calculating fusion protein concentration

To calculate the concentration of fusion protein produced by transformed *E. coli* cells, an aliquot of fusion protein and known quantities of BSA were electrophoresed and the gels were stained with Coomassie Brilliant Blue solution.

The following equation was used to calculate the concentration of fusion protein produced by transformed *E. coli* cells:

$$\frac{\text{ratio of intensity of fusion protein band compared to intensity of a known quantity of a BSA standard} \times \frac{\text{molecular weight of BSA}}{\text{molecular weight of fusion protein}}}{\text{volume of fusion protein electrophoresed}}$$

Sample calculation:

If the fusion protein band was half as intense as a BSA standard containing 4 μg protein, and since the molecular weights of BSA and GST-$CD4_{cyt}$ are 86 and 33 kDa respectively, and if 10 μl GST-$CD4_{cyt}$ were electrophoresed, then the concentration of GST-$CD4_{cyt}$ would be:

$$\frac{(½ \times 4\ \mu g\ BSA) \times (86\ kDa/33\ kDa)}{10\ \mu l} = 0.52\ \mu g/\mu l\ GST\text{-}CD4_{cyt}$$

VII. Making C600hflA and BL21 *E. coli* cells competent

An aliquot of C600hflA or BL21 cell glycerol stocks was used to establish cultures in 2 ml 2YT medium (without ampicillin) in 15 ml Falcon 2059 polypropylene tubes. The cells were cultured overnight at 37°C, 225 rpm in a G24 Environmental Incubator. The following day, 1 ml of the overnight culture was added to 15 ml Falcon 2059 polypropylene tubes containing 40 ml fresh 2YT medium without ampicillin. The cells were cultured for approximately 3 hr at 37°C, 225 rpm until the desired confluency was attained. The cells were centrifuged for 5 min at 4°C, 12 000 × g in the RC5C Sorvall Centrifuge (Dupont) following which they were resuspended with 20 ml cold, sterile 0.1 M $CaCl_2 \cdot 2H_2O$ (Sigma). The cells were incubated on ice for 20 min, then centrifuged for 5 min at 4°C, 12 000 × g, and resuspended with 2 ml cold, sterile 0.1 M $CaCl_2$. The cells were incubated on ice for 1 hr, then stored overnight on ice at 4°C prior to being transformed.

C. GENOTYPES OF *E. COLI* STRAINS

Epicurian Coli XL1-Blue:

recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]

C600hflA:

hflA 150 lacY1 leuB6 $mcrB^+$ supE44 thi-1 thr-1 tonA21 [chr :: Tn10 (Tet^r)] $F^- \lambda^-$

BL21:

B F^- dcm ompT $hsdS(rB^- mB^-)$ gal

IX. *CURRICULUM VITAE*

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Academic Degrees

University of Ottawa Ottawa, ON	B.Sc. Biology (honours Physiology) 1992

Scholarships and Awards

University of Ottawa Admission Scholarship ($1000)
1988-1989

1st prize - Ottawa-Carleton Regional Concours de français University Scholarship (tuition waived)
University of Ottawa, Ottawa, ON
1988-1989

Ontario Graduate Studies Scholarship ($11 859)
Sponsored by the Ontario Ministry of Education
University of Ottawa, Ottawa, ON
1993-1994

University of Ottawa Supplementary Scholarship (tuition waived)
1993-1994

National Health Ph.D. Fellowship (AIDS) Scholarship ($59 400)
University of Ottawa, Ottawa, ON
1994-1997

University of Ottawa Excellence Scholarship (tuition waived and $3000 per annum)
1994-1997

NSERC Post-Graduate Scholarship ($34 800); declined
1994-1996

Ontario Graduate Studies Scholarship ($11 859); declined
1994-1995

2nd prize - National Graduate Student Poster Competition - Ottawa Life Sciences Conference, Nov. 17-18, 1998. Ottawa, ON

3rd prize (tied) - National Graduate Student Poster Competition - Ottawa Life Sciences Conference, Nov. 1-3, 1999. Ottawa, ON

Employment History

summer student (Dr. L. Filion's laboratory)
University of Ottawa, Dept. of Microbiology & Immunology
May 1992 to August 1992

Teaching Experience

- Microbiology lab demonstrator (1993)

- TA for medical students' immunology poster session (1993, 1994)

- participant in the *Let's Talk Science* Program (1993-1996)

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Graziani-Bowering, G.M., Graham, J.M., Filion, L.G. A quick, easy and inexpensive method for the isolation of human peripheral blood monocytes. 1997. J. Immunol. Methods **207**: 157-68

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9th International Congress of Immunology, San Francisco, CA. July 23-29, 1995
XI International Conference on AIDS, Vancouver, BC. July 7-12, 1996
Ottawa Life Sciences Conference, Ottawa, ON. Oct. 29-30, 1996

Graziani-Bowering, G., Graham, J.M., Filion, L.G. A Quick, Easy and Inexpensive Method for Isolating Human Peripheral Blood Monocytes. Ottawa Life Sciences Conference, Ottawa, ON. Nov. 17-18, 1998 - *2nd prize for student posters*

Kozlowski, M., Lee, F., Yang, H., Zoueva, O., Ridsdale, A., Graziani-Bowering,G., Thibault, P., Kumar, A. GM-CSF Induces Direct Association of Tyrosine Phosphatase SHP-1 with Cytoskeletal Proteins in Normal and Motheaten Murine Macrophages. Cytokines, Hormones and Immunity: Hormones and Cytokines: Signalling Molecules in the Immune Neuroendocrine Cross Talk, Castelvecchio Pascoli, Italy. Sept. 25-30, 1999

Graziani-Bowering,G., Filion, L.G., Kozlowski, M. Stimulation of CD4 on Thp-1 Monocytic Cells Activates the PLC-γ and PI3-K Pathways. Ottawa Life Sciences Conference, Ottawa, ON. Nov. 1-3, 1999 - *3rd prize (tied) for student posters*

Yang, H., Lee, F., Zoueva, O., Ridsdale, A., Graziani-Bowering, G., Thibault, P., Kumar, A., Kozlowski, M. Tyrosine Phosphatase SHP-1 is a Regulator of GM-CSF Induced Cytoskeletal Rearrangements in Murine Macrophages. Ottawa Life Sciences Conference, Ottawa, ON. Nov. 1-3, 1999

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presentation