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The Expression and Function of a Novel CD8 Molecule on Alveolar Macrophages and Mast Cells

By

Nadir Hirji

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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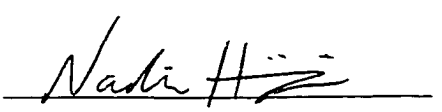

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January 14, 2000

The gem cannot be polished without friction,
nor man perfected without trial.

-Confucius

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, the thesis entitled "*The Expression and Function of a Novel CD8 Molecule on Alveolar Macrophages and Mast Cells*", submitted by Nadir Hirji in partial fulfillment of the requirements for the degree of Doctorate of Philosophy (Ph.D.) in Medical Sciences–Medicine .

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Abstract

CD8 is a cell surface glycoprotein that is best described on a subset of T lymphocytes with cytotoxic/suppressor functions and natural killer cells. It is composed of two chains that can form either an α/α homodimer or an α/β heterodimer. Initial studies on rat bronchoalveolar lavage cells identified a large number of CD8 positive cells. As alveolar macrophages (AM) are the most abundant cell type in lavage, we hypothesised that CD8 is expressed on AM. Flow cytometry determined that 63±5% of lavage cells (89±1% $OX41^+$,AM) were positive for the hinge region of CD8α (OX8) and 52±3% were positive for CD8β, but were negative (2±0%) for the Ig-like domain of CD8α (G28). Double staining, using OX41 and OX8, confirmed that AM were positive for CD8. Flow cytometry also suggested that CD8α on AM may differ from T lymphocyte CD8α, as an antibody to the immunoglobulin variable-like region of CD8α (G28) stained splenic lymphocytes but did not bind AM. Immunoprecipitation of CD8 confirmed that AM CD8α (40 kDa) differed from T lymphocyte CD8α (32 kDa). Functional studies on macrophage CD8 demonstrated that crosslinking either α or β chains of CD8 stimulated induction of iNOS protein and release of NO. Stimulation through CD8α, but not CD8β, also induced NO-dependent killing of the parasite *Leishmania major* in infected AM. Cell signalling inhibitors were used to elucidate the pathways of CD8 stimulation. CD8 induced iNOS and NO production was protein tyrosine kinase (inhibitor genistein), specifically *src*-kinase (inhibitor PP1), and PKC (inhibitors polymixin B and Ro 31- 8220) dependent, and PI3 kinase (inhibitor wortmannin) independent. Finally, the expression of CD8 on other cell types was analyzed. CD8 α (OX8) and β (341) were expressed on rat peritoneal macrophages and

mast cells (MC). However, these cells were negative for G28, an antibody that binds the Ig-like domain of CD8α. Cross linking MC CD8 (OX8) stimulated the production of iNOS protein and NO release. These results demonstrate, for the first time, that rat macrophages and MC express a unique CD8. This molecule plays an important role in stimulating effector functions of these cells and may be involved in regulating immune responses.

Preface

This thesis has been written in paper format according to guidelines of the University of Alberta. Each chapter stands alone as a separate document and is written in the style of the Journal of Immunology. With minor exceptions, the experimental data shown in this thesis was generated by Nadir Hirji. In the two instances where there were collaborators (Table 3.I and Figure 5.5), details of the collaborations are described in the figure legends.

The optimized concentrations of antibodies and inhibitors used in this thesis were generated from dose response studies. For flow cytometry experiments, concentrations ranged from 2-4 μg/ml of antibody. For cell stimulation experiments, antibody concentrations ranged from 0.5-10 μg/ml.

ACKNOWLEDGEMENTS

"It is not the destination, but the journey that is important" and throughout my Ph.D. I have had many people by my side as I made this journey.

First and foremost is my Supervisor, Dr. A. Dean Befus. Dean has been my teacher, not just by words, but actions as well. It is with his guidance, trust, and respect, that I have been able to mature, both scientifically and non-scientifically. Dr. Elyse Y. Bissonnette, my co-supervisor, was a tremendous resource within the laboratory. Dr. Tong-Jun Lin has been my scientific partner, who's collaborations and late night discussions were essential. Dr. Fiona Wills is a good friend whom I could trust to always bring me back to reality. Mr. Rene Dery, Mr. Mark Gilchrist, Dr. Grant Stenton, and the other laboratory members have been friends that I could rely on for help, advice, and a good laugh.

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Finally, without the support and love of my family, completing this Ph.D. would have been impossible. There are no words that can adequately describe how grateful I am to my mother, father, brother, and sister-in-law for their encouragement and understanding. They have been extremely supportive and loving. To my family all I can say is thank you, it was and is appreciated.

I have failed to personally acknowledge many people that I have met during my Ph.D., but please know that I am grateful for all the help I received and the friends that I made.

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Bi-directional communication between CD8+ cells and cells expressing ligand(s) for CD8.

List of Abbreviations

aa:	amino acid
Ab:	antibody
AM:	alveolar macrophages
APC:	antigen presenting cells
BAL:	bronchoalveolar lavage
bp:	base pairs
BSA:	bovine serum albumin
CD:	cluster designation
Cl2MDP:	dichloromethylene-diphosphonate
CTL:	cytotoxic T lymphocyte
DC:	dendritic cells
DMEM:	Dulbecco's modified culture medium
EtBr:	ethidium bromide
$F(ab)_2$:	fragment antigen binding
FACS:	fluorescence-activated cell sorter
FBS:	fetal bovine serum
Fc:	fragment crystallize
Fig:	figure
FITC:	fluorescein isothiocyanate
g:	gravity
GM-CSF:	granulocyte-macrophage colony stimulating factor
gp:	glycoprotein
h:	hour
IEL:	intraepithelial lymphocytes
IF:	immunofluorescence
IFN-γ:	interferon gamma
Ig:	immunoglogulin
IgG:	immunoglobulin G
IL:	interleukin
IL-1β:	interleukin one beta
INOS:	inducible nitric oxide synthase
ITAM:	immunoreceptor tyrosine-based activation motif
ITIM:	immunoreceptor tyrosine-based inhibitory motif
Jak:	Janus kinase
kDa:	kilodaltons
L. major:	*Leishmania major*

List of Abbreviations - Continued

LCMV:	lymphocytic choriomeningitis virus
LPS:	Lipopolysaccharide
MC:	mast cells
MCPI:	mast cell protease I
mg:	milligram
MHC:	major histocompatibility complex
mL:	millilitre
mmol:	millimolar
mRNA:	messenger ribonucleic acid
N. brasiliensis:	Nippostrongylus brasiliensis
$NaNO_2$:	sodium nitrite
NK:	natural killer
NO:	nitric oxide
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PE:	phycoerythrin
PI3:	phosphotidyl inositol 3
PKC:	protein kinase C
PLC:	phospholipase C
PMA:	phorbol myristate acetate
PMC:	peritoneal mast cells
PMSF:	phenylmethylsulfonyl fluoride
PVDF:	polyvinylidene difluoride
RNA:	ribonucleic acid
rpm:	revolutions per minute
RT:	reverse transcription
SEM:	standard error of the mean
STAT:	signal transduction and activators of transcription
TBS:	tris buffered saline
TCR:	T cell receptor
TGFβ:	transforming growth factor beta
TLTF:	T lymphocyte triggering factor
TNFα:	tumour necrosis factor alpha
μg:	microgram
μmol:	micromolar

CHAPTER 1

Introduction and Hypothesis

I. The Expression of CD8 (Table 1.I)

CD8 is a cell surface glycoprotein that is best known for its expression on a subset of T lymphocytes with cytotoxic/suppressor functions and on natural killer (NK) cells. It is composed of two chains that form either an α/α homodimer or an α/β heterodimer. The α/β heterodimer is best known to be associated with thymus-derived lymphocytes, whereas the α/α homodimer is associated with thymus-independent T lymphocytes (eg. TCR γ/δ+ CD8 α/α+), such as those found in the heterogeneous population of intraepithelial T lymphocytes (IEL), and with NK cells (Table 1.I). However, there is recent evidence that CD8 is expressed on other cell types, necessitating that our current paradigm of CD8 expression be revised.

CD8 on other lymphocyte subsets:

The expression of CD4 or CD8 on mature lymphocytes has been used as a marker to separate lymphocyte sub-populations. However, there is evidence that CD8 can be induced on CD4 T lymphocytes. Concanavalin A activated CD4 T lymphocytes, treated with the glucocorticoid dexamethasone, can express CD8α (1). Individuals with rheumatoid arthritis (2) and rats treated with cyclosporine (3) can also express CD8 on $CD4^+$ lymphocytes.

CD8 has also been demonstrated on B lymphocytes. HIV-1 infected individuals express CD8 on 7.2% of $CD19^+$ B lymphocytes (4). $CD19^+$ B lymphocytes from patients with chronic lymphocytic leukemia also express CD8α (68%) but not CD8β (5). Moreover, in at least one case of chronic lymphocytic leukaemia, CD8 expression on B lymphocytes was associated with an aggressive form of this disease (6).

Table 1.I The classical distribution of CD8 expression

Table 1. Classical Model of Cells Expressing CD8

Cell Type	CD8 α	CD8 β
Cytotoxic/Suppressor T Lymphocytes	+	+
Thymus Dependent IELs	+	+
Thymus Independent IELs	+	-
Natural Killer Cells	+	-

Abbreviations: IELs, intraepithelial lymphocytes

These results suggest that during certain inflammatory responses or disease states, cells that normally do not express CD8 can synthesize this molecule. This implies that CD8 may play an important role in regulating immune function and inflammatory responses. In addition, the expression of CD8 may also be associated with cells that are in different stages of their maturation or at different sites in the body, where they maybe required to perform unique tasks.

Expression of CD8 on Dendritic Cells

Dendritic cells (DC) are potent antigen presenting cells (APC) that originate from lymphoid or myeloid progenitors. When these cells encounter antigen, they can move to lymphoid organs and initiate an immune response (7,8).

Murine thymic and splenic DC subpopulations can express both mRNA and protein for CD8, at comparable levels to T lymphocyte CD8 expression (9,10). However, there is heterogeneity in CD8 expression. Thymic DC express both CD8α and β, whereas splenic DC express predominantly CD8α (9). These results suggest that thymic DC express CD8 heterodimer, whereas splenic DC express CD8α homodimer. However, thymic DC may also express some homodimers, as the α-chain is in excess compared to CD8β (10). The splenic DC population can also be sub-divided into the white pulp interdigitating cells and marginal DC, located at the border of the marginal zone and red pulp. Approximately 25% of freshly isolated splenic DC express CD8α and these cells appear to be predominantly interdigitating DC (11). This localized distribution of CD8+ DC may reflect different roles for these

two populations, CD8+ and CD8- DC. Marginal zone DC have greater phagocytic abilities and turnover than interdigitating DC (11).

Rat thymic DC also express CD8α (34%) protein, but have not been tested for CD8β expression (12). Interestingly, human thymic DC express high levels of CD4, but no detectable CD8α protein (10), which is opposite of murine thymic DC. However, these cells were not tested for CD8β expression (10).

Expression of CD8 on Macrophages

Progenitors of rat liver macrophages, pre-Kupffer cells, express CD8. RT-PCR analysis demonstrated that these cells make CD8α, but not CD8β, message (13). Protein analysis showed that 73% of pre-Kupffer cells express CD8α (13). However, only 7% of Kupffer cells express CD8α. These results suggest that, as pre-Kupffer cells mature to Kupffer cells, they down regulate CD8 expression.

In addition to developmental regulation of CD8 on liver macrophages, there are instances where an inflammatory response can generate a microenvironment conducive to macrophage CD8 expression (the following studies were published after our work appeared in the literature). Generation of focal cerebral ischaemia in rats leads to an influx of CD8 positive macrophages/microglia (14). These CD8+ macrophages express CD8 α and β message and protein. Interestingly, the appearance of CD8+ macrophages was dependent on the type of inflammatory response, as other models of nervous system injury, namely EAE (experimental autoimmune encephalomyelitis) or optic nerve injury, did not induce upregulation or an influx of CD8+ macrophages/microglia (14).

Expression of CD8 on Mast Cells

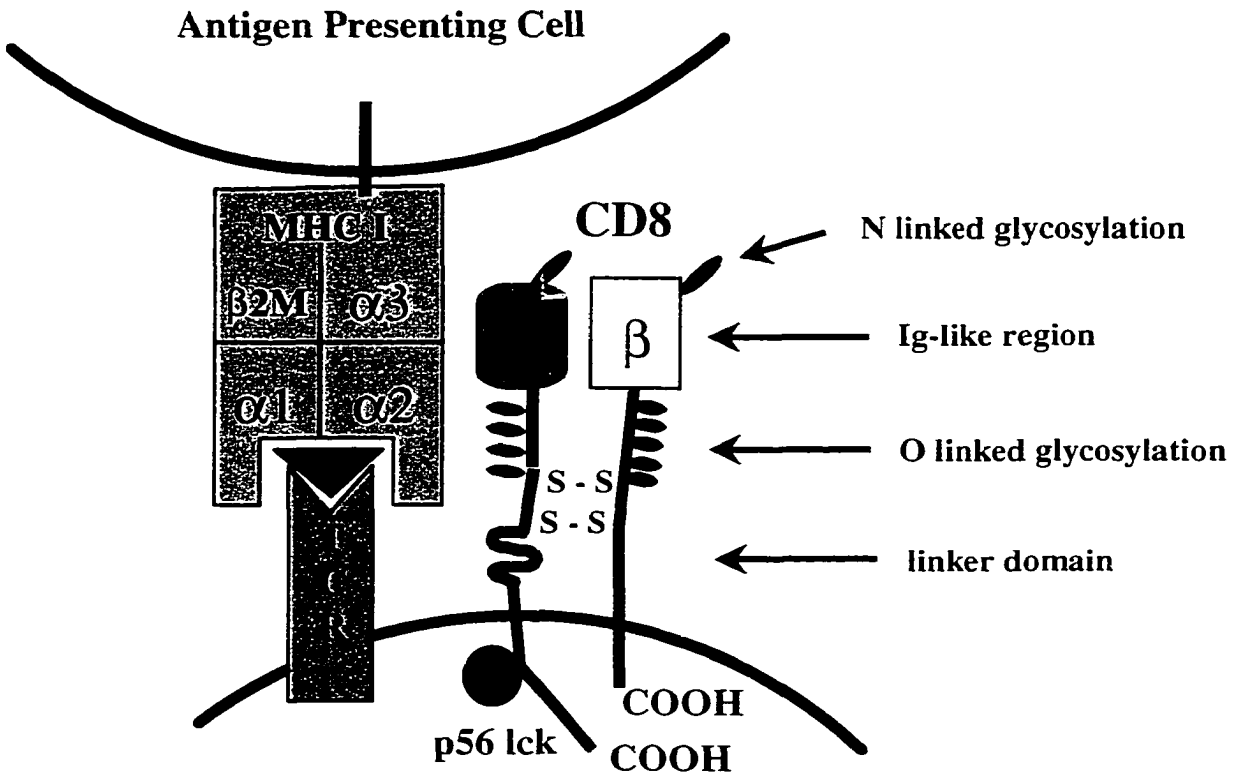
Mast cells play an important role in allergic and other inflammatory reactions by producing a plethora of mediators, such as histamine, and a variety of cytokines, including TNFα and IL-10 (15,16). Initial identification of CD8 on MC was made with a murine MC line which expressed mRNA and protein for CD8α, but not CD8β (17). However, as this line lacked cytoplasmic granules and contained low amounts of histamine, it may be a poor representative for *in vivo* MC.

II. The Structure and Function of CD8 (Fig. 1.1)

Structure of CD8

CD8 is composed of two chains that form either an α/α homodimer or an α/β heterodimer (Fig. 1.1). The structure of the α and β chains is similar and they are encoded by two genes that are linked (18). They both have an amino terminal immunoglobulin-like (Ig) variable domain, a "linker" region, transmembrane and cytoplasmic domains. The α-chain has a longer "linker" domain and is rich in prolines, serines, and threonines. The linker domain acts like a hinge or extension segment to facilitate α-chain interaction with MHC I (Fig. 1.1). The α-chain also has a longer cytoplasmic domain that contains a cysteine rich motif, allowing it to bind the src-family protein tyrosine kinase $p56^{lck}$ (reviewed in 19 and 20). Mouse CD8α is a 38 kDa surface protein, whereas CD8β is expressed as a 30 kDa protein (21-23). In rats, CD8α has a molecular weight of 32 kDa (24) and CD8β has a

Figure 1.1 The structure and function of CD8 on T lymphocytes. CD8 is composed of two chains that form either an α/α homodimer or α/β heterodimers, which is shown here. The Ig-like region of CD8α interacts with the α3 domain of MHC I. CD8 mediates it's signaling effects through the *src*-family protein tyrosine kinase $p56^{lck}$, which associates with the α-chain of CD8. The antibodies for rat CD8 α and β ,which have been used to characterize macrophage and MC CD8 in our studies, are also shown.



Antibodies to Rat CD8

OX8 - CD8α, linker domain
G28 - CD8α, Ig-like region
341 - CD8β

molecular weight of 37 kDa (25). Human CD8α and CD8β both have molecular weights of approximately 34 kDa (26-28).

In addition to differences between the chains, there are also multiple forms of CD8. CD8α can be produced as a full length transcript, encoding the domains described above, or as an alternatively spliced CD8α′ (29) transcript (mouse CD8α with a truncated cytoplasmic tail), or an alternatively spliced secreted CD8α molecule (24). Along with multiple spliced forms of CD8, there are differences in the types and amount of CD8 glycosylation. Mouse CD8α has three N-linked glycosylation sites, whereas rat has one (20,30). Human CD8α is not N-linked glycosylated in resting T lymphocytes, but when these cells are stimulated, CD8α expresses beta 1-6-branched N-linked oligosaccharides (31). However, all species have sites of O-linked glycosylation, mostly located within the linker/hinge region (20,26,30,32).

Ligands for CD8 (Fig. 1.1)

The classical and best studied ligand for CD8 is MHC I. During T lymphocyte interaction an antigen presenting cell (APC, eg. macrophage or dendritic cell), the immunoglobulin variable-like region of CD8α binds to the α3 domain of MHC I (33) and enhances formation of stable TCR/MHC I complexes (34). CD8 may also interact with the α1 and α2 domains of MHC I (35,36). In addition, the binding groove of MHC I can bind T lymphocyte CD8 (37). Therefore, because CD8 interacts with MHC I in many ways, this suggests that different CD8 expressing cells may interact with MHC I in unique ways.

In addition to MHC I, there have been novel ligands for CD8 recently identified. Gp 180, a glycoprotein expressed by human intestinal epithelial cells, can stimulate CD8+ T lymphocyte proliferation through the src-family kinase $p56^{lck}$ (38,39). This proliferation was not inhibited by anti-MHC I antibodies, but was inhibited by anti-CD8 antibodies (38). Further study on gp 180 determined that this glycoprotein exists in two forms, a GPI-anchored and/or transmembrane isoform (39).

The *Trypanosoma brucei* factor TLTF (T lymphocyte triggering factor) is another protein that has been shown to bind CD8 (40). TLTF induces CD8+ T lymphocytes to secrete IFN-γ (41,42). Moreover, CD8 knockout mice infected with trypanosomes survive longer, have decreased parasitemia, and do not produce as much IFN-γ (40).

Functions of CD8

The functions of CD8 and the roles of the individual α and β chains have been elucidated using anti-CD8 antibodies or gene targeted manipulation. CD8 acts as both an adhesion molecule and a co-stimulatory molecule in conjunction with the TCR (Fig. 1.1). CD8 binds to MHC I, in conjunction with the TCR, and delivers a co-stimulatory signal to the cell via the *src*-kinase $p56^{lck}$. Studies on CD8α knockout mice have shown that when this chain is removed, the β-chain cannot be expressed on lymphocytes (43). In addition, these mice have no MHC I restricted T lymphocytes and do not mount a CTL response against LCMV (43). To discriminate between the adhesion and signaling roles of CD8, gene targeted mice were developed that lacked the cytoplasmic domain of CD8α and thus could not signal via $p56^{lck}$. These mice

had decreased numbers of CD8+ cells. However, these cells could function as CTL, suggesting that signaling via the cytoplasmic domain of CD8α is not essential for maturation of CTL (44). Further studies on mutated CD8α, which is deficient in $p56^{lck}$ binding, confirmed that functionally active CD8+ CTL can develop in the absence of the CD8 signaling machinery (45).

CD8β knockout mice also had reduced numbers of CD8+ lymphocytes and expression of CD8α on these cells was decreased (46,47). Interestingly, there was no effect on the number of thymus-independent CD8+ IEL or their expression of CD8α (47). CD8β knockout mice had normal CTL function (46,47). However, studies on mice lacking the cytoplasmic domain of CD8β showed a role for CD8β in CD8+ cell development, which varied depending on TCR antigenic specificity (48). Therefore, it appears that CD8β is important in CD8+ lymphocyte maturation, but does not play a role in CTL cytotoxicity.

However, this model of CD8β was contradicted by Wheeler et al (1992) and Karaki et al (1992) who showed a functional role for CD8β in T lymphocyte interaction with MHC I and cytokine production. To examine the roles of CD8 α and β, CD8- mouse T lymphocytes were transfected with CD8α or CD8 α and β. Cells expressing CD8 α/β heterodimers produced more IL-2 when stimulated by L cells, compared to cells expressing CD8 α/α homodimers (49). In addition, when CD8 β–α hybrids (extracellular region of β and cytoplasmic domain of α) were transfected into CD8- mouse T lymphocytes, these cells produced increased levels of IL-2 when stimulated by L-cells, compared to untransfected cells (49). Cells transfected with

CD8 α/β bound MHC I molecules that CD8 α/α homodimers could not recognize (50). Finally, co-capping studies with TCR and CD8 demonstrated that anti-CD8β antibodies were more efficient than anti-CD8α antibodies in inducing co-localization of TCR with CD8 (51).

CD8 Signaling (Fig. 1.1)

CD8α contains a cysteine-X-cysteine-proline motif that allows the *src*-kinase $p56^{lck}$ to physically associate with the cytoplasmic domain of this chain (52,53). Moreover, zinc is required for the association of $p56^{lck}$ with CD8α (54). The β-chain of CD8 does not contain the cysteine-X-cysteine-proline sequence and does not associate with $p56^{lck}$ (55). Interestingly, CD8β appears to influence $p56^{lck}$ association with CD8α. Transfecting CD8β into a CD8α expressing T cell hybridoma resulted in a ten-fold increase in $p56^{lck}$ kinase activity, when stimulated by anti-CD8 antibodies, compared to cells expressing CD8α only (56). Moreover, CD8β appears to stabilize $p56^{lck}$ interaction with CD8α (56).

The signaling machinery of CD8 is used in conjunction with the TCR to stimulate T lymphocyte activation. The negatively charged sialic acids on CD8 may directly interact with positively charged lysine or arginine residues on TCR Vα (57), which could allow CD8 to influence TCR interaction with MHC I. CD8 can increase TCR interactions with MHC I (58), which may be due to reducing the off rate (34). CD8 has also been shown to be essential for T lymphocyte signaling when using low antigen concentrations (55). Soluble antigen+MHC I molecules can lead to a CD8 dependent increase in Ca^{2+} (59). In addition, anti-CD8 antibodies can inhibit both

adhesion and cytolytic killing of cytotoxic T lymphocytes (60). Therefore, the current model of TCR-CD8 signaling is that TCR and CD8 interact with the same MHC I molecule. $p56^{lck}$ on CD8 is dephosphorylated by CD45, which activates this kinase, and starts the signal transduction pathway, which includes Zap 70/Syk kinase, PLC, and PKC (61).

III. Origin and Function of Alveolar Macrophages

Rationale

Alveolar macrophages (AM) are unique in that they are the only macrophages in direct contact with the air and are situated between the air and tissue interface. These macrophages are the first line of defence against inhaled pathogens and play a critical role in regulating the immune response. Our initial investigations on lymphocyte subsets within the lumen of the lungs suggested that AM may express CD8. This observation is the main focus of this studies.

Origin of Alveolar Macrophages

AM are similar to other macrophages in that they share a common circulating monocyte precursor. However, it is the unique tissue microenvironment that leads to the different macrophage populations, e.g. Kupffer cells in the liver and microglial cells in the nervous system. There are several types of macrophages within the lungs, these include alveolar macrophages, interstitial macrophages, intravascular macrophages, and DC. Interstitial macrophages are located within the connective tissue of the lungs and appear to play a role in lung defence as well as antigen

presentation (62, 63). Compared to AM, interstitial macrophages are smaller and have decreased phagocytic activity (64). Intravascular macrophages are located on the endothelial cells of the capillaries facing the bloodstream (65,66). These cells are believed to play a role in debris removal from blood entering the lungs (67). DC are also located in the lung interstitial tissue. These cells do not express Fcγ receptors, but express high levels of MHC II on their surface, which would allow for increased interaction and antigen presentation with CD4+ T lymphocytes (68,69).

AM are obtained from the lumen of the lungs (washings known as bronchoalveolar lavage (BAL)). Few if any interstitial macrophages or DC are found in BAL. AM make up approximately 90% of the cells within the BAL (70,71). The majority of AM are derived from peripheral monocytes that home to the lung (72,73). This was demonstrated in leukaemia patients with bone marrow transplants, in which donor cells repopulated the alveoli (74,75). AM may also arise from interstitial macrophages that have migrated into the lumen of the lungs, where they differentiate into AM (76). Finally, there may be local proliferation leading to an increase in AM cell numbers (77). Normally, less than 1% of human AM incorporate thymidine, however, inflammatory conditions can increase proliferation up to 10 fold (77).

Functions of Alveolar Macrophages

AM play an important role in regulating the immune response in the lungs and have the ability to synthesize and secrete numerous mediators. Some of the cytokines that AM can secrete include tumour necrosis factor-α (TNFα) (78), interferon-γ (IFN-γ) (79), IL-1β (80), and TGFβ (81). AM can also secrete prostaglandins (82),

leukotrienes (83), fibronectin (84), proteases (85,86), oxygen metabolites (87), and nitric oxide (NO) (88). To date, over 100 products have been identified from AM (89).

The current model of the function of AM within the lungs is to down regulate immune responses, particularly to "non-threatening" antigens, thereby limiting damage that may occur to the lung by inflammatory mediators. The functions of AM have been examined in experimental systems where AM were eliminated by liposome-encapsulated dichloromethylene-diphosphonate (Cl_2MDP), which is taken up by AM leading to their elimination, without affecting the interstital macrophage population (90). Liposome Cl_2MDP treated mice, compared to controls, had greater numbers of B lymphocytes in the lungs and lymph nodes, when treated intratracheally with TNF-KLH (90). In models of allergic inflammation, in which rats have been primed and 2 weeks later AM were eliminated, AM depleted rats produced significantly higher levels of specific IgE, but not IgG, upon antigen exposure (91). Moreover, AM depleted rats had more than double the number of T lymphocytes in the lungs compared to controls (91).

Early *in vitro* studies on AM demonstrated that in co-culturing studies with lymphocytes, AM inhibited mitogen-induced proliferation of T and B lymphocytes (92). This was further demonstrated in studies showing that T lymphocyte proliferation by Con A or ovalbumin was inhibited by 50 to 80% when co-cultured with AM. Moreover, AM down regulation was time dependent, in that addition of AM 24h after stimulation did not inhibit T lymphocyte proliferation (93). This inhibition was reversible, so that if AM were removed 24h after stimulation, T

lymphocytes were able to proliferate without further stimulation (93). These "anergized" T lymphocytes were shown, in AM co-culturing experiments, to have decreased CD2 and TCR expression after stimulation (93). Moreover, there was less total protein phosphorylation in IL-2 stimulated lymphocytes that were co-cultured with AM (93).

The immunosuppressive effects of AM appear to be dependent on certain mediators, timing of mediator exposure, and maturity of macrophages. In macrophage – lymphocyte interactions, the use of a NO inhibitor demonstrated that AM down regulation of T lymphocyte proliferation was NO dependent (93, 94). The mechanism of NO inhibition may be through disruption of the Jak3/STAT 5 signaling pathway. NO leads to reduced tyrosine phosphorylation of Jak3 and STAT5 (95). In addition, inhibition by AM derived NO is dependent on guanylate cyclase activation (95). These results are consistent with experiments showing that AM do not inhibit CD28 and IL-2 receptor upregulation or IFN-γ and IL-2 secretion, but do inhibit T lymphocyte responses to IL-2 (96), which are dependent on the Jak3/STAT 5 signaling pathway.

Pre-incubation (24h), but not co-incubation, of AM with GM-CSF or GM-CSF + TNFα, but not TNFα alone, inhibits AM dependent down regulation of T lymphocytes (94). Moreover, TGF-β also inhibits AM dependent down regulation of T lymphocyte proliferation, when this cytokine is co-incubated in mixed cell cultures (94). TGF-β may mediate its affects by inhibiting AM NO production (94). In addition to modulation of AM function by cytokines, prostaglandin production by

AM is required for inhibition of T lymphocyte proliferation (97). Therefore, AM function appears to be highly regulated.

The maturation state of macrophages is also important in regulating T lymphocyte responses. Freshly recruited AM, day 3 after liposome Cl_2MDP elimination, were stimulatory for T lymphocyte proliferation, whereas day 5 AM were non-inhibitory. In contrast, day 10 AM regained the ability to inhibit T lymphocyte proliferation (94).

Direct down regulation of T lymphocyte proliferation and IL-2 receptor signaling may not be the only mechanism of AM inhibition of the inflammatory response. It has been shown that AM and DC may be located in similar areas within the lungs (98), therefore, AM may also inhibit DC function. Lung DC isolated from rats depleted of AM were better able to stimulate lymphocytes, as measured by thymidine incorporation (98). This upregulation was inhibited if AM were incubated with DC for 24h before lymphocyte stimulation (98). The ability of AM to inhibit DC induced T lymphocyte activation was enhanced by the addition of TNFα. However, addition of this cytokine alone could not inhibit DC function (98). Moreover, AM induced down regulation of DC activity is dependent on NO, as a NO synthase inhibitor reversed this downregulation (98).

These studies demonstrate that AM may play an important role in maintaining lung homeostasis. Potential inflammatory reactions to inhaled materials can be down regulated, thereby limiting lung damage.

IV. Summary

Our understanding of the expression of CD8 is no longer limited to T lymphocytes; other cells including B lymphocytes, mast cells, and some macrophages express CD8. We established preliminary evidence that AM express CD8. As CD8 plays an important role in T lymphocyte function and immune responses, it is essential to characterize the structure and elucidate the functions of CD8 on other cell types, particularly AM.

V. Hypothesis

We hypothesise that rat alveolar macrophages express both the message and protein for CD8 α and β. We further hypothesize that, kike T lymphocyte CD8, macrophage CD8 plays a role in regulating macrophage function.

VI. Objectives:

1. *To determine if rat alveolar macrophages express CD8.*

2. *To identify if CD8 expressed on macrophages is different from that on T lymphocytes.*

3. *To examine whether cross linking CD8 on AM stimulates the release of mediators, specifically NO.*

4. *To elucidate the signaling pathways involved in CD8-induced mediator release from AM.*

5. *To determine if CD8 is expressed on other cell types.*

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CHAPTER 2

A Novel CD8 Molecule Expressed by Alveolar and Peritoneal Macrophages Stimulates Nitric Oxide Production

PREFACE

This chapter deals with the expression of CD8 α and β on rat alveolar and peritoneal macrophages. At the time of this study (started in 1994), one study had been published on the expression of CD8 on macrophages, and no data was available as to the function of CD8 on non-T lymphocytes. This work suggests that macrophage CD8 may differ from T lymphocyte CD8 and demonstrates, for the first time, a possible functional role for this molecule. The contents of this chapter is published in the "Journal of Immunology", 1997, 158: 1833-1840, under joint authorship with Drs. Tong-Jun Lin and A. Dean Befus. The co-authors have provided a statement confirming that my contribution to this manuscript was significant.

This is to certify and confirm that Nadir Hirji made major and most significant contributions to the experimental work, scientific content, and writing of the paper entitled "A Novel CD8 Molecule Expressed by Alveolar and Peritoneal Macrophages Stimulates Nitric Oxide Production" by Nadir Hirji, Tong-Jun Lin, and A. Dean Befus, *The Journal of Immunology*, 1997, 158: 1833-1840.

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Introduction

CD8 is a cell surface glycoprotein found on a subset of T-lymphocytes with cytotoxic/suppressor functions and on NK cells. It is composed of two disulfide linked chains that form either a homodimer (α/α) or heterodimer (α/β). CD8 can act as both an adhesion molecule and as a co-stimulatory receptor in conjunction with the T-cell receptor (TCR). During T-lymphocyte interaction with an antigen presenting cell (APC), the α chain of CD8 binds to the $\alpha 3$ domain of MHC class I (on the APC) and delivers an activation signal to the T-lymphocyte through a pathway involving the *src*-related protein tyrosine kinase, p56-lck [reviewed in (1, 2)].

Alveolar macrophages, the most abundant cell type in the airways, play a central role in lung defence. They are involved in phagocytosis, clearance of particles and debris, and produce numerous cytokines and other mediators such as arachidonic acid metabolites, oxygen radicals, and nitric oxide (NO) [reviewed in (3-5)]. Alterations in the functions of alveolar macrophages are associated with several disease states. For example, patients with Sarcoidosis, a multi-system granulomatous disorder, have increased TNFα release by alveolar macrophages (6). Moreover, depletion of alveolar macrophages can be associated with an increased hyperresponsiveness to inhaled antigens (7, 8). One function often considered to be a central role of alveolar macrophages is their ability to inhibit T-lymphocyte activities in the lung. Recently, it has been shown that this inhibition involves the production of NO by macrophages (9).

In our studies of bronchoalveolar lavage (BAL) cells from normal male Sprague Dawley rats, we made the surprising observation that alveolar macrophages

express a surface CD8 protein, which was previously thought to be found exclusively on T-lymphocytes and NK cells. In this report, we describe the novel observation that alveolar and peritoneal macrophages express CD8, and that both macrophage cell types are positive for the α and β chains. In addition, CD8α mRNA is present in alveolar macrophages, suggesting protein synthesis of CD8. Furthermore, the N-terminal Ig-like region of CD8α appears to be altered on macrophages, in contrast to T-lymphocyte CD8, a difference that may be due to post translational modification of CD8α. Finally, we have demonstrated that ligation of CD8α on alveolar macrophages stimulates the production of NO.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats, 200 to 300 g, were obtained from Charles River Canada Inc. (Quebec, Canada) and maintained in an isolated room in filter-top cages to minimize unwanted infections. The animals were given food and water *ad libitum* and maintained on a 12 h (0700 h) -12 h (1900 h) light-dark cycle. No experimental procedures were performed on animals within the first week after arrival, decreasing the effects of stress associated with transport, new housing facilities and handling. All experimental procedures were approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Isolation of bronchoalveolar cells

Animals were anaesthetized by i.p. injection of 0.5 ml Rompun (Xylazine) and 0.5 ml Ketalean (Ketamine). The trachea was catheterized with a polyethylene tube and 12 X 5 ml of cold PBS was massaged into the lungs. Lavage cells were pelleted at 200 X g for 20 min and resuspended in PBS (10).

Peritoneal macrophage isolation

Peritoneal cells were obtained by lavage of the peritoneal cavity with 15 ml of cold HEPES-buffered Tyrode solution containing 0.1% BSA. They were layered on a two-step (30% 80%) discontinuous gradient of sterile Percoll (Pharmacia Ltd., Uppsala, Sweden) and separated as described earlier (11). Cells within the interphase (85 ± 5% CD11b (OX42), mean ± SEM, >95% viability) were washed and used as an enriched source of peritoneal macrophages.

Flow cytometric analysis

The following antibodies were used for cell analysis : pan T-lymphocytes [MRC OX52 (IgG2a) FITC or PE] (12) , CD45 B-lymphocytes [MRC OX-33 (IgG1) FITC] (13), alveolar macrophages [MRC OX-41 (IgG2a), confirmed by morphological staining of BAL cells] (14), CD11b (iC3b receptor) [MRC OX-42 (IgG2a) FITC] (14), CD4 [W3/25 (IgG1) PE] (15), CD8α hinge region [MRC OX-8 (IgG1) FITC] (15), CD8α immunoglobulin variable-like region [G28 (IgG2a) FITC] (16), CD8β [341 (IgG1) FITC] (16), and $F(ab)_2$ rabbit anti-mouse IgG (FITC and PE). All antibodies, except G28 and 341, were purchased from Serotec (Toronto, Canada); G28 and 341 were

purchased from Cedarlane (Hornby, Canada). The results with specific antibodies were compared to isotype matched controls (IgG1-FITC, IgG1-PE, IgG2a, IgG2a-FITC, and IgG2a-PE) purchased from Accurate Chemical and Scientific Co. (New York, USA).

In 96 well u-bottom plates, cells (5 X 10^5 cells per test) were preincubated in immunofluorescence (IF) buffer (PBS + 1% BSA + 0.2% sodium azide) + 10% normal mouse serum (for conjugated primary antibodies only) for 30 min before 1 hr antibody incubation at 4°C. Cells were washed three times (with IF buffer), resuspended in 400 ml of 1% formalin (IF buffer), and 10,000 cells were analyzed on a FACScan (Becton Dickinson, California). Three regions, based on forward and side scatter profiles, were defined for analysis, region R1 (lymphocyte enriched), R2, and R3 (macrophage enriched).

Reverse Transcription - PCR

Alveolar lavage cells were incubated with OX41-FITC and sorted (R3 and OX41+) on a Coulter EPICS Elite cell sorter (Coulter Electronics, Hialeah, FL) to obtain a population with <1% OX52 (T-lymphocyte) contamination (as determined by flow cytometry). Splenic CD8 T-cells were used as a positive control in PCR. Briefly, splenic cells were isolated from rat spleen and passed through a rat CD8 immunocolumn, according to the manufacturer's protocol (Biotex Laboratories Inc., Edmonton, Canada), to enrich for CD8 positive T-lymphocytes (81±3% OX52 (T-lymphocytes), 79±1% OX8 (CD8α), 73±3% G28 (CD8α), 63±4% 341 (CD8β)). Total RNA was extracted using TRIzol reagent (Gibco-BRL, Burlington, Canada) yielding 3.5 μg RNA/million cells from alveolar macrophages, with an OD 260/280 ratio of 1.8, and 0.8 μg RNA/million cells

from CD8 enriched splenic T-cells, with an OD 260/280 ratio of 1.7. mRNA (1 μg total RNA) was reversed transcribed (RT) by SuperScript RNase (Gibco-BRL) using a PTC-100 Programmable Thermal Controller (MJ Research, Massachusetts, USA) according to the manufacturer's protocols. To judge the success of the RT reaction, samples were subjected to a rapid paper chromatography process (17, 18). Briefly, 2 μL [α-32p]-dctp incorporated RT products were spotted on chromatography paper (Whatman 1, FisherScientific, Nepean, Canada) and run in buffer (3.5 M ammonium sulfate, 0.075 M sodium phosphate pH 6.8, 2% n-propanol) until the buffer front reached the top end of the paper (1 h). The paper was air-dried and applied to Kodak X-omat AR film (Eastman Kodak Co., Rochester, NY) to visualize the RT reaction.

PCR was a modification of the Gibco-BRL *Taq* DNA polymerase protocol, with changes in the concentration of dNTP's (1.23 mM) and 10X PCR buffer (67 mM Tris pH 8.8, 1.5 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, and 10 mM BME). Primers were designed to have similar annealing temperatures, ranging from 53°C to 56°C. The primers used were : (a) rat β-Actin (a gift from Dr. Linda Pilarski, University of Alberta), (b) rat CD8α Ig-like region 5' primer (nucleotide 136, within amino acid 5) : 5'-TCACCAAAGAAAGTGGAGGC-3' and 3' (nucleotide 370, within amino acid 123) primer : 5'-CTTGCTCAGGGTGAGGATGT-3' giving a 234 bp fragment. PCR conditions were optimized using temperature gradients. Twenty-five cycles of amplification were used for all primers (95°C for 45 s, 55°C for 1 min, and 72°C for 1.5 min). Products were run on a 2% agarose gel and stained with EtBr.

Measurement of NO production

FACS sorted, on the basis of their forward and side scatters (region R3), alveolar macrophages were incubated ($2X10^5$ cells/test) with 2-10 μg/ml OX8 (or IgG1 isotype control) for 24 h. Cell-free supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% N-(l-naphthyl)-ethylene-diamine dihydrochloride, 2.5% H_3PO_4) and incubated for 10 min at room temperature (19). Concentration of NO_2^- was determined by measuring the absorbance at 540 ηm with a Molecular Devices V max Kinetic Microplate Reader (Menlo Park, CA, USA). $NaNO_2$ was used as a standard.

Statistical analysis

Statistical significance of differences between any two groups was analyzed by Student's T-test, with $p<0.01$. Data in text represents mean ± SEM.

RESULTS

The distribution of CD8 positive cells within bronchoalveolar lavage

The cellular distribution in bronchoalveolar lavage was determined using flow cytometry. Cells were initially defined on the basis of their forward and side scatter profiles (Fig. 2.1) and separated into three regions for analysis. The distribution of various cell types differed among these regions (Fig. 2.2).

The majority of cells within R1 were lymphocytes; OX52 (T-lymphocytes) made up 37 ± 7% and OX33 (B-lymphocytes) 17 ± 5%. Of the T-lymphocytes,

Figure 2.1 Forward (size) and side (granularity) scatter light profiles of bronchoalveolar lavage cells from a pool of normal rats. Three regions were defined based on light scatter profiles and used for analysis.

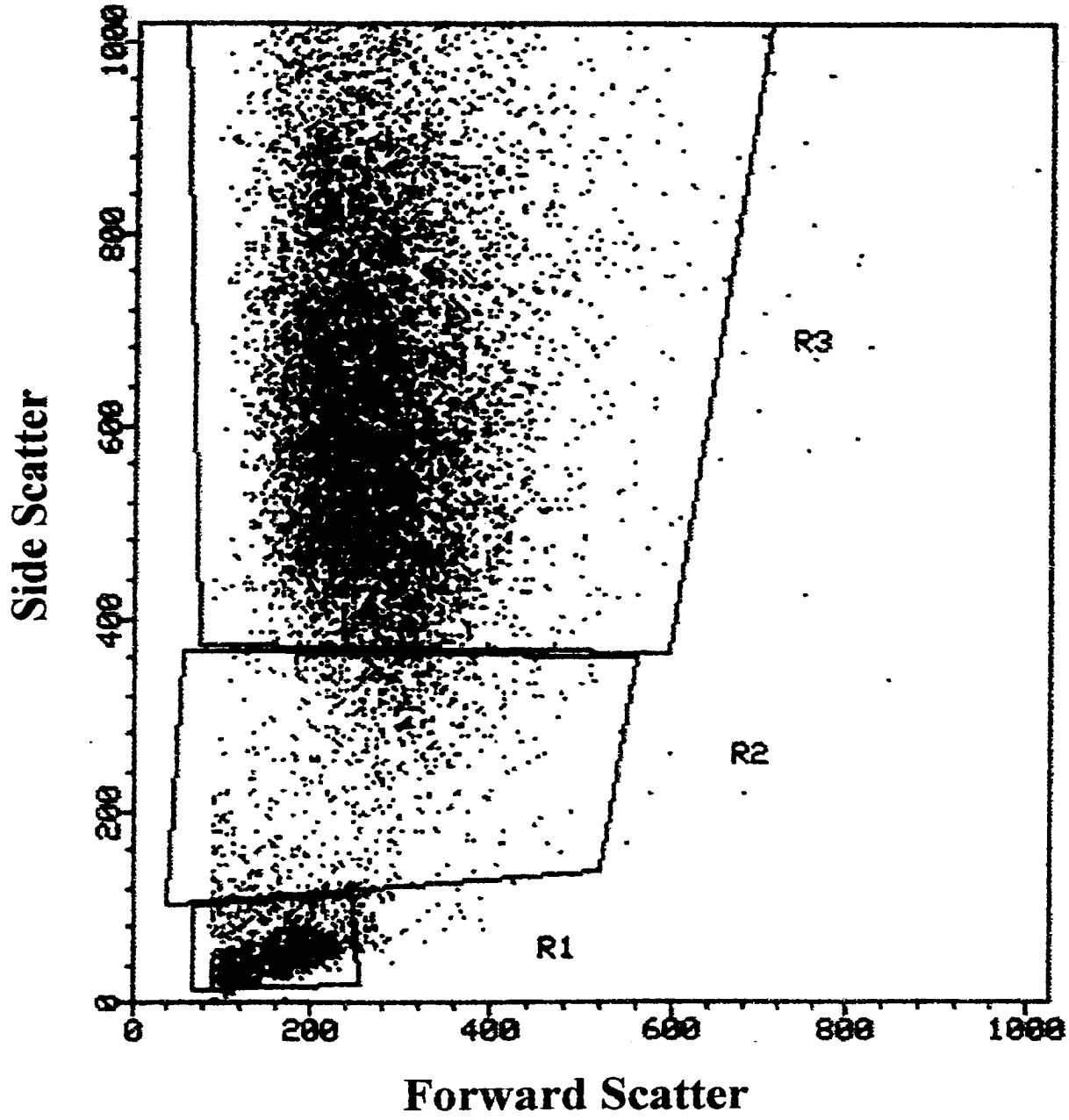
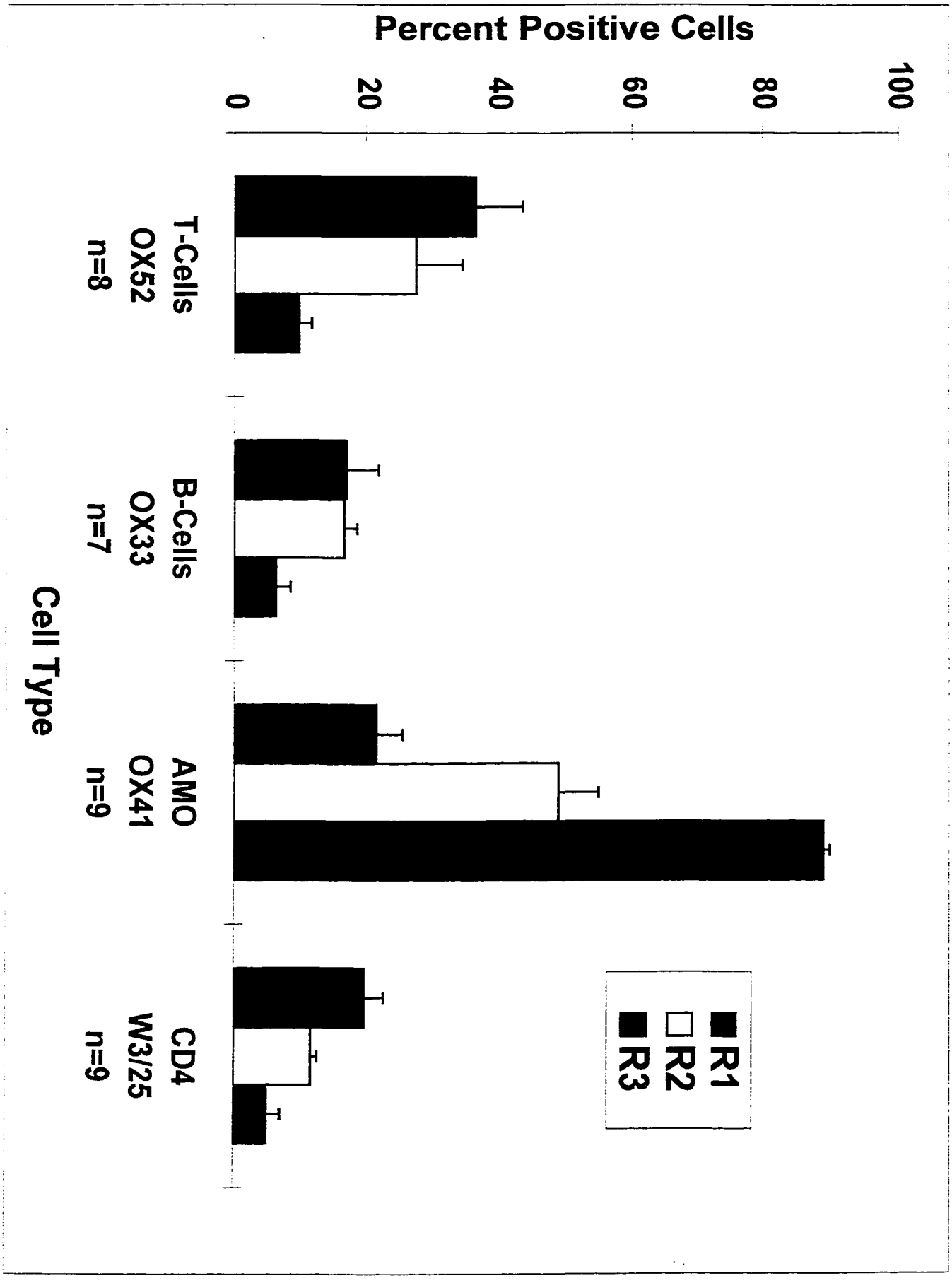


Figure 2.2 Differential distribution of T-lymphocytes (OX52), B-lymphocytes (OX33), alveolar macrophages (OX41) and CD4 (W3/25) positive cells within regions R1, R2, and R3, as defined in Fig. 2.1, of bronchoalveolar lavage. The percent represents mean ± SEM, n = number of experiments.



approximately half were W3/25 (CD4) positive (20 ± 3%). There was also a significant number of OX41 positive cells (alveolar macrophages; 22 ± 4%). Together, the number of lymphocytes plus alveolar macrophages did not equal 100% (76%), indicating that there are a number of null cells (using these markers) within R1.

Within R2, the percentage of lymphocytes decreased as the percentage of alveolar macrophages increased. OX52 (T-lymphocytes) dropped to 28 ± 7%, whereas the percentage of OX33 (B-lymphocytes) remained relatively constant at 17 ± 2%. The percentage of OX41 positive cells (alveolar macrophages) increased to 49 ± 6%. The percent of W3/25 (CD4) positive cells also decreased to 11 ± 1%. Together, the percentage of lymphocytes plus alveolar macrophages approximately equaled the total number of cells (94%), indicating that if there were null cells within R2, they made up a small proportion of the total.

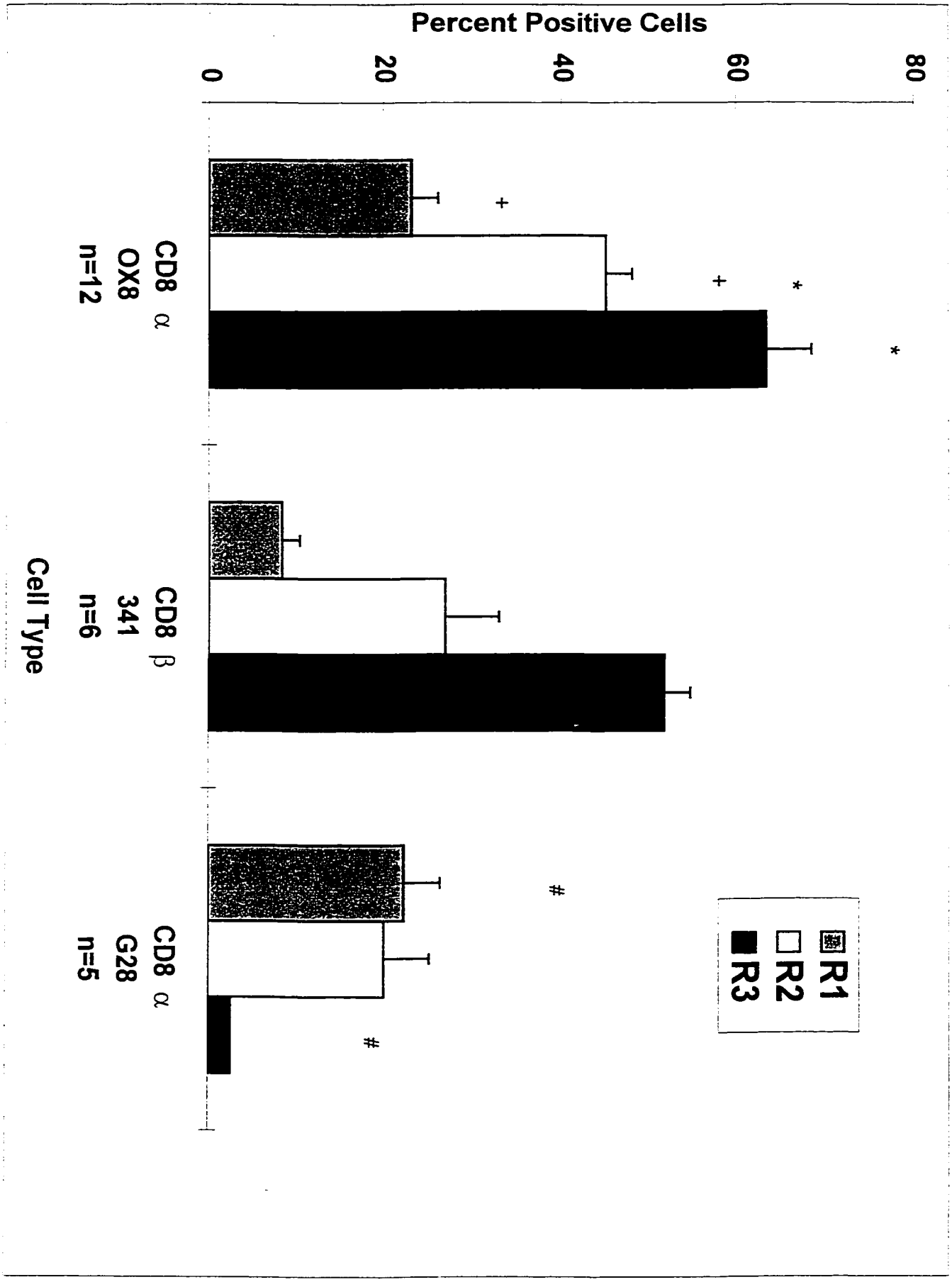
The majority of cells (89 ± 1%) within R3 were OX41 positive (alveolar macrophages, confirmed by morphological staining), with few lymphocytes. OX52 (T-lymphocytes) decreased to 10 ± 2% and OX33 (B-lymphocytes) to 6 ± 2%. Moreover, approximately 50% of the T-lymphocytes were W3/25 (CD4) positive, 5 ± 2%. Within this region, the combined percentages of lymphocytes plus alveolar macrophages was greater than 100% (105%). This discrepancy could be explained by individual variations among the experimental groups. However, we did not rule out the possibility that there may be a small percentage of cells expressing two or more of the surface markers. In particular, the marker for B-lymphocytes (OX33) identifies an isoform of the CD45 antigen which is believed to be exclusively expressed on B-lymphocytes. As CD45 is found on all hematopoietic cells, except erythrocytes, it is possible that a small

percentage of T-lymphocytes and/or alveolar macrophages also express this isoform of CD45 (20).

Unlike the distribution of lymphocytes within regions R1 to R3, CD8 positive cells increased from R1 to R3 (Fig. 2.3), similar to the proportion of alveolar macrophages. In R1, the percent of OX8 (CD8α hinge) and 341 (CD8β) cells were 23 ± 3% and 9 ± 2%, with OX8 (CD8α) significantly greater ($p<0.01$) than 341 (CD8β). The mean fluorescence intensity for isotype control stained cells (IgG1) was 3.8, the mean fluorescence intensity of cells stained with OX8 was 11.6 and it was 5.7 for 341 (representative values). In R2, the percent of CD8 cells increased, OX8 (CD8α hinge, 45 ± 3%) was significantly greater ($p<0.01$) than 341 (CD8β, 27 ± 6%). The mean fluorescence intensity for IgG1 stained cells was 16.1, compared to 86.6 for OX8 and 34.6 for 341 (representative values). Finally, greater than 50% of R3 cells were positive for CD8, OX8 (CD8α hinge) 63 ± 5% and 341 (CD8β) 52 ± 3%. The mean fluorescence intensity for IgG1 was 47.4, compared to 165.5 for OX8 and 129.8 for 341 (representative values). The percent of CD8 cells could not be accounted for by T-lymphocytes alone, as the latter cells are only 10 ± 2% (OX52) of the total within R3.

An antibody to the Ig-like region near the N-terminus of CD8α (G28) did not follow the pattern of staining that was observed using OX8 and 341, but was similar to that of T-lymphocytes. In R1, there was no significant difference between the percent of G28 (CD8α N-terminus) positive cells was 22 ± 4% compared to OX8 (CD8α hinge, 23 ± 3%). The mean fluorescence intensity of the isotype control (IgG2a) was 2.1, compared to 3.5 for G28. Within R2, the percent of G28 positive cells (20 ± 5%) was significantly ($p<0.01$) less than the percent of OX8 (45 ± 3%). The mean fluorescence

Figure 2.3 The di_stribution of CD8, using two antibodies to the α (OX8, G28) chain and one to the β (341) chain, within regions R1, R2 and R3, as defined in Fig. 2.1. The percent represents me=an ± SEM, n= number of experiments. + $p<0.01$ OX8 versus 341, * $p<0.01$ OX8 versus G28, # $p<0.01$ G28 versus 341.



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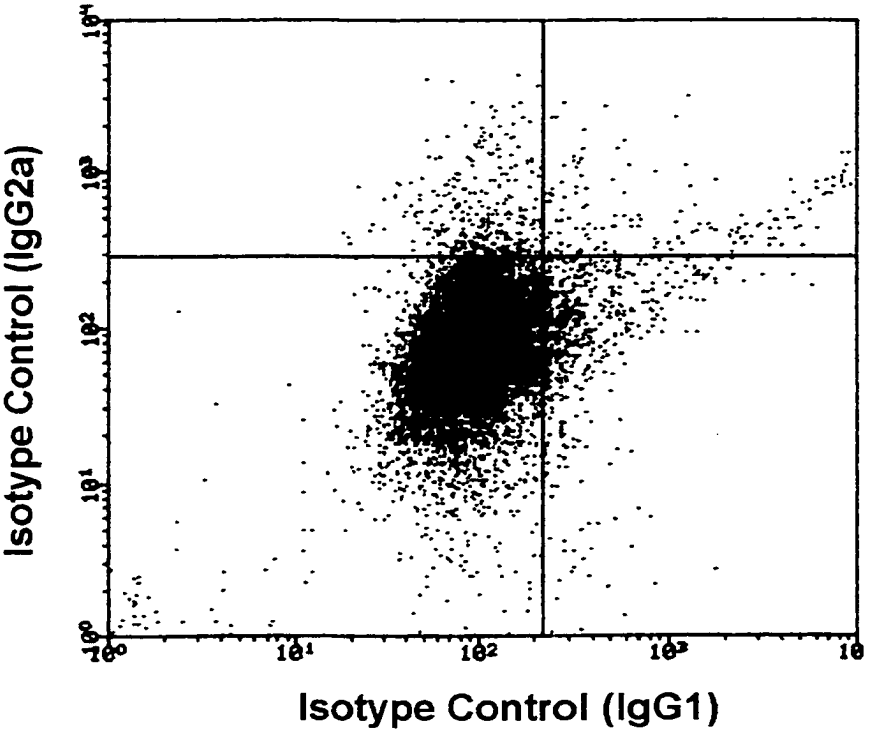
intensity of IgG2a was 6.2, compared to 13.5 for G28. In R3, G28 fell to 2 ± 0% and was significantly less ($p<0.01$) than OX8 (63 ± 5%). The mean fluorescence intensity of IgG2a was 35.2, compared to 40.7 for G28. These results indicate that there may be differences between T-lymphocyte and alveolar macrophage CD8.

To confirm the presence of CD8 on alveolar macrophages, lavage cells were double stained for both OX41 (alveolar macrophages) and OX8 (CD8α hinge) (Fig. 2.4). Eighty-five percent of the cells within R3 stained positive for both the alveolar macrophage marker (OX41) and CD8α (OX8).

PCR for CD8α

One possible explanation for the expression of CD8 on alveolar macrophages could be that these cells adsorb or endocytose/phagocytose soluble CD8 or CD8 bearing T-cells and re-express a modified T-cell CD8 antigen on their surface. Alternatively, alveolar macrophages may synthesize CD8 themselves. RT-PCR was used to determine if alveolar macrophages contain CD8 mRNA. After establishing that there was a positive RT reaction using paper chromatography, cDNA from FACS enriched alveolar macrophages (<0.7% OX52 (T-lymphocyte) contamination), and cDNA from immunocolumn enriched splenic CD8 lymphocytes (81% OX8) (positive control), were used in the PCR reaction to detect CD8α mRNA. Both alveolar macrophages and enriched CD8 splenic T-cells were positive for β-actin, indicating that both cells had usable cDNA (Fig. 2.5). Furthermore, both CD8 enriched splenic lymphocytes and alveolar macrophages were positive for CD8α mRNA (Fig. 2.5).

Figure 2.4 The scatter profile of double stained lavage cells for the alveolar macrophage marker (OX41 (IgG2a)) and CD8α (OX8 (IgG1)), compared to isotype controls, within region R3 (as defined in Fig. 2.1).



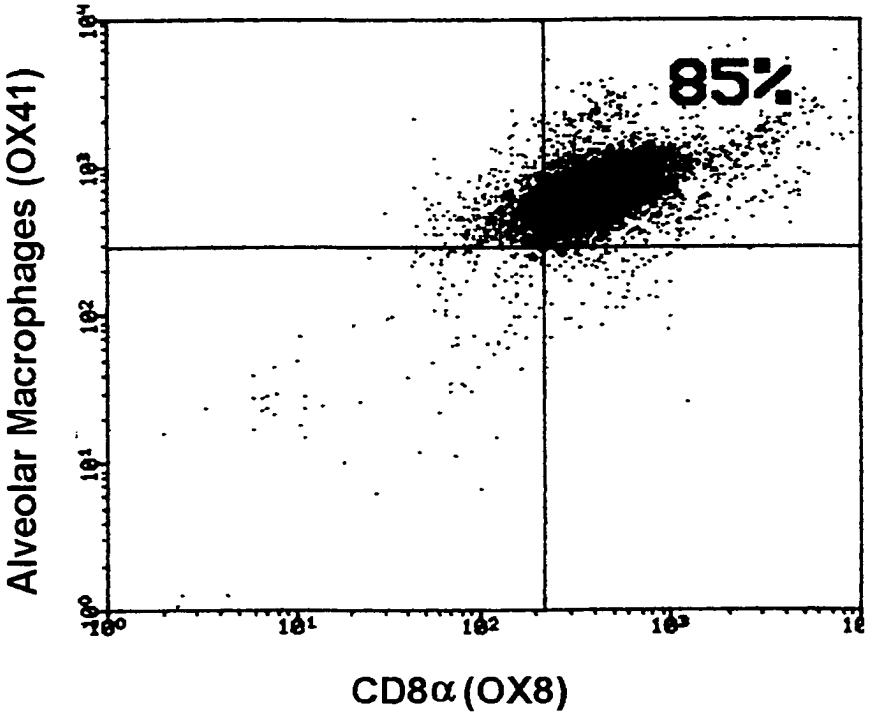
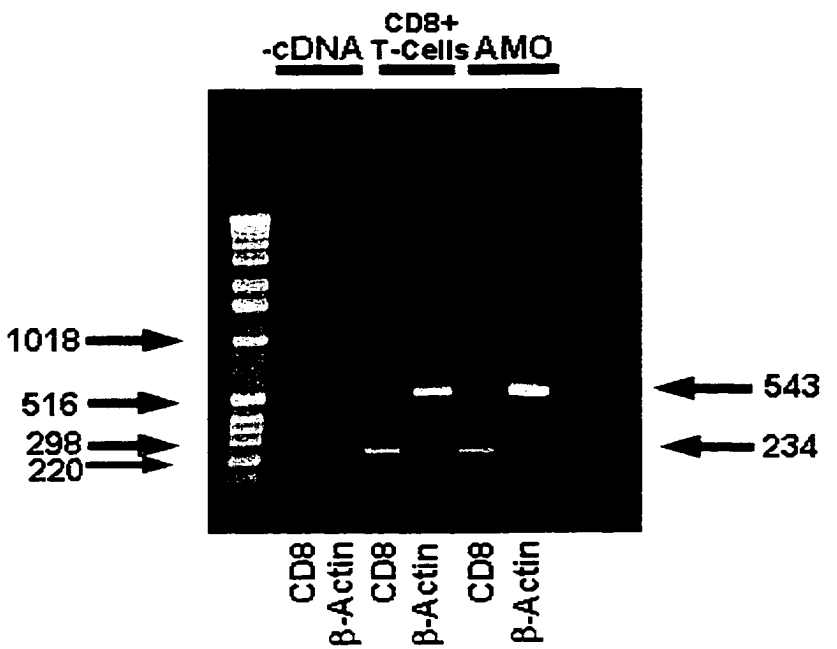


Figure 2.5 PCR analysis of FACS enriched alveolar macrophages (<1% T-lymphocyte (OX52) contamination), lanes 6 (CD8α) and 7 (β-actin), compared to CD8 enriched splenic T-lymphocytes, lanes 4 (CD8α) and 5 (β-actin). Negative controls using PCR reagents without cDNA were also run, lanes 2 (CD8α) and 3 (β-actin). Lane 1 is 1 kb DNA ladder.



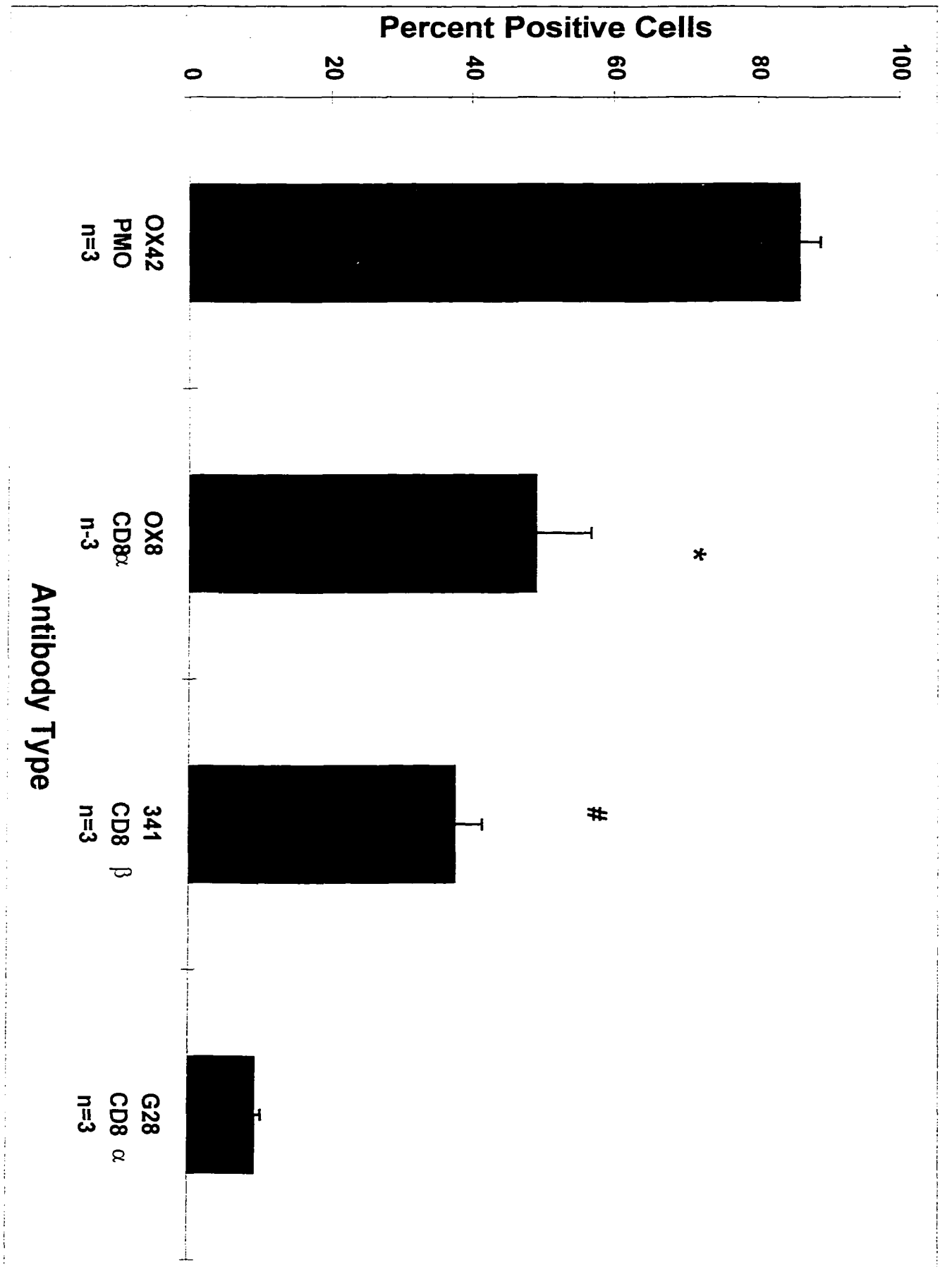
The primers used for the detection of CD8α mRNA encompassed the Ig-like region. Although this region appears to be lacking, truncated or masked in the CD8α protein expressed on alveolar macrophages, the mRNA detected includes this region.

Although contamination of the sorted alveolar macrophages with T-lymphocytes was small, it could be a problem in PCR. T-lymphocyte RNA was titrated and used in RT and PCR to determine the lowest level of RNA required for detection of a PCR product. A minimum of 3.13% (0.031 μg RNA) T-lymphocyte RNA was required to detect β-actin and 6.25% (0.0625 μg RNA) of T-lymphocyte RNA was needed to detect CD8α PCR product. As the amount of T-lymphocyte contaminating RNA was 0.7% (0.007 μg RNA), the CD8 product identified in the sorted alveolar macrophage population could not be accounted for by T-lymphocyte contamination.

Expression of CD8 on peritoneal macrophages

To examine the expression of CD8 on other macrophage populations, flow cytometry was used to determine the presence of CD8 on peritoneal macrophages (Fig. 2.6). The percent of CD11b (OX42, macrophages) within the 30%/80% Percoll interphase of peritoneal lavage was 86 ± 3% (OX42). Peritoneal cells also stained positive for both OX8 (CD8α hinge, 49 ± 8%) and 341 (CD8β, 37 ± 4%). In contrast, the antibody to the Ig-like domain of CD8α (G28) stained a small proportion of CD11b (OX42) peritoneal lavage cells (9 ± 1%). Therefore, like alveolar macrophages, peritoneal macrophages (CD11b, OX42) appear to express both the α and β chains of CD8. In addition, as with alveolar macrophages, these results suggest that there are

Figure 2.6 The distribution of macrophages (OX42, Cd11b) and CD8 (OX8, G28, 341) within Percoll separated peritoneal lavage cells. The percent represents mean ± SEM, n = number of experiments. * <0.01 OX8 versus G28. # p<0.01 G28 versus 341.



differences between the structure of CD8 on T-lymphocytes and peritoneal macrophages.

NO production from CD8α (OX8) stimulated alveolar macrophages

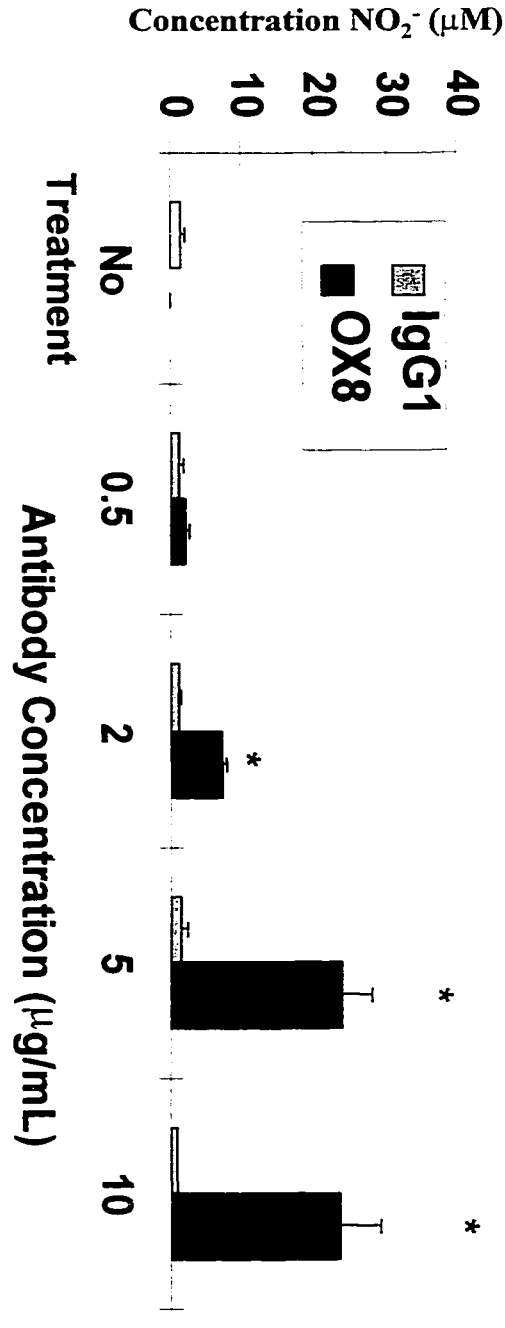
To test the functional significance of CD8 on macrophages, we stimulated alveolar macrophages with 0.5, 2, 5, or 10 μg/ml OX8 (24 h), and compared it with concentration-matched IgG1 isotype control antibody, and assessed the production of NO (Fig. 2.7). There was a significant ($p<0.01$) increase in NO production with 2 (7.1 ± 0.7 μM NO_2^-), 5 (23.8 ± 4.1 μM NO_2^-), and 10 (26.3 ± 8.2 μM NO_2^-) μg/ml OX8 compared to 2 (1.1 ± 0.5 μM NO_2^-), 5 (1.5 ± 0.9 μM NO_2^-), and 10 (0.8 ± 0.01 μM NO_2^-) μg/ml IgG1. Thus, alveolar macrophages express a functional CD8 molecule that, when crosslinked by OX8 antibody, stimulates NO production.

DISCUSSION

We have shown that rat alveolar and peritoneal macrophages express a novel CD8 molecule that differs from CD8 on T-lymphocytes. Flow cytometry of bronchoalveolar lavage (R3) identified a large number of CD8 positive cells for both the α (63 ± 5%, OX8) and β (52 ± 3%, 341) chains (Figs. 2.1-2.3). Double staining of lavage cells confirmed the presence of CD8α (OX8) on alveolar macrophages (OX41) (Fig. 2.4).

Because alveolar macrophages are potent phagocytotic cells (3, 4, 21, 22) one possible explanation for the presence of CD8 on alveolar macrophages could be that

Figure 2.7 The release of NO (using Greiss reagent) from FACS sorted alveolar macrophages (OX41) stimulated with anti-CD8α (OX8) for 24 h, compared with isotype control (IgG1). NO released represents $[NO_2^-]$ μM mean ± SEM, n=3 (number of experiments in duplicate), $2X10^5$ cells/sample were analyzed. * $p<0.01$ OX8 versus IgG1.



these cells take up CD8, either in a soluble form (23-25) or by ingesting CD8 positive T-lymphocytes, and re-expressing it on the cell surface. RT-PCR on FACS enriched alveolar macrophages (<1% OX52 (T-lymphocyte) contamination) and CD8 enriched splenic T-lymphocytes (positive control) showed the presence of CD8α mRNA in both cell populations. Thus, CD8 on alveolar macrophages is likely a product of protein synthesis and not due to re-expression of ingested CD8.

Flow cytometry identified differences in the abundance of CD8 α and β positive cells in both alveolar macrophages and T-lymphocytes. Within the lymphocyte rich region of bronchoalveolar lavage (R1, Fig. 2.3), the percent of CD8β positive cells (9 ± 2% (341)) was significantly less ($p<0.01$) than the number of CD8α cells (23 ± 3% (OX8), 22 ± 4% (G28), indicating that there are cells within R1 expressing CD8 α/β^- in addition to CD8 α/β. Given our evidence that the antibody to the N-terminal Ig-like domain of CD8α (G28) does not recognize alveolar macrophage CD8, as the percentages of OX8 and G28 are similar in R1, we believe the CD8 α/β^- cells within R1 are T-lymphocytes. Our results are consistent with those of others that have shown the presence of CD8 α/β^- T-lymphocytes at other mucosal sites. Within the gut mucosa of mice, up to 90% of intraepithelial lymphocytes are CD8 positive, with greater than 50% expressing the α chain only (26-29).

Within R2, the percent of CD8β (341) positive cells was also significantly less ($p<0.01$) than number of CD8α (OX8) positive cells, but not when stained with G28 (CD8α N-terminus). In addition, the numbers of cells positive for OX8 (CD8α hinge) was significantly greater than for G28 (CD8α N-terminus) ($p<0.01$). Therefore, it

appears that some alveolar macrophages in region R2 are CD8α/β$^-$ and may express the CD8 α/α homodimer. With R3, there was no statistical difference between the percent of CD8α (OX8, 63 ± 5%) and CD8β (341, 52 ± 3%) positive macrophages, indicating that they express the CD8 α/β heterodimer.

To examine the distribution of CD8 on other cells, flow cytometry studies on peritoneal macrophages were conducted. Peritoneal macrophages (86 ± 3%, CD11b (OX42)) were positive for both the α (OX8, 49 ± 8%) and β (341, 37 ± 4%) chains of CD8. In addition, there was no significant difference between the percent of CD8α (OX8) and CD8β (341) cells within peritoneal lavage, indicating that peritoneal macrophages express CD8α/β.

Flow cytometry studies on alveolar and peritoneal macrophages provided evidence that there are differences between macrophage and T-lymphocyte CD8. The antibody to the N-terminus of the Ig-like domain of CD8α (G28) did not appear to bind alveolar (OX41) or peritoneal macrophages (CD11b, OX42); the percentage of G28 (CD8α N-terminus) positive cells in regions R2, R3 (Fig.2.3) and peritoneal lavage (Fig. 2.6) was significantly less ($p<0.01$) than the percent of OX8 (CD8α hinge) positive cells. These differences could not be explained by technical variations in antibody fluorescence intensities, as both antibodies stained splenic lymphocytes and the lymphocyte rich region of bronchoalveolar lavage equally (no statistical difference between OX8 and G28 within R1, Fig. 2.3). Thus, although we have intriguing evidence for differences between CD8 on macrophages and on T-lymphocytes, the nature of the difference is unclear.

Differential splicing of macrophage CD8 mRNA, compared with T-lymphocyte CD8 mRNA, could lead to different isoforms of CD8 being expressed and explain the apparent lack of the G28 domain on macrophages. Johnson *et al* (30) and Lawlor *et al* (31) identified possible splicing sites for CD8 that could lead to novel mRNAs and thus novel proteins. Two different isoforms of CD8α have been identified; a mouse CD8α chain, CD8α′, which lacks the cytoplasmic tail (2) and human CD8α which lacks the transmembrane region (32). Therefore, the mRNA for macrophage CD8α could be missing all or part of the Ig-like region.

Changes in protein structure after translation of mRNA into protein (post-translational modification) could also be responsible for the novel CD8 molecule expressed by macrophages. The Ig-like domain of the CD8α chain is glycosylated (30, 33) and it is possible that differences in glycosylation could account for the apparent lack of G28 binding in macrophages. Interestingly, Barber *et al* (33) demonstrated the presence of various forms of CD8α using 2D electrophoresis that may be due to differences in sialylation or phosphorylation of the protein.

Our results suggest that there is post-translational modification of macrophage CD8α because the CD8α primer set, used in RT-PCR, encompassed the Ig-like domain of CD8α. Therefore, the Ig-like region appears to be altered at the protein level in alveolar macrophage CD8α, but is present at the mRNA level. However, as the sequence of macrophage CD8 is unknown, differences at the mRNA level between CD8 in macrophages and T-lymphocytes cannot be ruled out.

Our results are supported by those of others who have reported CD8 on cells other than T-lymphocytes. Hozumi *et al* (35) showed that progenitors of rat Kupffer

cells (macrophages from the liver) expressed both the protein and mRNA for CD8α. Furthermore, CD8α has also been shown on a murine mast cell line (37). However, our results differ from Hozumi et al (35) in that they could not detect CD8 on peritoneal macrophages or CD8β on pre-Kupffer cells, whereas we have demonstrated the expression of both CD8 α and β on alveolar and peritoneal macrophages. The reasons for these differences are unclear.

Even with the supporting evidence of *Hozumi et al* (35), the question of why no other groups have shown CD8 on macrophages is important. The commercially available antibodies to human and mouse CD8 identify epitopes within the Ig-like MHC I binding domain. It is our hypothesis that macrophage CD8 differs from T-lymphocyte CD8 within this domain (lack of G28 binding) and so antibodies widely used to study CD8 would not recognize macrophage CD8. This is further supported by the fact that OX8 is the only commercially available antibody that recognizes the hinge region of CD8.

Why CD8 has not been found previously on rat macrophages is unclear. *Ward et al* (36) used OX8 and did not find CD8 on rat tissue macrophages or Kupffer's cells. These studies used immunohistochemistry to look for CD8, and given that CD8 expression appears to be lower on macrophages compared to T-lymphocytes, it would be difficult to determine the presence of CD8 on macrophages, especially if the background was high. More sensitive and less subjective techniques such as flow cytometry and PCR, used by both ourselves and *Hozumi et al* (35), are likely able to identify cell surface proteins missed by other techniques.

Our findings that macrophages express CD8 and that this molecule may be different from that on T-lymphocytes, leads to the question of the function of macrophage CD8. On T-cells the Ig-like domain of CD8 interacts with the α3 domain of MHC I (1, 2, 38) to act as a co-stimulatory molecule. If macrophages are missing all or part of the Ig-like domain, or if there are amino acid or glycosylation differences within this region, interaction between MHC class I and CD8 may be affected. This could dictate that there are alternative sites of interaction between MHC I and CD8α, or that there are alternative, hither to unknown ligands for CD8 on macrophages. Even with the apparent altered ligand binding site, we have demonstrated that the CD8 molecule on alveolar macrophages is functional. Anti-CD8α (OX8) alone, compared to IgG1 isotype controls, significantly ($p<0.01$) stimulated the release of NO (Fig. 2.7). Studies using the NO inhibitor amino guanadine confirmed CD8α stimulated NO release (48). Moreover, studies on the β-chain of CD8 determined that crosslinking CD8β, in the absence of CD8α crosslinking, significantly stimulated the release of NO (48). These results further stress the differences between alveolar macrophage and T-lymphocyte CD8. CD8β is involved in the maturation of CD8+ T-lymphocytes, but it's function on mature T-lymphocytes is unknown as CD8β gene-targeted mice (lacking CD8β) appear to have normal CD8 effector function (38, 39). In addition, NO production could not be stimulated by an antibody to another cell surface marker (OX 41) on alveolar macrophages (48), indicating that NO release was specific to CD8 stimulation and not due to crosslinking any surface molecules. If CD8 is identified on human macrophages, the question of function will be important. Other studies on NO release in human macrophages have been inconclusive. Human macrophages have been

shown to stain for iNOS protein and message, but their ability to produce large amounts of NO has not been demonstrated (40, 41). Therefore, CD8 on human macrophages may play a role in NO production, but it's role in regulating other mediators may be more prominent. These findings further demonstrate the difference between macrophage and T-lymphocyte CD8. T-lymphocyte CD8 functions as a co-receptor, by phosphorylating the ζ chain of the CD3-TCR complex, after interacting with the same MHC molecule as the TCR (42). In contrast, alveolar macrophage CD8 stimulates the release of NO directly without any apparent interaction with other surface molecules.

The ability of CD8 on macrophages to also act as a co-stimulatory molecule cannot be ruled out. On T-lymphocytes, the co-stimulatory effects of CD8 are mediated through the association of the tyrosine kinase p56-lck to the α chain (2, 34, 42). Macrophages express receptors for the Fc portions of antibodies (Fc receptors) which share the ζ chain with the lymphocyte TCR complex (43, 44). Moreover, immunoprecipitation experiments with CD16 identified the association of $p56^{lck}$, along with other proteins, to this complex (45-47). Therefore, during the signaling cascade of Fc receptor cross linking, macrophage CD8 may play an important co-stimulatory role. In addition to Fc receptors, there are many other molecules on macrophages that CD8 could play a critical co-stimulatory role, including CD14 and CD45. However, it is possible that CD8 on macrophages may not interact with $p56^{lck}$.

In summary, we have demonstrated the presence of CD8 on both alveolar and peritoneal macrophages (Fig. 2.2-2.4) and mRNA for CD8α in alveolar macrophages (Fig. 2.5). Furthermore, CD8 on macrophages and T-lymphocytes differs, perhaps as a result of post-translational modification, at the MHC I ligand binding domain of CD8α.

Finally, a ligand for CD8 (anti-CD8α, OX8) can stimulate the release of NO by alveolar macrophages. We hypothesize that the identification of differences between macrophage and T-lymphocyte CD8, particularly within the ligand binding domain, may lead to the discovery of novel ligands for CD8, alternative methods of CD8 signaling, and elucidate the functions of CD8 on macrophages.

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Chapter 3

Mechanisms of Macrophage Stimulation Through CD8 : Macrophage CD8 α and β Induce Nitric Oxide Production and Associated Killing of the Parasite *Leishmania major*

PREFACE

This chapter deals with the function and signaling pathways of CD8 α and β on alveolar macrophages. At the time, we were the only group to have published that ligation of macrophage CD8α was able to stimulate cell function. As macrophages expressed both CD8 α and β, it was essential to understand the contributions of each of these chains to macrophage function. In addition, we needed to elucidate the signaling mechanisms involved in CD8 stimulation. As the signaling pathways of T lymphocyte CD8 are well established, we used inhibitors to determine if macrophage CD8 uses similar or unique pathways for cell signaling.. Moreover, as macrophages are important in host defense, we determined if CD8 could stimulate macrophages to kill the parasite *Leishmania major*. In addition to the published data, we have added to this chapter our recent work on the role of Fcγ receptors in CD8 induced NO production (Table 3.II). Taken together, this work demonstrates, for the first time, that both CD8 α and β play an important role in regulating macrophage mediator release and stimulating AM host defense. The majority of this chapter is published in the "Journal of Immunology", 1998, 160: 6004-6011, under joint authorship with Drs. Tong-Jun Lin, Elyse Bissonnette, Miodrag Belosevic, and A. Dean Befus. The co-authors have provided a statement confirming that my contribution to this manuscript was significant.

This is to certify and confirm that Nadir Hirji made major and most significant contribution to the experimental work, scientific content, and writing of the paper entitled "Mechanisms of Macrophage Stimulation Through CD8: Macrophage CD8α and CD8β Induce Nitric Oxide Production and Associated Killing of the Parasite *Leishmania major*" by Nadir Hirji, Tong-Jun Lin, Elyse Bissonnette, Miodrag Belosevic, and A. Dean Befus, *The Journal of Immunology*, 1997, 158: 1833-1840.

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Introduction

Nitric Oxide (NO) is an important mediator synthesized from the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). NO has been implicated in a variety of biological functions including neurotransmission, host defense, bronchodilation, and tumor cytotoxicity (1,2). When macrophages are stimulated with LPS or IFN-γ they produce copious amounts of NO, due to the upregulation of inducible NOS (iNOS) (1,2). In addition to paracrine and juxtacrine effects, macrophage NO also has an autocrine effect. The NO inhibitor NMMA increases macrophage production of IL-1β and IL-6 (3).

CD8 is a cell surface glycoprotein best known for its expression on a subset of T-lymphocytes with suppressor/cytotoxic functions and on NK cells (4 and 5). We have recently shown that rat macrophages contain both the message and protein for CD8 (6). Macrophage CD8 is a heterodimer composed of an α and β chain, and differs from T-lymphocyte CD8 within the ligand binding domain of the α chain (6).

Our initial studies on the function of macrophage CD8 determined that crosslinking the α chain, with the antibody OX8 (anti-CD8α), stimulated the release of NO (6). However, there is no understanding of the role of β chain in macrophage stimulation. On T-lymphocytes, the β chain of CD8 does not appear to play role in T-lymphocyte effector function, but may be important in their maturation (7). Because we have evidence for differences, both in structure and function, between macrophage and T-lymphocyte CD8, we investigated the role of CD8β on macrophage effector function (NO production).

To further understand the function of macrophage CD8, the pathway(s) involved in CD8 α and/or β stimulation of NO production was examined. As CD8α is linked to the protein tyrosine kinase $p56^{lck}$ in T-lymphocytes (8 and 9), and there is evidence that protein tyrosine kinase activity is involved in macrophage NO production (10 and 11), we used the broad spectrum protein tyrosine kinase inhibitor genistein (12) and an inhibitor of the *src*-family kinases, PP1 (13), to elucidate part of the pathway(s) involved in CD8 stimulated NO production. In addition to tyrosine kinase activity, we examined the roles of protein kinase C, inhibitors polymixin B (14) and Ro 31-8220 (15), and PI3 kinase, inhibitor wortmannin (16), on CD8 α (OX8) and β (341) stimulated NO production.

As the production of macrophage NO is dependent on the enzyme iNOS, we examined if crosslinking CD8 α and/or β upregulated the production of iNOS protein in alveolar macrophages. Moreover, the tyrosine kinase inhibitors, genistein and PP1, the PKC inhibitors, polymixin B and Ro 31-8220, and the PI3 kinase inhibitor, wortmannin, were used to elucidate the mechanisms of CD8 stimulated iNOS production.

Finally, given the important role of macrophages in host defense, we examined if CD8 could affect macrophage cytotoxicity. We used *Leishmania major*, an intracellular parasite that causes human disease ranging from self-healing ulceration to fatal systemic infection (17), as a model to test macrophage host defense. During the life cycle of *Leishmania*, the parasite replicates within macrophages (17). Therefore, we examined if infected macrophages could be induced to kill this parasite when stimulated with either anti-CD8α (OX8) or β (341).

Materials and Methods

Animals

Adult male Sprague-Dawley rats, 200 to 300 g, were obtained from Charles River Canada Inc. (Quebec, Canada) and maintained in an isolated room in filter-top cages to minimize unwanted infections. The animals were given food and water *ad libitum* and maintained on a 12 h (0700 h) -12 h (1900 h) light-dark cycle. No experimental procedures were performed on animals within the first week after arrival, decreasing the effects of stress associated with transport, new housing facilities and handling. All experimental procedures were approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Isolation of bronchoalveolar cells

Animals were anaesthetized by i.p. injection of 0.5 ml Rompun (Xylazine) and 0.5 ml Ketalean (Ketamine). The trachea was catheterized with a polyethylene tube and 12 X 5 ml of cold PBS was massaged into the lungs. Lavage cells were pelleted at 200 X g for 20 min and resuspended in PBS (6 and 18). Within rat alveolar lavage, the percent of alveolar macrophages, tested by esterase staining, was 96±0.4% (82.2±1.2 OX41 positive). The expression of CD8 on alveolar macrophages, using an enriched population (89±1% OX41 positive), was 63±5% for CD8α (antibody OX8) and 52±3% for CD8β (antibody 341) (6).

Measurement of NO production

Alveolar macrophages were incubated ($2X10^5$ cells/test) with 0.5-20 μg/ml of antibody for 24 h. Cell-free supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% N-(l-naphthyl)-ethylene-diamine dihydrochloride, 2.5% H_3PO_4) and incubated for 10 min at room temperature (19). Concentration of NO_2^- was determined by measuring the absorbance at 540 ηm with a Molecular Devices V max Kinetic Microplate Reader (Menlo Park, CA, USA). $NaNO_2$ was used as a standard. In experiments with genistein (Calbiochem, La Jolla, CA), PP1 (Calbiochem), wortmannin (Sigma), polymixin B (Sigma), and Ro 31-8220 (ROCHE, Welwyn Garden City, UK), inhibitors were added 10 min prior to addition of antibodies.

Western Blot Analysis of iNOS Protein

Alveolar macrophages ($1X10^6$ cells/mL) were incubated (24 h) with antibodies, OX8 (anti-CD8 α, 0.5 - 10 μg/mL) , 341 (anti-CD8 β, 0.5 - 10 μg/mL), IgG1 (isotype control, 0.5 - 10 μg/mL) and separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the proteins were blotted onto Hybond-C Super membrane (Amersham Life Science, Oakville, Ontario). The membrane was blocked over night in 10% instant skim-milk powder before incubation with antibodies. The membrane was incubated with rabbit anti-murine iNOS (kindly provided by Dr. J. Weidner, Merck Research Laboratories, Rahway, NJ) for 1 h, after which $F(ab)_2$ goat-anti rabbit HRP (Jackson Laboratory, Bar Harbor, ME) was used to identify specific proteins. Control experiments were run using secondary antibody alone. In addition, experiments with the peptide used to raise the rabbit anti-murine iNOS antibody

(peptide NO17, kindly provided by Dr. J. Weidner, Merck Research Laboratories, Rahway, NJ) were used to determine the specificity of the proteins identified by western blot analysis. Anti-iNOS was pre-incubated for 1 h with 1 μM of peptide before addition to membrane. Pre-stained rainbow molecular weight standards (Bio-Rad Laboratories, Mississauga, ON) were used as markers. Visualization of the HRP was done using Western Blot Chemiluminescence Reagent (DuPont, Boston, MA) according to manufacturer's protocol.

In experiments with genistein (12), PP1 (13), wortmannin (16), polymixin B (14), and Ro 31-8220 (15), inhibitors were added 10 min prior to addition of anti-CD8 α or β.

Densitometry of the iNOS protein was determined using ImageMaster 1D/2D gel analysis system (Pharmacia Biotech, Baie D'Urfe, Quebec, Canada).

Leishmania major parasite

The NIH 173 (WHOM/Ir/-/173) strain of *Leishmania major*, isolated from a patient in Iran (20), was used in this study. The amastigotes of *Leishmania major* were maintained by serial passage in BALB/c mice (50 μl containing $2X10^6$ amastigotes inoculated subcutaneously into footpads) (21,22). Four to 5 weeks after infection, monodispersed amastigotes were obtained by disruption of infected footpad tissue and passage through #50 stainless steel mesh screens into Dulbecco's Modified Culture Medium (DMEM) (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco) and gentamicin (Sigma, St. Louis, MO). Parasites were stained

with fluorescein diacetate (ICN, Cleveland, OH) and ethidium bromide (ICN) (23) and the number of viable amastigotes determined using a hemocytometer.

Intracellular parasite killing assay

Rat alveolar macrophages were suspended in DMEM at a concentration of $1X10^5$ cells/0.5 mL in polypropylene tubes (Falcon, Becton Dickinson, Lincon Park, NJ). To each culture, $4X10^5$ viable amastigotes were added and the cultures incubated for 2 h at 37°C and 5% CO_2. Cells were washed 2 times (0.5 mL DMEM, 200 g for 5 min) and resuspended in 0.5 mL DMEM. Appropriate concentrations of test solutions were added (0.1 mL) and the cells incubated for 72 h at 37°C in 5% CO_2. After incubation, 0.1 mL of the cell suspension was used to prepare cell smears using a cytocentrifuge (Shandon, Pittsburgh, PA) and stained with Wright stain (Leukostat, Fisher Sci., Orangeburg, NY). The percentage of infected cells out of 200 macrophages was determined by microscopic examination of stained cell smears under oil immersion (21,23). Experiments were conducted in quadruplicate sets, and repeated three times. The data shown is from one representative experiment.

Statistical analysis

Statistical significance between any two groups was analyzed by two tailed Student's T-test. Data in text represents mean ± SEM; n = separate experiments (mean of triplicate samples) using pooled cells from 2-6 rats.

Results

Anti-CD8 α and β stimulated NO production in alveolar macrophages

The production of NO (24 h) by alveolar macrophages was examined using the Griess reagent (Fig. 3.1). OX8 (anti-CD8α, 5 μg/mL) and 341 (anti-CD8β, 10 μg/mL) significantly ($p \leq 0.05$) stimulated the production of NO, compared to IgG1 (10 μg/mL) isotype controls. In addition, an antibody to another surface marker on alveolar macrophages (OX41, 10 μg/mL) did not stimulate NO production.

As macrophages are sensitive to LPS stimulation, antibodies were tested for endotoxin contamination (Limulus E-Toxate test, Sigma). OX8 and 341 were negative for endotoxin. As the lower limit of the test is 0.05-0.1 EU (≈ 0.02-0.04 ng/mL LPS) and with previous work on macrophages demonstrating that 1 ng/mL is not sufficient to induce significant NO release (24), OX8 and 341 stimulation of NO was not due to LPS contamination.

Anti-CD8 α and β stimulates the production of alveolar macrophage iNOS protein

Because stimulating alveolar macrophages with either anti-CD8 α or β induced NO production (Fig. 3.1), we examined if there was an increase in iNOS protein using western blot. Alveolar macrophages were stimulated with OX8 (anti-CD8α, 5 μg/mL) or 341 (anti-CD8β, 10 μg/mL) (24 h) and examined for the upregulation of iNOS protein (Fig. 3.2). Compared to untreated cells or IgG1 (isotype control, 10 μg/mL) stimulated cells, OX8 and 341 upregulated iNOS protein (Fig. 3.2A). Using densitometry (figure 3.2C), it was determined that both OX8 and 341 significantly

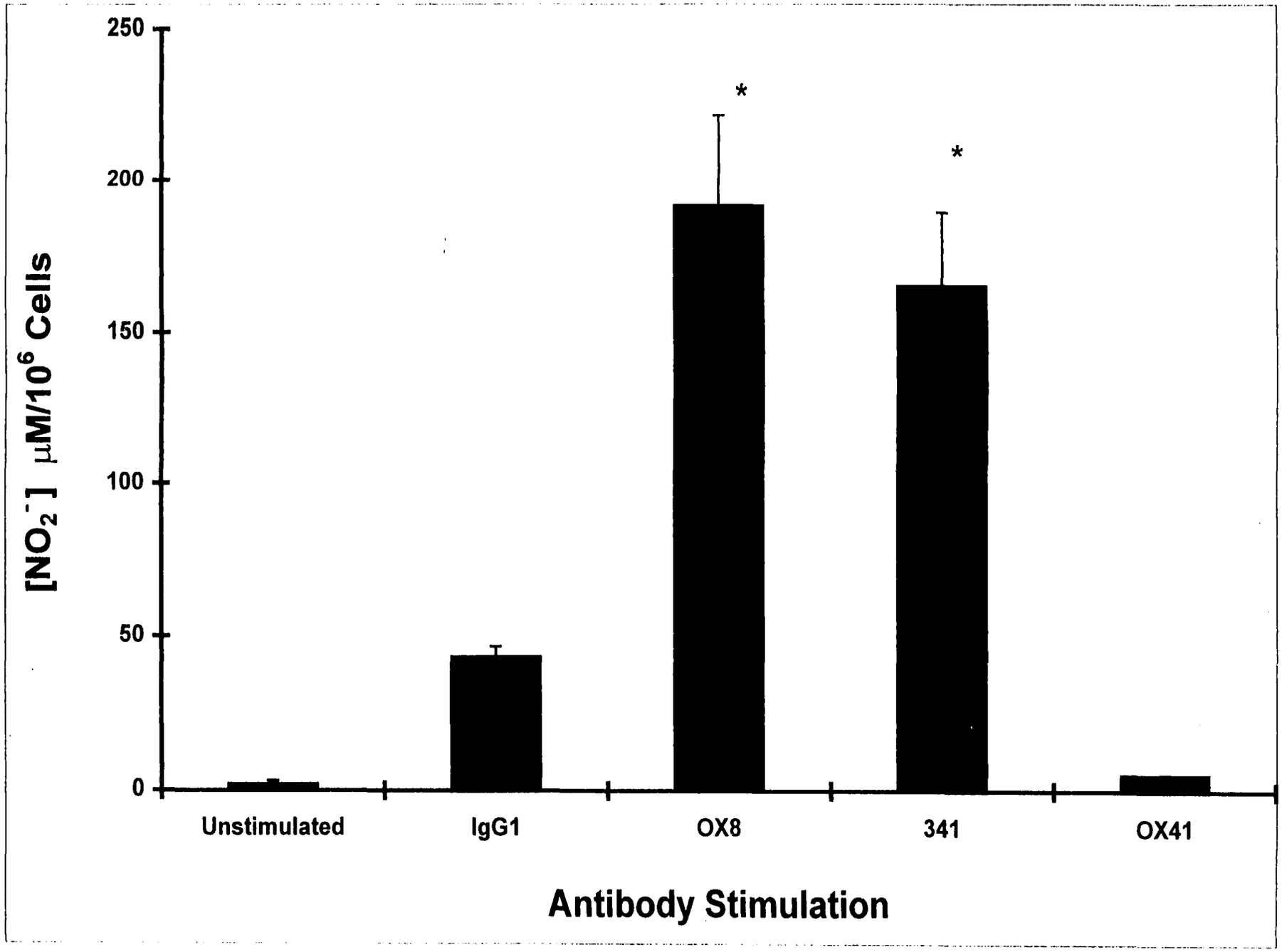
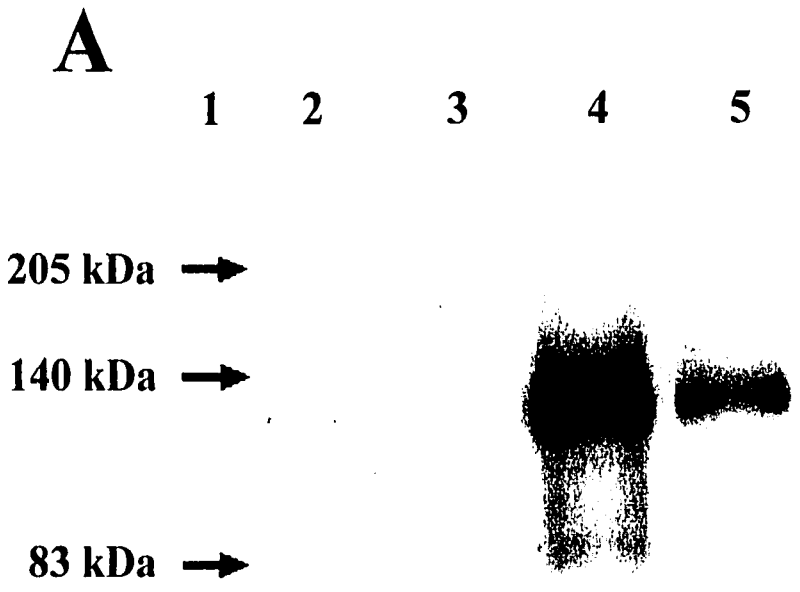
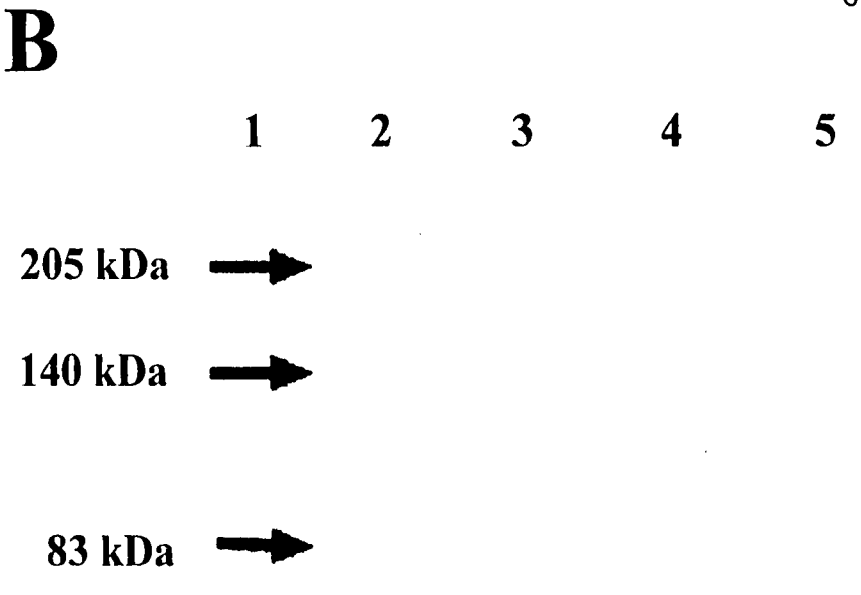
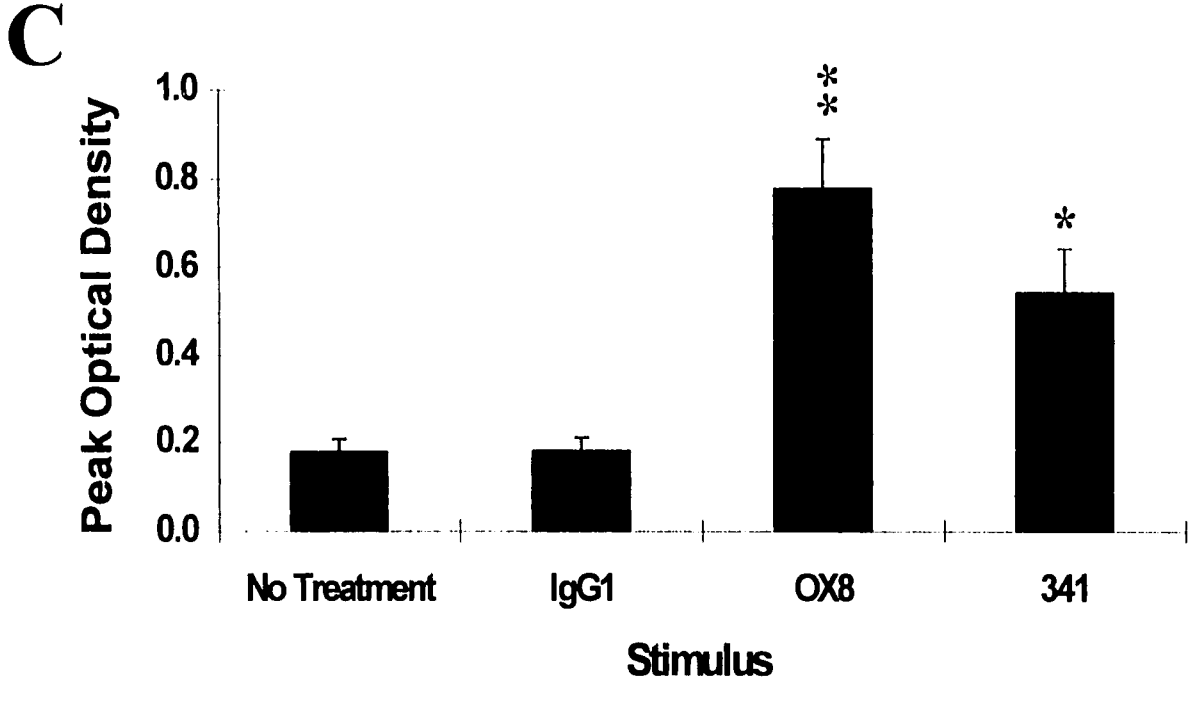


Figure 3.2 Anti-CD8 α (OX8, 5 μg/mL) and β (341, 10 μg/mL) stimulated (24h) upregulation of iNOS protein from AM. Figure A., lane 1. molecular weight standards, 2. no treatment, 3. isotype control (IgG1, 10 μg/mL), 4. OX8 (5 μg/mL), 5. 341 (10 μg/mL). Figure B. Anti-murine iNOS was pre-incubated (1 h) with NO17 (iNOS peptide, 1 μM) before use in western blot analysis. Lane 1. molecular weight standards, 2. no treatment, 3. isotype control (IgG1, 10 μg/mL), 4. OX8 (5 μg/mL), 5. 341 (10 μg/mL). Figure C., densitometry of iNOS production by anti-CD8 α or β. ** $p \leq 0.01$ OX8 versus IgG1, * $p \leq 0.05$ 341 versus IgG1, n=3.







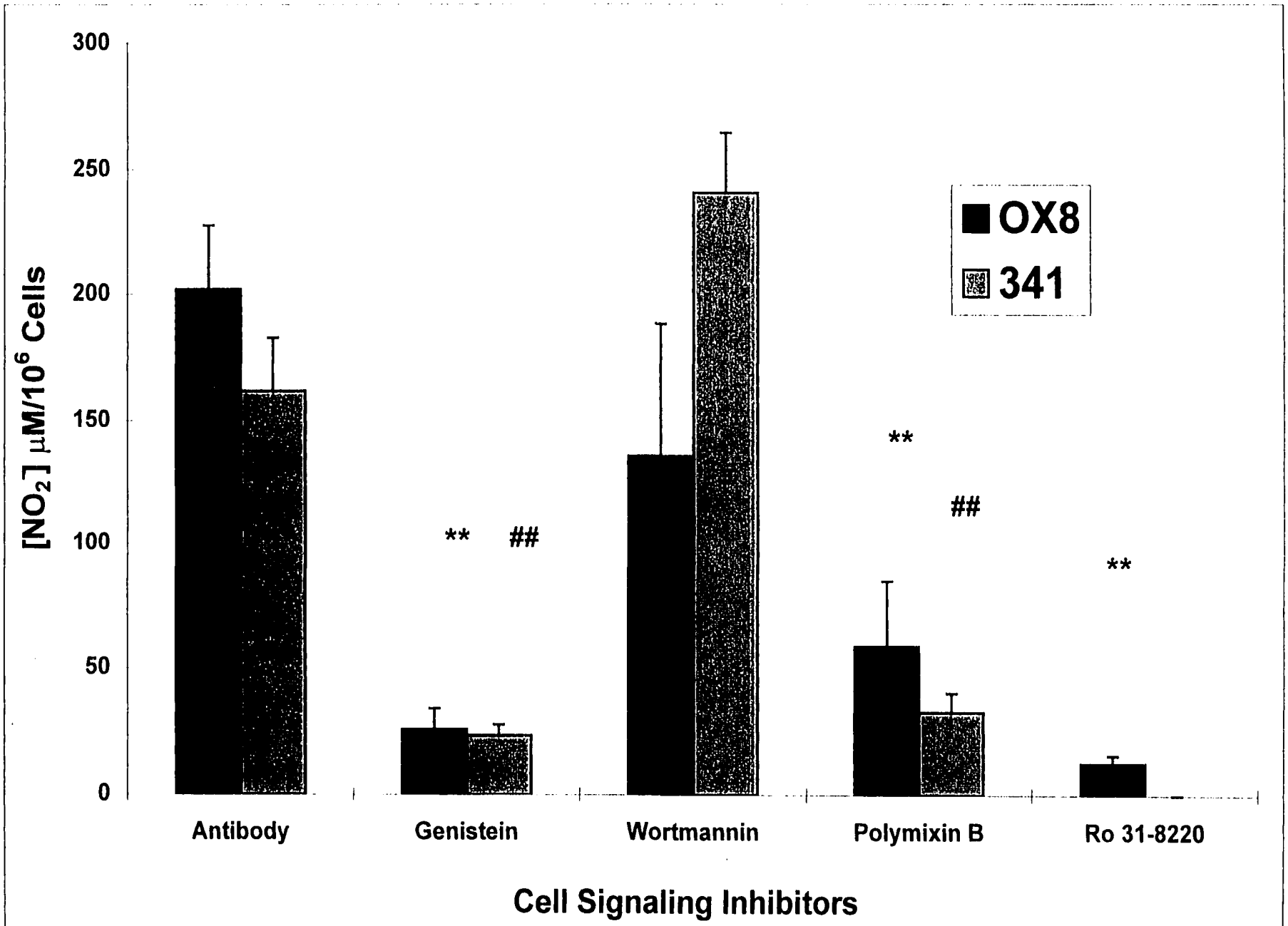
($p \leq 0.01$, $p \leq 0.05$) stimulated the production of iNOS protein compared to isotype control.

To determine if the 130 kDa band was iNOS, the anti-iNOS antibody was pre-incubated (1 h) with NO17 peptide (1 μM), which corresponds to the antigenic determinant identified by the anti-murine iNOS antibody, before use in western blot analysis (Fig. 3.2B). Using these conditions, the band corresponding to iNOS protein was not identified, indicating that the 130 kDa protein upregulated by OX8 and 341 is iNOS.

Determination of the mechanisms involved in CD8 α and β stimulated NO production and iNOS upregulation

Because CD8 in T-cells is associated with a protein tyrosine kinase (8 and 9) and there is evidence that NO production in macrophages is linked to protein tyrosine kinase activity (10 and 11), we examined the effects of a broad range protein tyrosine kinase inhibitor, genistein, on OX8 (5 μg/mL) and 341 (10 μg/mL) stimulated NO production by alveolar macrophages (Fig. 3.3). To further elucidate the pathway(s) of CD8 stimulation, inhibitors to PI3 kinase (wortmannin) and PKC (polymixin B) were also used. Dose response studies were used to identify optimal concentrations for each inhibitor. Genistein (10 μg/mL) and polymixin B (100 μg/mL) significantly ($p \leq 0.01$) inhibited (24 h) OX8 (5 μg/mL) and 341 (10 μg/mL) induced NO production (Fig. 3.3). As the antibodies used were shown to be LPS negative, the effects of polymixin B on OX8 and 341 stimulated NO release cannot be due to

Figure 3.3 Inhibition of anti-CD8 α (OX8, 5 μg/mL) and β (341, 10 μg/mL) stimulated (24h) NO production (Griess reagent, NO_2^- μM / 10^6 cells) by the inhibitors genistein (broad spectrum protein tyrosine kinase inhibitor, 10 μg/mL), wortmannin (PI3 kinase inhibitor, 1000 nM), polymixin B (PKC inhibitor, 100 μg/mL), and Ro 31-8220 (1 μM). ** $p \leq 0.01$ OX8 versus OX8 + genistein or OX8 + polymixin B, ## $p \leq 0.01$ 341 versus 341 + genistein or 341 + Polymixin B, n = 3-4.

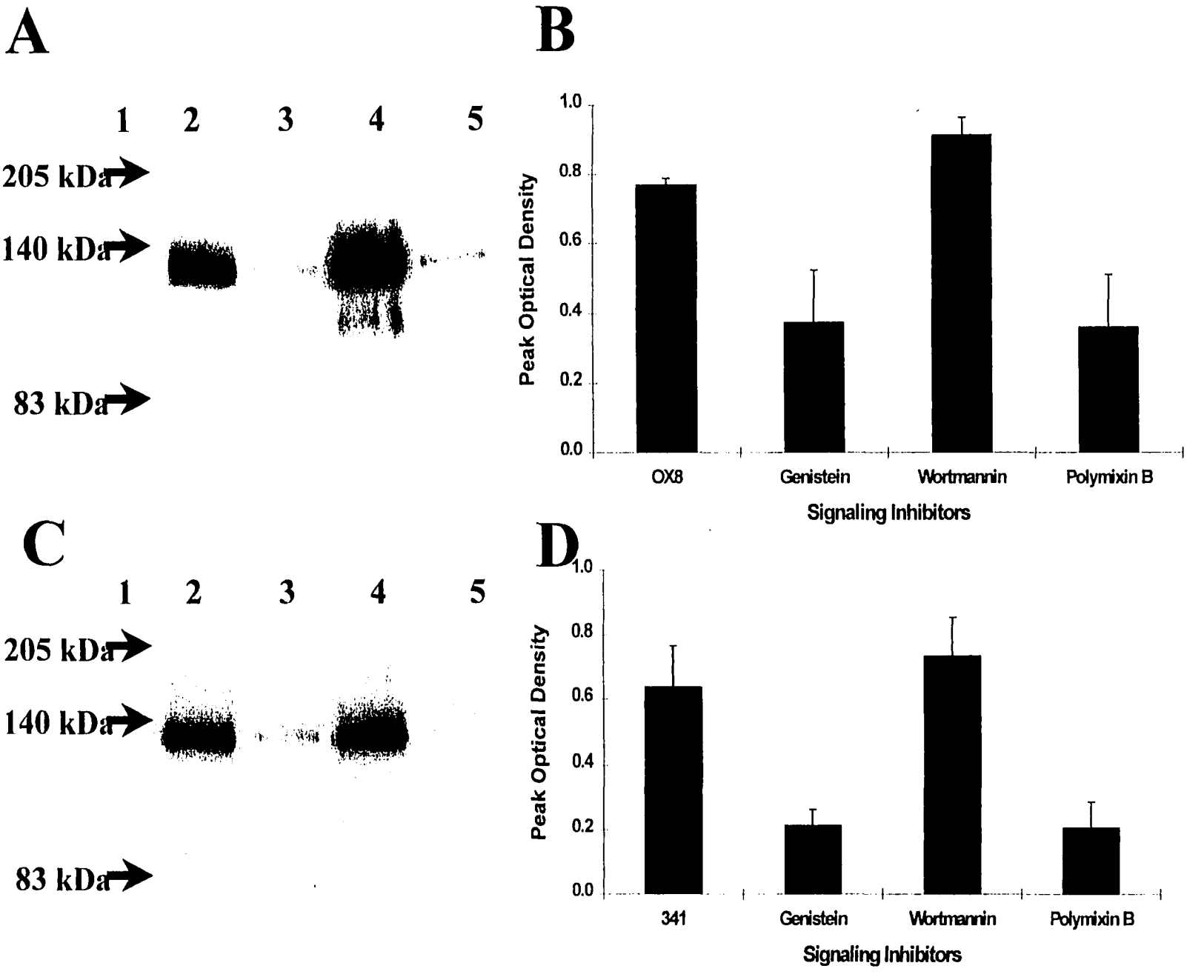


polymixin B binding contaminating LPS in the antibody preparations. In addition, as polymixin B has been shown to disrupt calmodulin-sensitive processes (25), a more selective inhibitor for PKC, Ro 31-8220 (15), was used to further examine the role of PKC in OX8 stimulated NO production. Ro 31-8220 (1 μM) inhibited ($p \leq 0.01$) OX8 (5 μg/mL) stimulated NO production (Fig. 3.3). In contrast, wortmannin (PI3 kinase inhibitor, 1 μM) did not inhibit OX8 (5 μg/mL) and 341 (10 μg/mL) stimulated NO production (Fig. 3.3). These results suggest that stimulating macrophages through the α (OX8) and/or β (341) chain(s) of CD8 signals NO production through a protein tyrosine kinase and/or PKC dependent pathway.

To further elucidate the pathways of macrophage CD8 stimulation, we used western blot analysis to determine if genistein, wortmannin, or polymixin B inhibited OX8 (Fig. 3.4A) and/or 341 (Fig. 3.4C) stimulated iNOS production. Genistein (10 μg/mL) and polymixin B (100 μg/mL) inhibited OX8 (5 μg/mL) and 341 (10 μg/mL) upregulation (24 h) of iNOS (Fig. 3.4 A and C). Consistent with the NO data in Fig. 3, wortmannin (1 μM) did not inhibit OX8 or 341 stimulated (24 h) iNOS production (Fig. 3.4 A and C).

The magnitude of the inhibition of iNOS upregulation was determined by densitometry (Fig. 3.4 B and D). Consistent with the NO data in Fig. 3.3, densitometry of iNOS (Fig. 3.4 B and D) confirmed that genistein and polymixin B inhibited the upregulation of OX8 (genistein 48% and polymixin B 47% inhibition) and 341 (genistein 33% and polymixin B 33% inhibition) stimulated iNOS.

Figure 3.4 Inhibition of anti-CD8 α (OX8, 5 μg/mL) and β (341, 10 μg/mL) stimulated (24h) iNOS production by genistein and polymixin B. Lane 1. molecular weight markers, 2. anti-CD8 α (figure A) or β (figure C), 3. antibody + genistein (broad spectrum protein tyrosine kinase inhibitor, 10 μg/mL), 4. antibody + wortmannin (PI3 kinase inhibitor, 1000 nM), and 5. antibody + polymixin B (PKC inhibitor, 100 μg/mL).). Figure B (anti-CD8 α) and figure D (anti-CD8 β) densitometry of iNOS production by anti-CD8 α or β + inhibitors, n = 3.



src-Family kinase(s) are involved in CD8 stimulated NO production and iNOS upregulation

Data in Figs. 3.3 and 3.4 determined that protein tyrosine kinase(s) are involved in CD8 α and β stimulated NO production and iNOS upregulation, but did not identify the family of these kinases. Because *src*-family kinases are involved in T-lymphocyte CD8 stimulation (8 and 9) and are upregulated in lipopolysaccharide (LPS) and interferon-γ (IFN-γ) stimulated macrophages (26 and 27), we examined the role of *src*-family kinase(s) in CD8 α and β mediated stimulation. PP1 (5, 10, and 20 μg/mL), an inhibitor of src-family kinases (13), dose dependently inhibited ($p \leq 0.01$) OX8 (5 μg/mL) and 341 (10 μg/mL) mediated NO production (Fig. 3.5).

To further explore the role of *src*-family kinase(s) in CD8 mediated stimulation, the effects of PP1 on OX8 and 341 upregulation of iNOS was examined using western blot analysis (Fig. 3.6). Similar to the results in Fig. 5, PP1 dose dependently inhibited OX8 (Fig. 3.6A) and 341 (Fig. 3.6C) stimulated iNOS production. The magnitude of the inhibition was determined using densitometry. Figs. 3.6B (OX8) and 6D (341) confirm the dose dependent inhibition of anti-CD8 α (OX8) and β (341) mediated iNOS production by PP1.

Enhanced anti-parasitic activity by anti-CD8 stimulated alveolar macrophages

The functional significance of alveolar macrophage CD8 was further examined using the protozoan parasite *Leishmania major*. Alveolar macrophages infected with

Figure 3.5 Inhibition of *src*-family kinases (using the inhibitor PP1) to assess their role in anti-CD8 α (OX8. 5 μg/mL) and β (341. 10 μg/mL) stimulated (24h) NO production. ** p≤0.01 OX8 versus OX8 + PP1. ## p≤0.01 341 versus 341 + PP1. n = 3.

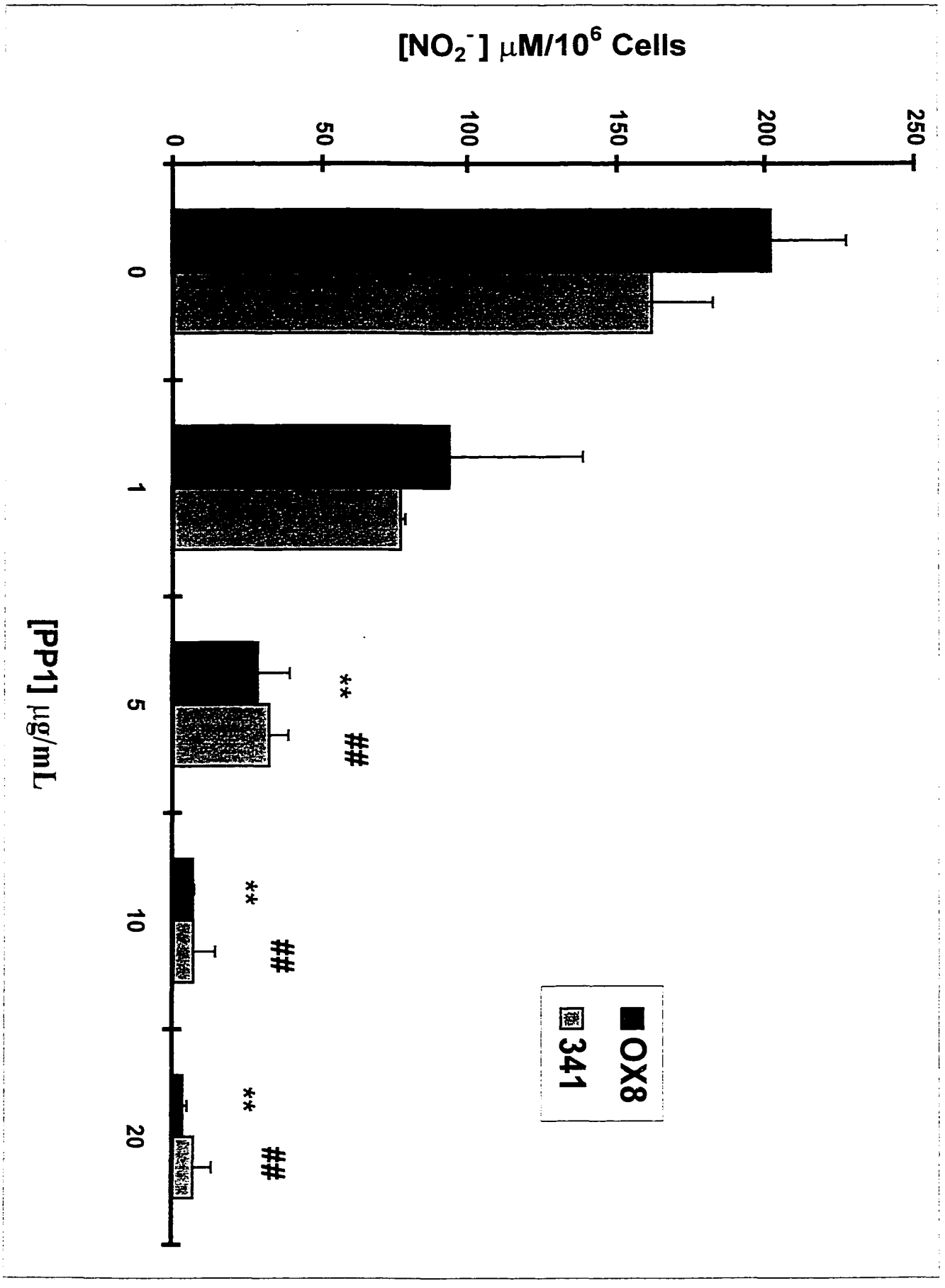
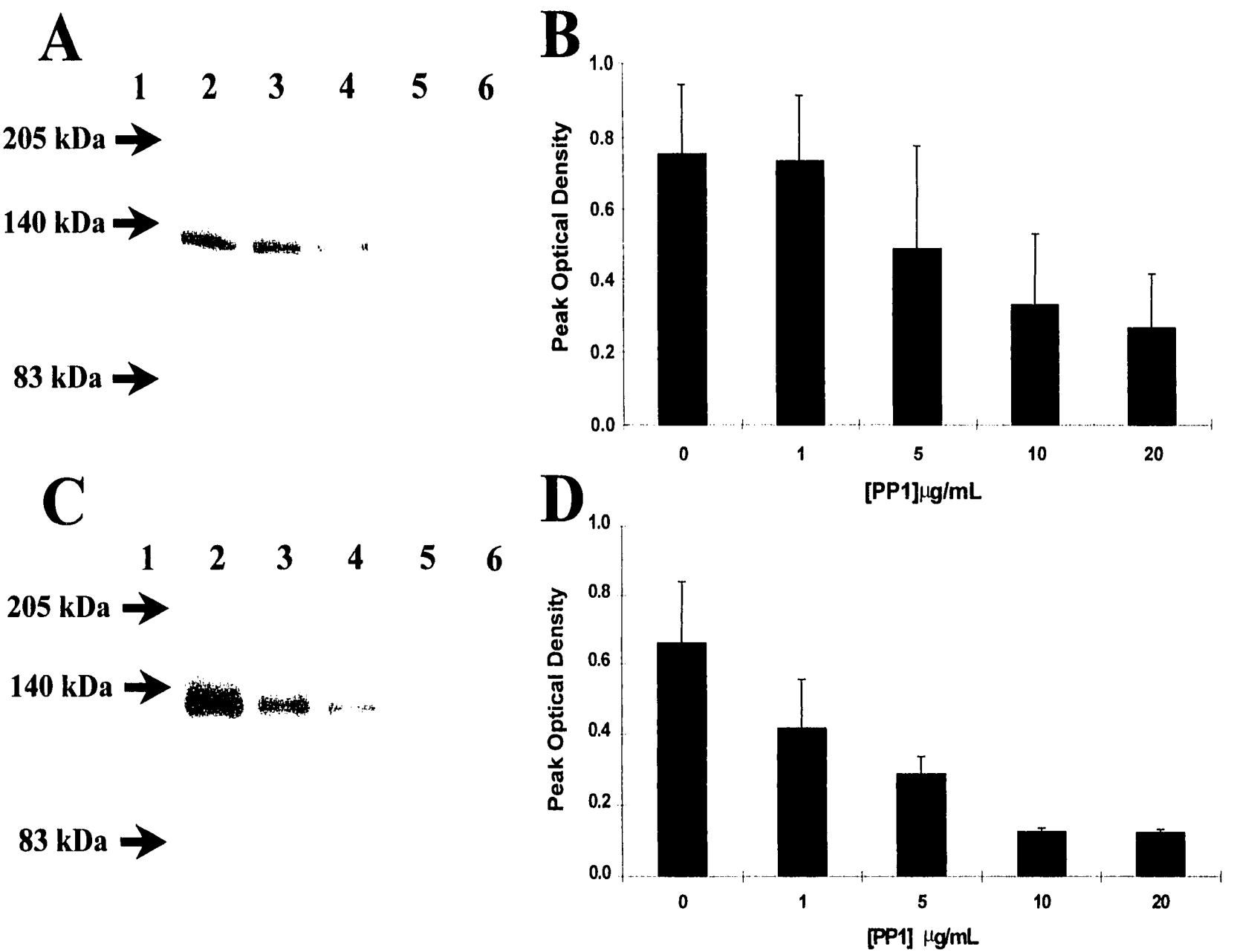


Figure 3.6 Src-family kinase(s) are involved in CD8 α (OX8, 5 μg/mL) and β (341, 10 μg/mL) stimulated (24h) iNOS production. Lane 1. molecular weight markers, 2. anti-CD8 α (figure A) or β (figure C), 3. antibody + 1 μg/mL PP1, 4. antibody + 5 μg/mL PP1, 5. antibody + 10 μg/mL PP1, 6. antibody + 20 μg/mL PP1. Figure B (anti-CD8 α) and figure D (anti-CD8 β) densitometry of iNOS production by anti-CD8 α or β + PP1, n = 3.



Leishmania major were stimulated for 72 h with either IFN-γ (control), OX8 (anti-CD8α), 341 (anti-CD8β), or IgG1 (isotype control), and the differences in infection were examined (Table 3.I). After 72 h, 82.3±4.4% of unstimulated alveolar macrophages were infected with the parasite. IFN-γ (200 U/mL) significantly ($p<0.001$) decreased the percent of parasite-infected alveolar macrophages, which correlated with an upregulation in NO production, compared to unstimulated cells. OX8 dose dependently (0.5 μg/mL, $p\leq0.05$, 2 μg/mL $p\leq0.01$, 5 and 10 μg/mL $p\leq0.001$) decreased the number of infected cells, compared to IgG1 isotype controls. In addition, there was a corresponding dose dependent increase in NO production. In this experiment, 341 (anti-CD8β) stimulated killing of *Leishmania*. However, this appeared to be independent of dose and NO production. Further experimentation using 341 failed to initiate a cytotoxic response in *Leishmania* infected macrophages. Therefore, CD8β appears to play a minor or limited role in stimulating macrophage cytotoxicity to *Leishmania*.

To determine if the protective effects of OX8 (anti-CD8α) were NO dependent, amino guanadine (a NO inhibitor) was used. Pre-treatment with amino guanadine (1 mM) reversed the protective effects of IFN-γ (63.5±11.5% infected cells), which was used as the internal control to verify activity. Amino guanadine also reversed OX8 (10 μg/mL, 78.0±4.1% infected cells) stimulated anti-parasitic activity, suggesting that the protective effects of OX8 (anti-CD8α) are NO dependent.

The role of Fcγ receptors in anti-CD8α induced NO release

These studies described above have used intact anti-CD8 α and β antibodies, along with isotype matched controls, to analyze NO release. As AM express Fc receptors, it is possible that the Fc ends of these antibodies interact with Fcγ receptors on AM to induced NO release.

To examine the role of Fcγ receptors, $F(ab)_2$ OX8 (Pharmingen, special order) was used to stimulate AM function (Table 3.II). This antibody was commercially made and tested by the supplier using gel electrophoresis and flow cytometric binding studies to T lymphocytes. Compared to a $F(ab)_2$ control ($F(ab)_2$ goat anti-mouse IgG), 2 to 10 μg/ml $F(ab)_2$ OX8 failed to stimulate AM NO release (Table 3.II, 10 μg/ml result shown). In addition, crosslinking $F(ab)_2$ OX8 (10 μg/ml) with a $F(ab)_2$ goat anti-mouse IgG H+L chain or $F(ab)_2$ goat anti-mouse $F(ab)_2$ (10 μg/mL) also failed to stimulate NO release (Table 3.II). Moreover, $F(ab)_2$ OX8 coated onto microsphere beads (10 μg/ml) (kind gift from Dr. Kevin Kane) did not stimulate NO production, compared to controls (Table 3.II). In these studies, control antibodies, which were from a different species, stimulated low amounts of NO release. Therefore, low levels of NO released by $F(ab)_2$ OX8 stimulated AM would not be detected in this study. To further test the involvement of Fc receptors, $F(ab)_2$ OX8 (10 μg/ml) was used to stimulate cells in conjunction with Fcγ receptor activation. The addition of soluble IgG1 (10 μg/ml) or crosslinked IgG1 (10 μg/ml IgG1 + 10 μg/ml anti-IgG), in conjunction with $F(ab)_2$ OX8 stimulation, failed to initiate NO release. Moreover, cross linking IgG1 (10 μg/ml IgG1 + 10 μg/ml anti-IgG) also failed to initiate NO release (Table 3.II).

Table 3.I Crosslinking CD8α stimulates anti-parasitic activity in AM, which is NO dependent. The percent infection of AM, infected *with Leishmania major*, after 72 h stimulation with IFN-γ (200 Units/mL), IFN-γ (200 Units/mL) + amino guanadine (AG, 1 mM), isotype control (IgG1, 0.5 - 10 μg/mL), OX8 (anti-CD8α, 0.5 - 10 μg/mL), 341 (anti-CD8β, 0.5 - 10 μg/mL), OX8 (10 μg/mL) + AG (1 mM), or 341 (10 μg/mL) + AG (1 mM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, percent infection OX8 or 341 versus IgG1. ¯ $p \leq 0.05$, ¯¯ $p \leq 0.01$, ¯¯¯ $p \leq 0.001$, percent killing IFN-γ, IgG1, OX8, or 341 versus unstimulated cells. Tests were carried out in quadruplicate using $1X10^5$ cells/sample, this is a representative experiment from three experiments which had similar results. This work was done in collaboration with Dr. M. Belosevic, who infected AM and counted the number of *Leishmania major* positive cells.

Table 3.I Cross linking CD8α stimulates antiparasitic activity in alveolar macrophages, which is NO dependent.

GROUP	INFECTION MEAN (%)	KILLING (%)	NO NO_2^- (μM)
Unstimulated	82.3 ± 4.4	0	3.4 ± 0.5
IFN-γ 200	21.7 ± 10.7	73.7^{+++}	71.6 ± 1.5
IFN-γ + AG	63.5 ± 11.5	22.8^{+}	5.3 ± 0.8
IgG1 0.5	81.2 ± 4.5	1.3	3.7 ± 0.4
IgG1 2	81.0 ± 4.1	1.5	4.4 ± 0.6
IgG1 5	83.7 ± 3.8	0	4.4 ± 0.4
IgG1 10	77.2 ± 4.4	6.1	4.5 ± 0.9
IgG1 + AG	79.2 ± 3.2	3.7	2.2 ± 0.2
OX8 0.5	76.5 ± 4.5*	7.0^{+}	2.9 ± 0.2
OX8 2	65.0 ± 6.4**	21.0^{++}	32.3 ± 1.1
OX8 5	34.0 ± 5.7***	58.7^{+++}	31.6 ± 1.6
OX8 10	16.7 ± 11.1***	79.7^{+++}	43.8 ± 2.4
OX8 + AG	78.0 ± 4.1	5.2	2.8 ± 0.1
341 0.5	65.5 ± 9.0**	20.4^{++}	19.8 ± 1.4
341 2	83.0 ± 5.9	0	7.0 ± 0.9
341 5	66.7 ± 6.4**	18.9^{++}	7.2 ± 0.4
341 10	80.7 ± 5.6	1.9	7.4 ± 1.0
341 + AG	79.5 ± 2.43	3.3	8.5 ± 1.1

We have concerns about the $F(ab)_2$ OX8 antibody used in these studies. Pre-incubation (1h) of BAL cells with NMS and $F(ab)_2$ OX8 (20 μg/ml) failed to inhibit OX8-FITC (3 μg/ml) binding, as determined by flow cytometery. In addition, pre-incubation (1h) of BAL cells with $F(ab)_2$ OX8 (20 μg/ml) failed to inhibit OX8 (5 μg/ml) induced NO release. The reasons why $F(ab)_2$ OX8 failed to inhibit OX8 binding and NO release is unclear. However, there may be avidity/affinity differences between the $F(ab)_2$ and intact antibodies that account for these results.

A series of blocking experiments were used to further elucidate the role of Fc receptors in CD8 induced NO release. Pre-incubation of AM with $F(ab)_2$ OX8 (30 μg/ml – 2h pre-incubation) failed to block OX8 induced NO release (Table 3.II). Pre-incubation (2h) of AM with BSA (3-10%) or mouse serum (10-20%) inhibited OX8 (5 μg/ml) induced NO release (Table 3.II). However, BSA and mouse serum pre-incubation also inhibited IFN-γ (200 U/ml) induced NO release (Table 3.II).

Discussion

The expression of CD8 is not exclusive to cytotoxic T-lymphocytes. Dendritic cells and macrophages also express CD8 (6, 28, and 29). CD8 plays an important role in cytotoxic T-lymphocyte adhesion and function (5 and 30). Previous work on alveolar macrophages demonstrated differences within the ligand binding domain (the α chain of CD8) of macrophage CD8 compared to T-lymphocytes (6). Initial work on the function of alveolar macrophage CD8 demonstrated that crosslinking CD8α stimulated NO production from alveolar macrophages (6).

Table 3.II Multiple experimental techniques to examine the role of Fcγ receptors in CD8 induced stimulation of NO release. AM were stimulated with $F(ab)_2$ OX8 (2-10 μg/ml, 10 μg/ml result shown here), cross linked $F(ab)_2$ OX8, or microsphere immobilized $F(ab)_2$ OX8 (10 μg/ml) for 24h. In addition, AM were pre-incubated (2h) with BSA (3-10%) or mouse serum (10-20%) before OX8 stimulation of NO release (24h). n=2-3.

Table 3.II Multiple experimental techniques to examine the role of Fcγ receptors in CD8 induced stimulation of NO release

TEST n=3-4	NO Release	Observations and Hypotheses
IgG1	43.3±3.3	Soluble antibody does not stimulate AM
OX8 (anti-CD8α), IgG1 isotype	191.2±30.4	Anti-CD8α stimulates NO release, which may not be due to non-specific stimulation through Fc receptors, as IgG1 does not stimulate NO release.
$F(ab)_2$ control (goat anti-mouse IgG)	26±15	$F(ab)_2$ does not non-specifically activate AM
$F(ab)_2$ OX8	2.5±1.44	OX8 affects may be partially related to the Fc portion of the antibody, suggesting a role for Fc receptors
$F(ab)_2$ (goat anti-mouse IgG) control+ $F(ab)_2$ goat anti-mouse $F(ab)_2$	152.8±108	Cross linking a $F(ab)_2$ control activates AM, the mechanisms for which is unclear
F(ab)2 OX8 + $F(ab)_2$ anti-IgG	30.7±17.7	Cross linking a $F(ab)_2$ control does not stimulate NO release, suggesting a role for Fc receptors in OX8 stimulation of AM
Microsphere beads	595.9±344	Beads non-specifically activate AM
$F(ab)_2$ control coupled to microsphere beads	611.5±353.1	Coupling a $F(ab)_2$ antibody to the beads does not enhance/suppress activation of AM
$F(ab)_2$ OX8 coupled to microsphere beads	558.3±322.4	Coupling $F(ab)_2$ OX8 to beads, to form a solid matrix, does not further enhance AM activation, suggesting that Fc receptors may be involved
$F(ab)_2$ control + IgG1	0*	Non-specific activation with a $F(ab)_2$ antibody, along with Fc signalling, does not activate AM
$F(ab)_2$ OX8 + IgG1	36.0*	$F(ab)_2$ stimulation of OX8 in conjunction with Fc receptor stimulation does not activate AM, suggesting that Fc receptors are not involved
$F(ab)_2$ OX8 + IgG1/anti-IgG	51.9*	$F(ab)_2$ stimulation of OX8 in conjunction with Fc receptor cross linking does not activate AM, suggesting that Fc receptors are not involved
$F(ab)_2$ OX8 + OX8	211*	$F(ab)_2$ OX8 does not block OX8 induced NO release.
IgG1/anti-IgG	5.5*	IgG1 cross linking does not activate AM, suggesting that OX8 activation is Fc receptor independent
BSA blocking of AM + OX8	-**	BSA blocking non-specifically inhibits OX8-induced AM activation
BSA blocking of AM + IFN-γ	-**	BSA blocking non-specifically inhibits AM activation
Serum blocking of AM + OX8	-**	Serum blocking non-specifically inhibits OX8-induced AM activation
Serum blocking of AM + IFN-γ	-**	Serum blocking non-specifically inhibits AM activation

* n=2, number represents a mean of the two experiments

** Multiple blocking procedures of AM were examined, therefore the data presented is a synthesis of these experiments

Further evidence for differences between alveolar macrophage and T-lymphocyte CD8 were demonstrated by experiments on CD8β. The antibody 341 (anti-CD8β) stimulated alveolar macrophage NO production and iNOS upregulation (Figs. 3.1 and 3.2). In contrast, experiments on T-lymphocyte CD8β have demonstrated that this chain plays a role in T-lymphocyte maturation. CD8β knockout mice and transgenic mice expressing "tailless" CD8β have decreased numbers of functionally active CD8 positive (31 and 32). Additional work has identified a role for CD8β in recognition of altered peptide ligands (33). These studies identify a role for T-lymphocyte CD8β in maturation and ligand binding. Our work demonstrates that CD8β is able to regulate, either directly or indirectly, macrophage effector function by stimulating mediator release.

The mechanisms of CD8 α and β stimulated macrophage function were examined using inhibitors to different signaling pathways. In T-lymphocytes, CD8 is associated with the *src*-family protein tyrosine kinase $p56^{lck}$ (8 and 9). Monocytes/macrophages express several *src*-family protein tyrosine kinases, including fgr, fyn, hck, and lyn (34). LPS and IFN-γ stimulated macrophages upregulate hck and lyn (26). In addition, hck is involved in TNF production by macrophages (35). Genistein, a broad spectrum tyrosine kinase inhibitor, inhibited OX8 (anti-CD8α) and 341 (anti-CD8β) stimulated NO production and iNOS upregulation (Figs. 3.3 and 3.4). To determine if the kinase(s) involved in CD8 mediated stimulation belong to the *src*-family, the inhibitor PP1 was used. PP1 dose dependently inhibited OX8 and 341 mediated NO production and iNOS upregulation (Figs. 3.5 and 3.6). These results suggest that *src*-family kinases are involved in CD8

mediated stimulation of alveolar macrophages, similar to T-lymphocyte CD8α mediated stimulation. However, the inhibition of CD8β stimulated macrophage function by PP1 further suggests differences between macrophage and T-lymphocyte CD8β. Macrophage CD8β may directly associate with a *src*-family kinase to stimulate mediator release independent of CD8α. Alternatively, macrophage CD8β may also function through CD8α to induce mediator release. T-lymphocyte CD8β has been shown to increase $p56^{lck}$ association with CD8α (36), thus enhancing the effector function of CD8α. Thuse, macrophage CD8β may work in a similar fashion.

Further studies on the signaling pathways of CD8 concentrated on PKC. Our studies using polymixin B and Ro 31-8220 (Figs. 3.3 and 3.4) demonstrated that CD8 α and β induced NO stimulation and iNOS upregulation is PKC-dependent. These results correlate with those of Paul *et al* (37) and Eason and Martin (15), who demonstrated that LPS and IFN-γ induced macrophage iNOS upregulation is PKC-dependent.

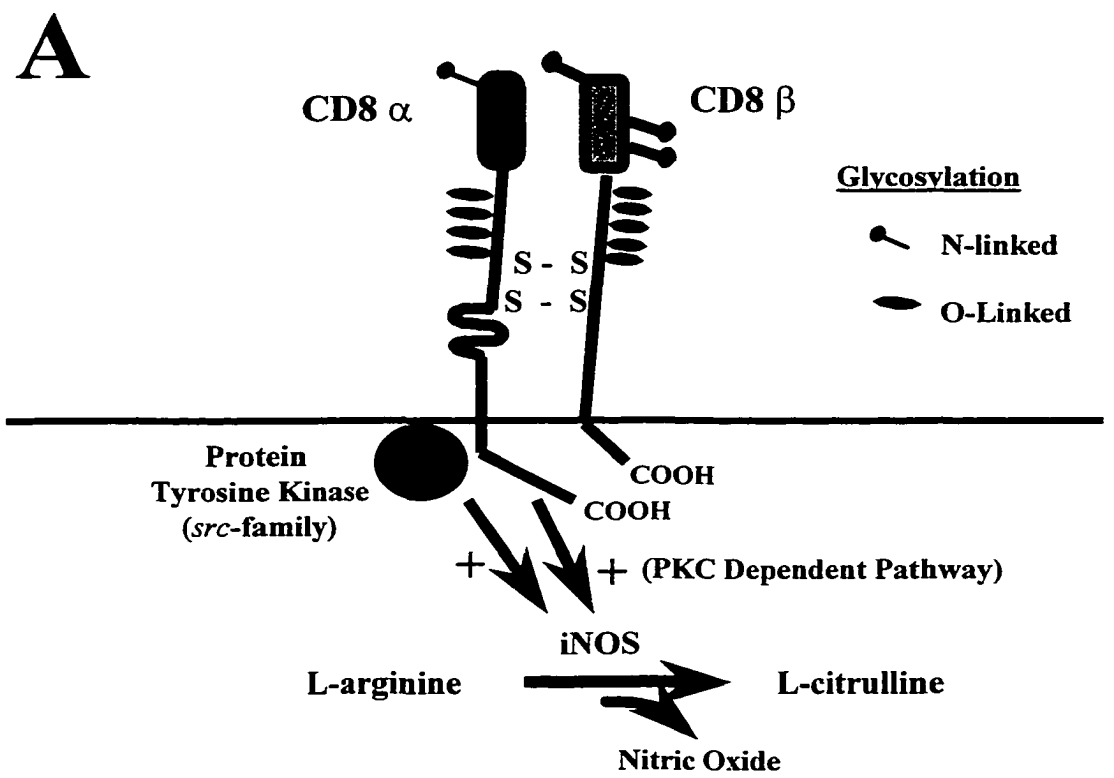
Intact OX8 or 341 have been used to stimulate AM. As AM express all three Fcγ receptor types, FcγRI (CD64), FcγRII (CD32), and FcγRII (CD16) (38) and stimulation through Fcγ receptors can induce macrophage NO release (39), it is possible that OX8 induced NO release could be partially or completely due to Fcγ receptor activation. $F(ab)_2$ OX8, or cross linked $F(ab)_2$ OX8, failed to NO release from AM (Table 3.II). This suggested that OX8 mediates its effects through the Fcγ receptor, which was surprising as IgG1 did not stimulate NO release. However, stimulating Fcγ receptors, via soluble or cross linked IgG1, in conjunction with $F(ab)_2$

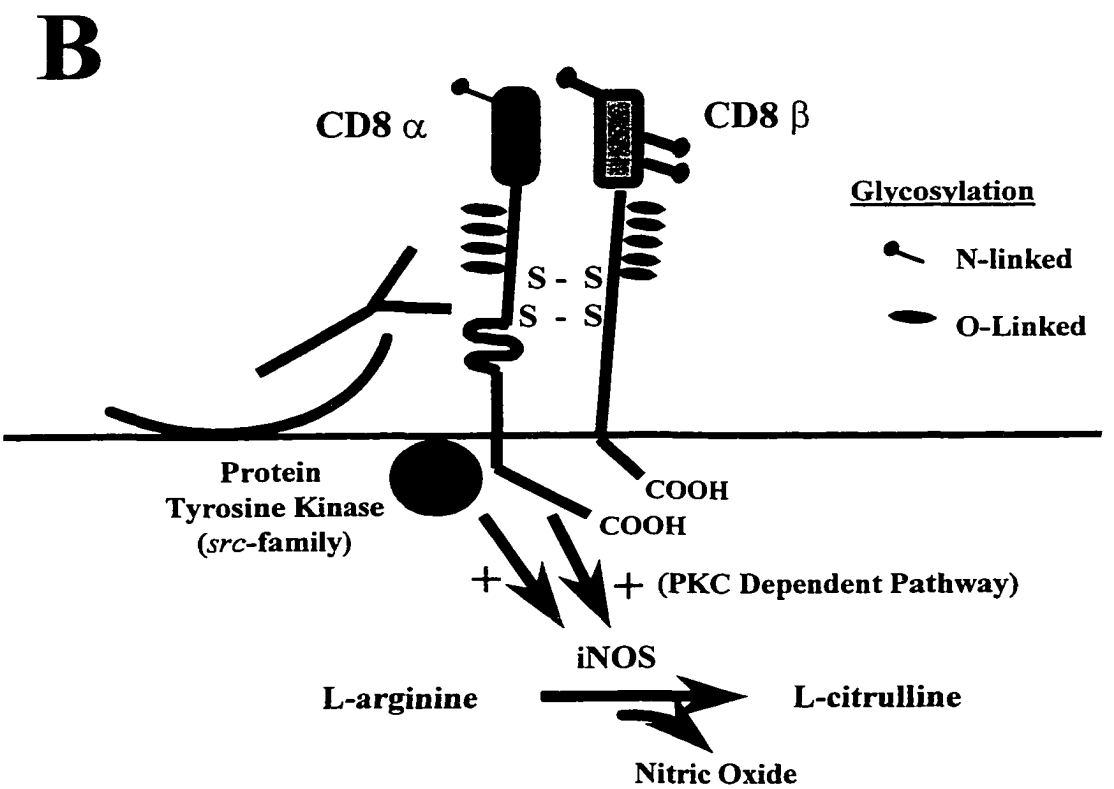
OX8 stimulation, did not induce NO release. These results suggest that Fc receptors are not involved in OX8 induced AM activation. OX8 blocking experiments, using $F(ab)_2$ OX8 to inhibit OX8 binding (Table 3.II), failed to clarify the involvement of Fcγ receptors in OX8 induced NO release, as $F(ab)_2$ OX8 did not inhibit OX8 induced NO release. This may be due to differences in binding avidity between $F(ab)_2$ OX8 and intact OX8. This was further suggested in flow cytometry studies that showed that $F(ab)_2$ OX8 stained AM had lower fluorescence than intact OX8 stained AM. Therefore, the role of Fcγ receptors in CD8 induced AM regulation remains unclear.

From these results, we have put forth two working models for CD8 stimulation (Fig. 3.7). In model one, CD8 may stimulate macrophage function directly through *src*-family kinase and PKC dependent pathway(s) (Fig. 3.7A). In model two, CD8 acts as a co-receptor for the Fcγ receptor(s) expressed by macrophages (Fig. 3.7B). Macrophages express the high affinity receptor FcγRI (38) and studies have shown that stimulating Fcγ receptors can induce the expression of iNOS in rat peritoneal macrophages (39). In addition, FcγRI receptors have been shown to associate with the *src*-family kinases hck and lyn (40). Therefore, our results may be explained by CD8 acting as a co-receptor in conjunction with Fcγ (Fig. 3.7B).

Experiments on NO stimulation using an IgG1 anti-IgG model, used to examine the role of Fcγ receptors, failed to initiate NO stimulation in AM (Table 3.II). In addition, studies have shown that PI3 kinase is involved in Fcγ receptor mediated signaling (41). Because we have evidence that PI3 kinase does not play a role in CD8-mediated NO stimulation or iNOS upregulation, and our data that IgG1

Figure 3.7 Working model for the function and signaling mechanisms of CD8 on AM.





does not stimulate AM NO release, we hypothesize that CD8 functions independently of the Fcγ receptor.

Independent of whether CD8 activates macrophages directly or as a co-receptor, we have demonstrated that CD8 plays an important role in stimulating macrophage function. *Leishmania major* infected alveolar macrophages were stimulated to kill this parasite, which was NO dependent, when crosslinked with anti-CD8α (OX8) (Table I). Moreover, anti-CD8α stimulated killing of *Leishmania* was not-significantly different from IFN-γ (200 U/mL) mediated killing.

Our studies using *L. major* infection showed differences in OX8 and 341 stimulated NO release (Table 3.I), compared to non-infected macrophages (Fig. 3.1), with OX8 stimulating more and 341 stimulating less NO/cell. One possible explanation for the differences in NO production, could be that expression of CD8 α and β is altered on infected macrophages, thereby changing the density of these surface molecules. Previous studies examining the expression of CD8 on uninfected macrophages (using an alveolar macrophage rich population, 89±1% OX41) showed that 63±5% stained positive for OX8 and 52±3% stained positive for 341 (6). In an attempt to determine if parasitic infection and associated pulmonary inflammation could modify the expression of CD8 on alveolar macrophages, we infected rats with the nematode *Nippostrongylus brasiliensis.* No significant difference in the expression of CD8 α or β was found. However, as *Leishmania* infects macrophages, we cannot rule out the possibility that this parasite may alter CD8 α and/or β expression or signaling.

These results stress the importance of CD8 expression on macrophages. Macrophage CD8 may interact with MHC I (ligand for T-lymphocyte CD8) to form a more stable interaction between the antigen presenting cell and T-lymphocytes and enhance macrophage adhesion. In addition, CD8 may also allow macrophages to modulate the immune response by inducing apoptosis in a subset of T-lymphocytes. Sambhara and Miller (42) demonstrated that precursors to cytotoxic and helper T-lymphocytes can be deleted, by apoptosis, when signaled through their TCR and MHC I molecule. To carry out efficient deletion of T-helper cells, Sambhara and Miller (42) postulated the need for a cell to express both MHC II and CD8, which macrophages have been shown to express. Alternatively, because macrophage and T-lymphocyte CD8 differ within the ligand binding domain (6), macrophage CD8 may interact with a novel ligand(s). This would suggest a hitherto unknown role for macrophage CD8. Recent work has demonstrated a non-MHC I ligand for T-lymphocyte CD8 (43), supporting the hypothesis for novel CD8 ligands. This ligand, gp180, is expressed on intestinal epithelial cells and it is able to stimulate CD8+ T lymphocyte proliferation (43).

For the first time, an effector function for both the α and β chains of macrophage CD8 is demonstrated. Macrophage CD8 can regulate mediator release and stimulate macrophage host defense. In addition, the β-chain of macrophage CD8 can also regulate effector function, supporting our previous work (6) demonstrating differences between macrophage and T-lymphocyte CD8. Additional studies on the signaling mechanisms and ligand(s) for macrophage CD8 will lead to a greater

understanding of macrophage regulatory mechanisms and the role of CD8 in modulating immune responses.

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CHAPTER 4

The Structure of Novel CD8 on Alveolar Macrophages

PREFACE

This chapter deals with the distribution and molecular weight of CD8 on rat AM. Our previous work (chapters 2 and 3) demonstrates that AM express CD8 α and β and that this molecule differs from T lymphocyte CD8, particularly within the Ig-like ligand binding domain of the α-chain. The work in this chapter shows that CD8+ AM express primarily α/α homodimers, with some α/β heterodimers. In addition, immunoprecipitation studies demonstrate that macrophage CD8α is larger (greater molecular weight) than T lymphocyte CD8α. Parts of this chapter will be submitted for publication, with me as senior author.

Introduction

CD8 is a cell surface glycoprotein that is expressed on cytotoxic/suppressor T lymphocytes and natural killer (NK) cells. It is composed of two chains that form either an α/α homodimer or α/β heterodimer. The α/β heterodimer is best known in association with thymus-derived lymphocytes, whereas the α/α homodimer is associated with NK cells and thymus-independent lymphocytes such as intraepithelial lymphocytes (IEL). The structure of the α and β chains is similar. They both have an amino terminal immunoglobulin-like (Ig) variable domain, a "linker" region, transmembrane and cytoplasmic domains. The α-chain has a longer cytoplasmic domain that contains a cysteine-rich motif, allowing it to bind the *src*-family protein tyrosine kinase, $p56^{lck}$ (reviewed in 1,2).

There are multiple forms of CD8. CD8α can be produced as a full length transcript, encoding the domains described above, or as an alternatively spliced α′ transcript (mouse CD8α with a short cytoplasmic domain), or an alternatively spliced secreted CD8α molecule (2). Along with the multiple spliced forms of CD8, there are differences in the types and amount of glycosylation of CD8 among species. Mouse CD8α has three N-linked glycosylation sites, whereas rat has one and human CD8α has no N-linked glycosylation sites (3-5). However, all species have sites of O-linked glycosylation, mostly located within the linker/hinge region (4,5).

We have recently identified a unique CD8 molecule on the surface of rat alveolar (AM) and peritoneal macrophages, and rat peritoneal mast cells (MC) (6-8). This molecule differs from T-lymphocyte CD8, specifically within the Ig-like ligand binding domain (6).

AM and MC CD8 have unique properties. Anti-CD8 α or β antibodies upregulate iNOS protein (7) and stimulate NO (6-7), TNFα (35), and IL-1β (35) release from AM. Moreover, anti-CD8α stimulates NO-dependent killing of *Leishmania major*, in infected AM (7). In MC, anti-CD8 α and/or β antibodies induces NO and TNFα release, without stimulating histamine release (8). Moreover, anti-CD8 α and β antibodies do not modulate antigen-induced mediator release from sensitized MC (8).

AM play an important role in regulating the immune response within the lungs. These cells release a plethora of mediators that protect the lung against invading pathogens and inhibit possible damaging immune responses (9). As AM CD8 plays a role in modulating mediator release, it is essential to elucidate the structure of this molecule on macrophages in order to understand the role of CD8 in modulating immune responses.

AM express CD8 α and β (6), but we do not know if these chains are expressed on the same cell or on different cells. Therefore, flow cytometry was used to examine CD8 α and β co-expression on AM. In addition, we used immunoprecipitation to examine the molecular weight of AM CD8α, and compared it with T lymphocyte CD8α, to further examine how these molecules differ.

Materials and Methods

Animals

Adult male Sprague-Dawley rats, 200 to 300 g, were obtained from Charles River Canada Inc. (Quebec, Canada) and maintained in an isolated room in filter-top cages to minimize unwanted infections. The animals were given food and water *ad libitum* and maintained on a 12 h (0700 h) -12 h (1900 h) light-dark cycle. No experimental procedures were performed on animals within the first week after arrival, decreasing the effects of stress associated with transport, new housing facilities, and handling. All experimental procedures were approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Isolation of bronchoalveolar cells

Animals were anaesthetized by i.p. injection of 0.5 ml Rompun (Xylazine) and 0.5 ml Ketalean (Ketamine). The trachea was catheterized with a polyethylene tube and 12 X 5 ml of cold PBS was massaged into the lungs. Lavage cells were pelleted at 200 X g for 20 min and resuspended in PBS (6,7). Within rat alveolar lavage, the percent of AM, as tested by esterase staining, was 96±0.4%, and 82.2±1.2 were $OX41^+$ as tested by flow cytometry.

Flow cytometric analysis

The following antibodies were used for cell analysis : CD8α [MRC OX-8 (IgG1) PE] (Serotec, Toronto, Canada) and CD8β [341 (IgG1) FITC] (Cedarlane, Hornby, Canada). The results with specific antibodies were compared to isotype

matched controls (IgG1-FITC, IgG1-PE) purchased from Accurate Chemical and Scientific Co. (New York, USA).

In 96 well u-bottom plates, cells (5 X 10^5 cells per test) were preincubated in PBS + 10% normal mouse serum for 30 min before 1 hr antibody incubation at 4°C. Cells were washed three times (PBS), resuspended in 400 μl of 1% formalin (PBS), and 10,000 cells were analyzed on a FACScan (Becton Dickinson, California) (6,7).

Immunoprecipitation

Cell Surface Biotinylation: Cells were resuspended (20 x 10^6 cells/ml) in labelling buffer (HBSS + glucose (200 mg/L)) and incubated with 800 μg/ml sulfo-nhs-biotin (Pierce) for 15 min over ice. Cells were washed 3 x DMEM (no additives) and lysed in lysine lysis buffer (10mM Tris-HCl PH 8.0, 3% NP-40, 150 mM NaCl, 10 mM EDTA, 5mM iodoacetamide, 0.5%BSA, 1 mM PMSF, 50 mM lysine) for 1 h at 4°C, after which nuclei were pelleted (10000 x g / 30 min). Mouse IgG1 (Pharmingin) was incubated (15 min) with lysate before addition of rabbit anti-mouse IgG (Serotec) for 15 min. Protein-A sepharose (Pierce) was added and solution was incubated for 1h, after which protein-A antibody complex was spun down at 1000 x g / 7 min (the precleared supernatant). The IgG1 precleared supernatant was incubated anti-CD8α (OX8), repeating the above immunoprecipitation procedure.

Total Protein Iodination: 10 x 10^6 cells per test were lysed in lysis buffer for 1 h at 4°C and the nuclei were pelleted (10000 x g / 30 min). One mCi I^{125} was added to supernatant along with 2 iodobeads (Pierce). Samples were incubated for 20 min, after which supernatant was passed over a PG10 column and washed with lysis buffer 1 (12 x

1 ml), where 1 ml fractions were collected. Protein fractions, compared to wash solution and free I^{125} were collected based on radioactive profile as determined by a Geiger counter (typically fractions 4-6). Samples were pooled and diluted with FBS buffer (1% NP40, 0.1% NaN_3, 0.25% Aprotinin, 10 mM iodoacetamide, 1mM PMSF) to 12 ml in a 15 ml conical tube. Supernatant was pre-cleared 3 x 200 µl MS (mouse serum)-Agarose (Sigma) x 30 min, 2 x Protein-A sepharose x 4 h. Sample was immunoprecipitated with IgG1 or OX8 (anti-CD8α) 5h, rabbit anti-mouse IgG 1h, Protein-A sepharose 8h. Samples were spun at 200 x g and Protein-A antibody complex was collected.

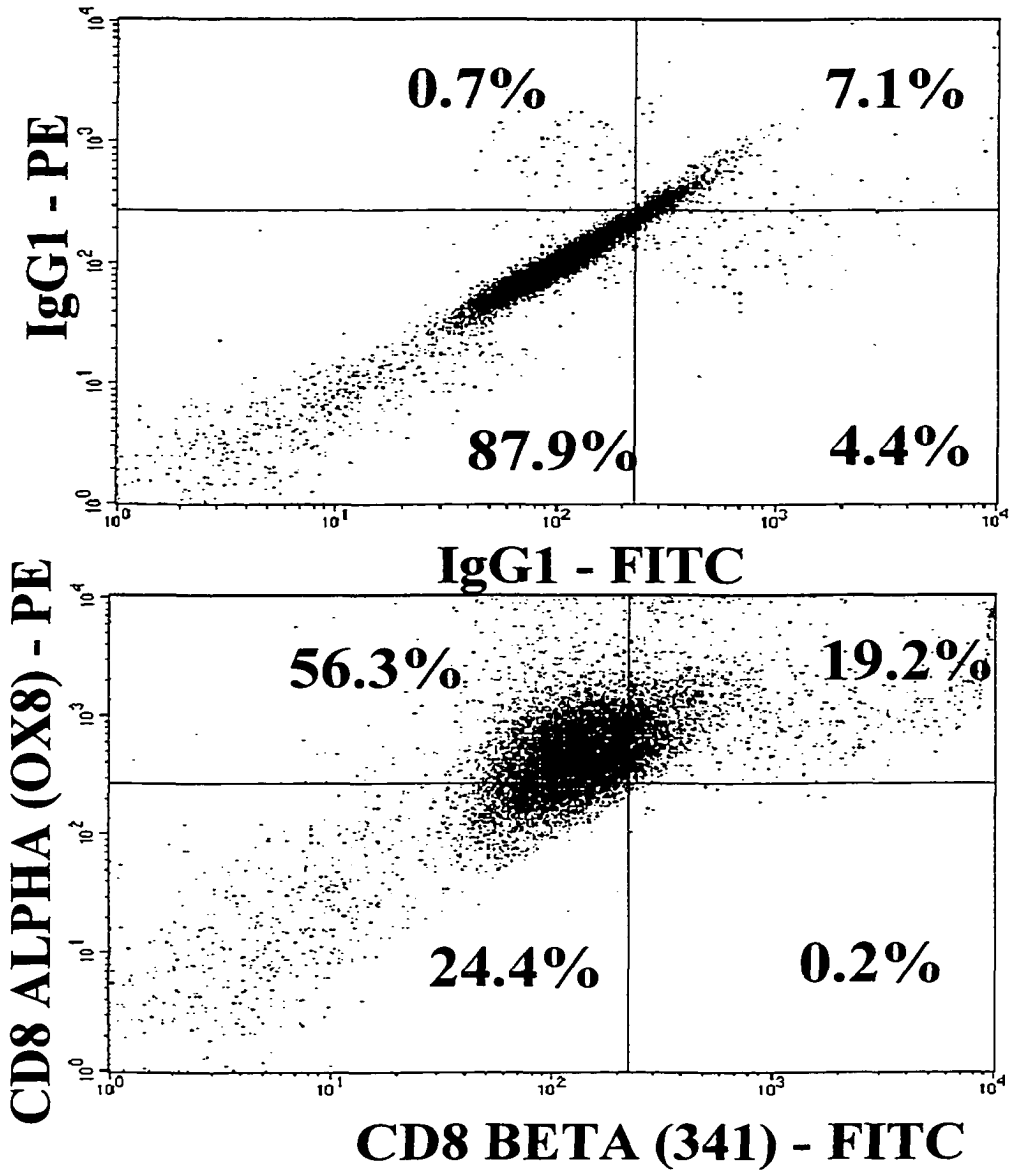
Protein Visualization – Surface and Total: Protein-A antibody complex was washed x 3 in wash buffer 1 (0.5 M NaCl, 5 mM EDTA, 50 mM Tris-Cl pH 7.5, 1% NP-40, 1% FCS) and x 1 in wash buffer 2 (wash buffer 1 minus NP-40). Complex was boiled in Laemmli sample buffer +BME, run on a 12% SDS PAGE gel and transferred to a PVDF (Amersham) membrane for biotinylated samples or dried for I^{125} samples. Dried gel and film were incubated (3-10 days) at –70°C for visualization. For biotinylated samples, membrane was blocked (5% instant skim milk in TBS-tween 2 h at room temperature) before addition of 1/5000 dilution Streptavidin-HRP. Protein was visualized with Dupont-NEN enhanced chemiluminescent kit (Dupont).

Results

Co-expression of CD8α and CD8β on AM

The co-expression of CD8 α and β on AM was examined using antibodies for the hinge region of CD8α (OX8) and the β-chain (341). The majority of AM are $CD8\alpha^+\beta^-$ (56.3%), with relatively equal numbers of $CD8\alpha^+\beta^+$ (19.2%) and $CD8^-$

Figure 4.1 The scatter profile, within the AM rich region, of double-stained lavage cells for CD8α (OX8-PE) and CD8β (341-FITC), compared to isotype controls (IgG1 PE and FITC). n=3, representative experiment.



(24.4%) AM (Fig. 4.1). However, no AM appear to be $CD8\alpha^{-}\beta^{+}$ (0.2%) (Fig. 4.1) (n=3, representative experiment). Interestingly, in recent experiments, we have seen a reduction in AM CD8β expression compared to our initial experiments (6). The percentage of $CD8\beta^{+}$ AM has dropped from 52% (Fig. 2.3, studies conducted in 1995-96) to 19% (Fig. 4.1 studies conducted in 1997). However, there has been no significant reduction in the percent of CD8α+ AM.

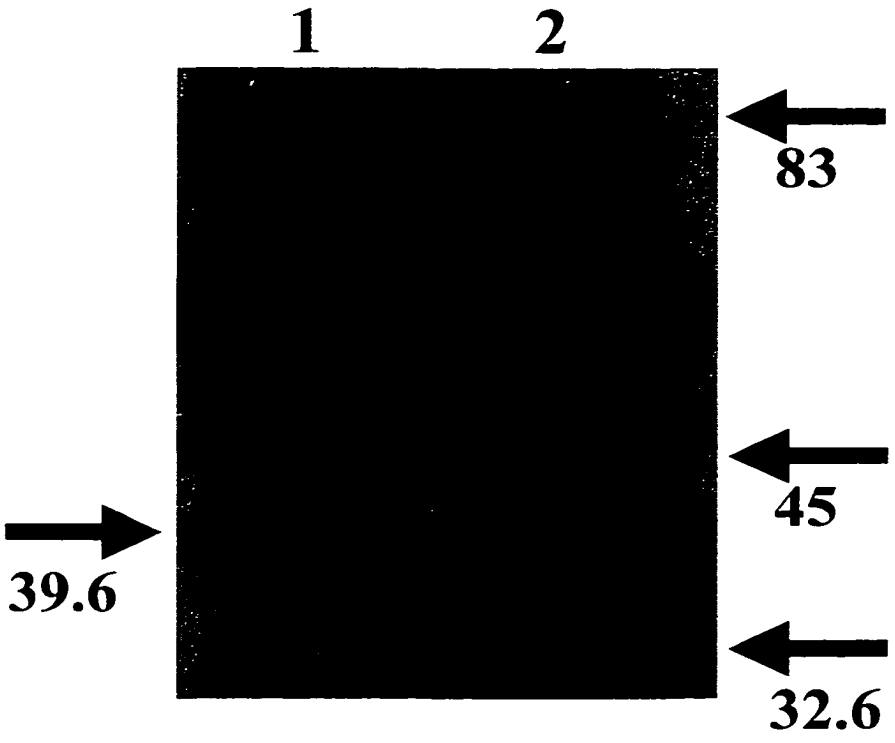
Immunoprecipitation of CD8α protein from AM

Our previous work (6) provided evidence that AM CD8 differed from T lymphocyte CD8. To further identify how these two molecules differ, AM CD8α was immunoprecipitated and compared to T lymphocyte CD8α. Immunoprecipitation of CD8 from I^{125} labelled AM, showed that OX8 identified a 40 kDa protein (n=3, representative experiment), that was not seen in isotype control (IgG1) cell lysates (Fig. 4.2A).

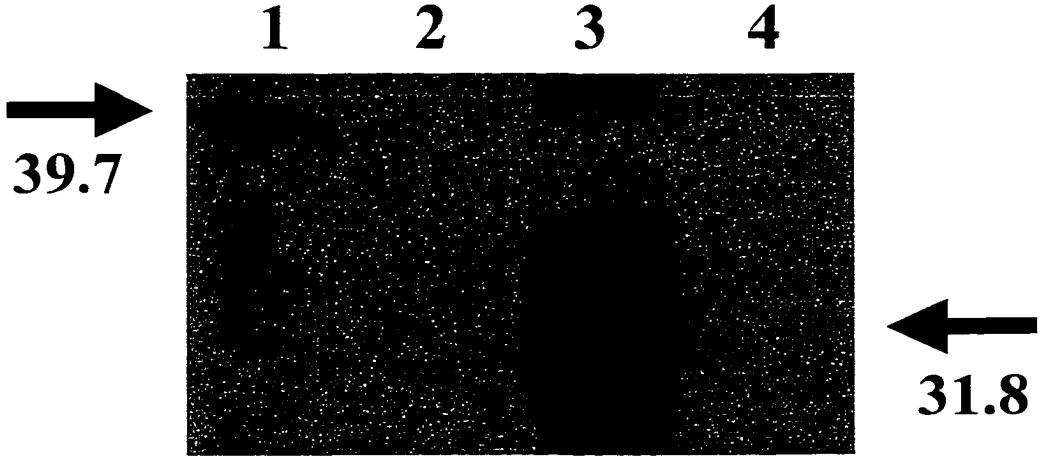
As CD8α on the cell surface may differ from that in total protein lysates, the OX8 antigen was immunoprecipitated from surface biotinylated AM. OX8 identified a 40 kDa protein from AM and a 32 kDa protein splenic T lymphocytes, which were not seen in IgG1 isotype controls (Fig. 4.2B) (n=3, representative experiment). Occasionally, weak bands ranging from 32 kDa to 40 kDa were seen in AM OX8 immunoprecipitates, which were not observed in isotype controls.

Figure 4.2 Immunoprecipitation of CD8 from AM and T lymphocytes. A. Total protein was I^{125} labelled from AM and immunoprecipitated with OX8 (lane 1) or IgG1 isotype control (lane 2), n=3. B. Surface protein of AM (lanes 1 and 2) or T lymphocytes (lanes 3 and 4) was biotinylated and immunoprecipitated with either OX8 (lanes 1 and 3) or isotype control (lanes 2 and 4), n=3.

A



B



Discussion

Our results show that the majority of CD8+ AM are CD8 $\alpha^+\beta^-$ (56%) compared to CD8$\alpha^+\beta^+$ (19.2%) (Fig. 4.1). However, compared to our initial work on CD8 (6), we have seen a decrease in CD8β expression by AM. Our initial work showed that AM were 63±5% CD8α^+ and 56±3% CD8β^+ (Fig. 2.3). However, in this recent study AM were 19.2% CD8β^+ (Fig. 4.1). The explanation for a reduction in CD8β expression is unclear. There are instances when specific infections can alter the expression of CD8. Chickens infected with Marek's disease have decreased CD8 molecules on CD4-CD8+ and CD4+CD8+ thymocytes (12). Infections agents can also selectively regulate CD8β expression. Cats in the acute stages of feline immunodeficiency virus (FIV) infection expand a population of CD8+ cells that are CD8$\alpha^+\beta^{lo}$ (13). Moreover, anti-FIV activity was associated with these CD8$\alpha^+\beta^{lo}$ cells (13). In addition to viral infections, CD8 expression can be differentially regulated by cytokines. TNFα and TGFβ can induce CD8α, but not CD8β, expression on pre-T lymphocytes in fetal thymus (CD3+CD4-CD8- or CD3+CD4+CD8-) (14,15). However, these cytokines did not induce CD8 expression on splenic (CD4+CD8-) T lymphocytes (14). Finally, we have evidence that NO can upregulate mRNA and protein for CD8 in MC (personal communication, Dr. O. Nohara). Therefore, there are multiple complex mechanisms that regulate CD8 expression. We do not believe the changes in AM CD8β expression were due to infection, as our animals were in virus and pathogen free housing and sentinel animals were routinely examined for infections. Therefore, the explanation for decreased AM CD8β expression, in our current studies, remains unclear.

The percent of CD8α$^+$ AM has remained relatively unchanged from our initial studies (6) to our current ones. Moreover, we confirmed our initial evidence that CD8α on AM was different from T lymphocyte CD8α (6). In our previous studies, an antibody to the Ig-like region of CD8α bound T lymphocytes, but did not bind AM (6). We extended these observations and showed that AM CD8α (40 kDa) is larger than T lymphocyte CD8α (32 kDa) (Fig. 4.2). Alternate splicing of CD8 mRNA may account for differences between AM and T lymphocyte CD8. Multiple spliced forms of CD8, within the N-terminal domain of rat CD8, have been postulated to exist by several groups (16,17). In mice, two different isoforms of CD8α have been identified, mouse CD8α′ which lacks the cytoplasmic tail (18) and a secreted form of human CD8α that lacks the transmembrane region (19). However, cDNA sequence analysis of the Ig-like domain of AM CD8α showed that there were no significant nucleotide differences between rat T lymphocyte and rat AM CD8α, within the Ig-like domain (personal communication, Mr. Mark Gilchrist). Therefore, we postulate that the increased size of AM CD8α is due to post-translational modifications that lead to an alternatively glycosylated form of CD8α.

The Ig-like domain of rat CD8α is glycosylated (16,20), further supporting the hypothesis that this site maybe differentially glycosylated on different cell types. Alternatively glycosylated forms of CD8 have been identified on T lymphocytes. Barber et al (1989) demonstrated, by 2D gel electrophoresis, that CD8α has multiple sialic acid and phosphorylated forms (21). In addition, differences in CD8β sialylation have been observed. Resting T lymphocyte CD8β is more heavily

sialylated than thymocyte and activated T lymphocyte CD8β (21). Thus, alterations in glycosylation within the Ig-like domain of CD8α could change the antigenicity within this site, resulting in a loss of binding by antibodies with epitopes within this region (eg. G28) in AM, compared to T lymphocytes (6). Alterations in glycosylation, either N or O –linked, within the Ig-like domain of CD8α may also explain the apparent lack of CD8 on human macrophages. Normally, human CD8α is not N-linked glycosylated, but, stimulated T lymphocytes can express beta 1-6 branched N-linked oligosaccharides on CD8α (22). To our knowledge, all the commercially available antibodies for human CD8 identify epitopes within the Ig-like domain. Therefore, if these domains are altered on human macrophages, antibodies which identify human T lymphocyte CD8 may not bind human macrophage CD8. Thus, antibodies to hinge or other parts of the CD8 molecule may be required to identify CD8 on human macrophages. Moreover, as CD8 is found on other cell types, including dendritic cells (DC) (23,24) and mast cells (MC) (8), it is essential to determine if this molecule is similar to that on T lymphocytes or on AM.

We have previously shown that crosslinking AM CD8 stimulates iNOS and NO release (6,7). However, as we have evidence that the Ig-like ligand binding domain of AM CD8α differs from T lymphocyte CD8α, there may be novel ligands for this molecule or CD8 may interact in a unique way, compared to T lymphocyte CD8, with its classical ligand MHC I. During T lymphocyte interaction with APC, the Ig-like domain of CD8α interacts with the α3 domain of MHC I (25,26). However, T lymphocyte CD8 also interacts with α1 and α2 domains of MHC I (27,28) and can also interact with the binding groove of MHC I (29). Therefore, AM

CD8 may interact with MHC I in a similar way to T lymphocyte CD8, or in a unique manner, to upregulate iNOS and NO release in AM (6,7).

There are also novel, non-MHC I ligands for CD8, that have been recently identified. Gp 180 is a glycoprotein expressed in human epithelial cells that can stimulate CD8+ T lymphocytes, through a CD8 dependent pathway (30,31). This stimulation leads to $p56^{lck}$ dependent T lymphocyte proliferation (30). Further studies on gp 180 demonstrated that this glycoprotein exists in two forms, a GPI-anchored and/or transmembrane isoform (31). Secondly, the *Trypanosoma brucei* factor TLTF (T lymphocyte triggering factor) is another protein that can bind T lymphocyte CD8 (32). TLTF can stimulate CD8+ T lymphocytes to secrete IFN-γ (33,34). It has not been examined if CD8 on macrophages or DC binds to gp 180 and/or TLTF. Therefore, gp 180 and/or TLTF may be ligands that stimulate AM and/or DC and regulate the immune response via CD8 on these cells.

In summary, we have demonstrated that AM express CD8 α or α and β. However, it is not known if these molecules are expressed as dimers, as co-immunoprecipitation studies or non-reducing gels have not been done. The CD8α molecule expressed by AM is larger than T lymphocyte CD8α, and this increase in size may be due to alterations in glycosylation. Finally, we hypothesize that AM CD8 interacts with MHC I and/or other recently identified CD8 ligands to regulate cell function. It will be essential to identify the ligand(s) for AM CD8 if we are to understand the role of CD8 on different cell types and within multiple microenvironments.

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CHAPTER 5

Mast Cells Express a Novel CD8 Molecule that Mediates Nitric Oxide Production and iNOS Upregulation

PREFACE

This chapter deals with the expression of CD8 α and β on rat peritoneal mast cells (PMC). Our previous work focused on alveolar macrophage (AM) CD8 and its role in regulating cell function. A previous study had shown that MC express CD8, however, the expression of this molecule was demonstrated on an unusual mouse mast cell line. As this line is a poor representative of *in vivo* derived mast cells, the expression of CD8 on MC remained unclear. This chapter shows that rat PMC express CD8, and like AM, MC CD8 differs from T lymphocyte CD8 within the Ig-like ligand binding domain of the α-chain. This chapter also demonstrates that CD8 can regulate the release of MC NO. Therefore, this work broadens our understanding as to which cells express CD8, and suggests that this molecule may play an important role in regulating the function of many cell types. Parts of this chapter are published in the "Journal of Immunology", 1998, 161: 6265-6272, under joint authorship with Dr. Tong-Jun Lin, Dr. Osamu Nohara, Dr. Grant R. Stenton, Mr. Mark Gilchrist, and Dr. A. Dean Befus. The co-authors have provided a statement confirming that my contribution to the work described in this chapter was significant.

This is to certify and confirm that Nadir Hirji made major and most significant contribution to the experimental work, scientific content, and writing of the sections described below, in which part appear in the paper entitled "Mast Cells Express Novel CD8 Molecules That Selectively Mediate Mediator Release" by Tong-Jun Lin, Nadir Hirji, Osamu Nohara, Grant R. Stenton, Mark Gilchrist, and A. Dean Befus. *The Journal of Immunology*, 1998, 161: 6265-6272.

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Introduction

Mast cells are highly granulated cells that play a prominent role in allergic and inflammatory diseases, such as asthma (1,2) and inflammatory bowl disease (3). These cells contain numerous mediators that can be preformed and immediately released, e.g. histamine, or newly synthesised. The newly synthesised mediators include cytokines, such as IL-3, 4, 5, 6, TNFα, and IFN-γ (4), arachidonic acid metabolites (5), and nitric oxide (NO) (6,7).

The classical pathway of mast cell stimulation involves the cross linking of IgE antibodies on the surface of mast cells. Specifically, IgE antibodies bind, via their Fc ends, to the high affinity FcεR1 receptors on mast cells (8,9). Antigen cross-links IgE on the surface of the mast cell, which triggers a signaling cascade that includes the src-family kinase Lyn (10,11), Syk (10,11), phospholipase C (PLC) (12), and protein kinase C (PKC) (13), and leads to mast cell degranulation.

Mast cells can also be activated via an IgE independent pathway. Cationic compounds (48/80), the neuropeptide substance P, bee venom peptides, and complement, activate mast cells in a non-Fcε dependent pathway (14-16). Mast cells can also be stimulated by bacterial products. Lipopolysaccharide (LPS) stimulates TNF release from mast cells (17), suggesting that mast cells may play an important role in host defence.

Recently, we have identified a unique CD8 molecule on the surface of rat alveolar and peritoneal macrophages (18), that can regulate mediator release and stimulate host defence against *Leishmania major* through multiple signaling pathways (19). Classically, CD8 is thought of as a cell surface glycoprotein found on a subset of

T-lymphocytes with cytotoxic/suppressor functions and on NK cells. It is composed of two disulfide linked chains that form either a homodimer (α/α) or heterodimer (α/β). During T-lymphocyte interaction with an antigen presenting cell (APC), the α chain of CD8 binds to the α3 domain of MHC class I (on the APC) and delivers an activation signal to the T-lymphocyte through a pathway involving the *src*-related protein tyrosine kinase, p56-lck [reviewed in (20,21)].

In addition to the expression of CD8 on T lymphocytes and macrophages, there is growing literature that CD8 is expressed on other cell types, including dendritic cells (22,23) and B lymphocytes (24). Mast cells have also been shown to express CD8 (25), however, this was work was done on a murine mast cell line. Therefore, this work will investigate if CD8 is expressed on rat peritoneal mast cells and determine if this molecule is able to regulate cell function.

Materials and Methods

Animals

Adult male Sprague-Dawley rats, 200 to 300 g, were obtained from Charles River Canada Inc. (Quebec, Canada) and maintained in an isolated room in filter-top cages to minimize unwanted infections. The animals were given food and water *ad libitum* and maintained on a 12 h (0700 h) -12 h (1900 h) light-dark cycle. No experimental procedures were performed on animals within the first week after arrival, decreasing the effects of stress associated with transport, new housing facilities and handling. All experimental procedures were approved by the University of Alberta Animal Care

Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Peritoneal mast cell (PMC) isolation

Peritoneal cells were obtained by lavage of the peritoneal cavity with 15 ml of cold HEPES-buffered Tyrode solution containing 0.1% BSA. They were layered on a two-step (30%/80%) discontinuous gradient of sterile Percoll (Pharmacia Ltd., Uppsala, Sweden) and separated as described earlier (26). The purity of mast cells was ≥98% as determined by Toludine blue staining and/or cell morphology under microscope.

Flow cytometric analysis

The following antibodies were used for cell analysis : CD8α [MRC OX-8 (IgG1) FITC and PE] (Serotec, Toronto, Canada), CD8α [G28 (IgG1) FITC] and CD8β [341 (IgG1) FITC], purchased from Cedarlane (Hornby, Canada). The results with specific antibodies were compared to isotype matched controls (IgG1-FITC, IgG1-PE) purchased from Accurate Chemical and Scientific Co. (New York, USA).

In 96 well u-bottom plates, cells (5 X 10^5 cells per test) were preincubated in PBS + 10% normal mouse serum for 30 min before 1 hr antibody incubation at 4°C. Cells were washed three times (PBS), resuspended in 400 ml of 1% formalin (PBS), and 10,000 cells were analyzed on a FACScan (Becton Dickinson, California) (18,19).

Confocal microscopy imaging of CD8

In a 96-well U-bottom plate, cells (5 X 10^5 per test) were preincubated in HTB (hepes tyrode buffer) + 10 % normal mouse serum for 30 min before incubation for 1 h with OX8-FITC at 4°C. Cells were washed three times, and cytospins of PMC were made by vortexing slides in a Cytospin 2 (Shandon, UK) at 600 rpm for 3 min. Antibleaching solution (10 mM n-proply gallate (Sigma), 8.1 M glycerol, in Tris-buffered saline) was dropped onto slides before coverslip attachment. Cells were examined with a Leica confocal laser-scanning microscope (Heidelberg, Germany). The results with OX8-FITC were compared to isotype matched controls.

Immunoprecipitation for iNOS

Mast cells (1 X 10^6 cells/ml) were incubated for 24 h with 10 μg/ml OX8. Cells, 16 x 10^6 per test, were lysed in lysis buffer (10mM Tris-HCl PH 8.0, 3% NP-40, 150 mM NaCl, 10 mM EDTA, 5mM iodoacetamide, 0.5%BSA, 1 mM PMSF) for 1 h at 4°C and the nuclei were pelleted (10000 x g / 30 min). 1 mCi I^{125} was added to supernatant along with 2 iodobeads (Pierce). Samples were incubated for 20 min, after which supernatant was passed over a PG10 column and washed with lysis buffer 1 (12 x 1 ml), where 1 ml fractions are collected. Protein fractions, compared to wash solution and free I^{125}, were collected based on radioactive profile as determined by a Geiger counter (typically fractions 4-6). Samples were pooled and diluted with FCS buffer (1% NP40, 0.1% NaN_3, 0.25% Aprotinin, 10 mM iodoacetamide, 1mM PMSF) to 12 ml in a 15 ml conical tube. Supernatant was pre-cleared 3 x normal mouse serum-agarose x 6 h, 3 x Protein-A sepharose x 6 h. Sample was immunoprecipitated with rabbit anti-mouse IgG for 6 h followed by Protein-A sepharose 8 h. Protein-A antibody complex was

collected, spun at 200 x g, and the supernatant was re-immunoprecipitated with anti-iNOS (a gift from Dr. J. Weidner, Merck Research Laboratories, Rahway NJ).

Protein Visualization – Protein-A antibody complex was washed x 3 in wash buffer 1 (0.5 M NaCl, 5 mM EDTA, 50 mM Tris-Cl pH 7.5, 1% NP-40, 1% FCS) and in wash buffer 2 (wash buffer 1 minus NP-40) x 1. Complex was boiled in Laemmli sample buffer + BME, run on a 12% SDS PAGE gel, and dried. Dried gel was incubated with autoradiographic film at –70°C for visualization.

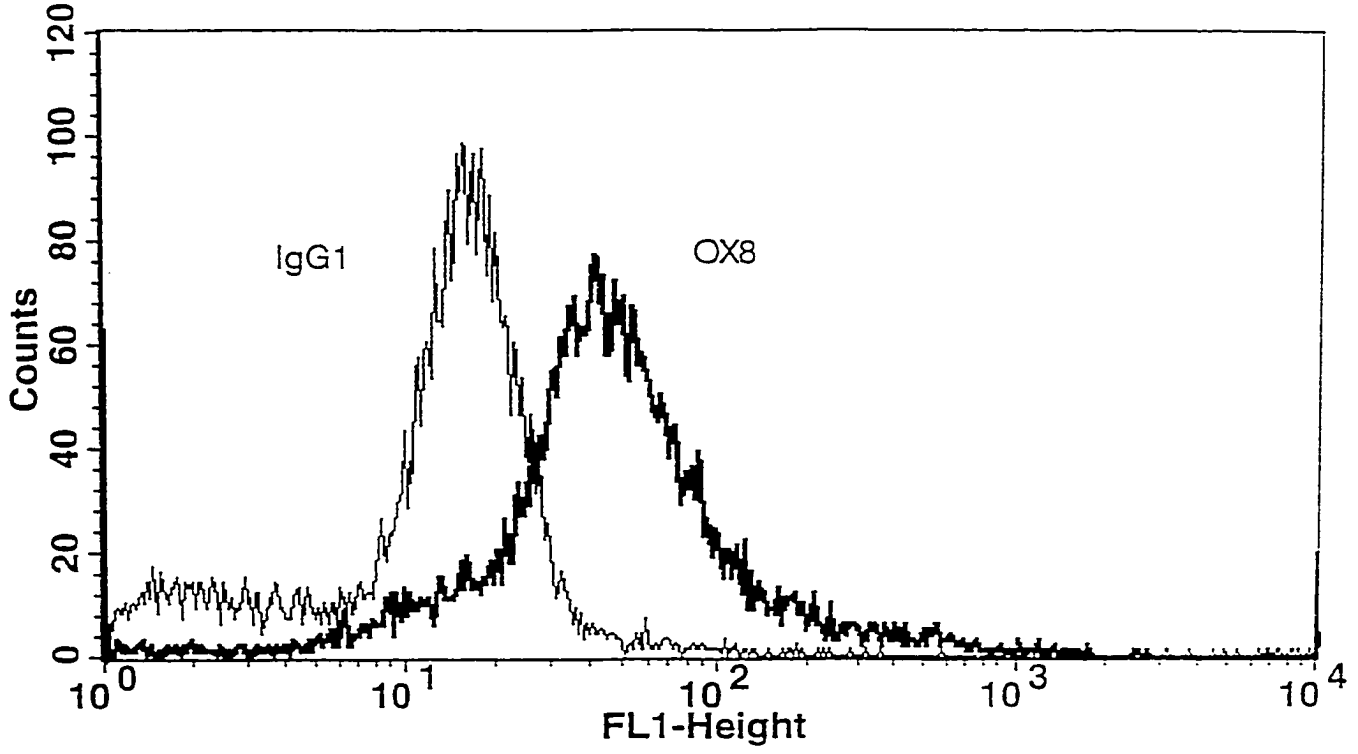
Results

Flow cytometry and confocal microscopy of CD8 expression on rat PMC

To examine if mast cells, PMC and RBL-2H3, express CD8, antibodies to the hinge region (OX8) and the Ig variable-like region (G28) of CD8α, and CD8β (341) were used. Surprisingly, the majority of PMC (67.5±9.5%, n=4) were OX8 positive (Fig. 5.1A and 5.2B). There were also a significant number of 341 (27.8±2.3%, n=4) positive cells (Fig. 5.1B and 5.2A). Interestingly, few PMC were positive for the Ig variable-like region of CD8α (G28) (7.3±1.1%, n=4), in contrast to T lymphocytes, which were similarly positive for both OX8 (79±1%) and G28 (73±3%). Few PMC were positive for CD4 (W3/25) (2±0.9%, n=3).

To determine if other mast cell populations express CD8, the same antibodies were used to examine CD8 expression on RBL 2H3 cells (Fig. 5.2B), a mast cell line with mucosal mast cell like properties (27). The majority of RBL 2H3 cells were positive for both OX8 (87.7±1.9%) and 341 (81.7±1.9%) (n=3). Similarly to PMC, few RBL 2H3 cells were positive for G28 (5.7±3%, n=3).

Figure 5.1 Detection of CD8α and CD8β on rat PMC, stained with OX8-FITC (CD8α) (A), 341-FITC (CD8β) (B), or isotype control IgG1-FITC, by flow cytometric analysis.



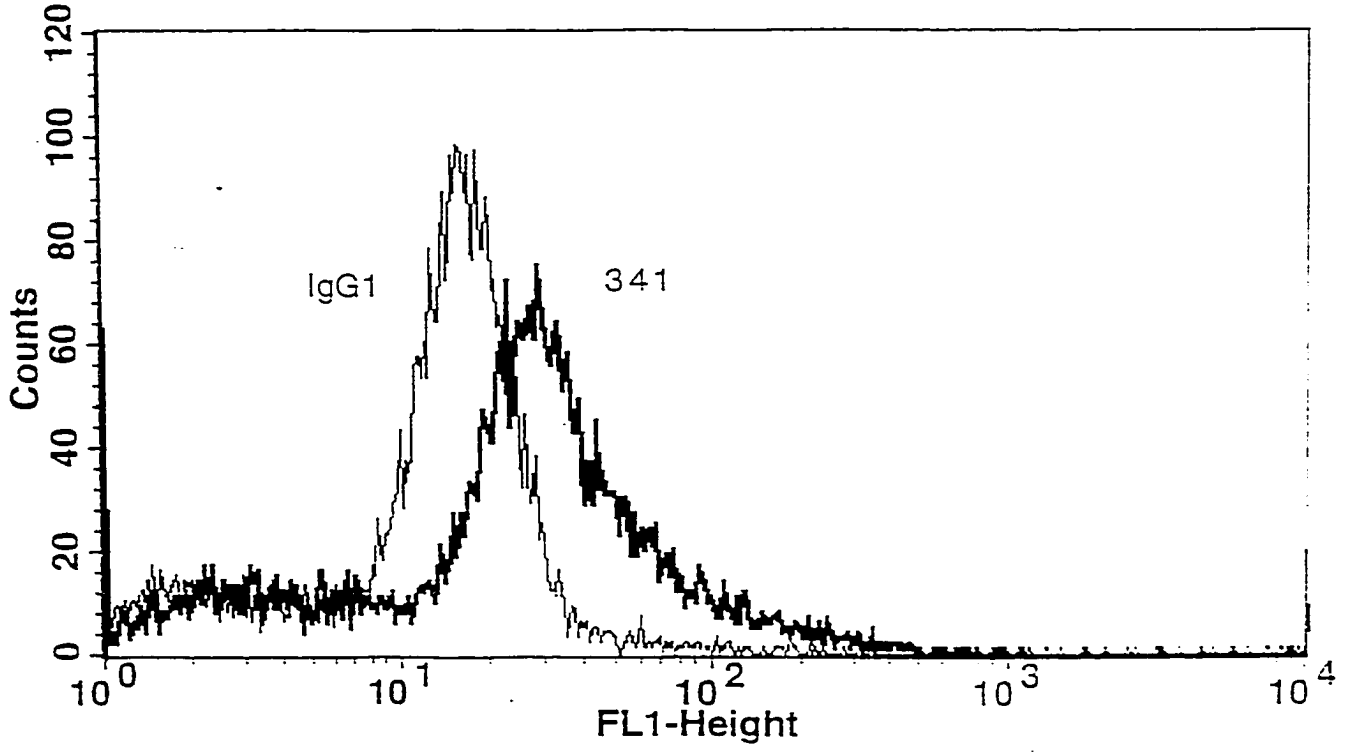


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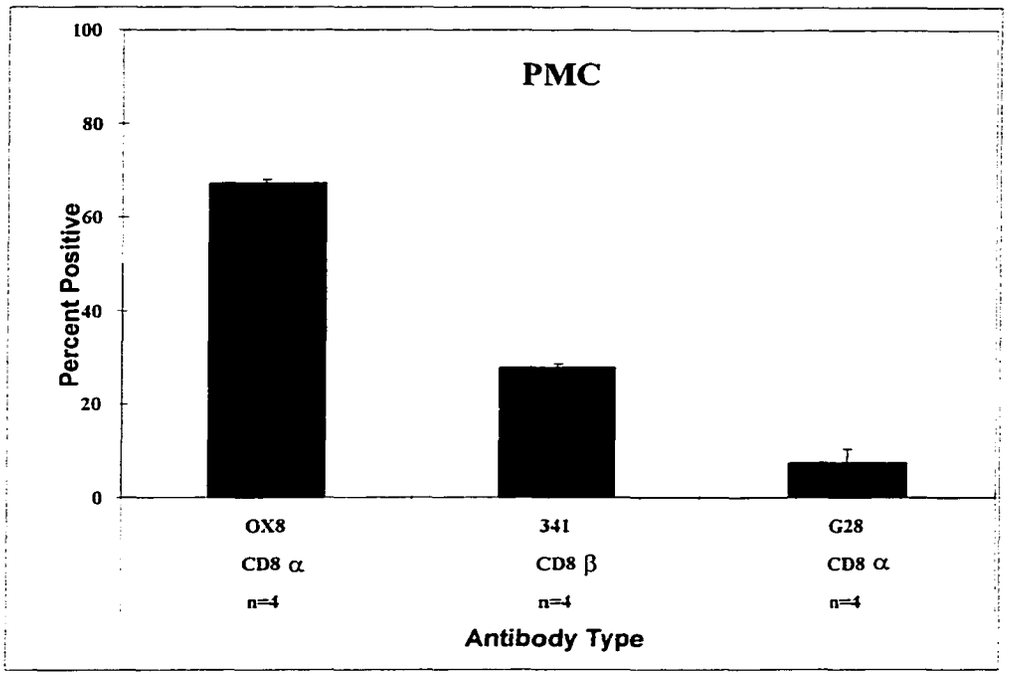
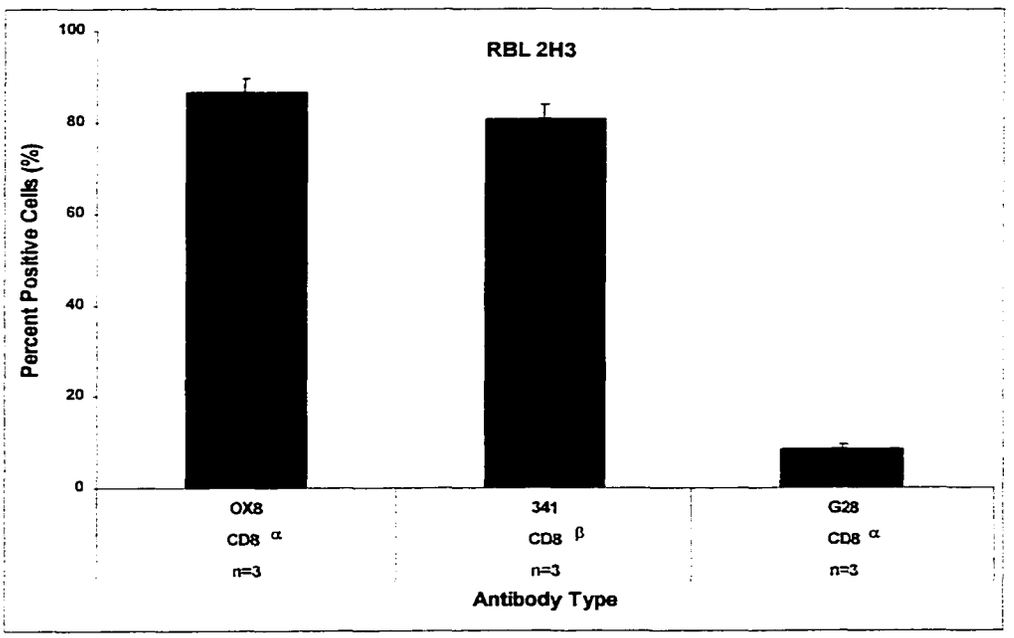
Figure 5.2 The abundance of OX8 (CD8α hinge region), G28 (CD8α Ig-like domain), and 341 (CD8β) positive cells in rat PMC (A) and RBL 2H3 (B). Results are mean±SEM for four (A) and three (B) experiments.

A



B



To further confirm the expression of CD8 on MC, PMC were stained with OX8 and examined by confocal microscopy. Consistent with the flow cytometry results, PMC were positive for OX8 (Fig. 5.3).

As PMC are positive for both CD8α (OX8) and CD8β (341), we stained cells with OX8-PE and 341-FITC to determine if CD8 α and β are expressed on the same cells (Fig. 5.4). PMC were composed of two populations, CD8 α^+ (26.4%) and CD8 $\alpha^+\beta^+$ (24.2%), with virtually no CD8 $\alpha^-\beta^+$ (0.5%) cells (Fig. 5.4). These experiments were done approximately eight months after the initial flow cytometry work described in Fig. 5.1 and 5.2. We have observed a lower number of CD8β-positive cells in these latter experiments, an observation that we do not understand.

Immunoprecipitation of iNOS

Previous work in our laboratory has shown that PMC release NO when stimulated with anti-CD8α (OX8) antibody (28). Therefore, we wanted to determine if this release is due to an upregulation of the inducible form of nitric oxide synthase (iNOS). OX8 (10 μg/ml) stimulated (48 h) PMC (lane 2) and peritoneal macrophages (positive control, lane 5) both expressed iNOS protein (Fig. 5.5), which was not detected in cells treated with OX8 isotype control (lane 1 PMC, lane 5 peritoneal macrophages). As a positive control for immunoprecipitation, mast cell protease I (MCPI) was isolated from PMC (Fig. 5.5, lane 3).

Figure 5.3 Confocal microscopy analysis of rat PMC for OX8 (CD8α) staining, compared to isotype-matched control IgG1.

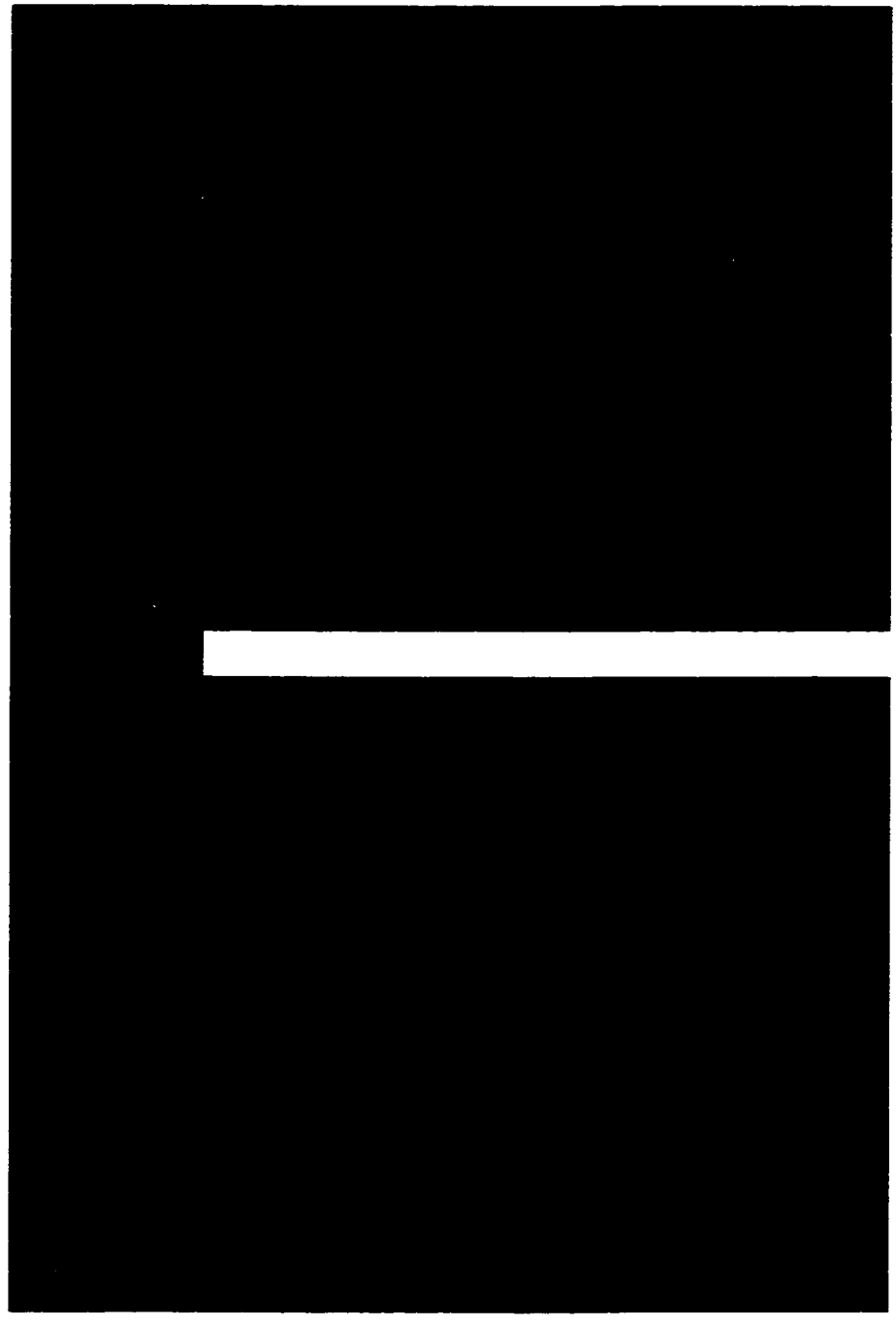
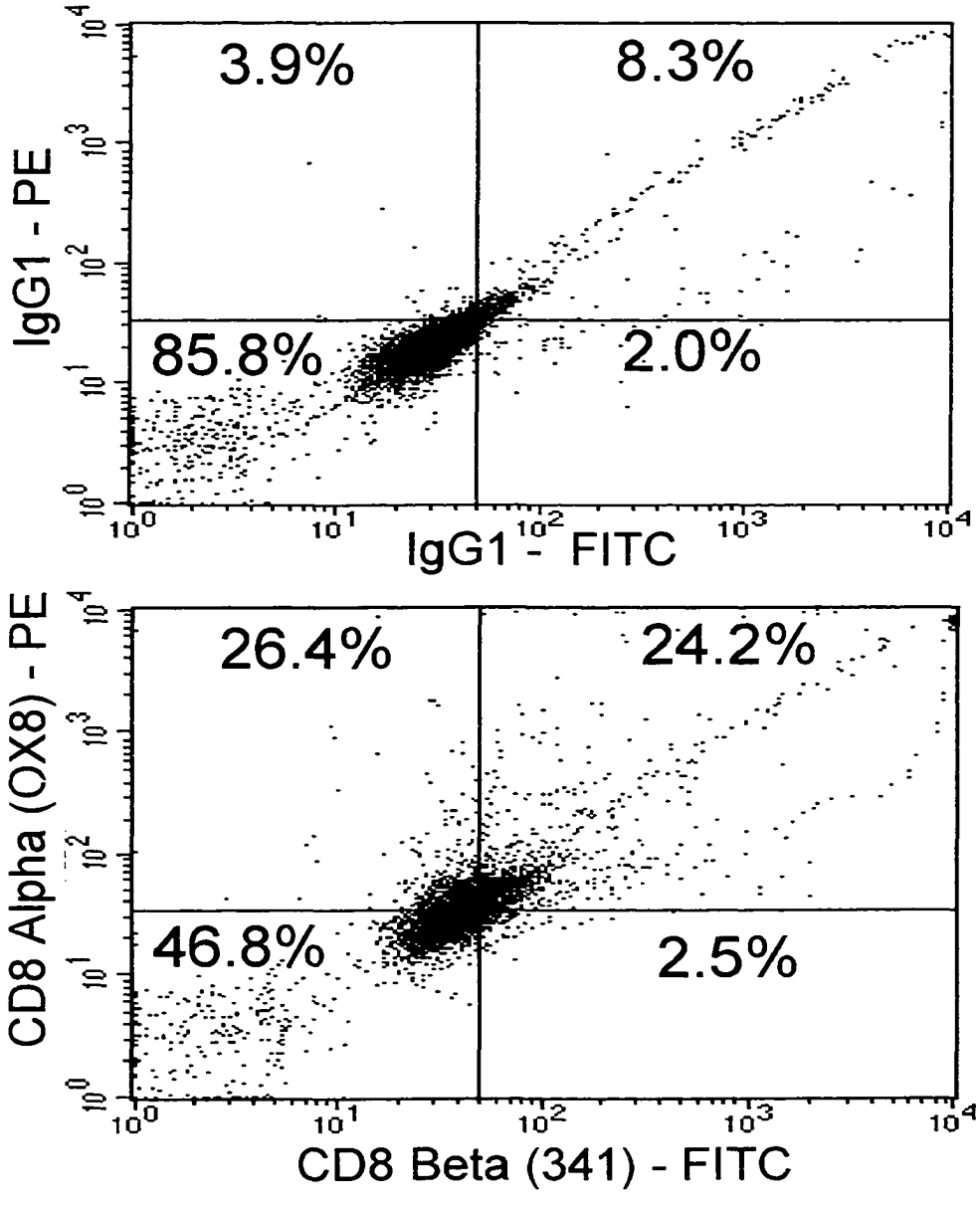


Figure 5.4 Double staining of PMC for CD8α (OX8-PE) and CD8β (341-FITC), compared with isotype controls (IgG1-PE and IgG1-FITC).



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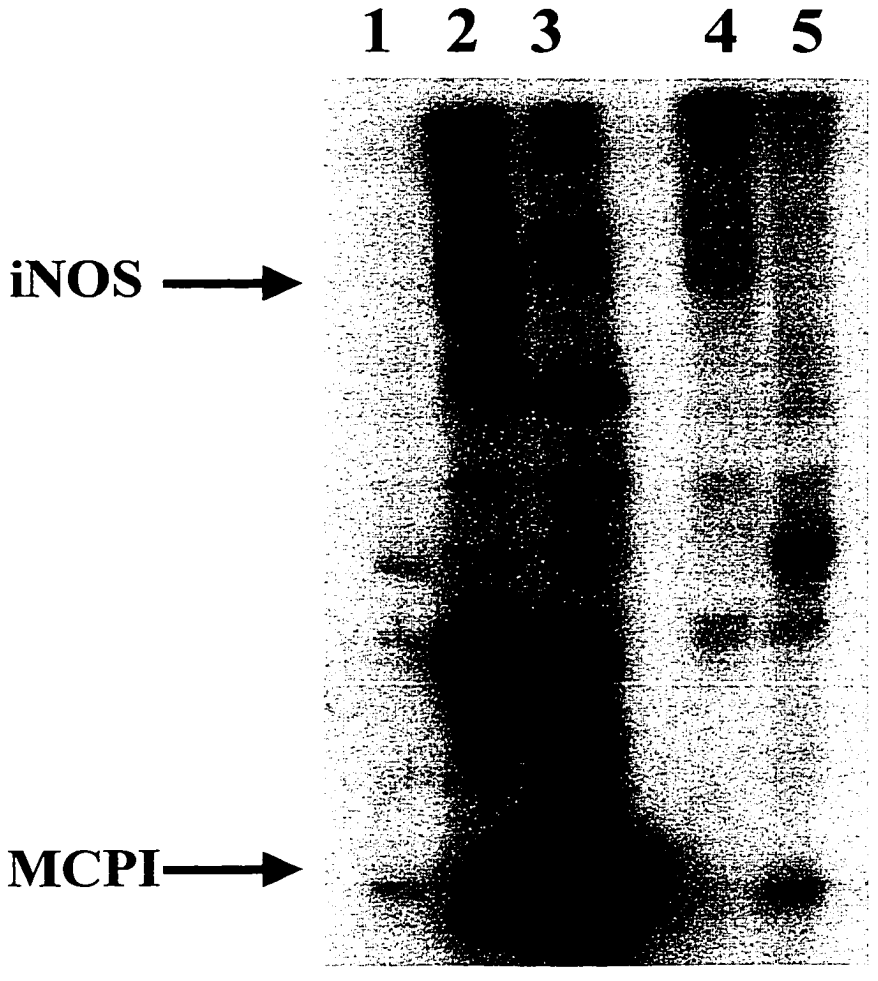
Discussion

This work shows that freshly isolated rat PMC and RBL 2H3 express CD8 α and β (Figs. 5.1 and 5.3), which supports previous work demonstrating that PMC and RBL 2H3 express the message for CD8 (28). These results further suggest that there may be differences between MC types. RBL 2H3 also appear to express equal levels of CD8 α (87.7±1.9%) and β (81.7±1.9) (Fig. 5.2), suggesting that the majority of these cells are CD8 $\alpha^+\beta^+$. In contrast, a greater percent of PMC express CD8 α (67.5±9.5%) compared to β (27.8±2.3%). Double staining PMC for CD8 confirmed that there are two populations, CD8 $\alpha^+\beta^+$ (24.2%) and CD8 $\alpha^+\beta^-$ (26.4%) (Fig. 5.4). Furthermore, as not all MC are $CD8^+$, within each population there may be further divisions between $CD8^+$ and $CD8^-$ MC populations. This heterogeneity in MC is consistent with previous work showing that rodent and human MC can be sub-divided. Rodent MC can be divided into mucosal MC or connective tissue MC, depending on their mediator content and staining properties. Specifically, rat connective tissue MC express rat MC proteinase (RMCP) 1, 5, 6, 7, and greater amounts of histamine, whereas rat mucosal MC express RMCP-2 and lower levels of histamine (29-31). Human MC can also be subdivided into tryptase and chymase (TC) positive or chymase (C) positive (32).

The flow cytometry results also suggest that CD8 expressed on PMC and RBL 2H3 may differ from that expressed on T lymphocytes (Fig. 5.1 – 5.3). All three cell types were positive for the hinge region of CD8α (OX8). However, T lymphocytes were equally positive for the Ig variable-like region (G28), whereas PMC and RBL 2H3 are not. This is similar to macrophages, in which these cells were positive for

Figure 5.5 Immunoprecipitation of iNOS from OX8 (10 μg/ml) (lane 2) or isotype control (IgG1 10 μg/ml) (lane 1) stimulated PMC. Anti-mast cell protease I (MCPI) (lane 3) and peritoneal macrophages simulated with OX8 (lane 5) or isotype control (lane 6) were used as positive controls for immunoprecipitation. This work was done in collaboration with Mr. M. Gilchrist; we worked in tandem to immunoprecipitate iNOS.



OX8, but negative for G28 (18). This difference is in the ligand binding domain, which recognizes MHC I. PCR data and sequencing of AM CD8 cDNA identified that this difference may be due to post-translational modification, as there were no significant differences in cDNA sequence between macrophages and T lymphocytes (personal communication, Mr. M. Gilchrist). Alterations in the ligand binding domain of CD8 suggest that there may be novel ligands for MC CD8 and/or that MC CD8 interacts with MHC I in a way distinct from T lymphocytes. There is recent evidence for a non-MHC I ligand for CD8. Epithelial cells are able to stimulate CD8+ T lymphocyte proliferation, which was CD8 dependent, through the non MHC-I ligand gp 180 (33,34).

Regardless of whether MC CD8 interacts with MHC I, gp 180, or a novel ligand, it is able to stimulate cell function. Cross linking CD8 α or β on MC is able to stimulate TNFα and NO secretion (28). Cross linking CD8α also upregulated iNOS, the inducible form of the enzyme that produces NO from the conversion of L-arginine to L-citrulline (Fig. 5.5). CD8 also selectively regulates MC mediator secretion. Cross linking CD8 did not induce histamine release or increase antigen induced TNFα, NO, and histamine secretion (28).

Our work shows that MC express CD8 and that different sub-populations of MC differentially express CD8 α and β. MC CD8 differs from T lymphocyte CD8, suggesting that there may be novel ligands for CD8. In addition, this molecule selectively regulates mediator release (28). Therefore, CD8 may play an important role in stimulating MC function. Identifying ligands for this molecule will be essential if we are to understand the role and function of CD8 on MC.

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CHAPTER 6

Discussion and Future Directions

I. Review

This thesis deals with the expression and function of CD8 on rat alveolar (AM) and peritoneal (PM) macrophages and mast cells (MC). In chapters two and five, we showed that AM and MC express the message and protein for CD8 α and β. Analysis of CD8 expression suggests that AM express both α/α and α/β dimers, with the majority of AM expressing the α/α homodimers (chapter 4). However, it appears that there has been a decrease in CD8β expression on AM over time (chapters 2 and 4). MC express approximately equal numbers of CD8 α/α and α/β positive cells (chapter 5). Functional studies showed that crosslinking α or β chains of CD8 upregulate iNOS production and stimulate NO secretion from AM and MC (chapters 2-5). In addition, anti-CD8α antibodies induced parasite killing in *Leishmania major* infected AM (chapter 3). Studies on CD8 signaling pathways showed that protein tyrosine kinases, particularly *src*-kinases, and PKC, but not PI3 kinase, are involved in the CD8 signal cascade (chapter 3). Thus, CD8 modulates macrophage and MC function, which may be an important mechanism by which these cells regulate immune responses.

II. Does CD8 Function as a Co-receptor?

The studies on CD8 function used intact anti-CD8 α and β antibodies and isotype matched IgG, to analyze cell function. These antibodies, in addition to binding CD8, may also interact with Fcγ receptors via their Fc ends. Three types of Fcγ receptors have been identified, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (1). There are multiple genes that encode for the three different Fcγ receptors (1-5). FcγRI

binds monomeric IgG with high affinity, whereas FcγRII and FcγRIII bind monomeric IgG with low affinity, but bind aggregated IgG (1,2). Based on their abilities to bind IgG, FcγRI is termed the high affinity receptor, whereas FcγRII and Fcγ RIII are classified as low affinity IgG receptors.

The structure of the Fcγ receptors varies among the isoforms. The extracellular region of FcγRI has three Ig-like domains, whereas FcγRII and FcγRIII have two Ig-like domains (1,2). It is the third Ig-like domain of FcγRI that may confer higher binding affinity. The homology between the first two Ig-like domains of FcγRI and the Ig-like domains of FcγRII is approximately 45% (6). Studies on chimeric proteins, which included the removal of domain three from FcγRI or the addition of domain three to FcγRII, showed that domain three is involved in regulating FcγRI affinity (7). Specifically, removal of domain three converted FcγRI receptors into FcγRII-like receptors based on their affinity (7).

In addition to the structure, the signaling machinery for Fcγ receptors also differs among the three isoforms. FcγRI and FcγRII, which are transmembrane proteins, and FcγRIII, which can be expressed as both a transmembrane and GPI- linked form, can all associate with the common γ-chain (8), which has been shown to be involved in receptor signaling (see below). In addition, FcγRII also has ITAM (immunoreceptor tyrosine-based activation motif) or ITIM (immunoreceptor tyrosine-based inhibitory motif) domains (see below), which have been implicated in mediating receptor signaling. The γ-chain is a member of the ζ-chain family (9), which has been shown to play an essential role in TCR expression and signaling (10,11). The γ-chain can associate as a

homodimer and contains an ITAM (12). This chain plays a role in Fcγ receptor signaling, where it has been shown to be phosphorylated after receptor clustering (13) and to interact with Syk kinase, which is important in the Fcγ receptor signaling cascade (14). It has been shown that surface expression of FcγRIII, and to a lesser degree FcγRI expression, is dependent on the γ-chain (15). Moreover, mouse knockout studies showed that FcγRI signaling and phagocytosis, but not binding to IgG-opsonized red blood cells, is dependent on the γ-chain (15).

Downstream of γ-chain, Syk and PI3 kinase are involved Fcγ receptor signaling (reviewed in 16 and 17). Initially, upon FcγRI aggregation, there was rapid phosphorylation of the γ-chain subunit (13). In addition, within 30s of FcγRI receptor cross linking, there was a significant increase in protein tyrosine phosphorylation, with increases in calcium mobilization seen after 1 to 2 min, which were greater than after stimulation through FcγRII (18). Further analysis determined that the *src*-kinase hck (19) and Syk kinase were proteins that were rapidly phosphorylated after FcγRI and FcγRIII activation. In addition, Syk kinase can also associate directly with FcγRI (20).

Syk kinase binds the ITAM domain of FcγRII (21) and plays an important role in FcγRI and FcγRII receptor signaling (22). Antisense to Syk kinase demonstrated that Fcγ receptor-mediated phagocytosis is Syk dependent (23). In addition, COS cells transfected with Fcγ receptor extracellular domains and Syk, showed that these molecules are sufficient to initiate Fcγ receptor dependent phagocytosis (24).

PI3 kinase is another signaling molecule that plays an important role in Fcγ receptor signaling. Like Syk kinase, PI3 kinase directly binds the ITAM region FcγRII

(21). General stimulation of Fcγ receptors or specifically crosslinking FcγRI or FcγRII, stimulated phosphorylation of PI3 kinase (25), which is indicative of increased kinase activity. Wortmannin, an inhibitor of PI3 kinase, inhibited Fcγ receptor mediated phagocytosis (25). Transfection of COS cells with the extracellular domain of FcγRI fused to PI3 kinase can initiate Fcγ receptor-mediated phagocytosis (26).

Stimulation of macrophages through their Fcγ receptors initiates the production of numerous mediators, including NO (27), H_2O_2 (28), superoxide (28), TNFα (28,29), IL-10 (30), and chemokines (30). As AM express all three types of Fcγ receptors, FcγRI, FcγRII, and FcγRIII (31,32), it is possible that in our anti-CD8 α and β experiments, interactions with the Fcγ receptors occurred, which could account for some of the effects attributed to CD8.

We used many experimental approaches to examine the involvement of Fcγ receptors in CD8 induced stimulation (Table 3.II). AM were incubated with mouse serum or IgG1 (OX8 isotype) control to block the Fcγ receptors , or BSA to block non-specific protein interactions. Anti-CD8α failed to stimulate NO release from these blocked AM. However, these cells also failed to respond to IFNγ induced stimulation (Table 3.II). Inhibition of cell activation after IgG binding has been demonstrated in other systems. Macrophages immobilized on IgG failed to respond to zymosan, a potent stimulator of macrophages (33). This inhibition was reversed when macrophages were detached from IgG and allowed to bind to plastic (33). Furthermore, these blocked macrophages produced superoxide when activated by PMA (33), suggesting that these cells are sensitive to down-stream activators. Thus, binding of monomeric IgG, while

not sufficient to stimulate mediator release, may induce the sequestering of essential early signaling molecules leaving the macrophage in a state of anergy.

To directly assess the role of Fcγ receptors, $F(ab)_2$ anti-CD8α (OX8) was used to stimulate cell function (Table 3.II). $F(ab)_2$ OX8 antibody, microsphere-immobilized $F(ab)_2$ OX8, or cross-linked $F(ab)_2$ OX8, did not stimulate NO release from AM (Table 3.II). In addition, studies in which AM were stimulated by $F(ab)_2$ OX8 and through Fcγ receptors, which were activated by IgG1/anti-IgG, NO release was not observed (Table 3.II). Moreover, $F(ab)_2$ anti-CD8α (OX8) did not block OX8 binding, as determined by flow cytometry, nor did it inhibit OX8 induced NO release from AM (Table 3.II). Thus, these experiments are inconclusive as to the role of Fcγ receptors in anti-CD8 mediated stimulation.

There is evidence that Fcγ receptors may not involved in CD8 induced NO release. IgG1 (OX8 isotype) alone, or IgG1/anti-IgG, was unable to stimulate NO release from AM (Table 6.I). Thus, if Fcγ receptors were responsible for OX8 (anti-CD8α) induced macrophage stimulation, NO should have been produced by IgG1/anti-Ig activation (Table 6.I). In addition, PI3 kinase, which is an important signaling molecule in the Fcγ receptor signaling cascade (see above), is not involved in anti-CD8 (OX8) induced NO, TNFα, or IL-1β (Table 6.I) release. Moreover, Syk kinase, another essential signaling molecule in the Fcγ receptor signaling pathway (see above), was not involved in OX8-induced IL-1β release (Table 6.I). However, AM Fcγ receptors are functionally active. IgG2a/anti-IgG stimulates NO and TNFα release from AM (personal communication, Dr. G. Stenton). Moreover, soluble IgG1 stimulates PGE2 release from these cells (personal communication, Dr. G. Stenton). Therefore, this data

suggests that CD8 and Fcγ induced AM stimulation are distinct and that CD8 activation is not Fcγ receptor dependent, in our system. Thus, we hypothesize that CD8 functions as an independent receptor which regulates cell function (the data on OX8 induced TNFα and IL-1β release is currently in a manuscript that has been accepted for publication within the *Journal of Immunology*, T.J. Lin, N. Hirji, G.R. Stenton, *et al* authors). However, we can not rule out the possibility that OX8 specifically/uniquely interacts with Fc receptors, in a fashion that can not be mimicked by isotype control antibodies. Thus, it may be the specific interaction of OX8 with Fc receptors and the interaction with CD8 that is required for cell activation. In this situation, Fc receptors would be involved in OX8-induced NO release.

III. Expression of CD8

The expression of CD8 on multiple cell types (Table 6.II), that were not previously recognized to express CD8, the recent identification of new CD8 ligands, and our work showing CD8 stimulation of cell function, suggest that CD8 plays an important role in regulating immune responses.

Jander et al (1998) found that induction of focal cerebral ischaemia in rats leads to an influx of CD8+ macrophages/microglia at day 3, which peak at day 6, before returning to normal levels (34). Interestingly, this contrasted with CD4+ macrophages which were virtually undetectable on day 3, but steadily increased thereafter (34). The appearance of CD8+ macrophages/microglia infiltration was dependent upon the type of ischaemic injury and inflammatory responses, as other models of nervous system injury,

Table 6.I Experimental evidence that CD8 functions independent of Fc receptor stimulation

Table 6.I Evidence that CD8 induced regulation of AM function is independent of Fcγ receptor activation

Stimulus n=3-4	**NO Release ($\mu M/10^6$ cells)**	**Hypotheses**
OX8 (anti-CD8α)	191.2±30.4	Anti-CD8α stimulates NO release from AM
IgG1 (OX8 isotype)	43.3±3.33	Isotype control antibodies do not stimulate NO release, suggesting that OX8 activation of AM is not mediated through Fc receptors
IgG1/anti-IgG	5.5*	IgG1 cross linking does not activate AM, suggesting that OX8 activation is Fc receptor independent
OX8 + Wortmannin (PI3 kinase inhibitor)	135.6±52.6	Since PI3 kinase plays an important role in Fcγ receptor signalling, and as it is not involved in OX8 stimulation of AM, this suggests that Fc receptors are not involved in OX8 induced AM stimulation
Stimulus n=3	**IL-1β Release ($\mu M/10^6$ Cells)**	**Hypothesis**
IgG1	2.2±0.5	Isotype control does not stimulate IL-1β release from AM
OX8	40.9±4.2	OX8 stimulates AM IL-1β release
OX8 + Syk antisense	27.5±4.6	Antisense to Syk kinase, an important kinase in the Fc receptor signalling cascade, only partially inhibits OX8 induced IL-1β release, suggesting that OX8 activation is, in part, independent of Fc receptors

* n=2

e.g. experimental autoimmune encephalomyelitis or optic nerve damage, did not induce influx of CD8+ macrophages/microglia (34).

There is further evidence that CD8 may be expressed on sub-populations of macrophages with specialized function. In neo-intimal hyperplasia, which is an excessive thickening of the graft vein after vein-to-artery graft, monocytes/macrophages are present in high numbers and appear to play a role in the thickening process (35,36). Depletion of macrophages reduces neo-intimal hyperplasia in vein grafts (37,38). In a model of neo-intimal hyperplasia in which two strains of rats were used, the spontaneously hypertensive rat (SHR) and the normotensive control Wky rat, a difference in the numbers of CD8+ macrophages was seen. CD8+ macrophages were more prevalent in Wky rats, in contrast to SHR where CD4+ macrophages were more prominent (personal communication with Dr. Alec J. Redwood, The University of Western Australia). SHR have increased neo-intimal hyperplasia, suggesting that CD8+ macrophages may play a protective role in vein grafts. NO can limit graft neo-intimal hyperplasia and suppress smooth muscle proliferation, and because we have shown that crosslinking macrophage CD8 induces NO production, CD8 may play a role in protection against neo-intimal hyperplasia. Therefore, it may be that the expression of CD8 relates to a cell's function within a specific tissue or during precise microenvironmental stimuli.

IV. Structure of CD8

There is evidence that different cell populations, e.g. T lymphocytes, macrophages, MC, and DC, express either CD8 homo or heterodimers, a combination of

Table 6.II The expression of CD8 on multiple cell types

Cell Type	CD8 α	CD8 β	Reference
Rat Macrophages			
Alveolar	+	+	Chapter 2
Peritoneal	+	+	Chapter 2
Microglia	+	+	14*
Pre-Kupffer	+	-	14*
Mouse DC			
Thymus	+	+	9-11*
Spleen	+	-	9-11*
Rat Thymic DC	+	NT	12*
Mouse MC	+	-	17*
Rat MC	+	+	Chapter 5

Abbreviations: NT, not tested; DC, dendritic cells; MC, mast cells

*References are found in Chapter 1

the two, or perhaps even monomers (as no linkage studies have been done). It is essential to elucidate the structure and sequence of the individual chains and to determine if they are linked. In chapter two and four, we demonstrated that macrophage CD8 differs from T lymphocyte CD8. An antibody that binds the Ig-like domain of T lymphocyte CD8, does not bind AM or MC CD8 (chapter 2 and 5). Moreover, immunoprecipitation studies showed that macrophage CD8 (40 kDa) is larger than T lymphocyte CD8 (32 kDa) (chapter 4). However, cDNA sequence analysis of AM CD8α, compared to T lymphocytes, showed that the Ig-like domains are similar between these cell types (personal communicaton, Mr. Mark Gilchrist). Therefore, we hypothesize that macrophage CD8 is post-translationally processed differently than T lymphocyte CD8, which could involve alterations is glycosylation.

Differences in CD8 glycosylation may be one explanation why CD8 has not been demonstrated on human macrophages, MC, or DC. If there are differences in glycosylation, particularly within the Ig-like domain, antibodies that bind T lymphocyte CD8 may not bind CD8 on other human cells. This would similar to our results with rat CD8, in which an antibody to the Ig-like domain of T lymphocyte CD8 does not bind AM and/or MC CD8. Alternative glycosylated and phosphorylated forms of CD8 have been shown. Barber et al (1989) showed, that CD8 α and β have multiple sialic acid and phosphorylated forms (39).

To date, there is little data on the structure of CD8 α and β, and their dimers, on cells other than T lymphocytes. We have postulated several possible structures for CD8 on other cell types (Fig. 6.1). In this figure, we suggest that CD8 may be expressed as a disulfide linked dimer, two non-associated chains, or as a potentially secreted form. In

addition, CD8 may interact with Fcγ receptors, act independently, or lack the signaling machinery. Moreover, the expression of these possible different forms of CD8 may be cell specific, ie. T lymphocytes express CD8 dimers only where as AM and DC express a combination of these structures. Elucidating the structure of CD8 will lead to a further understanding as to the role of this molecule on macrophages, MC, and DC.

V. Function of CD8: Cross Signaling Between CD8 and its Ligands

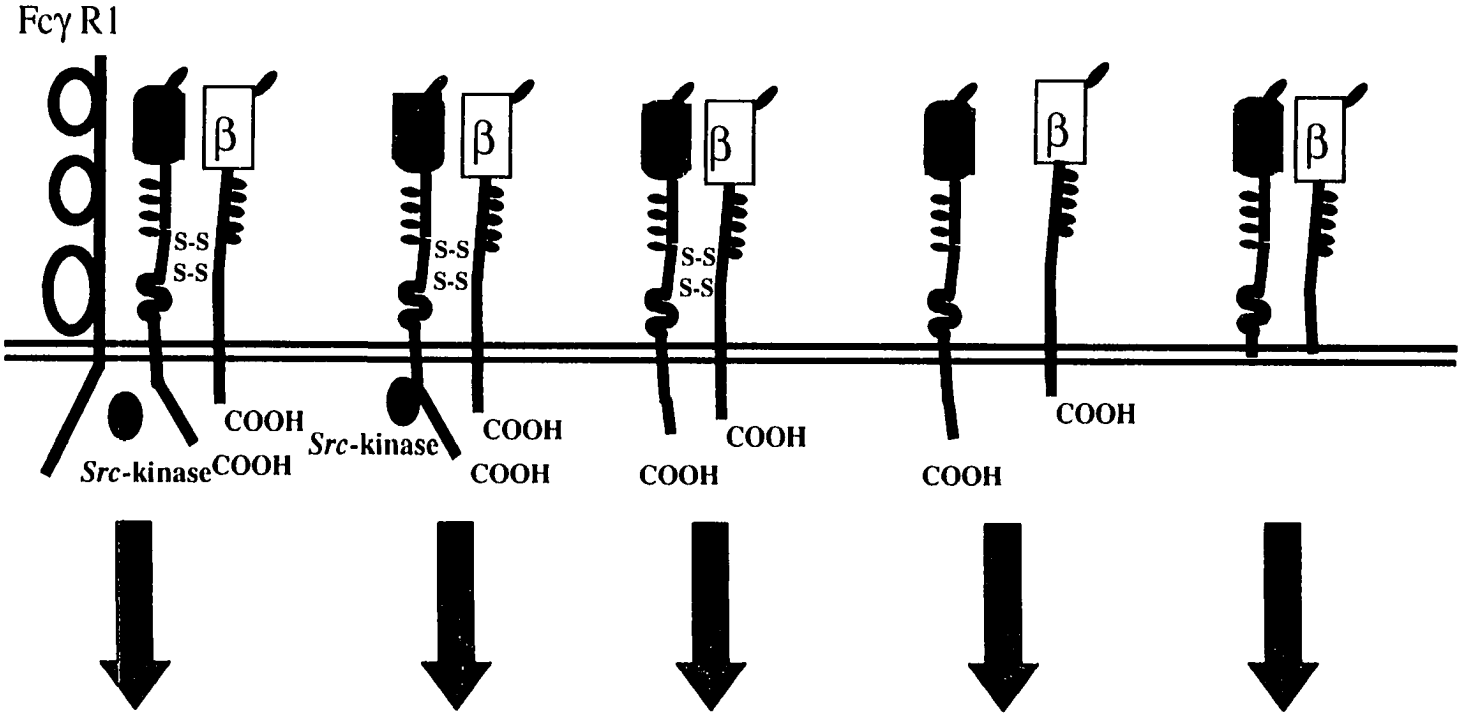
In addition to the known functions of CD8 (chapters 2-5), there may be undiscovered functions of CD8, depending on the cell type that expresses it, the specific microenvironment, and/or the maturation of this cell. Thus, it is essential to elucidate the structure and signaling pathways of CD8 on these cells, if we are to gain a greater understanding into its function.

Further analysis on CD8 function determined that cross linking this molecule on AM can also stimulate the release of TNFα and IL-1β (unpublished results). However, greater concentrations of anti-CD8β antibody were required to stimulate equivalent amounts of release compared to anti-CD8α. In addition, sub-optimal doses of anti-CD8 α and/or β showed that these molecules do not act synergistically, suggesting that the β-chain may mediate it's functions via the α-chain (personal communication, Dr. T.J. Lin). In MC, both anti-CD8 α and β, at similar levels, stimulated equivalent levels of NO and TNFα (40). Moreover, CD8 stimulation did not affect the release of preformed mediators, such as histamine, or antigen-induced (IgE dependent) stimulation of NO, TNFα, and histamine (40).

Our results on CD8 signaling cascade suggest that there are distinct signaling pathways for CD8-induced release of NO, TNFα, and IL-1β release (Fig. 6.2). CD8 induced NO release is *src*-kinase, Syk kinase, and PKC dependent, whereas TNFα is *src* and Syk kinase dependent, but PKC independent, in contrast with IL-1β which is *src*-kinase- and PKC-dependent, but Syk kinase-independent.

In addition to the signaling and stimulatory events regulated by CD8, this molecule may also interact with its ligands to stimulate cells bearing the CD8 ligands. Figure 6.3 is a synthesis of these ideas of CD8 function, where interaction between CD8 and its ligands may stimulate both CD8 and CD8 ligand expressing cells. Stimulating cells through MHC I can regulate cell function. Jurkat cells (human T lymphocyte leukemia cell line) had reduced proliferation and IL-2 production when stimulated through TCR, when they were treated with anti-MHC I (epitope α3 domain) antibodies (41). Crosslinking MHC I on Jurkat cells activated tyrosine kinases, including ZAP 70, and elevated intracellular free calcium (42). Interestingly, $p56^{lck}$ is phosphorylated after MHC I ligation and is associated with the induction of apoptosis (42). In anti-MHC I induction of apoptosis in Jurkat cells, Stat-3 was shown to be phosphorylated and translocated to the nucleus (43). In B lymphoma cells, p53/56lyn and p72syk, two important signaling molecules, are phosphorylated after MHC I crosslinking (44). Other studies have also shown that activation through MHC I can stimulate cells. T lymphocytes activated with immobilized anti-MHC I antibody had increased proliferation and increased expression of TCR/CD3 and CD28 molecules (45). The explanations for these differing results, eg.cell activation or apoptosis after anti-MHC I administration, are unclear. However, it is intriguing to postulate that CD8 molecules

Figure 6.1 The possible structure of CD8 on macrophages, MC, and DC. CD8 may act as a co-receptor, functioning in conjunction with Fcγ to mediate its effects. CD8 may also stimulate cells independently. A truncated form of CD8α may be expressed which lacks the ability to associate with *src*-family kinases; thus CD8 would facilitate cell-cell interaction without directly initiating any signaling events. As there is no formal data on the structure or dimerization of macrophage, MC or DC CD8, it is also possible that these molecules are expressed as non-linked monomers. Finally, as soluble CD8 has been demonstrated in many disease states, macrophage, MC, DC CD8 may be released in a soluble form to mediate paracrine or autocrine effects.



from different cell types may interact with MHC I to differentially regulate cell function (Figure 6.3).

This interaction between CD8 and MHC I may be one mechanism by which CD8+ DC regulate their functions. DC are potent stimulators of T lymphocytes, however, they also down regulate T lymphocyte function. Specifically, CD8+ DC can induce apoptosis in CD4+ T lymphocytes via the Fas pathway (46). However, CD8+ DC can also inhibit CD8+ T lymphocyte responses, but this is through a Fas independent mechanism (47). CD8+ DC downregulate CD8+ T lymphocyte proliferation and IL-2 production (47). Direct DC – T lymphocyte interaction is required for this inhibition (48) suggesting that DC CD8 may bind to a molecule on T lymphocytes, which may or may not be MHC I (Fig 6.3).

This hypothesis that CD8 protein on DC, or other cell types, can regulate T lymphocyte proliferation is known as the "veto" model. This model proposes that CTL and T helper cells, that are stimulated through their TCR, can be downregulated and/or eliminated by signaling through MHC I, thus suppressing the immune response. This model was demonstrated by Sambhara and Miller (1991) who showed that non-mature CTL's or T helper cells, that were stimulated through their TCR, could be triggered into apoptosis if stimulated through the $\alpha 3$ domain of MHC I (49). Early evidence for the importance of CD8 in regulating the immune response was shown in studies using CD8+ and CD8- stimulator cell lines. The expression of CD8 on these irradiated cloned stimulators inhibited T lymphocyte proliferation in a mixed lymphocyte reaction (50). Moreover, the signaling machinery of CD8 was not required for this inhibition, as GPI-

Figure 6.2 Distinct signaling pathways for CD8 induced NO, TNFα, and IL-1β release in AM

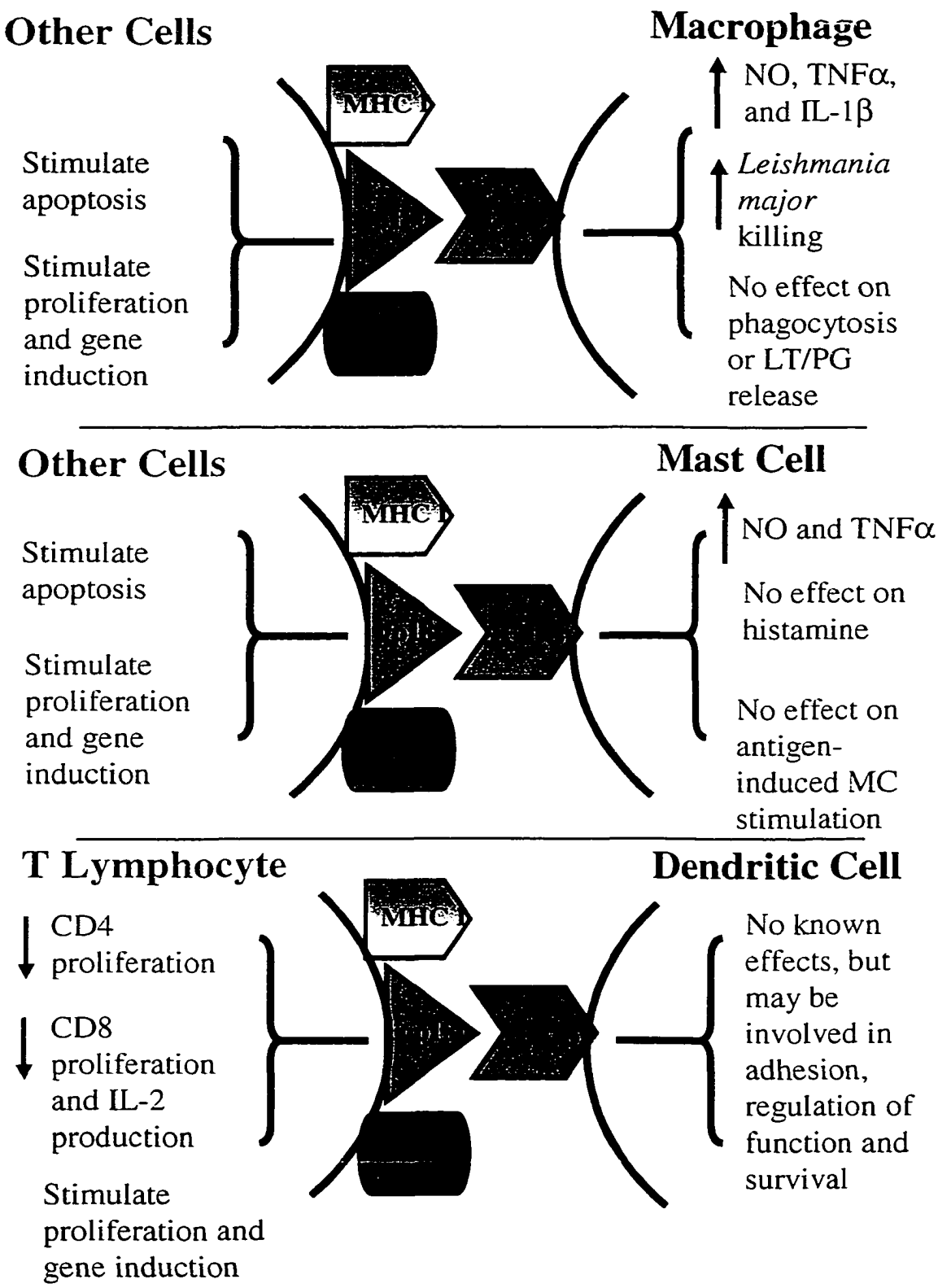
linked CD8 or fixed cells expressing CD8 could mediate this inhibition (51), suggesting that CD8 binding is sufficient to mediate these responses.

The veto affect may play an important role in graft rejection. Addition of allogeneic donor bone marrow, cells with a CD2+ CD3- CD8+ CD16+ phenotype, induces tolerance of renal allografts and deletion of recipient CTL precursors (52,53). Furthermore addition of antibodies to block CD8 – MHC I interactions results in the loss of tolerance and CTL inhibition (54).

There may be multiple mechanisms by which CD8 on donor bone marrow cells can induce tolerance and inhibit CTL. Even though evidence has shown that CD8 signaling is not essential for tolerance (51), CD8 induced signal transduction and subsequent mediator release cannot be ruled out. To test this model, CD8 was crosslinked on bone marrow cells that were either CD3-CD16+ or CD3+CD16+, that were previously shown to induce tolerance (55). Crosslinking CD8 upregulated TGFβ mRNA and mediator release in both cell subsets (55). The role of TGFβ in the veto process was demonstrated in experiments showing that TGFβ induces apoptosis in PHA activated T lymphocytes (55). It should also be considered that this process of "veto" may not be limited to lymphocytes. Initial studies showed that anti-MHC I antibodies induced MC death, and to a lesser extent, AM death (Table 6.III). Thus, other cells may also be regulated through MHC I - CD8 binding.

These studies demonstrate that CD8 plays an important role in regulating the immune response through the veto effect. Our results, showing that crosslinking CD8 can stimulate macrophage and MC mediator release, and others demonstrating that DC can regulate T lymphocyte proliferation (46-48), support the veto hypothesis and expand

Figure 6.3 Bi-directional communication between CD8+ cells and cells expressing ligand(s) for CD8. CD8 can stimulate AM to selectively release mediators, such as TNFα, NO (nitric oxide), and IL-1β, and upregulate macrophage cytotoxicity, but it does not affect LT (leukotriene) or PG (prostaglandin) release. MC CD8 can also selectively stimulate release of *de novo* synthesized mediators such as NO and TNFα. Macrophage, MC, and DC CD8 may also interact with cells expressing CD8 ligands (MHC I, gp 180, or novel ligands), such as T lymphocytes, to stimulate apoptosis, gene induction and proliferation.



it so that CD8 on AM and MC may also play a role in the veto effect (Fig. 6.3). However, studies in knockout mice suggest that CD8 may not be involved in DC regulation of immune responses. A DC marker that correlates with CD8 expression was used to identify the population of DC in CD8- mice that would normally express CD8. These experiments show that both CD8+ DC and the "same" population from CD8- mice, had similar T lymphocyte proliferative responses (56). However, it may be that CD8 null mice were able to compensate for the lack of CD8 expression. In addition, as DC are professional antigen presenting cells, CD8 may play a more prominent regulatory role in macrophage and MC than it does in DC. Despite the CD8 knock-out work on DC CD8, our experiments and data from other labs suggests that CD8 plays a dual role in regulating immune responses (Fig. 6.3). When stimulated, CD8 initiates a signal transduction pathway leading to cell mediator release (chapters 2-5). Moreover, CD8 binding activates signal transduction pathways in CD8 ligand expressing cells, which may lead to decreased proliferation and apoptosis.

VI. Function of CD8 on Alveolar Macrophages

AM play an essential role in regulating lung immune responses as they are the first line of defence within the lungs. One hypothesis about AM function is that these cells down regulate the inflammatory response so as to minimize lung damage (chapter 1 and reviewed in 57). In addition, numerous mediators have been associated with AM-dependent immune regulation, including NO (chapter 1 and 58-60) and TNFα (chapter 1 and reference 61). As our results show that CD8 crosslinking on AM stimulates NO and TNFα release, it is intriguing to hypothesize that AM CD8 may bind to a ligand

expressed on lung epithelium. This interaction would stimulate AM to secrete mediators and down regulate inflammation, thereby maintain lung homeostasis.

I propose a model in which AM CD8 interacts with gp 180 on lung epithelial cells, and that it is this interaction which is essential for AM to maintain their functions (Fig. 6.3). Gp 180, a glycoprotein first identified on human intestinal epithelial cells, can stimulate CD8+ T lymphocyte proliferation through the src-family kinase $p56^{lck}$ (62,63). This proliferation was inhibited by anti-CD8 antibodies, but not by anti-MHC I antibodies (62). Further study on gp 180 determined that this glycoprotein exists in two forms, a GPI-anchored and transmembrane isoform (63).

Airway epithelial cells may express an altered form of gp 180, as there are two antibodies that recognize this molecule, but only one binds airway epithelial cells (64). Interestingly, these antibodies see glycosylated proteins, suggesting that this discrepancy in Ab binding may be due to alterations in gp 180 glycosylation (62). Incubation of airway epithelial cells with one of the anti-gp 180 antibodies, but not anti-MHC I, inhibited T lymphocyte proliferation (64), suggesting that the gp 180 molecule on airway epithelial cells is involved in the modulation of T lymphocyte activity. This inhibition correlated with diminished T lymphocyte tyrosine phosphorylation (64). However, there may be other mechanisms, which are gp 180-independent, that account for these findings. In the lung, it has been shown that airway epithelial cells stimulate both CD4+ and CD8+ lymphocytes (64), and thus if this was a gp 180-mediated effect, CD4+ cells should not be affected. Moreover, this anti-gp 180 antibody immunoprecipitated a 50 kDa protein from the lung, suggesting that there may be a different protein, with shared gp 180 epitopes, that could be mediating these affects (Dr.

Table 6.III Anti-MHC I induced MC death after 24h incubation

Table 6.II Stimulation of MC death by anti-MHC I incubation

MC Stimulus (24h Incubation)	Percent live cells (Trypan Blue) Representative Experiment n=3
Cells	95
Isotype control	87
Anti-MHC I	15
Isotype + anti-IgG	89
Anti-MHC I + anti-IgG	3
Antigen (*N. brasiliensis*)	94
Antigen + Isotype	95
Antigen + anti-MHC I	5

L. Mayer, personal communication). Therefore, the expression and function of gp 180 on lung epithelial cells remains unclear. However, as this protein is glycosylated, there may be novel glycosylated forms of gp 180 expressed on lung epithelial cells, which would not be detected by the available reagents.

Interestingly, in the intestine, gp 180 has been shown to co-immunoprecipitate with the CD1 (non-classical MHC I) (65). Moreover, anti-CD1 antibody inhibits intestinal epithelial cell induced T lymphocyte proliferation and $p59^{fyn}$ activation, but does not affect $p56^{lck}$ upregulation (65). Therefore, these studies suggest that CD8+ T lymphocytes interact with the CD1-gp 180 complex on epithelial cells, which stimulates $p59^{fyn}$ and $p56^{lck}$ to activate T lymphocytes.

I propose that a similar model may also occur with CD8+ AM and lung epithelial cells. In this model, CD8 on AM binds to gp 180 or the CD1-gp 180 complex, which activates AM to secrete NO, TNFα, and other mediators to regulate (inhibit) the immune response. Thus, a down regulation or loss of gp 180 may lead to an increase in inflammation. This may contribute to the pathogenesis of inflammatory bowel disease, where patients with ulcerative colitis and Crohn's disease have been shown to have low gp 180 expression (66).

In addition to activation of CD8+ AM, gp 180 may also activate signal transduction pathways in epithelial cells, leading to mediator release from these cells. The lung epithelium, particularly in the upper airways, is a multi-cell layer that forms a physical barrier in the lung between the body and external environment. It is composed of multiple cell types, including columnar cells, secretory cells, basal cells, serous cells, Clara cells, and immune cells. Columnar cells form the majority of cells in the

epithelium and contain cilia, which is essential for mucus clearance. Secretory cells secrete mucus, which is important for trapping foreign substances. Basal cells may be progenitors of columnar cells and play a role in cell anchoring. Clara cells produce surfactant, which plays a role in oxygen exchange, preventing alveoli from collapsing, and anti-microbial activity (reviewed in 67). These different cell types form a network that makes up the epithelial layer.

Our understanding of epithelial cell function is expanding. Epithelial cells can release numerous mediators that can regulate immune responses. Cytokines, such as IL-1,6,10 and TNFα; chemokines, which include RANTES, MCP-1, and IL-8; stimulating factors such GM-CSF, M-CSF, and CSF-1; and oxygen metabolites such as NO, allow epithelial cells to play a role in regulating immune responses (68-69). Thus, in expanding the model of the function of AM CD8, it is possible that this molecule interacts with gp 180 on epithelial cells, which could initiate signal transduction pathways that regulate mediator release from different epithelial cell types.

Therefore, AM CD8 and gp 180, or the CD1-gp 180 complex, interact to stimulate macrophages to maintain lung homeostasis and regulate mediator secretion from epithelial cells (Fig. 6.3).

VII. Future Directions

Our data and the literature demonstrate that CD8 should no longer be thought of as a molecule largely restricted to a subset of T lymphocytes. CD8 plays important roles on a variety of cell types, including macrophages, MC, DC, and NK cells. We

postulate and provide evidence that the expression of CD8 is related to the differentiation state and specific microenvironment of these cells. In addition, CD8 may interact with ligands on other cell types to stimulate apoptosis and regulate inflammatory responses (Fig. 6.3).

These results also suggest that many of the concepts in the literature, using rat models, that have been developed using anti-CD8 antibodies *in vivo* will have to be re-evaluated. Such studies have been interpreted focusing exclusively on CD8 expression of T lymphocytes. For example, Olivenstein *et al* (70) and Huang *et al* (71) used i.v. anti-CD8α antibodies to modulate the allergic airway response in rats. These researchers found an increase in bronchial hyperresponsiveness (70) and increased numbers of AM, neutrophils, and lymphocytes (71) after allergen challenge, in anti-CD8 treated rats. These changes were attributed to a loss of CD8+ T suppressor cells. In another example, anti-CD8α treatment significantly reduced the severity of induced arthritis in rats, but did stimulate IgE production and IFNγ upregulation (72). Again, these results were interpreted as T lymphocyte specific, which may not be the case. It may be that anti-CD8α stimulated CD8+ cells, such as macrophages, MC, and DC to release mediators. Therefore, NO, TNFα, IL-1β, and other mediators released by these cells, after anti-CD8α activation, would modulate the immune response and may be responsible for the observed effects.

As this field is in its infancy, there are many basic questions left unanswered. Are CD8 molecules expressed by non-T lymphocytes similar in structure to T lymphocyte CD8? Are these novel molecules linked as dimers, or can they be expressed as unique single chain forms? When, and what stimuli, initiate and

regulate the expression of CD8 on the surface of cells? What is/are the ligands for this molecule and are they unique for each CD8 isoform? Does CD8 – ligand (eg. MHC I or gp 180) interaction initiate any signal transduction pathways in CD8 ligand expressing cells? Is CD8 expressed on human and/or murine macrophages and MC and does it function in a similar manner to rat CD8?

The answers to these questions will provide insight and understanding as to the role(s) that CD8 plays within immune and inflammatory responses. The expression of CD8 on the different cell types may provide important markers for immune responses elicited, and may allow us to better understand, and therefore treat, patients with autoimmune and inflammatory diseases.

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Appendix I Curriculum Vita

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Education

B.Sc. Cellular Molecular Microbial Biology (major) and Psychology (major)
University of Calgary
09/88 to 04/93

Ph.D. Experimental Medicine – 2000
Thesis Title: The Expression and Function of a Novel CD8 Molecule on Alveolar Macrophages and Mast Cells.
Supervisor: Dr. A. Dean Befus, Department of Medicine, University of Alberta
Cumulative GPA: 8.2/9

Awards

Amoco Canada Petroleum Ltd. University Scholarship - 1988 to 1992
G.H. Wood - 1993 to 1994
75th Anniversary Studentship - 1994 to 1995
Canadian Society For Immunology Travel Award - 1995
Alberta Respiratory Symposium Presentation Award - First Place - 1995
Alberta Lung Association Studentship - 1996
Mary Louise Imrie Travel Award – 1996
Canadian Society For Immunology Travel Award - 1997
Alberta Lung Association Studentship - 1998
Canadian Society for Immunology Travel Award - 1998
Mary Louise Imrie Travel Award - 1998
10th International Congress of Immunology Merit (Presentation) Prize (first place) –1998
Selected to represent the U of A (2/428) at a National MRC Competition - 1999
Gold Medal – National medical research competition (1 of 4 given nationally) – 1999

Teaching Experience

Problem Solving Skills – Medical Students
University of Alberta, 1999

- Assist students in defining questions necessary to attain their diagnostic goal
- Help students with patient-doctor interactions so that they are able to identify areas that need to be further probed
- Set up a diagnostic checklist that students can use on multiple patients
- Teach students how to determine if their goals were met

Leadership Activities

Graduate Student Representative, Department of Medicine
University of Alberta, 1994 to Current

- Relate student concerns and liaison between the graduate students and the Department of Medicine
  - Successfully lobbied the Department to subsidize graduate student tuition fees.
- Initiated student representation on Departmental committees
- Developed and implemented annual orientations for new graduate students
- Increase communication between the Department of Medicine and the graduate students

Graduate Students Association Voting Council Member
University of Alberta, 1994 to Current

- Help develop university wide graduate student policy

Canadian Society for Immunology Travel Awards Committee, 1999

- Judged student abstracts for travel awards

Hockey Coach, YMCA
Calgary 1991 to 1993, Edmonton 1993 to 1997

- Coach children ages 6 to 8 and 9 to 11

Mentor
Boys and Girls Club of Edmonton, Norwood School
Edmonton 1998 to Current

- Help children with reading, self esteem, and life skills

Papers

1. **Hirji, N.S.**, T.J. Lin, and A.D. Befus. 1997. A Novel CD8 Molecule Expressed by Alveolar and Peritoneal Macrophages Stimulates Nitric Oxide Production. The Journal of Immunology, 158:1833-1840.

2. **Hirji, N.S.**, T.J. Lin, R. Sigurdson, M. Belosevic, and A.D. Befus. 1997. Function of a Novel CD8 Molecule on Macrophages and Mast Cells: Stimulation of Nitric Oxide and TNFα Release. In: Mucosal Solutions: Advances in Mucosal Immunology, (Alan J. Husband, Kenneth W. Beagley, Andrew M. Collins, Robert L. Clancy, Allan W. Cripps and David L. Emery, eds), University of Sydney, Sydney, Volume 1:163-171.

3. **Hirji, N.S.**, T.J. Lin, E.Y. Bissonnette, M. Belosevic, and A.D. Befus. 1998. Mechanisms of Macrophage Stimulation Through CD8: Macrophage CD8α and CD8β Induce Nitric Oxide Production and Associated Killing of the Parasite *Leishmania major*. The Journal of Immunology, 160:6004-6011.

4. Lin, T.J., **N.S. Hirji**, O. Nohara, G. Stenton, M. Gilchrist, and A.D. Befus. 1998. Mast Cells Express Novel CD8 Molecules that Selectively Modulate Mediator Secretion. The Journal of Immunology, 161:6265-6272.

5. **Hirji, N.S.**, T.J. Lin, M. Gilchrist, G. Nault, O. Nohara, B.J. Grill, M. Belosevic, G.R. Stenton, A.D. Schreiber, and A.D. Befus. 1999. Novel CD8 Molecule on Macrophages and Mast Cells: Expression, Function, and Signaling. International Archives of Allergy and Immunology, 118:180-182.

6. ‡Lin, T.J., **N.S. Hirji**, G.R. Stenton, M. Gilchrist, B.J. Grill, and A.D. Befus. Activation of Macrophage CD8: Pharmacological Studies of TNFα and IL-1β Production. The Journal of immunology, *In Press*.

7. Nohara, O., M. Gilchrist, G.R. Stenton, **N.S. Hirji**, and A.D. Befus. 1999. Reverse Transcriptase (In Situ) Polymerase Chain Reaction of mRNA Expression in Rat Mast Cells and Macrophages. The Journal of Immunological Methods, 226:147-158.

8. Gilchrist, M., M. Savoie, **N.S. Hirji**, O. Nohara, F. Wills, J. Wallace, and A.D. Befus. Nitric Oxide Synthase Expression and Production in *In-Vivo* Derived Mast Cells. *Submitted.*

9. Stenton, G.R., M.K. Kim, P.H. Hwang, J.G. Park, O. Nohara, **N. Hirji**, F.L. Wills, M. Gilchrist, W. Finlay, R.L. Jones, A.D. Befus, A.D. Schreiber. Aerosolized Syk Antisense Suppresses Syk Expression, Mediator Release from Alveolar Macrophages and Pulmonary Inflammation. *Submitted.*

‡ denotes that first three authors contributed equally to the manuscript.

Abstracts

1. **Hirji, N.S.**, and A.D. Befus. Rat alveolar macrophages express a low molecular weight CD8 like molecule. The Ninth Spring Meeting of the Canadian Society for Immunology, Lake Louise, Alberta, March 24-27, 1995.

2. **Hirji, N.S.**, and A.D. Befus. Alveolar macrophages transcribe and express CD8. The 2nd Annual Alberta Respiratory Symposium, Jasper, Alberta, October 20-22, 1995.

3. ***Hirji, N.S.** and A.D. Befus. Alveolar macrophages express both the message and protein for the T-cell marker, CD8. American Thoracic Society/American Lung Association International Conference, New Orleans, Louisiana, May 10-15, 1996.

4. Befus, A.D., **N.S. Hirji**, R. Sigurdson, and T.J. Lin. Function of the novel CD8 molecule on macrophages and mast cells : stimulation of nitric oxide and TNFa release. 9th International Congress of Mucosal Immunology, Sydney, Australia, January 27-31, 1997.

5. Lin, T.J. **N.S. Hirji**, and A.D. Befus. Novel CD8 Molecules Expressed by Rat Mast Cells Regulate Mediator Release. American Association of Immunologists International Conference, San Francisco, California, February 21-26, 1997.

6. **Hirji, N.S.**, T.J. and A.D. Befus. iNOS Message and Protein is Upregulated Following Ligation of CD8 on Alveolar Macrophages. Canadian Society for Immunology Spring Meeting, Lake Louise, Alberta, March 14-17, 1997.

7. ***Hirji, N.S.**, T.J. Lin, and A.D. Befus. The Newly Discovered CD8 Molecule on Alveolar Macrophages Stimulates Nitric Oxide Production through a Protein Tyrosine Kinase(s). American Thoracic Society/American Lung Association International Conference, San Francisco, California, May 17-21, 1997.

8. ***Hirji, N.S.**, T.J. Lin, M. Gilchrist, G. Nault, O. Nohara, B.J. Grill, M. Belosevic, G. Stenton, A. Schreiber, and A.D. Befus. Novel CD8 Molecule on Macrophages and Mast Cells: Expression, Function, and Signaling Mechanisms. Collegium Internationale Allergologicum, Corfu, Greece, September 12-16, 1998.

9. **Hirji, N.S.**, T.J. Lin, M. Gilchrist, G. Nault, B.J. Grill, M. Belosevic, and A.D. Befus. Novel CD8 Molecule on Macrophages: Expression, Function, and Signaling Mechanisms. 10th International Congress of Immunology, New Delhi, India, November 1-6, 1998.

10. Gilchrist, M., M. Savoie, **N.S. Hirji**, O. Nohara, F. Wills, J. Wallace, and A.D. Befus. Inducible Nitric Oxide Synthase Production in Rat Mast Cells. 55th American Academy of Allergy, Asthma and Immunology, Orlando, USA, February 26-March 3, 1999.

11. Stenton, G.R., M.K. Kim, P.H. Hwang, O. Nohara, **N.S. Hirji**, F. Wills, M. Gilchrist, A.D. Befus, and A.D. Schreiber. Syk Antisense Oligodeoxynucleotide Treatment Inhibits Fcγ-Receptor Mediated Alveolar Macrophage Function and Pulmonary Inflammation. 55th American Academy of Allergy, Asthma and Immunology, Orlando, USA, February 26-March 3, 1999.

12. Nohara, O., F.L. Wills, **N.S. Hirji**, M. Gilchrist, G.R. Stenton, and A.D. Befus. Regulation of CD8 Expression in Rat Peritoneal Mast Cells by Nitric Oxide. 55th American Academy of Allergy, Asthma and Immunology, Orlando, USA, February 26-March 3, 1999.

13. Gilchrist, M., M. Savoie, **N. Hirji**, O. Nohara, F. Wills, J. Wallace, and A.D. Befus. The Expression and Characterization of Inducible Nitric Oxide Synthase in Rat Mast Cells. The 13th Annual Spring Meeting of the Canadian Society for Immunology, Lake Louise, Alberta, March 5-8, 1999.

14. **Hirji, N.S.**, T.-J. Lin, M. Gilchrist, G. Nault, B.J. Grill, M. Belosevic, and A.D. Befus. Molecular Mass and Signaling Mechanisms of CD8 on Macrophages. The 13th Annual Spring Meeting of the Canadian Society for Immunology, Lake Louise, Alberta, March 5-8, 1999.

* Indicates abstract was selected for oral presentation.