MOLECULAR MECHANISM OF IFN-γ-INDUCED MACROPHAGE ACTIVATION

By

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ABSTRACT

Macrophages are considered as one of the early effector cells involved in the immunological response against infection. Following macrophages interaction with pathogens, macrophages become activated and attempt to eliminate the invader. Interferon gamma (IFN-γ) is one of the most important activators of macrophage function. In macrophages IFN-γ is promoting the expression of major histocompatibility complex class II (MHC-II) molecules and other proteins directly involved in the process of containing infection.

The effect of the *Nramp1*, on macrophage function is investigated in this thesis. Nramp1 has been shown to determine the resistance or susceptibility of mice to infections with several intracellular microorganisms, including *Mycobacterium bovis* BCG. Although the precise mechanism of Nramp1 action is unknown, there are several well-established effects associated with the *Nramp1*. In general, it has been shown that macrophages derived from mice susceptible to infections with *M. bovis* BCG are less efficient in the production of nitric oxide (NO), reactive oxygen intermediates (ROI), TNF-α, and MHC-II antigens in response to IFN-γ.

Using macrophage cell lines derived from mice that are either resistant (B10R) or susceptible (B10S) to *M. bovis* BCG infection, we have demonstrated that lower levels of IFN-γ-induced expression of MHC-II antigens is correlated with less efficient phosphorylation of the STAT1 protein in B10S macrophages compared to B10R macrophages. We have shown that low levels of MHC-II expression in B10S macrophages correlate with less efficient expression of CIITA (Class II Transactivator). We have observed that infection of macrophages with *M. bovis* BCG has an inhibitory effect on both CIITA and MHC-II expression in macrophages stimulated with IFN-γ.

We have also studied the effect of lipopolysaccharide (LPS) on MHC-II expression in macrophages. We have found that the inhibitory effect of LPS on CIITA gene transcription does not involve changes in the binding of STAT1 to CIITA promoter IV. We have also demonstrated that unlike *M. bovis* BCG, the inhibitory effect of LPS on MHC-II expression is mediated by Toll-like receptor 4 (TLR4). In addition, we have shown that inhibitory effects of both LPS and *M. bovis* BCG depend on the adaptor protein MyD88.

We have also analyzed the regulation of IFN-γ- or/and LPS-stimulated expression of TLR2 in macrophages. We have shown that regulation of TLR2 expression by IFN-γ depends on TLR4 expression. We have also determined that the phenol extractable fraction present in the commercial preparations of endotoxins from Gram-negative bacteria is able to synergize with IFN-γ and activate TLR4-deficient macrophages.

Overall, we believe that these studies significantly contribute to the understanding of the molecular mechanism of the process of macrophage activation.

RÉSUMÉ

Les macrophages sont reconnus comme étant parmis les premières cellules effectrices durant la réponse immunitaire contre une infection. Suite à l'interaction des macrophages avec les pathogènes, ceux-ci deviennent activés et tente d'éliminer l'intru. L'interféron gamma (IFN-γ) est une des cytokines les plus efficaces pour activer les macrophages en augmentant l'expression du complexe majeur d'histocompatibilité de classe 2 (CMH-II) ainsi que d'autres protéines jouant un rôle direct dans le contrôle de l'infection.

L'effet du gène *Nramp1* sur la fonction des macrophages est aussi adressé dans cette thèse. Chez la souris, *Nramp1* contrôle la résistance ou la susceptibilité à plusieurs espèces de pathogènes intracellulaires telles que *Leishmania donovani, Salmonella typhimurium* ainsi que *Mycobacterium bovis* BCG. Le méchanisme d'action de *Nramp1* est toujours incertain, cependant plusieurs effets ont été associés à celui-ci. Généralement, les macrophages dérivés de souris susceptible à *M. bovis BCG* produisent moins d'oxyde nitrique (NO), de radicaux d'oxygène, de TNF-α et de CMH-II, spécialement en réponse à l'IFN-γ.

En employant des lignées cellulaires dérivées de souris résistantes (B10R) ou susceptibles (B10S) à *M. bovis BCG*, nous avons démontré que l'expression réduite de CMH-II par l'IFN-γ dans les macrophages de souris B10S est associée à une plus faible phosphorylation de la protéine STAT1. Nous avons aussi démontré que la réduction de l'expression de CMH-II dans les macrophages B10S est liée à une réduction de l'expression de CIITA. Nous avons observé que l'infection de macrophages avec *M. bovis BCG* inhibe l'expression de CIITA et de CMH-II suite à une stimulation avec IFN-γ.

Nous avons aussi étudié l'effet de LPS sur l'expression de CMH-II dans les macrophages. Nous avons découvert un effet inhibiteur de LPS sur la transcription du gène CIITA qui n'est pas associé à un changement dans l'interaction de STAT1 avec le promoteur IV de CIITA. Nous avons démontré que cet effet, contrairement à ce qui est observé pour *M. bovis BCG*, utilise la voie de signalisation de ``Toll-like receptor 4`` (TLR4). De plus, nous avons montré que l'effet inhibiteur de LPS et *M. bovis BCG* utilise la molécule adaptrice MyD88.

La régulation de l'expression de TLR2 dans les macrophages par LPS et IFN-γ a aussi été analysée. Nous avons démontré que la régulation de TLR2 par IFN-γ requiert l'expression de TLR4. Nous avons aussi déterminé qu'une fraction soluble dans le phénol présente dans les préparations commerciales de LPS a un effet de synergie avec IFN-γ et que cette fraction peut activer des macrophages dérivés de souris TLR4-KO.

Globalement, nous croyons que l'étude présentée contribue de façon significative à la compréhension du mécanisme moléculaire de l'activation des macrophages.

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ABBREVIATIONS

α	alpha
β	beta
γ	gamma
κ	kappa
μ	micro
°C	degree Celsius
A	adenine
Ab	antibody
Ag	antigen
AP-1	activator protein 1
APC	antigen presenting cell
Arg	arginine
Asp	aspartate
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BBEC	bovine brain endothelial cells
BCG	Bacillus Calmette-Guérin
Bcg^r	*M. bovis* BCG resistant
Bcg^s	*M. bovis* BCG susceptible
BLS	bare lymphocyte syndrome
bp	base pair

BSA	bovine serum albumin
BUEC	bovine umbilical endothelial cells
C	cystosine
CBP	CREB binding protein
CD	cluster of differentiation
cDNA	complementary DNA
CIITA	Class II Transactivator
CMV	cytomegalovirus
CNS	central nervous system
cpm	counts per minute
CR3	complement receptor type 3
CREB	cAMP responsive element binding protein
Cu	copper
D	aspartate
DC	dendritic cells
dCTP	deoxy-cytidine-5'-triphosphate
dI:dC	deoxy-inosinic-deoxy-cytidilic acid
DMEM	Dulbecco's modified Eagle medium
DMT	divalent metal transporter
DNA	deoxyribonucleic acid
DPBS	Dulbecco's PBS
dsRNA	double stranded RNA
DTT	dithiotreithol

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
Fc	fraction crystallizable
FcR	Fc receptor
Fe	iron
Fig	figure
FITC	fluorescein isothiocyanate
FP	free probe
Fura-6-FF-triethylester	COOH-FF6[COOEt]$_4$
g	gram
G	guanine
GAF	interferon gamma activated factor
GAPDH	glyceraldehydes phosphate dehydrogenase
GAS	gamma interferon activated site
GBP	guanylate-binding protein
GI	gastro-intestinal
Gly	glycine
GM-CSF	granulocyte-macrophage colony stimulating factor
GTP	guanosine-5'-triphosphate
h	hour

HCl	hydrochloric acid
HDF	human dermal fibroblasts
HEC	human endothelial cells
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
hsp	heat shock protein
HSV	herpes simplex virus
hTAF	human TATA-binding polypeptide-associated factor
ICAM	intracellular adhesion molecule
ICSBP	interferon consensus sequence binding protein
IFN	interferon
Ig	immunoglubulin
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK	IL-1R associated kinase
IRF	interferon-responsive factor
ISG	interferon stimulated genes
ISGF3	interferon stimulated gene factor 3
ISRE	interferon stimulated response element
JAK	Janus tyrosine kinase

JNK	c-Jun N-terminal kinase
KCl	potassium chloride
kDa	kilodalton
KO	knockout
l	liter
LAM	lipoarabinomannan
Lamp1	lysosome-associated membrane glycoprotein
LBP	LPS binding protein
LCMV	lymphocytic choriomeningitis virus
LFA	lymphocyte function-associated antigen
LMP	low molecular weight proteasome
LPS	lipopolysaccharide
LRR	leucine rich repeat
LTA	lipoteichoic acid
LTR	long terminal repeat
m	milli-
M	molar
mAb	monoclonal antibody
MAL	MyD88-adapter-like
MALP-2	macrophage-activating lipoprotein 2 kDa
MAP kinase	mitogen-activated protein kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MCMV	murine cytomegalovirus

MDHM	high molecular weight material
ME	mercaptoethanol
MEF	mouse epithelial fibroblasts
MEKK	MAP kinase kinase
$MgCl_2$	magnesium chloride
MHC-II	major histocompatibility complex class II
min	minutes
Mn	manganese
mRNA	messenger RNA
MV	measles virus
MyD88	Myeloid Differentiation protein 88
MΦ	macrophage
n	nano
Na_3VO_4	sodium orthovanadate
NaCl	sodium chloride
NaF	sodium fluoride
NF	nuclear factor
NIK	NFκB-inducing kinase
NK	natural killer
NO	nitric oxide
NP-40	Nonidet P-40
Nramp	natural resistance associated macrophage protein
P/S/T	proline/serine/threonine

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	PBS tween-20
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol 3-kinase
PIAS	protein inhibitor of activated STAT
PKC	protein kinase C
PKR	dsRNA-activated protein kinase
PMSF	phenylmethylsulfonyl fluoride
PRR	pattern-recognition receptor
PSM	phenol-soluble modulin
PTP	protein tyrosine phosphatases
PVDF	polyvinylidene difluoride
R	receptor
RFX	regulatory factor X
RFXANK	regulatory factor X with ankyrin repeats
RFXAP	RFX associated protein
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
RPM	revolutions per minute
rRNA	ribosomal RNA
S	serine

SDS	sodium dodecyl sulfate
SFV	Semliki forest virus
SHP	*Src* homology 2 containing tyrosine phosphatese
Slc11a1	solute carrier family 11 member 1
SNAP	S-nitroso-N-acetyl-penicillamine
SOCS	suppressors of cytokine signaling
SR-A	scavenger receptor, types I and II
SSC	sodium chloride/sodium citrate
STAT	signal transducer and activator of transcription
T	thymine
TAP	transporter associated with antigen presentation
TCR	T-cell receptor
TFII	transcription factors class II
TGF	transforming growth factor
TIR	Toll/IL-1 receptor homology domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIL	Toll-like receptor 4 independent ligand
Tris	tris(hydroxymethyl)aminomethane
Tyk-2	tyrosine kinase 2
Tyr	tyrosine

U	unit
unLPS	unpurified LPS
USF-1	upstream stimulatory factor 1
UTR	untranslated region
UV	ultra violet
VSV	vesicular stomatitis virus
VZV	varicella-zoster virus
WHO	World Health Organization
X2BP	X2 binding protein
Zn	zinc

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PREFACE

In accordance with the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research, manuscripts of papers which have been published or which have been submitted for publication have been incorporated in the thesis. This format for the thesis preparation has been approved by the Division of Experimental Medicine, Department of Medicine. The following is quoted directly from the Guidelines:

> "Candidates have the option, subject to the approval of the Department, of including as a part of their thesis, copies of the text of a paper(s) submitted for publication, or a clearly duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as a separate chapter or section: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and the objectives of the study, (4) a comprehensive general review of the background literature to the subject of study, when this review is appropriate, and (5) a final overall conclusion and/or summary".

"Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g., in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers".

Each manuscript included in the thesis (chapters II, III, and IV) possess their own Summary, Introduction, Materials and Methods, Results, Discussion and Bibliography. Prefaces, used to connect the chapters, are present at the beginning of each chapter. Furthermore, an Summary (iii), Introduction (Chapter I), and General Discussion (Chapter V) section is included in the thesis in order to present an overall view of the subject matter contained in the thesis. References are present at the end of each chapter.

The studies which are described in the thesis have been carried out by the author of the thesis under the guidance and supervision of Dr. Danuta Radzioch at the Montreal General Hospital Research Institute. The author, Dr. Danuta Radzioch and Dr. Emil Skamene are co-authors of all manuscripts included in the thesis. Studies presented in Chapter II have been published in The Journal of Immunology[1] (*Copyright 1999. The American Association of Immunologists, Inc.*). The author of the thesis is first author of the paper. Dr. Juan DeSanctis

participated in the generation of preliminary FACS analyses of MHC-II in B10R and B10S macrophages. Studies presented in Chapter III have been submitted in December 2001 to The European Journal of Immunology. The author of the thesis is first author of this paper. Ariana Murata contributed to the paper presented in Chapter III by preparing RNA samples from MyD88-KO cells, used for I-A_β and CIITA Northern blot analysis. She also performed analysis of I-Aβ and CIITA mRNA stability and FACS analyses of MHC-II expression in TLR4-Del cells. Results of these experiments were not included in the manuscript but were quoted as "data not shown". Jacques Moisan participated equally with the author in obtaining data from FACS analyses of MHC-II expression included in figures 1 and 6, as well as analysis of MHC-II expression in iNOS-KO macrophages, which was not included in the manuscript but was quoted as "data not shown". Aihao Ding participated by providing the authors with the bone marrow from C57B10/SnCr and MyD88 gene knockout mice which were used to generate TLR4-Del and MyD88-KO macrophage cell lines, respectively. Studies presented in Chapter IV are prepared for publication in The European Journal of Immunology. The author is first author of this manuscript. Ariana Murata contributed by obtaining TLR2 DNA probe, establishing the protocol for TLR2 Northern and Western blot analyses and performing preliminary studies of TLR2 mRNA and protein expression in macrophage cell lines. Dr. Craig Daniels performed silver staining of LPS fractions included in figure 3. Dr. Joseph S. Lam provided guidance and assistance in analysing LPS fractions.

1. Wojciech Wojciechowski, Juan DeSanctis, Emil Skamene and Danuta Radzioch. Attenuation of MHC Class II Expression in Macrophages Infected with *Mycobacterium bovis Bacillus*

Calmette-Guerin Involves Class II Transactivator and Depends on the *Nramp1* Gene. *The Journal of Immunology,* 1999, 163: 2688–2696. (Chapter II).

2. Wojciech Wojciechowski, Ariana Murata, Jacques Moisan, Aihao Ding, Emil Skamene and Danuta Radzioch. Inhibition of IFN-γ induced Major Histocompatibility Complex class II expression by lipopolysaccharide and *Mycobacterium bovis* BCG occurs in a Myeloid Differentiation protein 88 (MyD88) dependent manner and involves attenuation of Class II Transactivator mRNA expression. The European Journal of Immunology (submitted December 2001). (Chapter III).

3. Wojciech Wojciechowski, Ariana Murata, Craig Daniels, Joseph S. Lam, Emil Skamene and Danuta Radzioch. IFN-γ induces prolonged TLR2 protein expression in TLR4 deficient but not in wild type macrophages. Prepared for submission to The European Journal of Immunology (Chapter IV).

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CHAPTER I

GENERAL INTRODUCTION

Biology and function of macrophages

Macrophages play an important role in the process of immunological response both to infectious agents and to transformed cells. Their basic function is to recognize and remove dead cells or fragments of dead tissue as well as invading pathogens and foreign objects such as dust particles in the lungs. Their ability to migrate and colonize different kind of tissues as well as the capacity to ingest relatively large objects makes them very effective components of the first line of defense in the immunological system. Macrophages not only eliminate infectious agents, they also help to develop an immunological response against them. Although granulocytes are much more efficient in killing and eliminating microorganisms from the site of infection than macrophages, the macrophages are able to activate other cells of immune system more efficiently than granulocytes. This feature of macrophages is associated with their ability to process and present antigens to specific T-cells as well as to produce a variety of soluble mediators like cytokines, chemokines as well as chemoattractants and lipid mediators. In addition, they take part in processes like chemotaxis, formation of the blood clot, activation of the complement and the acute phase that take place during the host response to infection (1). Macrophages are not the only cell type able to perform the above functions. In fact, other cells might be much more efficient in performing particular functions. For example dendritic cells are much better antigen presenting cells, neutrophils have more efficient cytotoxic properties and T-cells are able to regulate immunological response in more specific manner. Macrophages, are an indispensable part of the host defense system because of their presence in virtually every type of tissue, their capacity to contain the majority of infections in the early phase of their development, and their ability to mount specific immunological responses (2, 3).

Basic characteristic of macrophages

Macrophages constitute a non-homogenous population of so-called mononuclear phagocytes, which in the past were collectively considered as reticulo-endothelial system (3). Most of the tissue macrophages are derived from the progenitor cells of the myeloid lineage which proliferate in the bone marrow. The early myelomonocytic stem cell develops into a monoblast, then into a promonocyte, and finally into a monocyte circulating in the blood stream that represents a functionally mature cell capable of phagocytosis and antigen presentation (2). Some of the monocytes adhere to the walls of blood vessels' sinuses in such organs as the liver, the spleen, the lymph nodes, the pituitary and adrenal glands where they intercept pathogens trying to infect those organs. Other monocytes leave the blood at random and infiltrate surrounding tissue. Once they cross the blood barrier, they transform into macrophages. This process is accompanied by an increase in their original size by 5 to 10 times and by an augmentation of their phagocytic capacity. The production of hydrolytic enzymes, which is facilitated by the enlargement of the Golgi apparatus and an increase in the number of mitochondria, is also characteristic of macrophages but not monocytes (1). The primary functions of macrophages are immunological surveillance, active phagocytosis and killing of pathogenic microorganisms, transformed cells or cells infected with viruses, as well as removal of the debris and the remains of dead cells or damaged tissue. The phagocytosed objects are usually degraded using hydrolytic enzymes. The short peptides generated this way are presented to specific T-cells. The initial steps in the process of phagocytosis require specific recognition of the object which should be eliminated. The recognition and the initiation of physical contact are facilitated by the presence of an array of receptors on the surface of the macrophage (4). There are two general types of receptors which take part in phagocytosis of microorganisms. Receptors

from the first group recognize and bind cellular ligands present on the surface of microorganisms and therefore are sometimes called cellular receptors (1). The second group of receptors binds proteins produced by the host, which are able to recognize specific structures on the surface of the pathogens (5).

The so-called pattern-recognition receptors (PRR) are cellular receptors and include surface lectins such as the mannose-specific macrophage receptor, scavenger receptors and the Toll-like family of receptors (4, 6, 7). The pattern-recognition receptors are able to distinguish between host and microbial cells due to the fact that they bind structures present only on the surfaces of yeast, bacteria and some multi-cellular pathogens which are vital for their survival and therefore very conserved and 'resistant' to mutations. Recent interest in Toll-like receptors is mainly due to the fact that they bind bacterial products including lipopolysaccharides, peptidoglycans, lipoproteins and other small molecules which are important activators of mammalian cells. Although the involvement of Toll-like receptors (TLR) in phagocytosis has not been shown so far, their role in intracellular signaling and regulation of this and other functions of macrophages as well as other cell types makes them important molecules in the process of both innate and adaptive immunity(8-17).

The complement and Fc receptors bind proteins, produced by the host (i.e. opsonins, immunoglobulins), which are involved in recognition of pathogens and their phagocytosis (1, 2, 4). In contrast to PRR, these receptors do not directly bind antigens on the surface of the microorganism but rather recognize host proteins which are able to bind to those antigens. For example the C3b fragment is fixed to the carbohydrates on the surface of the pathogen and later it is recognized by the C3b receptor present on the macrophage. This process triggers

phagocytosis. The most specific selection of targets for phagocytosis by macrophages is facilitated by the Fc receptors, which are able to bind the Fc portion of immunoglobulins.

All receptors taking part in initiation and progression of phagocytosis are also able to activate expression of genes involved in perpetuating the initial response at the site of infection, which involves chemoattraction and activation of more macrophages and other cells from the surrounding tissue and the blood stream (3). Phagocytosis is important for both non-adaptive and adaptive immunity (4). It facilitates isolation of the pathogen in the phagosome, where ingested organisms are killed and their components are digested and prepared for presentation by the MHC complex (2). The positive recognition of the antigen presented by macrophages and other antigen-presenting cells triggers a cascade of events leading eventually to clonal proliferation of specific T-lymphocytes. Activated T-cells in turn produce cytokines able to activate additional cells, including macrophages. IFN-γ represents the main macrophage activator produced by T_H1, NK cells as well as NK receptor-positive T-cells [(NK)T-cells] (18, 19), (20). When activated by IFN-γ, macrophages secrete pro-inflamatory cytokines such as IL-1, IL-6, IL-8 and TNF-α, release proteases and undergo a respiratory burst, which results in the production of reactive oxygen intermediates (1, 2, 21). IFN-γ-activated macrophages also secrete large quantities of NO, especially when co-stimulated with TNF-α or bacterial cell wall products such as LPS (22, 23). Development of the immunological response depends on the efficient induction of MHC-II antigen expression in IFN-γ-activated macrophages (24, 25). The mediators produced by macrophages help to mobilize more cells into the site of infection and eventually allow clearance of the infection. As soon as the infection is cleared, the inflammatory reaction needs to subside; otherwise it could result in damage of infected tissue. There are several mechanisms that allow efficient down-regulation of the inflammatory response (26, 27). Activated T-cells and (NK)T-

cells produce IL-4 and IL-13 which are able to down-regulate most of the effects of IFN-γ on macrophages, with the exception of stimulation of MHC-II proteins and Fc receptors (2), (28). IL-10 secreted by T_H2 and B-cells as well as by monocytes, macrophages and keratinocytes represents a potent anti-inflammatory cytokine that is particularly effective in inhibiting production of many inflammatory cytokines produced by activated macrophages (21). Overall, the regulation of macrophage function is achieved at multiple levels and depends on the interaction of the macrophage with the pathogen, other host cells and soluble mediators.

Subpopulations of macrophages.

As previously mentioned, tissue macrophages are derived from monocytes which crossed the blood barrier and became either resident or migrating macrophages. The difficulties in formulating the general definition of macrophage function arise from the fact that cells collectively referred as macrophages belong to the broad spectrum of sub-populations which, although originate from common developmental linage, differ substantially in their phenotypes and specific functions (3, 29). It is believed that specific microenvironments modulate macrophage properties (e.g. expression of surface receptors) in such a way that their function differs depending on the organ or tissue in which they reside. For example, macrophages present in the lymphocyte proliferation organs are mainly responsible for removal of the dead cells. It is however possible, that they take part in the negative and positive selection of B- and T-lymphocytes. Even in particular organ like the spleen or the lymph node, several sub-populations of macrophages can be distinguished based on the expression of specific surface markers. For example, in the mouse, spleen macrophages present in red pulp express high levels of F4/80, a

macrophage-specific antigen, but no Mac-1/CR3 (receptor for complement fragment C3bi), also known as the CD11b/CD18 macrophage marker (30, 31). Macrophages present in the marginal zone, which surrounds the white pulp of the spleen, on the other hand, express CR3, SR-A (Scavenger Receptor A) and other receptors involved in phagocytosis, but no F4/80. Most likely it is correlated with their basic function as antigen-presenting cells in marginal zone and removing red blood cells in the red pulp. Therefore, macrophages isolated from the spleen, which is a very common source of cells for experiments, are not homogenous and may lead to difficulties in interpretation of the results. Macrophages found in other organs also exhibit some distinct and characteristic features. For example, macrophages present in Peyer's patches are also F4/80 negative, sharing similar characteristics with macrophages found in other T-cell rich organs like the thymus or the spleen white pulp. It is suggested that those macrophages take part in antigen presentation and lymphocyte activation. The F4/80 positive cells found along GI track are, on the other hand, believed to initiate mucosal immunity. Similarly, most of the macrophages found in the liver (Kupffer cells), the dermis, neuroendocrine and reproductive organs are F4/80 and CD68 (macrosialin) positive. Alveolar macrophages in lungs are, on the other hand, F4/80 negative and thus constitute a separate population.

One of the better characterized subpopulations of mononuclear phagocytes are microglial cells which comprise a highly differentiated group of macrophages of the central nervous system (CNS) (32). Microglial cells do not express many of the macrophage markers but can be easily distinguished from other tissue macrophages by their characteristic constitutive expression of CR3, stimulation of which leads to their apoptosis. Interestingly, the natural ligand for CR3 is not normally present in CNS. Microglia are very good examples of how the specific microenvironment created by the presence of the blood-brain barrier determines macrophages

phenotype and functionality. Except for microglia, tissue macrophages are believed to originate from monocytes circulating in the blood stream. The microglial cells come from special progenitor cells which are believed to colonize the CNS in very early stages of fetal development.

It is not clear whether monocytes are colonizing particular tissues at random or that perhaps their specialized features and final destination as macrophages are pre-determined before they begin circulating in the blood stream (3). The most distinct and specialized subpopulation of macrophages, named osteoclasts, participate in bone mass resorption during skeleton formation. They develop from the same lineage of myeloid precursor cells as other macrophages, but they do not participate in immunological response (33). The diversity of macrophage populations and high dependence of their specific function on the microenvironment in which they exist creates difficulties in defining precise characteristics of macrophage function in a given organ or tissue. The descriptive characterization of phenotypic features of the cell, like expression of surface markers, is fairly easy using histo-chemical methods. The analysis of the cell to cell interaction or time dependent responses to intercellular mediators, as well as molecular mechanisms of biochemical processes, may be more technically challenging. Isolation of macrophages from a tissue and their maintenance in culture, often in the presence of growth factors, may affect the cell phenotype. Therefore, many studies concerning macrophage function are done using cell lines. The use of a homogenous population of macrophages allows precise characterization of their functional and biochemical parameters with a high degree of reproducibility usually difficult to obtain with freshly isolated cells. On the other hand, cell lines may exhibit a phenotype that is characterized by dysfunction of regulatory proteins and transcription factors involved in cell cycle regulation, which is unique for them and generated as the result of their

transformation. The results obtained using established cell lines however can be easily verified *in vitro* using freshly isolated cells and then *in vivo*.

Macrophages in innate immunity

The role of the macrophage in innate immunity depends mainly on their ability to recognize and eliminate pathogens using phagocytosis to isolate infectious agents inside their vacuole, where a pathogen can be killed using a combination of proteolytic enzymes and toxic chemicals. Macrophages are usually the first to encounter invaders that crossed the first line of host defense, consisting of skin and mucosal surfaces (1). The successful recognition and ingestion of the pathogen depends on the presence of the pattern recognition receptors on the surface of macrophages (11, 12, 34). It is believed that they facilitate both physical contact between the cells and trigger signals which lead to the initiation and progression of phagocytosis as well as production of proteolytic enzymes, reactive oxygen and nitrogen intermediates through the process of oxidative burst. In addition, macrophages activated by contact with microorganisms release a number of soluble mediators, which stimulate and attract more immuno-competent cells. The efficiency of macrophages to eliminate the pathogen and activate other cells strongly depends on host genetic factors as well as certain properties of the microorganism itself. In fact, many pathogenic bacteria, protozoa and viruses have developed strategies to evade macrophage-mediated killing that enable them to proliferate inside macrophages. These intracellular pathogens include *Mycobacteria*, *Chlamydia*, *Listeria*, *Francisella,* certain species of *Salmonella*, as well as protozoa like *Leishmania* (35-41). Many of above listed microorganisms, due to special opportunistic adaptations, are able to colonize an ecological niche of macrophages that by definition should be a very hostile environment for

them. It was shown that *Mycobacteria* interfere with the normal process of phagosome maturation and can also cause a decrease in antigen-presenting ability of the macrophage (42). *Chlamydia* also interferes with antigen presentation by decreasing the stability of the RFX transcription factor involved in the expression of MHC-II antigens (43). It is also able to inhibit IFN-γ responsiveness of infected cells by destabilizing upstream stimulatory factor 1 (USF-1). Down-regulation of the IFN-γ signaling pathway was also observed in macrophages infected with *Leishmania*. The necessity of IFN-γ, but not IL-4, in order to eliminate *Leishmania* infection was demonstrated in numerous studies (44, 45). It seems that successful propagation of several different species of intracellular pathogens infecting macrophages depends upon inhibition of IFN-γ-induced effects. IFN-γ is a very strong activator of macrophages, which is able to enhance responses triggered by microbial products or other cytokines. *In vitro* studies reveal that IFN-γ, when given alone to macrophages, strongly induces MHC-II expression as well as other genes, but is inducing only very low levels of production of nitric oxide (NO) and other reactive oxygen intermediates (ROI), as well as certain pro-inflammatory cytokines like TNF-α or IL-1β (22, 46, 47). The relatively weak effect of IFN-γ on expression of NO and pro-inflammatory cytokines by macrophages is tremendously enhanced by various molecules (for example lipopolysaccharides) derived from bacteria and parasites (48). This process is often referred to as priming of macrophages, which once pre-exposed to IFN-γ become very responsive to a second stimulus.

The role of Nramp1 in intracellular infections

One of the genetic factors known to be involved in innate resistance to intracellular pathogens in mice is *Lsh/Ity/Bcg* locus. The susceptibility of certain strains of mice to infection with *Mycobacterium bovis* BCG characterized by poorly-controlled proliferation of the pathogen, was first observed in 1981 (49, 50). It was shown that the genetic factor located on chromosome 1 determined resistance of mice to infection with unrelated facultative intracellular pathogens *Mycobacterium bovis* BCG, *Salmonella typhimurium* and *Leishmania donovani* (51). In 1993, by the positional cloning approach, the gene responsible for natural resistance of mice to the stated above pathogens was identified and named Natural Resistance Associated Macrophage Protein 1 (*Nramp1*, now designated as *Slc11a1* for a solute carrier family 11 member 1) (52, 53). The Nramp1 protein encoded by the *Slc11a* gene appears to be a member of a highly conserved family represented in such distinct evolutionary species as bacteria and mammals, including humans (54). The analysis of *Nramp1* gene expression revealed that its mRNA is only present in monocytes/macrophages in mouse (52, 55), but it can be found in both human monocytes and neutrophiles (56). The second member of that family was cloned in rodents and humans, and was named *Nramp2* (*Slc11a2*). Nramp2 protein, the product of the *Slc11a2* gene, is now designated DMT1 for Divalent Metal Transporter 1, to better reflect its function as a transmembrane transporter of iron (57-59). A mutation in the *Nramp2* gene, which results in a Gly to Arg substitution at the position 185 of the Nramp2 protein, leads to severe microcytic anemia found in the *mk* strain of mice and Belgrade rats (60). The elevated levels of *Nramp2* mRNA were found in a variety of tissues but especially in the duodenum and kidneys

from animals exposed to diet containing low amounts of iron (61). The complementation studies of yeast's homolog of *Nramp1* gene *SMF1* demonstrated that Nramp2 acts as a manganese transporter (62). Interestingly, the impaired homeostasis of that element in Belgrade rats was also reported (63). Although Nramp2 was not shown to be directly implicated in the immunity against microorganisms, the studies on its function helped to characterize the properties of the mouse Nramp1 protein. Nramp2 and Nramp1 (now also referred to as DMT2) share 64% sequence identity (78% similarity) and have the analogical structural organization of a transporter protein composed of 12 hydrophobic transmembrane domains. The predicted molecular weight of Nramp1 is 53 kDa, however in murine macrophages this protein appears as a phosphoglycoprotein of apparent molecular weight between 90-100 kDa (64, 65). The presence of several potential glycosylation and phosphorylation sites allowed predicting the orientation of the Nramp1 protein in the membrane. According to this prediction, both N- and C-terminal domains are positioned on the cytoplasmic side of the membrane. This orientation of the protein places the highly conserved transport motif, present in the loop between 8^{th} and 9^{th} transmembrane domains, on the cytoplasmic face of the membrane as well (66). A similar transport motif was found in many eukaryotic transport proteins and it was shown to be involved in intra-subunit interaction, which was also described for many bacterial periplasmic permeases. The N-terminal portion of the Nramp1 protein also contains a proline rich sequence with potential SH3 domain-binding properties (53). The functional relevance of this domain has not been established yet.

The susceptibility of certain strains of mice to infections with *Mycobacterium bovis* BCG was shown to correlate with a single Gly to Asp substitution at position 169 of the predicted amino-acid sequence of the Nramp1 protein (52). The introduction of a charged residue in the

predicted transmembrane domain 4, where the substitution occurs, is believed to disrupt protein structure leading to the absence of the Nramp1^{Asp169} (Nramp1^{s}) in the lysosome/late endosome where wild type Nramp1^{Gly169} (Nramp1^{r}) protein is found. The presence of the *Nramp1*^{s} allele was shown to be associated with significantly lower IFN-γ responsiveness of macrophages, which leads to lower expression levels of several genes involved in the immunological response. Macrophages from *M. bovis* BCG susceptible mice, in response to *in vitro* stimulation with IFN-γ, express lower levels of MHC-II antigens and produce lower amounts of nitric oxide (NO) and reactive oxygen intermediates (ROI), secrete less TNF-α as compared to IFN-γ-treated macrophages from *M. bovis* BCG resistant mice (22, 67, 68). It was also reported that macrophages carrying susceptible allele of the *Nramp1* gene, express lower levels of chemokines KC (mouse growth-regulating oncogen alpha – GRO alpha) and JE (mouse monocyte chemoattractant protein 1 – MCP-1) mRNA in response to the stimulation with mycobacterial lipoarabinomannan (LAM) (69). The observed differences in responsiveness to IFN-γ together with the fact that it was shown that lower levels of IFN-γ could be detected in mice from *M. bovis* BCG susceptible strains led to the original hypothesis that inadequate activation of macrophages expressing *Nramp1*^{s} gene is responsible for uncontrolled proliferation of intracellular pathogens in susceptible mice. However, this does not explain why only a limited variety of intracellular pathogens are under the *Nramp1* gene control. In fact, it was shown that some intracellular bacteria, which also infect macrophages like *Francisella tularensis*, proliferate more rapidly in mice carrying *Nramp1*^{r} gene and, reciprocally, infection with this bacteria is efficiently controlled in mice expressing *Nramp1*^{s} (38). Also, infection of mice with virulent strains of *M. tuberculosis* does not seem to be under the control of the *Nramp1* gene (70). It suggests that the pathological outcome of infection with particular microorganisms depends

equally on host genetic factors and specific survival adaptations of the pathogen (71). This more complicated model of macrophage – pathogen interaction is supported by studies of the effect of macrophage depletion on the outcome of infection. For example, mice depleted of alveolar macrophages using liposome-encapsulated dichloromethylene diphosphonate were completely protected from otherwise lethal infection with *M. tuberculosis* (72). On the other hand, similar depletion of alveolar macrophages rendered normally mycoplasma-resistant C57BL/6 mice unable to control infection with *Mycoplasma pulmonis* (73). Finally, infection of macrophage-depleted mice with *Salmonella typhimurium* led to the conclusion that macrophages are responsible for the pathology of infection in naïve mice, but are necessary for efficient T-cell dependent immunity in pre-exposed animals (74). These experiments combined with the knowledge of macrophage biology and function suggest that, depending on the type of infection, macrophages are crucial for pathogen elimination; they also may be responsible for its dissemination. Therefore, it is possible that sometimes a more efficient activation of macrophages may lead to a uncontrolled spread of infection if the pathogen is able to survive inside the macrophage phagosome. Therefore, the studies of macrophage function in the context of genetic background should be followed by investigation of specific microbial factors involved in host responsiveness to infection. So far, studies concerning Nramp1 function have been focused on determining the functional differences between the two forms of the protein. It has been established that mouse Nramp1, and likewise its homologues from other species, acts as a pH-dependent transporter of divalent cations (75, 76). However, the precise mechanism of that transport as well as the transported molecule has not been well-defined and is the subject of certain controversy. It is well-documented that Nramp1 is localized on the lysosome or late endosome membrane where it co-localizes with Lamp1 protein, a biochemical marker of this

cellular compartment (77-79). The presence of the wild type Nramp1 protein was associated with normal acidification of phagosomes containing live *Mycobacteria*, whereas phagosomes from macrophages expressing *Nramp1*^{s} did not acidify upon infection of the cells (80). Low pH of the phagosome is required for the normal activation of several proteolytic enzymes as well as for Nramp1-facilitated transport. Several studies using electrophysiological methods to measure electrostatic potential across the membrane showed that Nramp1 might be involved in iron transport through the phagosomal membrane (75, 81-84). However, there is controversy as for the direction of this transport. One of the hypotheses suggests that removal of iron from the phagosome restricts bacterial growth (81). A completely different hypothesis proposed by Zwilling and colleagues(84), as well as another group results obtained using *Xenopus* oocytes transfected with Nramp1, postulate that iron is transported by Nramp1 into the phagosome where it is used for Fenton/Haber-Weiss reaction which produces hydroxyl radicals, facilitating killing of phagocytosed microorganisms (85). Recently published data obtained using zymosan particles labeled with the fluorescent molecular probe Fura-6-FF-triethylester, whose fluorescence is quenched by divalent metals, have shown that Nramp1 is involved in pH dependent manganese cation transport from the phagosome to the cytoplasm (76). Mn^{++} as well as Zn^{++}, Cu^{++} and Fe^{++} are all essential microelements required by bacteria and other organisms, which also represent potential Nramp1 substrates. Manganese is a cofactor of superoxide dismutases used by *Salmonella*, *Leishmania* and *Mycobacterium* that allow neutralizing of toxic superoxide ions and hydroxyl radicals. Therefore, availability of manganese may directly affect microbial survival.

The role of macrophages in acquired immunity

The function of macrophages in host defense is not limited to innate immunity but extends to processes characterized as acquired immunity. The basic role of macrophages in the development of T-cell-mediated immunological response depends on their ability to present foreign antigens in the context of MHC-II molecules to CD4 positive T-lymphocytes (2). In addition to presenting antigen to the T-cell where it is recognized by binding to the TCR (T-Cell Receptor), macrophages trigger additional secondary co-stimulatory signals provided by interactions between several molecules expressed on the surfaces of the two interacting cell types (CD40-CD40L, CD28-CD80 and CD86, ICAM-LFA-1) (2, 3). Macrophages also secrete several cytokines including IL-12 and IL-18, which promote the development of T_H1-type of response by stimulating IFN-γ production by NK cells and T-cells (21). Produced by lymphocytes, IFN-γ activates macrophages leading to even stronger activation of specific T-cells. IFN-γ is also able of "priming" macrophages. Primed cells become highly responsive to stimulation with a variety of stimuli such as lipopolysaccharides from Gram-negative bacteria. Although bacterial (as well as other pathogens and parasites) products are able to induce production of pro-inflammatory mediators in macrophages, the addition or pre-exposure to IFN-γ substantially augments this response. Such super-stimulation of macrophages may lead to septic shock, which in many cases leads to death (86).

The role of IFN-γ in activation of macrophages

Analysis of IFN-γ knockout mice demonstrated that the presence of IFN-γ is necessary during the course of infection especially with intracellular pathogens (87). IFN-γ knockout mice were shown to be extremely susceptible to *Mycobacterium, Listeria* and *Salmonella* infections. IFN-γ and type I interferons (IFN-α/β) were first described as antiviral agents with anti-proliferative activity. More detailed studies revealed that IFN-γ has a more broad activity (18, 19, 88). Mice with a disrupted IFN-γ-R gene were more susceptible to infection with vaccinia virus than wild type littermates. IFN-γ-R knockout mice were however resistant to other viral infection including Semliki forest virus (SFV), vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV). Interestingly, IFN-α-R knockout mice were susceptible to infection with all the above viruses (89, 90). These observations suggest that although biological activities of both types of interferon are partially overlapping, there are substantial differences in their effector mechanisms. In addition, IFN-γ or IFN-γ-R knockout mice show impaired regulation of MHC expression and nitric oxide production (91). The analysis of the IFN-γ signal transduction pathway and the discovery of transcription factors activated by IFN-γ stimulation allowed identification of genes directly regulated by this cytokine.

IFN-γ signal transduction pathway

In its active form IFN-γ exists as a non-covalently bound homodimer of a glycosylated protein of approximately 50 kDa. Only dimerized IFN-γ has a biological activity and it has been shown to be species-specific (21). The IFN-γ signal transduction pathway (Fig.1) is initiated

when the cytokine is bound by a receptor complex composed of a pair of two subunits designated as IFN-γR1 (also known as IFN-γ receptor α chain) and IFN-γR2 (IFN-γ receptor β chain) (91). The IFN-γR1 is expressed ubiquitously at various levels depending on the cell type, including macrophages. The constitutive levels of IFN-γR2 expression are usually very low but they can be up-regulated by many stimuli. It is believed that IFN-γR2 expression determines a cell's responsiveness to IFN-γ. In macrophages, IFN-γR2 expression can be upregulated by several stimuli, including phorbol ester and TNF-α (92). It was demonstrated that IFN-γ-producing T_H1-cells do not express IFN-γR2 and are unresponsive to IFN-γ stimulation (93). In contrast, T_H2-cells do respond to IFN-γ and express high levels of IFN-γR2. The IFN-γ itself has the ability to downregulate expression of IFN-γR2 in T-cells but not in fibroblasts. Two Janus family tyrosine protein kinases, JAK1 and JAK2, and a cytoplasmic protein named Signal Transducer and Activator of Transcription 1 (STAT1) were shown to be involved in formation of the IFN-γ signaling pathway (94). The cytoplasmic domains of IFN-γR1 and IFN-γR2 subunits are associated with JAK1 and JAK2, respectively, whereas STAT1 is recruited to the receptor complex after its activation (91, 95). The IFN-γ homodimer interacts directly with two IFN-γR1 chains, which leads to receptor oligomerization. In addition to binding the ligand, IFN-γR1 subunits are also responsible for signal transduction through the cell membrane and interaction with the STAT1 protein. It has been shown, however, that the presence of the second subunit IFN-γR2 is absolutely necessary for activation of STAT1 and further signal transduction. The dimerization of the IFN-γR1 provides species-specific recognition sites for two IFN-γR2 subunits. The formation of IFN-γ receptor complex containing two of each R1 and R2 subunits facilitates interaction between JAK1 and JAK2, which results in their activation by tyrosine

cross-phosphorylation (94). The activated JAK2 kinase is then responsible for phosphorylation of the IFN-γR1 chain on a tyrosine residue at position 440 (91, 93). It is believed that JAK1 plays a more structural than enzymatic role in the process of IFN-γR1 activation. It was demonstrated that cells expressing a dominant-negative mutant of JAK1 are still able to respond to IFN-γ. In contrast, cells expressing a dominant-negative mutant of JAK2 were unable to respond to IFN-γ and the IFN-γ signal transduction mechanism was impaired in these cells (96). The tyrosine phosphorylation of IFN-γR1 subunits of the receptor complex provides a binding site for the STAT1 protein, which recognizes a specific sequence containing a phosphorylated tyrosine, using its SH2 (src homology domain 2) domain. The activated JAK kinases quickly phosphorylate STAT1 on the Y_{701} residue. The phosphorylated STAT1 is recognized by another STAT1 molecule (via its SH2 domain) to form a homodimer (21, 94). After dimerization, STAT1 is serine phosphorylated on S_{727} by an unidentified kinase. The p38 MAP kinase was shown to be required for phosphorylation of STAT1 S_{727} in response to stress, but it was not responsible for serine phosphorylation of STAT1 after IFN-γ stimulation (97). Both the phosphatidylinositol 3-kinase (PI3K), and one of its downstream effector kinases the serine-threonine kinase Akt (PKB), were demonstrated to be involved in the process of IFN-γ-induced phosphorylation of STAT1 at S_{727} (98). It was demonstrated that serine phosphorylation of STAT1 at position 727 is required for it full transcriptional activity. Serine phosphorylation of STAT1 was also observed in cells stimulated with IFN-α, UV irradiation, LPS, PDGF, phorbol esters, IL-2, IL-12, TNF-α and cross-linking of B- and T-cell receptors (99). The STAT1 dimer is quickly translocated to the nucleus, where it regulates transcription by binding to so called GAS (gamma interferon activated site) elements. Although the above mentioned general model of IFN-γ signal transduction pathway is valid, recently published data point to the crucial role of

IFN-α/β signaling in efficient IFN-γ-stimulated responsiveness of the cells (100). The discovery of the crosstalk between the two types of interferon pathway was prompted by the observations that genetic disruption of IFN-α receptor subunit 1 (IFNAR1) leads to diminished responsiveness of mouse epithelial fibroblasts (MEF) to IFNγ. This was manifested by the lack of antiviral activity as well as a less efficient binding of the STAT1 homodimer to GAS elements. In addition, as a result of the lack of IFNβ production, the IFNγ stimulation of cells did not result in generation of ISGF3 (the complex composed of STAT1, STAT2 and IRF9) (94). Generation of the ISGF3 complex normally occurs in response to IFNα/β stimulation, but it can also be observed in the cells exposed to IFN-γ, although at a lower level. These effects were also observed in splenic cells and could be reversed by re-introduction of IFNAR1. On the other hand, the IFN-α/β responsiveness were not affected by knocking-out the IFN-γ receptor subunit 1. Using gene knockout technology as well as transfection and co-immunoprecipitation, it was shown that subthreshold levels of IFN-α expression present in normal cells allow maintaining minimum levels of IFNAR1 activation. The receptor complex for IFN-α/β contains two subunits: IFNAR1 and IFNAR2, whose cytoplasmic domains are associated with Tyk2 and JAK1 protein tyrosine kinases, respectively (101). The binding of the ligand by IFNAR1 induces phosphorylation of tyrosine in position 455 (mouse), which provides a docking site for STAT2 (94). Following STAT2 phosphorylation, STAT1 can bind to the complex by recognizing the phosphorylated residue on STAT2. Next, STAT1 becomes phosphorylated, and a heterodimer between STAT1 and STAT2 can be formed. After recruiting IRF9, the interferon stimulated gene factor 3 (ISGF3) is ready to enter the nucleus and bind ISRE sequences. During this process, STAT1 homodimers (also known as GAF – interferon gamma activated factor) are also formed. In the proposed model of the crosstalk between IFN-α/β and IFN-γ signaling pathways,

the IFNAR2 and IFNGR2 subunits interact with each other. This way, STAT1, which is associated with the activated IFNAR1 subunit, is phosphorylated by JAK kinases associated with IFN-γ receptor. The constant presence of activated IFNAR1 and its association with IFNAR2/IFNGR2 complex therefore facilitates the availability of STAT1 for the IFN-γ signaling. In order for IFNAR1 to be phosphorylated, it has to be bound to IFN-α (101). Depending on the presence of IFN-β (which augments expression of IFN-α) very low basal levels of IFN-α in the cells can be detected. Once cells are exposed to IFN-γ, the activation of IRF1 is induced, which in turn activates the IFN-α gene and consequently enhances IFN-γ signaling. STAT1 was shown to be crucial for the IFN-γ signal transduction pathway and the activation of so-called interferon stimulated genes (ISG), whose transcriptions are upregulated within 15 to 30 minutes after IFN-γ stimulation (102). The list of genes activated directly by IFN-γ through STAT1 action or indirectly as a result of activation of transcription factors includes: dsRNA-activated protein kinase (PKR), guanylate-binding protein (GBP), inducible nitric oxide synthetase (iNOS), receptors for TNF-α, IL-2 and GM-CSF, Fc-receptors, MHC class I and II and transcription factors IRF1 and ICSBP. IFN-γ stimulation of macrophages also leads to upregulation of STAT1 expression (unpublished data). The STAT1 protein also plays a central role in determining the specificity of the IFN-γ response. This was demonstrated by using STAT1 knockout mice, in which only IFN-γ and IFN-α/β signal transduction pathways and subsequent biological responses were impaired. Despite the fact that STAT1 is a part of signaling pathways of other cytokines such as IL-6, IL-10, EGF, as well as growth hormons, responses to these mediators were not affected by the lack of STAT1 (103). These results may also suggest a certain level of redundancy for some signal transduction pathways. In addition to the central role which STAT1 protein plays in IFN-γ signaling, another group of transcription

factors which belong to the family of interferon-responsive factors (IRF) was shown to mediate IFN-γ biological effects. Recognized by IRFs DNA sequences called IRF-E can be found in regulatory elements of many IFN-γ inducible genes. The IRF1 is one of the best studied members of this group and was originally shown to be involved in the regulation of IFN-α/β expression (94). IRF1 provides a positive transcription signal whereas IRF2 regulates transcription of the IFN-α/β genes in a negative fashion, although they both bind the same sequence called ISRE (for Interferon Stimulated Response Element). Some data concerning IFN-γ dependent gene expression strongly suggests that both IRF1 and IRF2 are able to contribute to the positive regulation of certain genes. One of the examples of such co-operation is the expression of human class II trans activator (CIITA) gene (104). Another important member of the IRF family is called Interferon Consensus Sequence Binding Protein (ICSBP/IRF8). The expression of this protein, also activated by IFN-γ, was shown to regulate expression of IL-12 in macrophages. The IRF8 is able to bind GAS elements and provide a second wave of transcriptional activation independently of STAT1 activation. Despite the importance of STAT1 in mediating IFN-γ signaling, there are several biological effects which are induced by this cytokine in STAT1 deficient cells (105, 106). Those effects include regulation of proliferation in both fibroblasts and macrophages as well as regulation of certain gene expression in both a positive and a negative manner. Interestingly, IFN-γ treatment affected expression of some genes the same way in STAT1-/- as in wild type cells, suggesting that the STAT1-independent pathway plays an important role in the regulation of expression of certain genes.

In addition to stimulating various effects in cells mainly related to host defense, IFN-γ also induces activation of mechanisms which downregulate the IFN-γ signaling pathway. The existence of such mechanisms is very important since over-activation of the immune system may

be sometimes more harmful than beneficial (26). The negative regulatory mechanisms which affect IFN-γ signaling include: 1) A direct effect on the transcription of key molecules; for example, IFN-γ activation induces expression of IFN-α/β leading to activation of IRF2, which, in turn, downregulates the expression of IFN-α/β lowering the efficiency of IFN-γ signaling; 2) The deactivation of the components of the IFN-γ signal transduction pathway, mainly JAK kinases, by protein tyrosine phosphatases (PTP), including SH2-domain-containing phosphatase SHP1(27). SHP1 binds to the tyrosine-phosphorylated IFN-γ receptor subunit 1 and deactivates JAK kinases by dephosphorylation. The expression of SHP-1 is not regulated by IFN-γ; 3) The prevention of the STAT1 homodimer binding to the DNA by Protein Inhibitors of Activated STAT1 (PIAS-1) (107). Proteins belonging to the PIAS family are able to bind C-terminal domains of specific STAT proteins, preventing interaction of the transcription factor with regulatory elements. The precise mechanism of this interaction is not known, but it only occurs following STAT phosphorylation; 4) Inactivation of JAK kinases by suppressors of cytokine signaling 1 (SOCS-1, known also as JAB or SSI-1) (108). SOCS-1 expression is upregulated by IFN-γ. The SOCS-1 protein binds JAK2 and blocks its enzymatic activity by an unknown mechanism. The importance of suppression of the signal transduction pathways activated by IFN-γ as well as other cytokines was demonstrated by generation of SOCS-1 knockout mice. These mice were growth retarded and died within 3 weeks after birth. In addition, an abnormally high percentage of lymphocyte apoptosis, monocytic infiltration of several organs as well as fatty degeneration of the liver and loss of mature B-lymphocytes in the bone marrow, the spleen and peripheral blood was observed in SOCS-1 -/- mice. At the molecular level, cells from SOCS-1-/- mice displayed high levels of constitutively activated STAT1. Interestingly, many of the above physiological defects were also observed in IFN-γ knockout mice (109).

The role of LPS in macrophage activation

As previously mentioned, many of the biological effects of IFN-γ on macrophages are strongly augmented by exposure of cells to certain bacterial or microbial products. In the last few years, the role of the Toll-like receptor system in mediating this synergy and in general responsiveness of cells to bacteria has been described and studied in great detail. Mammalian Toll-like receptors (TLR) are the homologs of an extremely conserved evolutionary family of proteins described originally in the fruit fly (*Drosophila melanogaster*) and shown to be involved in both early larval development and antifungal defense (110). Ten TLRs have been cloned and described so far in human and nine of their close relatives were also found in mice. Functionally and structurally, TLRs may be classified as pattern recognition receptors, since they bind characteristic structures of microbial origin using leucine rich domains of their extracellular domains (111). The molecular patterns recognized by TLRs are usually unique to microorganisms, thus providing a very important system for the discrimination of self and non-self antigens with a high degree of specificity. The cytoplasmic portion of TLRs contain a conserved Toll/IL-1 receptor homology domain (TIR domain), which is found in all TLR as well as in the IL-1 and IL-18 receptors as well as in a number of cytoplasmic proteins which take part in TLR signaling (112). The TIR domain mediates the homophilic interaction of proteins containing this domain. In a general model of TLR-mediated signal-transduction (Fig.2), the TIR domain of the receptors interacts with a cytoplasmic adaptor protein called MyD88 (myeloid differentiation protein) (113-115). MyD88 recruits the serine-threonine protein kinase IRAK (IL-1R associated kinase) to the receptor complex using the so-called death domain (13, 116). The

activation of TLR by binding the ligand leads to autophosphorylation of IRAK and its dissociation from the receptor complex. Activated IRAK phosphorylates and activates another cytoplasmic protein, TRAF6 (TNF receptor-associated factor 6) (117), which in turn activates the so-called IKK signalosome (IκB kinase), a high molecular weight complex of two kinase subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO) (118-120). IKK directly phosphorylates the inhibitory protein IκB, leading to its ubiquitination and degradation by the proteosome system. Degradation of IκB releases the transcription factor NFκB (nuclear factor κB) which allows its translocation to the nucleus and activation of transcription. The mechanism of activation of IKK by TRAF6 is not clear and at least two hypothetical mechanisms were proposed (16). The first suggests that mitogen activated protein kinases MAP3K, namely NIK and MEKK1, are responsible for mediating the TRAF6 effect on IKK. This hypothesis, however, was not confirmed by the studies using NIK and MEKK1 knockout mice. The second, alternative model assumes that TRAF6 works as a ubiquitin ligase and mediates polyubiquitination of IKK, which leads to its autophosphorylation and activation. TRAF6 also activates AP-1 transcription factor by a MAP kinase (p38)-dependent activation of JNK (c-Jun N-terminal kinase) (12). The above general pathway is used by all known TLRs as well as IL-1 and IL-18 signaling. The specificity of stimulated responses is achieved at the level of ligand binding, assembly of the receptor complex and presence of additional adaptor protein or alternative pathways. The analysis of ligands recognized by TLR revealed a high degree of specificity for both the type and the origin of the bound compounds. In general, the two best studied Toll-like receptors – TLR4 and TLR2- are categorized as binding stimuli of Gram-negative and Gram-positive bacterial origin, respectively (121). More detailed studies showed that TLR4 is predominantly able to bind lipopolysaccharides and that this receptor, but not TLR2 as previously reported, is responsible

for cell responsiveness to LPS. The TLR2 was, however, reported to bind lipopolysaccharides from *Leptospira interrogans* and *Porphyromonas gingivalis,* but the structure of those molecules are distinct from "classical" Gram-negative bacteria-derived LPS (122, 123). TLR2 seems to be able to interact with lipoproteins, lipoarabinomannan as well as with lipoteichoic acid and peptidoglycans from Gram-positive bacteria (10, 124). The importance of TLR2 was clearly demonstrated in its engagement in biological responses to *Mycobacteria*, *Mycoplasma*, yeasts (by binding zymozan), *Treponema* (glycolipid) and *Trypanosoma cruzi* (glycophosphatidylinositol anchor). Interestingly, TLR2 was shown to interact with the so-called phenol-soluble modulin (PSM) from *Staphylococcus epidermidis* (125), the outer surface protein A lipoprotein from *Borrelia burgdorferi* and to heat-stable and protease-resistant material collected from *M. tuberculosis*-conditioned culture (126, 127). The presence of molecules with similar characteristics in LPS preparations derived from various Gram-negative bacteria resulted in the earlier conclusion that both TLR2 and TLR4 participate in responsiveness to lipopolysaccharides. There are major differences between TLR2 and TLR4 in the mechanism of ligand binding, binding specificity, and receptor complex assembly. Binding of LPS by TLR4 requires interaction between the receptor homodimer, the soluble LPS binding protein (LBP), and the membrane-anchored CD14 (128). The secreted MD-2 protein may be involved in stabilization of LPS-TLR4 complex on the plasma membrane (129-131). TLR2, on the other hand, was shown to form homo- or heterodimers with other Toll-like receptors in a ligand-specific fashion. For example, TLR2 together with TLR6 are required for peptydoglycan binding. When overexpressed, TLR1 can compete with TLR6 in formation of heterodimers with TLR2 and inhibit cell responsiveness to peptidoglycan which is mediated by the TLR2-TLR6 heterodimer (5). On the other hand, responsiveness to soluble factors from *Nesseiria*

meningitides is mediated by the TLR2/TLR1 complex (132) and binding of lipopeptides requires the TLR2 homodimer. Interestingly, it was demonstrated that association of TLR2 with MD-2 enables TLR2-dependent responsiveness to protein free LPS and lipid A (133, 134). This finding suggests that binding of TLR to the ligands is strongly dependent on accessory molecules. As demonstrated in the studies on B-cells, the regulation of the specificity for ligand binding might be cell-type dependent. These studies showed that LPS responsiveness is mediated by TLR4 interacting with CD14 and Toll-related protein RP105 (135). RP105 lacks a TIR cytoplasmic domain, but contains a characteristic ligand-binding leucine-rich extracellular domain. It was shown that biological responses mediated by TLR4 differ from the responses mediated by TLR2. The binding of the LPS by TLR4 stimulates additional, MyD88 independent signaling pathway mediated through the recently cloned TIRAP/MAL protein (TIR domain-containing Adaptor Protein / MyD88-Adapter-Like) (136, 137). The TIRAP/MAL protein is expressed in a variety of cell types and, similarly to MyD88, contains the TIR domain that is essential for its function. TIRAP/MAL possibly mediates the activation of NFκB utilizing pathway which involves the GTPase Rac-1, the p85 subunit of phosphoinositide 3-kinase and activation of the Akt protein kinase. Interestingly, this pathway is able to activate the Rel protein p65 in an IκB-independent manner through direct phosphorylation (138). MyD88 deficient cells are unresponsive to stimuli which interact with TLR2, TLR9, IL-1R and IL-18R, but are able to respond to LPS which primarily binds to TLR4. TIRAP/MAL does not mediate all LPS responses. The stimulation of dendritic cells (DC) from MyD88 knockout mice with LPS results in activation of NFκB, cell differentiation, and expression of surface markers, although DC fail to produce cytokines in response to LPS (9, 139, 140). In addition, responses to LPS are delayed and less intense in MyD88 deficient cells compared to wild type cells. Using a peptide containing the TIR domain

from TIRAP/MAL, it was demonstrated that the responsiveness to LPS and other TLR4 ligands is required for normal TLR4-mediated responses. The binding of the TIR domain by the TIRAP/MAL peptide most likely disrupted specific interaction between TIRAP/MAL and its substrate (141). This interaction seems to be absolutely required for LPS responsiveness since cells treated with the TIRAP/MAL peptide became unresponsive to LPS. It was also shown that MyD88 mediated LPS signaling requires both IRAK-1 and IRAK-2 kinases whereas the TIRAP/MAL pathway is regulated by IRAK-2 only (137).

The Toll-like system appears to be responsible for detecting, in a relatively specific manner, microbial infection and mediating signals which stimulate the function of the early effector cells, thereby linking innate with adaptive immunity (10). The large variety of molecules recognized by TLRs allows efficient immune defense against the majority of infections without engaging the adaptive response. In addition to molecules secreted by the microorganisms or present on the surface of the pathogens, unmethylated bacterial CpG DNA sequences can also be recognized by TLR9 (142). Along with TLR3, which recognizes dsRNA (143), TLR9 as well as TLR1, TLR2, and TLR6 are recruited to the phagosome/endosome membrane where they can send stimulatory signals after recognizing their ligands (5, 144). Recently, TLR5 was shown to be engaged in the response to bacterial flagellin, adding another characteristic molecule to the repertoire of ligands recognized by the Toll system (145). In addition, TLR7 was recently demonstrated to mediate cell responsiveness to small anti-viral compounds belonging to the imidazoquinoline group, including imiquimod and R-848 (146).

Macrophages express TLR4 constitutively, however at relatively low level. The prolonged exposure of cells to LPS downregulates TLR4 expression (147). In contrast, the expression of TLR2 is induced by LPS, IFN-γ, and other cytokines (148, 149). The effect of LPS

can be further amplified *in vivo* since LPS induces IFN-γ production (21). The importance of the crosstalk between IFN-γ and TLRs pathways in the induction of both beneficial and harmful biological responses is well recognized. The molecular mechanisms involved in the activation and regulation of macrophage function that involve TLRs pathways activation require further studies due to their complexity and interactions with IFN-γ and other cytokines pathways.

MHC class II regulation in macrophages

As mentioned before, one of the important functions of macrophages which links innate and adaptive immunity is their ability to process and present various antigens to T-lymphocytes. This process is regulated by IFN-γ mainly by activating phagocytic activity of macrophages and by induction of expression of MHC-II molecules (24). The MHC-II molecules are used by antigen presenting cells (APCs) to stimulate specific T-cells during the early phase of development of adaptive immunity. MHC-II molecules are constitutively expressed in B-cells and mature dendritic cells. Other cell types, such as macrophages, endothelial cells, fibroblasts, glial cells, astrocytes and others express high levels of MHC-II antigens only upon IFN-γ stimulation (150). The MHC-II molecules are glycoproteins formed by the non-covalent association of α and β chains. In humans, there are three different known isotypes of MHC class II molecules (HLA-DR, -DQ and -DP), which are encoded by highly polymorphic A and B genes, for α and β chain, respectively (151). The two genes are part of gene cluster that includes HLA-DM, TAP and LMP genes. Products of these genes together with the invariant chain (Ii) take part in the assembly and loading of the MHC-II/antigen complex. In mice, there are two MHC-II isotypes: IA and IE. All MHC-II genes are regulated at the transcriptional level. The

promoter regions of all MHC-II genes contain several highly conserved *cis*-acting elements, including S (also referred to as W or Z), X, X2 and Y boxes that are present in the 150 bp proximal region of the MHC-II genes promoters (24, 25, 150, 152). The nucleotide sequence, orientation, relative order, and spacing between particular elements are all highly conserved and necessary for successful transcription. The presence of these regulatory regions is both necessary and sufficient for expression of the MHC genes. It was demonstrated, however, that the additional enhancer elements located further upstream from the promoter region might be required for achieving the optimal level of MHC-II genes expression.

As in many other cases, the mechanisms of regulation of MHC-II genes were discovered during studies on the mechanisms of rare genetic disorder characterized by the lack of expression of MHC-II. Human major histocompatibility complex class II deficiency (official WHO nomenclature), also known as the bare lymphocyte syndrome (BLS), leads to severe immunodeficiency characterized by recurrent infections and, very often, death in early childhood (151). The lack of MHC-II molecule expression results from mutations in at least one of four essential regulatory proteins which are involved in MHC-II transcription. From the scientific point of view, the BSL represents a very interesting model for studies on MHC-II gene regulation. First, a phenotype and subsequently common clinical symptoms observed in the BSL result from dysfunction in any of the four different genes located on different chromosomes. Secondly, the specificity of transcription factors responsible for development of BSL and their requirement during the MHC-II transcription process seems to be uncommon among "classical" transcription factors. The BSL patients could be categorized into four complementation groups (A-D) based on the characteristic of cell lines derived from them. The genetic and biochemical analysis of those cell lines led to the description of four transcription factors involved in MHC-II

expression regulation (151). Three of these factors are subunits of the RFX complex characterized by their binding of the X regulatory element in the MHC-II gene promoter (153). The fourth factor is a regulatory molecule which does not bind DNA directly, but rather coordinates binding and assembly of other factors. It was designated as Class II Trans-Activator (CIITA) (154). CIITA is a high molecular weight protein composed of two functionally distinct regions (151). The first is responsible for recruitment to the MHC-II promoter and for protein-protein interaction. It contains a GTP-binding region localized in the middle part of the protein and a leucine rich repeat domain (LRR) located at the C-terminal, both of which were implicated in nuclear localization of CIITA (155). The second region of CIITA is required for transcriptional activation and is composed of an acidic domain and a proline-, serine- and threonine- (P/S/T) rich region (156, 157). The nuclear localization sequence present in CIITA is essential for functional activity of this protein. The mutations in CIITA which affect its function result in BSL, which is classified as complementary group A (158). The three subunits of the RFX complex bind to DNA and interact with CIITA (151). RFXANK is characterized by the presence of a domain rich in ankyrin repeats, responsible for RFX complex binding and their assembly (159). Mutation or deletion within this domain leads to BSL, with biochemical characteristics of the complementary group B (160). The second subunit called RFX5 contains a proline-rich DNA-binding domain sufficient and required for both DNA-binding activity and the assembly of the RFX complex (161). It also contains a domain responsible for interaction with the NFY nuclear factor, which binds the Y element. The presence of these two domains is also necessary for interaction with CIITA. Any deletion within these domains impairs MHC-II expression. Dysfunction of RFX5 is characteristic for BSL complementation group C (151, 162). RFXAP contains a C-terminal glutamine-rich acidic domain that is responsible for binding and

assembly of RFX complex (163). The absence of this domain found in patients with BSL is classified as complementary group D (164). All of these factors are equally important in the assembly of a functional enhanceosome on the MHC-II promoter (165). In fact, binding of RFX is required for subsequent binding of NF-Y and X2BP to Y and X2 regulatory elements, respectively (151). The X2BP transcription factor was identified as CREB (166). It was shown that other members of the RFX family of transcription factors, namely RFX1 to 4, cannot substitute for RFX5 because they fail to interact with NF-Y and X2BP (151). These findings illustrate that the interactions between the components within the MHC-II enhanceosome complex are highly specific.

The role of CIITA in MHC-II expression.

CIITA is a very intriguing regulatory protein which does not bind DNA directly; yet its absence or loss of its function due to mutation completely impairs MHC-II gene transcription. The mechanism of CIITA action is not fully understood (151). Studies using the deletion mutants of the protein revealed that the N-terminally-located acidic domain and the PST region may serve as transcription activation domains (156). There have been three proposed models of transactivating domain action. The first model postulates that CIITA directly interacts with general transcription factors such as TFIIB, $hTAF_{II}32$, and $hTAF_{II}70$ (167, 168). The second model proposes that CIITA has the ability to interact with TFIIH or P- TEFb, promoting promoter clearance and/or elongation of transcription (169). The third model takes into consideration that CIITA is interacting with the histone acetyltransferase CBP; therefore, it postulates that CIITA may initiate chromatin remodeling, making it accessible to the general

transcription factors (166, 170, 171). It is possible that CIITA is involved in all these functions since some evidence for each of these hypotheses exist. CIITA is recruited to the MHC-II promoter through its interaction with transcription factors already bound to the promoter. The presence of all factors bound to S, X, X2 and Y regulatory boxes is necessary (151, 165). It is known that CIITA interacts directly with RFXANK and RFX5 subunits of the RFX complex as well as with CREB and the B and C subunits of NFY. The type of protein–protein interaction between these transcription factors and CIITA is determined by CIITA's C-terminal portion containing a leucine-rich region (LRR) (172). The importance of this leucine-rich region is evident from the fact that mutations in this region are involved in development of BSL. The nuclear localization of CIITA was shown to be regulated by three regions (158, 172-174). The independently acting nuclear localization signal was mapped to the C-terminus of the protein and it was also shown to be deleted in patients with BSL (158). The involvement of a GTP-binding domain and LRR in nuclear transport and localization was also demonstrated. It is, however, unclear whether these three regions cooperate in the nuclear localization process or if their effects are independent from each other. In addition to the apparently natural function of CIITA as a regulator of MHC-II expression, CIITA was also shown to have some unexpected effects on apparently unrelated phenomena. The ability of CIITA to interact with CBP (CREB-binding protein) leads to the competition between CIITA and the transcriptional complex involved in the expression of IL-4. An increase in CIITA binding to CBP in the IL-4 promoter decreases transcription of this cytokine (175). CIITA was also reported to induce transcription of HIV LTR promoter (176). Although, these observations do not contribute directly to our understanding of CIITA function as regulator of transcription of MHC-II, however they enrich our knowledge regarding CIITA structural and functional properties.

Studies on CIITA function are complicated since its effector function depends on simultaneous cooperation between several functional domains. In addition, CIITA gene transcription is regulated in a very particular manner. Analysis of both CIITA mRNA and its DNA sequence revealed the existence of four independent promoters, designated PI to PIV, in the human CIITA (177). The murine CIITA gene contains only three promoters homologous to human PI, PIII and PIV. The transcription initiated from PI and PIII generates transcripts containing unique sequences from the first exon and common second exon sequence with PIV. The product of human PII has not been identified yet in any of the analyzed cell types. It was demonstrated that PI and PIII are responsible for constitutive expression of CIITA in dendritic cells and B-lymphocytes, respectively (178-181). The IFN-γ-induced expression of CIITA is mediated by PIV and its transcript could be found in a variety of IFN-γ-stimulated cells including macrophages, fibroblasts, astrocytes, hepatocytes, endothelial and epithelial cells (179, 182-184). The IFN-γ-stimulated expression of CIITA from PIV strongly depends on GAS, IRF and E-box regulatory elements, which bind IFN-γ-induced STAT1, IRF1 and ubiquitously expressed USF-1 transcription factors respectively (177, 185-188). In addition, the requirement for IRF-2 for IFN-γ-stimulated expression of CIITA was shown using cells derived from mice with a targeted deletion for that transcription factor (104, 189). Recently, the involvement of PIII in IFN-γ-dependent expression of CIITA was also demonstrated (190). This could explain results obtained using mice knocked-out for PIV (191). Macrophages as well as microglia from these mice still express CIITA in response to IFN-γ, although this was not detected in other cell types. Overall regulation of CIITA expression seems to determine the transcription of MHC-II genes. Therefore, detailed analysis of expression and CIITA function may provide knowledge of crucial importance for understanding the process of antigen presentation and immunity.

The general objectives of the thesis

The main objective of the work presented in this thesis was to establish the molecular mechanisms of different factors affecting IFN-γ-induced macrophage activation. The activated state of the macrophage is manifested at multiple levels and involves several different functions such as phagocytosis, production of soluble mediators, and antigen presentation. We decided to focus our studies on specific aspects of molecular mechanisms of regulation of expression of MHC class II molecules. The second chapter demonstrates the role of the *Nramp1* gene in IFN-γ-induced expression of MHC class II in macrophages and the effect of *M. bovis BCG* infection on MHC-II expression. The third chapter illustrates the studies of the effects of a product of Gram-negative bacteria, lipopolysaccharide (LPS), on IFN-γ-induced expression of MHC class II and on CIITA. The possible mechanisms of LPS-induced abrogation of MHC-II expression is also addressed in this chapter. Since the commercial preparations of LPS are not homogenous, it was important to establish whether the effect of LPS on MHC-II can be attributed to LPS itself or to (an) additional ligand(s) we found to be present in these preparations. Therefore, the fourth chapter describes our results on the characterization of a new ligand (called TRIL) present in LPS preparations that works in a TLR4-independent fashion. The potential role of TLR2 in response to TRIL is also explored in this chapter. Overall, our results significantly contribute to the understanding of the molecular processes regulating macrophage activation that lead to the expression of particular macrophage functions, specifically antigen presentation.

FIGURE LEGENDS

FIGURE 1. Signal transduction and transcriptional regulation in IFN-α/β and IFN-γ systems.

IFN-γ and IFN-α/β systems signal transduction pathway

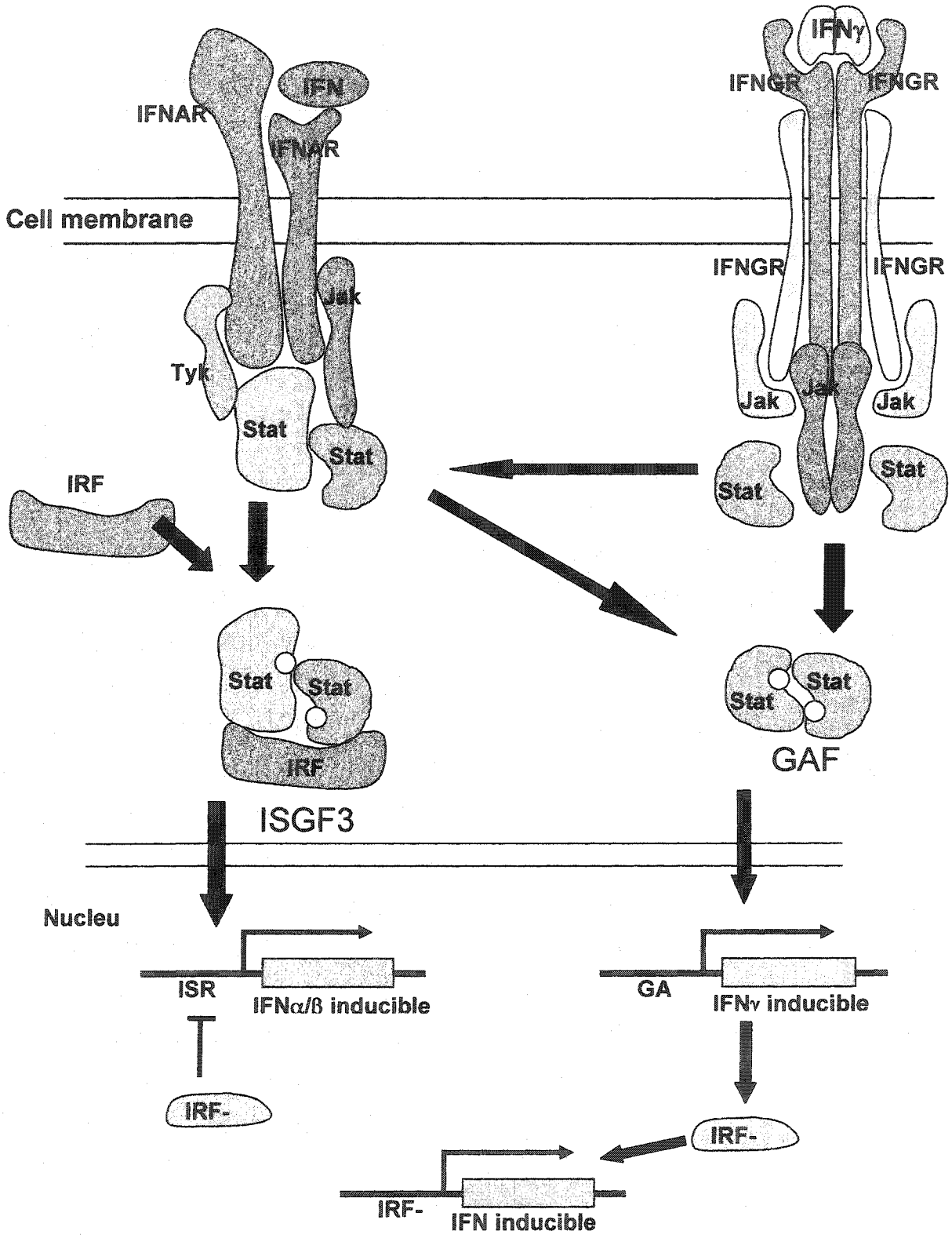
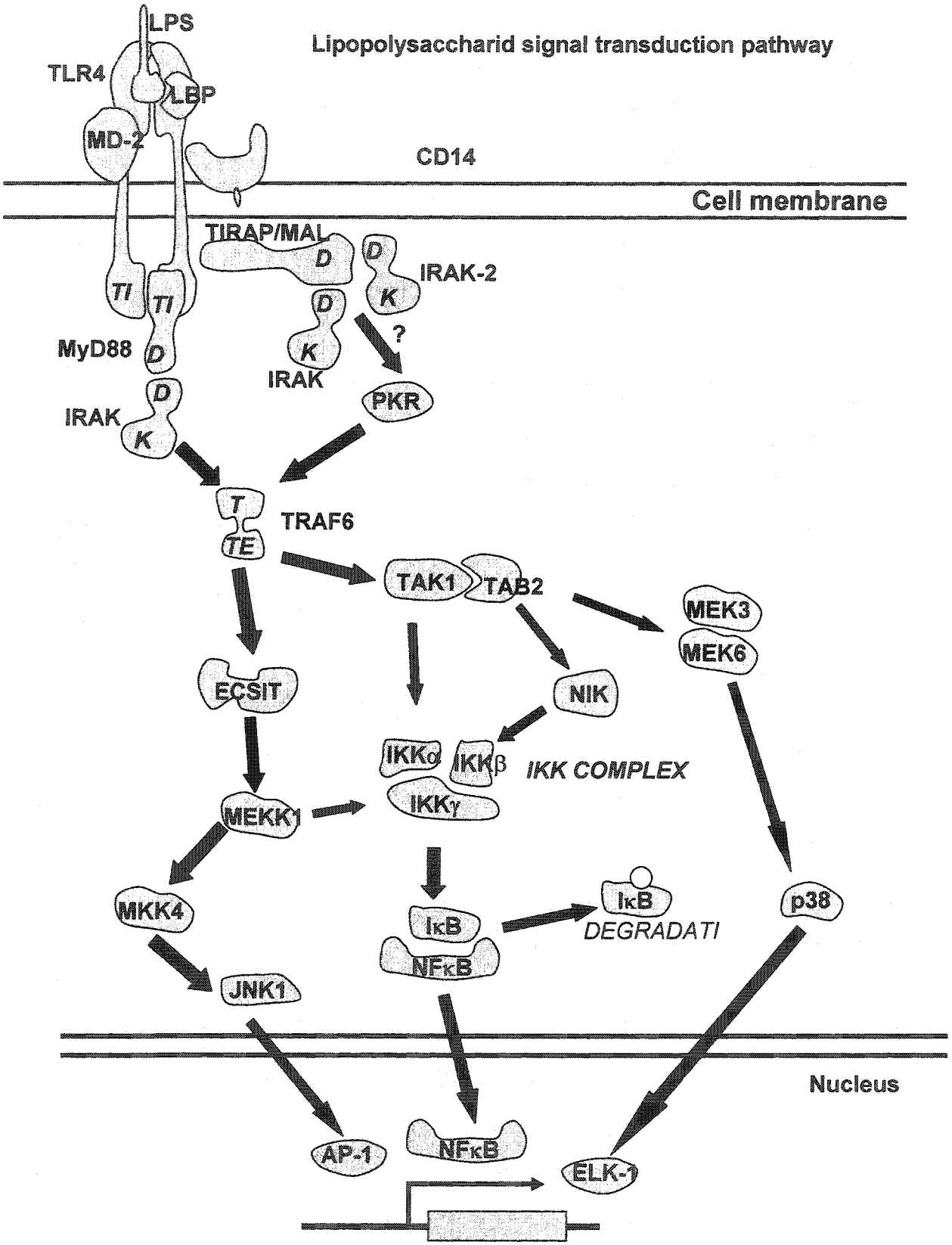


FIGURE 2. Signal transduction pathway of Toll-like receptors system.


Lipopolysaccharid signal transduction pathway
LPS
TLR4
LBP
MD-2
CD14
Cell membrane
TIRAP/MAL
D
D
IRAK-2
K
TI
TI
D
K
?
MyD88
D
IRAK
K
IRAK
PKR
T
TE
TRAF6
TAK1
TAB2
MEK3
MEK6
ECSIT
NIK
IKKα
IKKβ
IKK COMPLEX
IKKγ
MEKK1
MKK4
IκB
NFκB
IκB
DEGRADATI
p38
JNK1
Nucleus
AP-1
NFκB
ELK-1
Transcription Immune Response Genes

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CHAPTER II

ATTENUATION OF MHC CLASS II EXPRESSION IN *M. BOVIS* BCG INFECTED MACROPHAGES INVOLVES CIITA.

Role of the Nramp1 gene in IFN-γ-induced MHC class II gene expression.

PREFACE

In this chapter we investigate the molecular basics of impaired responsiveness of macrophages derived from mice susceptible to infection with *M. bovis* BCG to stimulation with IFN-γ. This defect is manifested by lower levels of MHC-II expressed by macrophages from susceptible mice in response to IFN-γ when compared to macrophages derived from resistant animals. We document that STAT1 protein phosphorylation, which is one of the early events during IFN-γ signaling, is less efficient in susceptible macrophages. The IFN-γ is a very important activator of macrophages and defect in its signaling pathway may have dramatic consequences for the host resistance, especially to the intracellular pathogens. We show that genetic factors determine the outcome of macrophage – pathogen interaction. In addition, our results by showing that infection of IFN-γ-stimulated macrophages with *M. bovis* BCG leads to down-regulation of MHC-II expression suggest that certain microorganisms might have developed mechanisms to modulate macrophage function.

SUMMARY

The natural resistance associated macrophage protein 1 *(Nramp1)* gene determines the ability of murine macrophages to control infection with a group of intracellular pathogens, including *Salmonella typhimurium*, *Leishmania donovani*, and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). The expression of the resistant allele of the *Nramp1* gene in murine macrophages is associated with a more efficient expression of several macrophage activation-associated genes, including class II MHC loci.

In this study, we investigated the molecular mechanisms involved in IFN-γ-induced MHC-II expression in three types of macrophages: those expressing a wild-type allele of the *Nramp1* gene (B10R and 129/Mϕ), those carrying a susceptible form of the *Nramp1* gene (B10S), and those derived from 129-*Nramp1*-knockout mice (129/*Nramp1*-KO). Previously, we published results showing that Ia protein expression is significantly higher in the IFN-γ-induced B10R macrophages, compared with its susceptible counterpart. In this paper, we also show that the higher expression of Ia protein in B10R cells is associated with higher I-A_β mRNA expression, which correlates with a higher level of IFN-γ-induced phosphorylation of the STAT1-aprotein and subsequently with elevated expression of class II transactivator (CIITA) mRNA, compared with B10S. Furthermore, we demonstrate that the infection of macrophages with *M. bovis* BCG results in a down-regulation of CIITA mRNA expression and, consequently, in the inhibition of Ia induction. Therefore, our data explain, at least in part, the molecular mechanism involved in the inhibition of I-A_β gene expression in *M. bovis* BCG-infected macrophages activated with IFN-γ.

INTRODUCTION

The natural resistance associated macrophage protein 1 (*Nramp1*) gene belongs to a family of proteins that are extremely conserved throughout evolution. Members of the Nramp protein family can be found in such distinct evolutionary species as bacteria (including *Mycobacterium*), yeast, plants, insects, worms, birds, mammals, as well as humans (1-6)

Interestingly, it has been shown that a single substitution of glycine with aspartic acid at position 169 of the predicted amino acid sequence of Nramp1 protein is responsible for loss of Nramp1 function in macrophages from bacillus Calmette-Guérin (BCG)-susceptible mice (7-9). Northern blot analysis has revealed that, in mice, the *Nramp1* gene is expressed exclusively by macrophages. Although the exact function of the Nramp1 protein has not been clarified yet, an examination of the sequence of the gene indicates that Nramp1 is a transmembrane protein with a potential transport function (10). It has 12 highly hydrophobic regions that may form transmembrane domains, two potential N-linked glycosylation sites, several consensus protein kinase C (PKC) phosphorylation sequences, a putative intracytoplasmic consensus transport signature, and a proline/serine-rich putative Src homology 3 (SH3) binding domain (7, 11).

The evidence obtained from the analysis of *Nramp1* homologues in yeasts SFM1 and SFM2 (from *Saccharomyces cerevisiae)* suggests that the Nramp1 protein may be involved in the transport of divalent cations, such as Mn^{2+}, Zn^{2+}, or Fe^{2+}, across the membrane (12). The Nramp2 protein, the second member of Nramp family in mammals, has been shown to transport iron in many different tissues (13). Overall, the above evidence, together with the experiments showing

the intracellular localization of the Nramp1 protein in the late phagosomal/lysosomal membrane (14), and an involvement of Nramp1 protein in phagosomal acidification (15) suggest that Nramp1 controls and/or regulates the intraphagosomal environment.

The *Nramp1* gene has been shown to be directly involved in determination of the resistance of mice to infections with several unrelated pathogens, including *Leishmania donovani*, *Salmonella typhimurium*, and *Mycobacterium bovis* BCG (7, 11, 16). In the case of infection with *M. bovis* BCG, a susceptible phenotype is characterized by the inability of the host's macrophages to control the growth of the microorganism in the early phase of infection (7, 17-20). The inefficient bactericidal activity of macrophages derived from mice susceptible to infection with *M. bovis* BCG seems to be associated with an inadequate magnitude of activation (21-28). It has been demonstrated that *Nramp1*, through a number of pleiotropic effects, influences the process of macrophage activation. These effects include the differential expression of IL-1ß, TNF-α, and (IL-10-sensitive gene) KC, inducible NO synthase (iNOS) resulting in variable production of reactive nitrogen intermediates (RNI), the respiratory burst, as well as MHC-II genes (27, 29, 30).

It has been well documented that the expression of MHC-II genes is essential for the development of an immune response (32). The MHC-II molecule is encoded by two genes in the mouse, denominated as I-A and I-E, which are located in two subregions on murine chromosome 17. The glycoproteins encoded by the I-A and I-E genes (called Ia surface proteins in mouse) are able to bind and present foreign peptides to competent T cells. Both I-A and I-E are expressed as heterodimers formed by the noncovalent association of α- and β-chains on the surface of APC, including macrophages, B-cells, thymic epithelial cells, glial cells, and dendritic cells (33). The expression of MHC II molecules can be either constitutive or inducible, depending on the cellular

type (34). IFN-γ is a potent inducer of MHC-II expression in macrophages. Although MHC II gene expression is under strict and highly complex transcription regulation involving interaction of several regulatory elements with specific transcription factors (31, 34, 35), it seems that the entire process is controlled by a single master regulator called class II transactivator (CIITA) (36-39). CIITA is necessary for both constitutive and inducible expression of MHC II genes. It does not bind directly to DNA, but rather interacts with transcription factors bound to the promoter of the MHC genes perhaps via the N-terminal activation domain (40-42). CIITA also contains a GTP-binding domain, which is absolutely essential for its function (43), a proline/serine/treonine-rich region of unknown function, as well as a leucine-rich region and two leucine charged domains most likely responsible for direct interaction with nuclear factors (39, 40).

The induction of CIITA mRNA expression by IFN-γ requires the presence of the functional STAT1α protein (44, 45). STAT1α is part of a well-described IFN-γ signal transduction pathway. The binding of IFN-γ to IFN-γ receptors on the surface of macrophages leads to the phosphorylation and activation of two tyrosine kinases termed Jak1 and Jak2, which associate with the IFN-γRα (IFNGR-1) and IFN-γRβ (IFNGR-2) subunits, respectively (46). The two Jak kinases rapidly induce the tyrosine phosphorylation of the α-subunit of IFN-γ receptor providing docking site for STAT1α (47). The cooperation of the two Jak kinases results in STAT1α protein phosphorylation, which is required for STAT1α release from the receptor, STAT1α/STAT1α homodimer formation, and translocation to the nucleus, where it can bind to DNA and modulate gene expression (48).

The role of the Nramp1 protein in the regulation of the response of macrophages to IFN-γ has not been elucidated yet. In this report, we present evidence indicating that the higher level of the Ia antigens, expressed by macrophages carrying the resistant allele of the *Nramp1* gene

(B10R), compared with susceptible macrophages (B10S), correlates with the higher level of CIITA expressed by those macrophages. The difference in CIITA expression also correlates with the superior capability of B10R macrophages to phosphorylate the STAT1α protein in response to IFN-γ stimulation, compared with B10S macrophages.

Furthermore, this study also shows the effect of *M. bovis* BCG infection on I-A_β and CIITA mRNA expression and, consequently, on Ia protein production in IFN-γ-stimulated macrophages.

MATERIALS AND METHODS

Reagents

DMEM, penicillin/streptomycin, Dulbecco's PBS (DPBS), and Trizol reagent were purchased from Life Technologies (Grand Island, NY). FBS, characterized for low level of endotoxin, was obtained from HyClone (Logan, UT). Recombinant murine IFN-γ was purchased from Amgen (Thousand Oaks, CA). Nonidet P-40 and PMSF were purchased from United States Biochemicals (Cleveland, OH). Sodium deoxycholate, sodium orthovanadate, sodium fluoride, Tween 80, BSA, aprotinin, leupeptin, *p*-nitrophenyl guanidinobenzoate, and rabbit anti-mouse IgG polyclonal HRP-conjugated Ab were purchased from Sigma (St. Louis, MO). Anti-murine p91 mouse mAb (C-111), anti-murine p84/p91 rabbit polyclonal Ab (M-22), anti-phosphotyrosine mouse mAb (PY-20), protein A/G PLUS-Agarose, and goat anti-human/murine CD64 (Fc receptor type I) Ab (N-19) were purchased from Santa Cruz Biotechnology (Santa

Cruz, CA). Ab against murine Fc receptor type II and III (FcBlock), FITC anti mouse I-A^b (used to analyze the Ia protein expression of cells derived from 129/J mice), and FITC anti mouse I-A^k Ab (used to analyze the surface Ia expression of cells derived from B10A mice) were obtained from PharMingen (Mississauga, ON, Canada). [γ-^{32}P]dCTP and ECL chemiluminescent reagent were purchased from Amersham (Amersham, U.K.). Middelebrook 7H9 broth was purchased from Difco Laboratories (Detroit, MI). BBL Middelebrook OADC Enrichment was purchased from Becton Dickinson (Mississauga, ON, Canada).

Bacteria

M. bovis BCG substrain Montreal was cultivated using constant rotation at 37°C for 2 wk in Middelebrook 7H9 broth supplemented with 10% Middelebrook OADC Enrichment and containing 0.05% Tween 80. After culture reached concentration of 0.6–1.0 OD_{600}, the cells were collected and briefly sonicated to break down bacterial clumps and filtered through a 5-μm syringe filter to eliminate remaining clumps. After estimation of cell concentration, the culture was aliquoted and frozen in 15% glycerol solution.

Cell lines

Macrophage cell lines were derived from the bone marrow of B10A.*Bcg*^{r} and 129/J mice expressing the wild type of the *Nramp1* gene (B10R cell line and 129.Mϕ cell line, respectively), from B10.A mice carrying a mutated *Nramp1* gene (point mutation at D169; B10S macrophage cell line), and from 129/*Nramp1* gene knockout mice with genetically disrupted *Nramp-1* (49) gene in the 129/J embryonic stem cells (129/*Nramp1*-KO cell line), as previously described (50). All mice were homozygous at the *Nramp1* locus. Cell lines were cultured in DMEM

supplemented with 10% heat inactivated FBS and penicillin/streptomycin antibiotic mixture. The subconfluent cell cultures were used for all of the experiments.

FACS analysis of cell surface Ia expression

Macrophage cell lines were plated at a concentration of 0.5–1 x 10^6/ml and treated with IFN-γ and/or *M. bovis* BCG for 48 h. Cells were removed from the flasks, washed in DPBS, and resuspended in DPBS containing 5% BSA and 0.1% sodium azide. FcR were blocked for 15 min at 4°C using Ab against FcR type II/III (FcBlock; PharMingen) and anti-murine/human CD64 Abs against FcR type I (N-19; Santa Cruz Biotechnology). Cells were washed three times and incubated for 15 min at 4°C with strain-specific anti I-A^k (B10R and B10S) and I-A^b (129 Mϕ) Abs directly labeled with FITC (PharMingen). After washing, cells were fixed in 1% solution of paraformaldehyde in PBS for 30 min at room temperature. Stained cells were analyzed using a dual laser FACStar^{Plus} flow cytometer (Becton Dickinson). A green fluorescence histogram of 1000 channel resolution was collected from 10,000 cells counted for each sample analyzed.

DNA probes

The pGEM-A_β containing a 500-bp *Pst*I fragment of the I-A_β^d gene was kindly provided by Dr R. Germain (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The GAPDH probe was generated by PCR amplification of a 456-bp cDNA fragment using the following oligonucleotide primers: sense primer, 5'-CCC TTC ATT GAC CTC AAC TAC ATG G-3'; antisense primer, 5'-AGT CTT CTG GGT GGC AGT GAT GG-3'. The PCR product was subcloned in pBluescript KS^+ and sequenced. The CIITA probe was obtained by PCR amplification of the 341-bp fragment of macrophage cDNA using the

following oligonucleotide primers: sense primer, 5'-CTT CTG GCT TCA CCT TCA CG-3' and anti-sense primer, 5'-ATT AAG GAC TCA GGG CTC CC-3'. The products of PCR amplification were subcloned into pGEM-T-Easy vector (Promega, Madison, WI).

Northern blot analysis

To isolate total cellular RNA, ~10 million cells, treated with IFN-γ and/or *M. bovis* BCG, were lysed using either guanidinium isothiocyanate solution or Trizol reagent, and RNA was isolated as previously described (51). A total of 15–20 μg of total cellular RNA extracted from B10R and B10S macrophages was always separated on the same agarose gel (1.2%) containing 2.2 M formaldehyde. The membranes were then hybridized for 18 h at 42°C with labeled probe (10^6 cpm/ml). Subsequently, the membranes were washed three times with 2x SSC, 0.1% SDS (10 min, 43°C), and then three times with 0.1x SSC, 0.1% SDS (10 min, 55°C) before autoradiography. The densitometry analysis data was obtained using PhosphoImager (Storm 860; Molecular Dynamics, Sunnyvale, CA) and analyzed using ImageQuant image analysis software (Molecular Dynamics). The presented results represent the averages of the relative values from the three independent experiments, standardized against GAPDH mRNA levels.

Immunoprecipitation of STAT1α protein

Analysis of STAT1α phosphorylation was performed according to the immunoprecipitation protocol provided by Santa Cruz Biotechnology technical services. Briefly, IFN-γ-stimulated cells were lysed in RIPA buffer (PBS (pH 7.5) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 10

μg/ml aprotinin, 1 μg/ml leupeptin, 100 μg/ml PMSF, and 0.025 mM *p*-nitrophenyl guanidinobenzoate), mixed with rabbit polyclonal anti-p84/p91 Abs and incubated overnight at 4°C. The immunocomplexes were precipitated for 2 h at 4°C using protein A/G agarose beads. After washing the agarose beads with RIPA buffer, STAT1 proteins were eluted from immunocomplexes by heating at 95°C for 5 min in SDS-PAGE sample buffer. Proteins were subjected to SDS-PAGE using 8 or 10% running gel, according to standard protocols, and transferred to the polyvinylidene difluoride membranes. Nonspecific binding was blocked overnight at 4°C using 10% FBS in PBS containing 0.05% Tween 20. To assess the level of STAT1α phosphoprotein, the membranes were incubated in a solution of mouse mAbs against phosphotyrosine (at a 1:1000 dilution in blocking buffer) at room temperature for 1 h, followed by incubation with a solution of anti-mouse IgG HRP-conjugated Abs (1:10,000 dilution in blocking buffer, 1 h at room temperature). The signal was visualized using ECL reagent. Subsequently, the anti-phosphotyrosine Abs were removed from membranes by a 30-min incubation at 55°C in buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 70 mM 2-ME, and the total amount of the immunoprecipitated STAT1α was assessed using mouse mAbs against STAT1α protein, following the same protocol as used for anti-phosphotyrosine Abs. The densities of the bands corresponding to phosphorylated STAT1α protein levels were determined and normalized against the total amount of immunoprecipitated STAT1 protein. The exposure times for loading controls used in normalization were always within the linear range of the film.

RESULTS

Expression of I-A_β mRNA in macrophages infected with M. bovis BCG.

We have previously shown that the induction of the I-A_β gene occurs 12–18 h post IFN-γ stimulation and then continues to rise for at least another 36 h (31). Therefore, in the experiments described below, we tested I-A_β mRNA expression 24 h after IFN-γ stimulation of macrophage cell lines derived from the bone marrow of B10A.*Bcg*^{r} (B10R) and from B10.A mice carrying the susceptibility associated mutation of *Nramp1* gene (point mutation at D169; B10S). As shown in Fig. 1, following 24 h of treatment with 10 U/ml of IFN-γ, B10R macrophages expressed a high level of I-A_β mRNA, whereas B10S macrophages expressed I-A_β mRNA at a very low level. The results of the densitometric analysis of four independent experiments are presented in Fig. 1*B*. Infection with *M. bovis* BCG decreased the ability of macrophages to express I-A_β mRNA in response to IFN-γ by ~75% in B10R cells when a 10:1 bacteria to macrophage ratio was used (Fig. 1). I-A_β mRNA expression in IFN-γ-activated B10S macrophages infected with *M. bovis* BCG was also reduced, compared with uninfected IFN-γ-stimulated B10S macrophages; however, it is difficult to estimate precisely the level of reduction because the expression level was already very low.

Analysis of Ia protein expression.

To establish whether the observed difference in the level of I-A_β mRNA expression between macrophages expressing either the *Nramp1*^{r} or the *Nramp1*^{s} allele of the *Nramp1* gene

was similarly reflected at the level of MHC-II surface protein (Ia) expression, we performed FACS analysis using specific Ab against Ia molecules. As shown in Fig. 2, *A* and *B*, the stimulation of B10R macrophages with 10 U/ml of IFN-γ resulted in much higher surface Ia protein (99.8%) expression, compared with B10S macrophages (15.6%).

Consistent with the results of Northern blot analysis showed in Fig. 1, the infection of macrophages with *M. bovis* BCG decreased the ability of both B10R and B10S macrophages to express surface Ia, as determined by FACS analysis. As shown in Fig. 2, *A* and *B*, infection of macrophages with *M. bovis* BCG suppressed the ability of IFN-γ-stimulated macrophages to express surface Ia protein. The expression of Ia in BCG-infected macrophages decreased from 99.8% to 75.8% in B10R and from 15.6% to 9.3% in B10S cells.

Differential tyrosine phosphorylation of STAT1α protein in resistant and susceptible macrophages.

The activation of STAT1α protein requires its phosphorylation at a specific tyrosine residue. Only phosphorylated STAT1α is capable of forming homodimers that are subsequently translocated to the nucleus. Therefore, macrophages carrying the resistant allele of the *Nramp1* gene (B10R and 129.Mϕ) and macrophages that do not express intact Nramp1 protein (B10S), as well as macrophages derived from *Nramp1* knockout mice (129/*Nramp1*-KO), were examined for their ability to phosphorylate STAT1 protein in response to IFN-γ stimulation. Macrophages were stimulated with 10 U/ml of IFN-γ for 5, 15, and 30 min, lysed, and STAT1 protein was immunoprecipitated with anti-STAT1 Abs and subjected to Western blot analysis using anti-phosphotyrosine Ab.

As shown in Fig. 3*A*, significantly higher levels of the phosphorylated form of STAT1 were present in B10R, compared with B10S, macrophages. The densitometric analysis of the level of phosphorylated STAT1 compensated for total immunoprecipitated STAT1 protein were plotted. In Fig. 3*B*, the results show that the level of the phosphorylated STAT1α protein induced by IFN-γ in B10R macrophages was at least 4-fold higher, compared with the B10S macrophages. Similar results were obtained using 129.Mϕ macrophages (carrying *Nramp1*^{r} allele) and macrophages derived from *Nramp1* gene knockout mice on the same genetic background. As shown in Fig. 4, *A* and *B*, 129.Mϕ macrophages expressed 2.5–3 times more phosphorylated STAT1, compared with the 129/*Nramp1*-KO macrophages.

Since our immunoprecipitation analyses shown in Figs. 3 and 4 seem to be more efficient for B10R macrophages than for 129/Mϕ (both carrying the resistant allele of the *Nramp1* gene), we decided to compare the level of I-A_β mRNA and Ia surface protein expression in the two cell lines following activation with 10 U/ml of IFN-γ. We have found no significant differences between IFN-γ-activated B10R and 129/Mϕ, either at the level of I-A_β mRNA expression or at the level of surface Ia protein (Fig. 5). Therefore, we can conclude that the apparent difference at the level of STAT1α expression was most likely due to different efficiency of immunoprecipitation between the two sets of experiments illustrated in Figs. 3 and 4.

Effect of infection with M. bovis BCG on IFN γ-induced CIITA mRNA expression.

Recently, it has been shown that phosphorylated STAT1α is acting as a transactivating factor that is able to induce transcription of the CIITA gene (52). Since we have observed a

significant difference in STAT1α phosphorylation between B10R and B10S macrophages, it was important to test whether the higher level of STAT1α phosphorylation observed in B10R cells correlated with higher level of CIITA mRNA. We analyzed the IFN-γ-induced CIITA mRNA expression in B10R and B10S macrophages. We found that the expression of CIITA mRNA was induced as early as 3 h post IFN-γ stimulation, reaching highest level at 12–18 h of stimulation both in B10R and B10S macrophages (data not shown). At each of the time points analyzed, the expression of CIITA was a few-fold higher in B10R than in B10S macrophages. Since the expression of I-A_β mRNA was analyzed 24 h post IFN-γ stimulation, we also analyzed the CIITA mRNA expression at this time point following IFN-γ stimulation. As shown in Fig. 6, *A* and *B*, and in Fig. 5*B*, stimulation with 10 U/ml of IFN-γ induced CIITA mRNA expression both in B10R and 129/Mϕ (both carrying the resistant allele of the *Nramp1* gene), as well as in B10S macrophages (carrying the susceptible allele of the *Nramp1* gene). However, the level of induction was ~10-fold higher in B10R, compared with B10S, macrophages. Interestingly, infection of macrophages with *M. bovis* BCG suppressed the ability of macrophages to augment CIITA mRNA in response to IFN-γ stimulation. In the B10R macrophages infected with *M. bovis* BCG, the induction of CIITA mRNA with IFN-γ was diminished by 70%, compared with IFN-γ-stimulated noninfected macrophages. The similar effect of *M. bovis* BCG infection on the induction of CIITA by IFN-γ was observed in B10S macrophages; however, since the level of CIITA mRNA induction by IFN-γ was a few-fold lower in B10S macrophages, compared with B10R macrophages, the level of CIITA in the BCG-infected B10S macrophages was barely detectable.

DISCUSSION

In mice, resistance to the early growth of *M. bovis* BCG is controlled by the *Bcg* gene. The *Bcg* gene, now termed natural resistance associated macrophage protein 1 (*Nramp1*), was cloned in 1993 and shown to encode a phagocyte-specific membrane protein, which bears significant structural similarity to transporter proteins (49). Recently, it has been shown that the Nramp1 protein is localized in the late phagosomal/lysosomal membrane, and that it is involved in regulation of iron balance (14, 53, 54). It has been suggested that Nramp1 may also be involved in the transport of other metal ions, such as Mn^{2+}, Zn^{2+}, and Mg^{2+} (53, 55). Alternative alleles of the *Nramp1* gene (*Nramp1*^{r} conferring resistance and *Nramp1*^{s} conferring susceptibility to infection of mice with *M. bovis* BCG, *L. donovani*, *S. typhimurium*) are expressed in mice by mature tissue macrophages.

The *Bcg/Lsh/Ity* gene was shown to be associated with regulation of macrophage activation (9, 30, 56). This has been demonstrated by a wide range of pleiotropic effects, including regulation of KC chemokine, iNOS and RNI production, respiratory burst, and IL-1β and TNF-α levels (27, 29, 30). RNI were shown to play a decisive role in the control of the replication of intracellular pathogens (21-28).

A proper analysis of the molecular basis of macrophage activation for effective bactericidal function requires access to homogenous cell populations of defined genetic background. We have generated macrophage cell lines from mouse strains that carry either the *Nramp1*^{r} or *Nramp1*^{s} allele (50), and we have made a detailed comparison of these cell lines (27, 31, 58, 59). In addition, we have generated macrophage cell lines from the *Nramp1* gene

knockout mice on the 129/J genetic background and from their 129/J littermate controls that carry the *Nramp1*^{r} allele.

Recently, using our macrophage cell lines, we have found that PKC-specific activity was significantly more increased in the cytosolic fractions derived from *Nramp1*^{r}, compared with *Nramp1*^{s}, macrophages. Furthermore, during the course of macrophage activation, particulate fractions from *Nramp1*^{r} macrophages contained significantly greater PKC activity, compared with *Nramp1*^{s}. The differences in PKC activity between *Nramp1*^{r} and *Nramp1*^{s} macrophages contributed to altered responsiveness to IFN-γ that resulted in more efficient production of RNI by *Nramp1*^{r} macrophages. *Nramp1*^{r} macrophages also had a superior ability to phosphorylate endogenous substrate compared with *Nramp1*^{s} macrophages (59).

Previously, we found that macrophages carrying the susceptible allele of *Nramp1* expressed much lower levels of MHC-II surface proteins and I-A_β mRNA when compared with macrophages with resistant allele (31). We also demonstrated a significantly reduced amount of produced nitrates, a decreased production of TNF-α, and a decrease in the level of MHC-II in response to IFN-γ stimulation in macrophages transfected with *Nramp1* antisense ribozyme *(Nramp1-Rb)*, compared with the controls transfected with mock vector (50). Overall, these studies supported the hypothesis that the *Nramp1* gene is involved in the regulation of the early signaling that occurs in macrophages activated with IFN-γ.

In this paper, we have focused on the mechanism of IFN-γ-induced expression of MHC-II Ags by macrophages carrying either the resistant or susceptible allele of *Nramp1* and macrophage cell lines derived from *Nramp1*-knockout mice. We also analyzed the effect of *M. bovis* BCG infection on the expression of MHC-II using these cell lines. We found that infection

with *M. bovis* BCG leads to a very significant inhibition of IFN-γ-induced I-A_β mRNA and Ia protein expression in tested macrophage cell lines.

The observed inhibition of I-A_β gene expression by *M. bovis* BCG infection may represent a protective mechanism that allows the pathogen to persist longer in the host. This phenomenon does not seem to be unique for the infection of macrophages with *M. bovis* BCG. It was previously reported that the protozoan *Toxoplasma gondii* is able to inhibit MHC-II expression in infected macrophages. The mechanism of that inhibition is still unknown, but does not seem to be related to the increased production of prostaglandin E2, IL-10, TGF-β, or NO (60). The infection of macrophages with *L. donovani* also leads to the inhibition of IFN-γ-induced MHC-II gene expression (61-63). Similarly, it was shown that murine CMV (MCMV) was able to inhibit MHC-II transcription (64). Macrophages infected with MCMV were not able to express Ia in response to IFN-γ and, consequently, failed to present Ags and activate $CD4^+$ T lymphocytes. The CMV virus most likely interferes with the expression of transcription factors involved in I-A_β gene expression. In addition, Wadee et al. (65, 66) showed that a 25-kDa glycoprotein encoded by *M. tuberculosis* was able to inhibit MHC-II expression in monocytes.

IFN-γ has been shown to be a potent inducer of MHC II molecules in a variety of cell types. The analysis of IFN-γ-knockout mice showed that they were able to develop normally in the absence of pathogens, but their macrophages were unable to produce antimicrobial products and had reduced expression of MHC-II genes. Thus, IFN-γ-deficient animals were extremely sensitive to infection and died shortly after administration of the pathogen, i.e., *M. bovis* (67). Similarly, the lack of IFN-γ receptor expression in knockout mice led to an inability of the animals to control *Mycobacterial* as well as other types of infection. Macrophages derived from

IFN-γ receptor-deficient mice produced much lower levels of TNF-α and NO, making them inefficient in killing bacteria (68). IFN-γ-induced signal transduction requires activation of specific receptor coupled to the JAK/STAT signal transduction system. This primary effect leads to induction of expression of a number of genes, including many transcription factors and regulatory proteins. One of them, CIITA, was characterized as a master regulator of MHC genes, and it was shown to be essential for both constitutive and inducible expression of MHC-II genes (41, 69-71). CIITA belongs to a family of IFN-γ-inducible genes. Interestingly, it was shown that STAT1 phosphorylation is required for the expression of CIITA gene. The aberrations in constitutive or infection-induced levels of CIITA expression may lead to severe immunological disorders or chronic infections.

Our studies demonstrate that, after IFN-γ stimulation, CIITA expression is elevated in macrophages carrying either resistant or susceptible allele of *Nramp1*, but the level of expression of CIITA was higher in macrophages carrying the "r" allele of *Nramp1*. Since IFN-γ-inducible expression of CIITA is STAT1α-dependent, we decided to evaluate its phosphorylation in response to IFN-γ stimulation. Our results demonstrate that, also at this level, *Nramp1*^{r} macrophages are superior to the *Nramp1*^{s} macrophages and were able to express much higher levels of the tyrosine-phosphorylated form of STAT1α in response to IFN-γ stimulation. A higher level of phosphorylated STAT1α protein would lead to a higher level of STAT1α homodimer being translocated to the nucleus and bound to the GAS element of the CIITA promoter in *Nramp1*^{r}, compared with *Nramp1*^{s}, macrophages. Consequently the lower level of CIITA gene expression in susceptible macrophages results in less efficient transcriptional activation of MHC-II gene expression. Our data clearly support this hypothesis and explain our

previously published findings, showing a difference between *Nramp1*^{r} and *Nramp1*^{s} macrophages at the level of transcription activation of I-A_β (31).

Overall, the presented results indicate that the differential MHC-II expression observed between *Nramp1*^{r} and *Nramp1*^{s} macrophages results from differences found at the level of transcription activation of the I-A_β gene controlled by CIITA. Alternative explanations, such as translational control and Ia protein stability, have also been proposed (72-75). Recent evidence suggests that one possible function of the Nramp1 protein is the transport of iron out of the bacteria/parasite-containing phagosome (14, 76). Therefore, it is possible that transport of iron by Nramp1 also has an impact on an iron homeostasis in macrophages. Iron and other divalent ions (e.g., Zn^{2+}), were shown to regulate the activity of various transcription factors and other regulatory proteins involved in posttranscriptional regulation of gene expression. The exact link between the regulation of iron metabolism by the *Nramp1* gene and transcriptional and posttranscriptional regulation of MHC-II expression remains to be established, and more studies are required to further characterize the molecular mechanism that regulates this important function of macrophages in immunity against bacterial infection.

FIGURE LEGENDS

FIGURE 1. Expression of I-A_β mRNA in B10R and B10S macrophages. *A*, B10R and B10S cells were stimulated with 10 U/ml of IFN-γ either alone or in combination with *M. bovis* BCG at ratio 10:1 (bacteria to macrophage) for 24 h. Total RNA was purified and the I-A_β mRNA expression was determined by Northern blot analysis. The GAPDH mRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of four replicative experiments. *B*, Scanning densitometry of the autoradiographs shown in *A*. The density of the bands corresponding to I-A_β mRNA expression was determined and normalized against GAPDH mRNA expression. There was significant statistical difference in the level of I-A_β mRNA expression between *M. bovis* BCG-infected IFN-γ-treated, compared with uninfected IFN-γ-treated cells ($p < 0.011$ for B10R cells and $p < 0.035$ for B10S cells, by paired two-tailed *t* test).

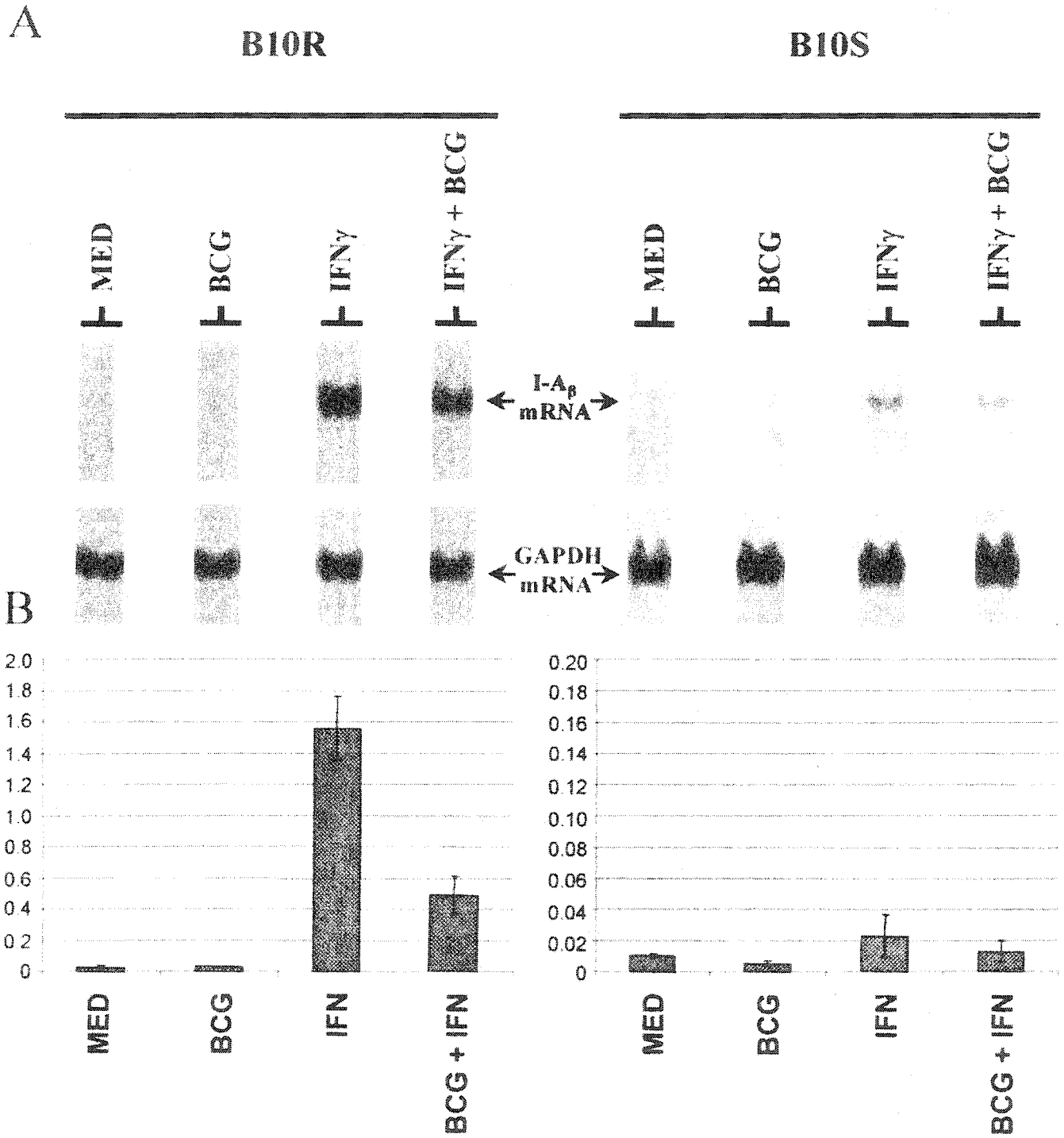

A
B10R
B10S
MED
BCG
IFNγ
IFNγ + BCG
MED
BCG
IFNγ
IFNγ + BCG
I-Aβ mRNA
GAPDH mRNA
B
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
MED
BCG
IFN
BCG + IFN
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
MED
BCG
IFN
BCG + IFN

FIGURE 2. FACS analysis of Ia Ags by macrophages stimulated with IFN-γ and/or infected with *M. bovis* BCG. Macrophages were stimulated for 48 h with 10 U/ml IFN-γ alone or infected with *M. bovis* BCG at the ratio 10 bacterial cells per 1 macrophage, or cells were infected with BCG and then stimulated with IFN-γ. The cells were than labeled with anti-I-A^b Abs conjugated to FITC and analyzed by FACS. *A*, B10R; and *B*, B10S.

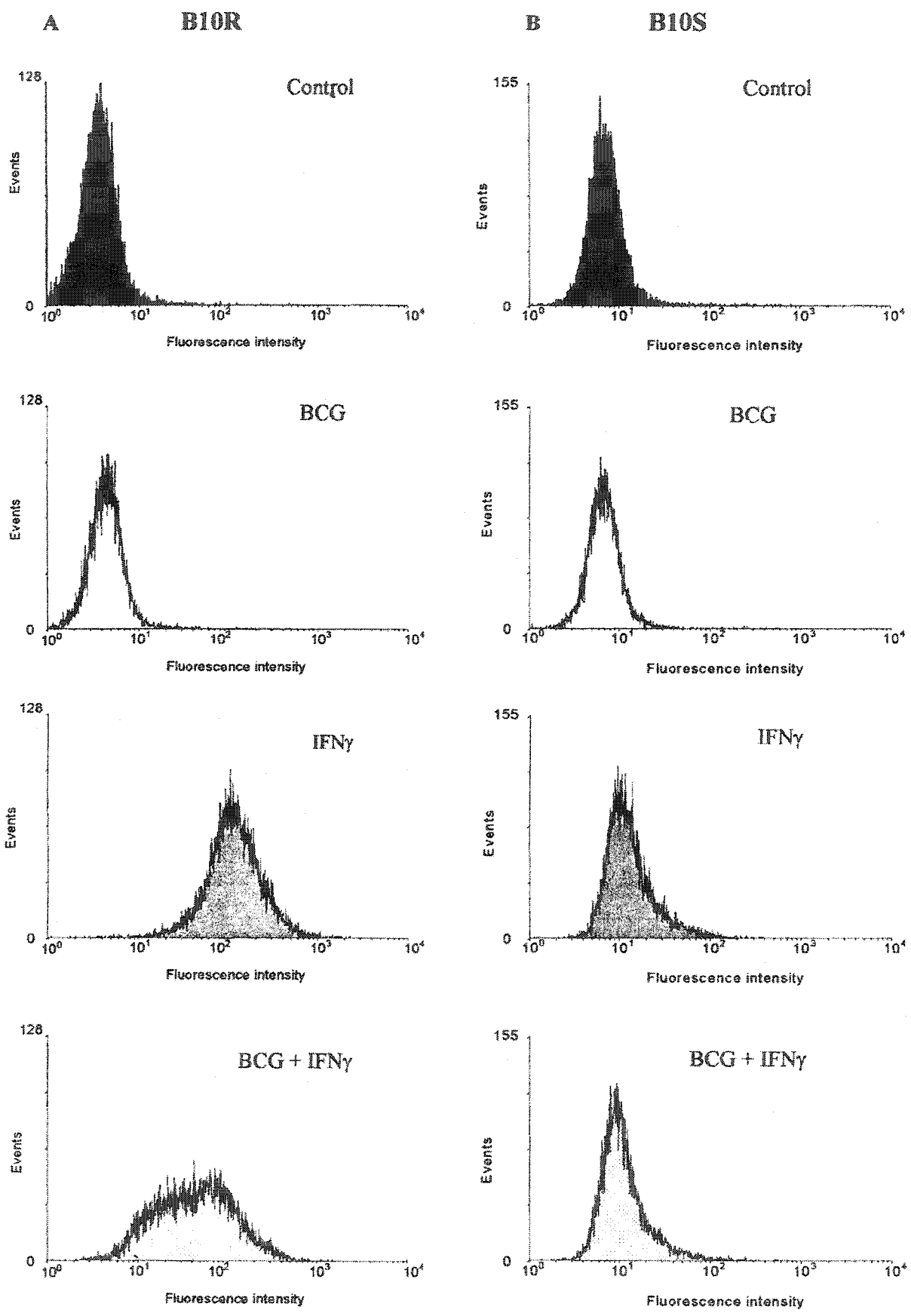
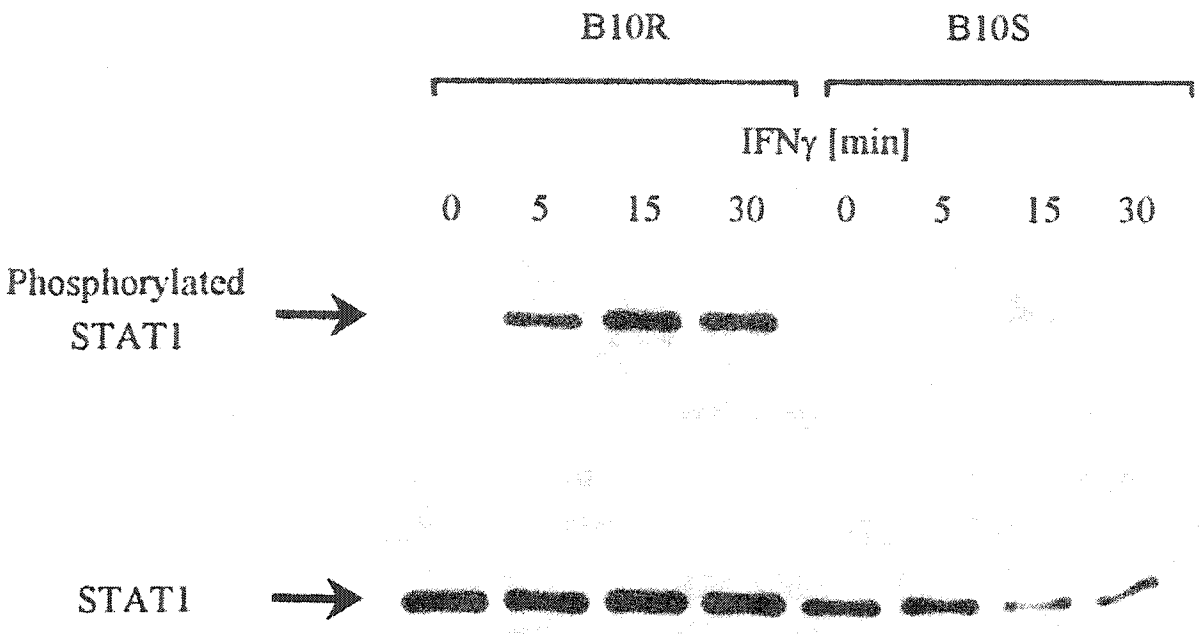

A
B10R
B
B10S
Control
Control
BCG
BCG
IFNγ
IFNγ
BCG + IFNγ
BCG + IFNγ
Events
Fluorescence intensity
128
155
0
10^0
10^1
10^2
10^3
10^4

FIGURE 3. Tyrosine phosphorylation of STAT1 protein in B10R and B10S macrophages. *A*, B10R and B10S macrophages were stimulated with 10 U/ml of IFN-γ for 5, 15, or 30 min. STAT1 protein was then immunoprecipitated using anti-STAT1 Ab and subjected to the Western blot analysis using anti-phosphotyrosine Ab. The expression of phosphorylated STAT1α protein was quantified by densitometric analysis (panel *B*). The KIOD values illustrating the levels of phosphorylated STAT1α protein were normalized against for the total amount of immunoprecipitated STAT1 protein. The results are representative of three independent experiments.


B10R
B10S
IFNγ [min]
0 5 15 30 0 5 15 30
Phosphorylated STAT1
STAT1

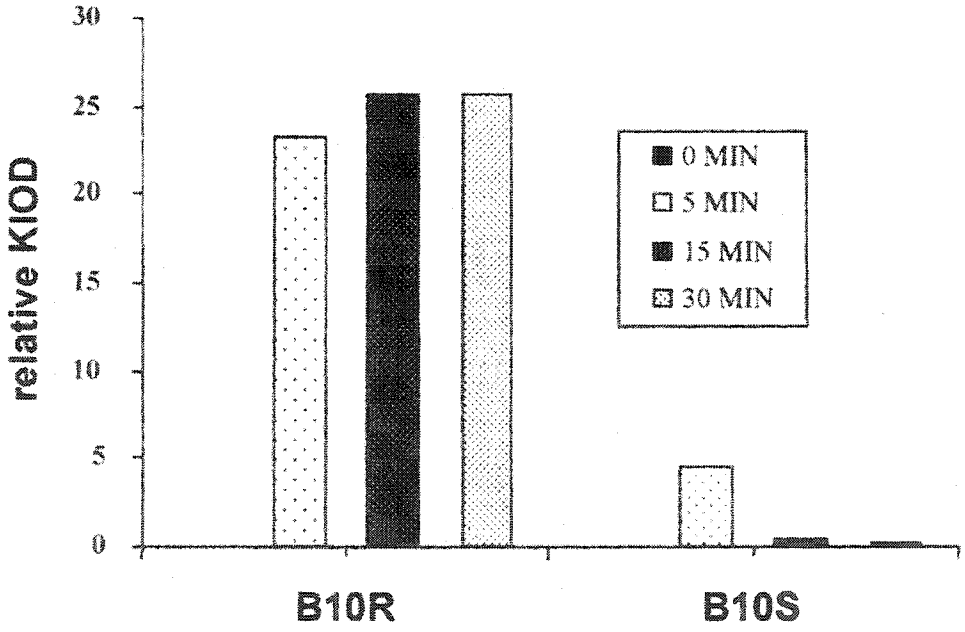
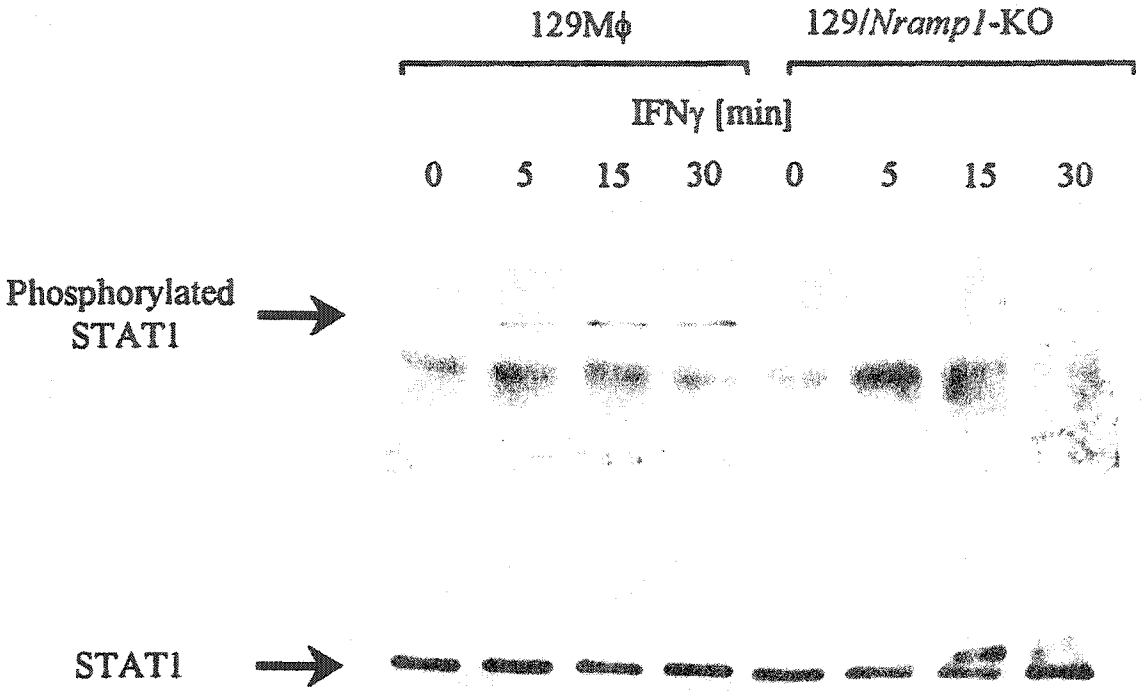

relative KIOD
30
25
20
15
10
5
0
0 MIN
5 MIN
15 MIN
30 MIN
B10R
B10S

FIGURE 4. Tyrosine phosphorylation of STAT1 protein in 129.Mϕ and 129/*Nramp1*-KO macrophages. *A*, 129.Mϕ and 129/*Nramp1*-KO macrophages were stimulated with 10 U/ml of IFN-γ for 5, 15, or 30 min. STAT1α protein was then immunoprecipitated using anti-STAT1α Ab and subjected to the Western blot analysis using anti-phosphotyrosine Ab. The expression of phosphorylated STAT1α protein was quantified by densitometric analysis (panel *B*). The KIOD values illustrating the levels of phosphorylated STAT1α protein were normalized against for the total amount of immunoprecipitated STAT1 protein. The results are representative of three independent experiments.


129Mϕ
129/Nramp1-KO
IFNγ [min]
0 5 15 30 0 5 15 30
Phosphorylated STAT1
STAT1

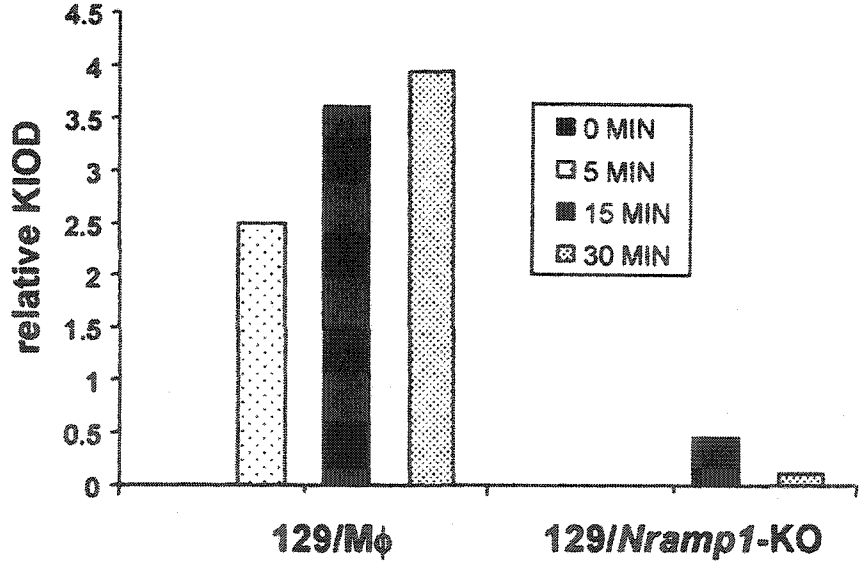
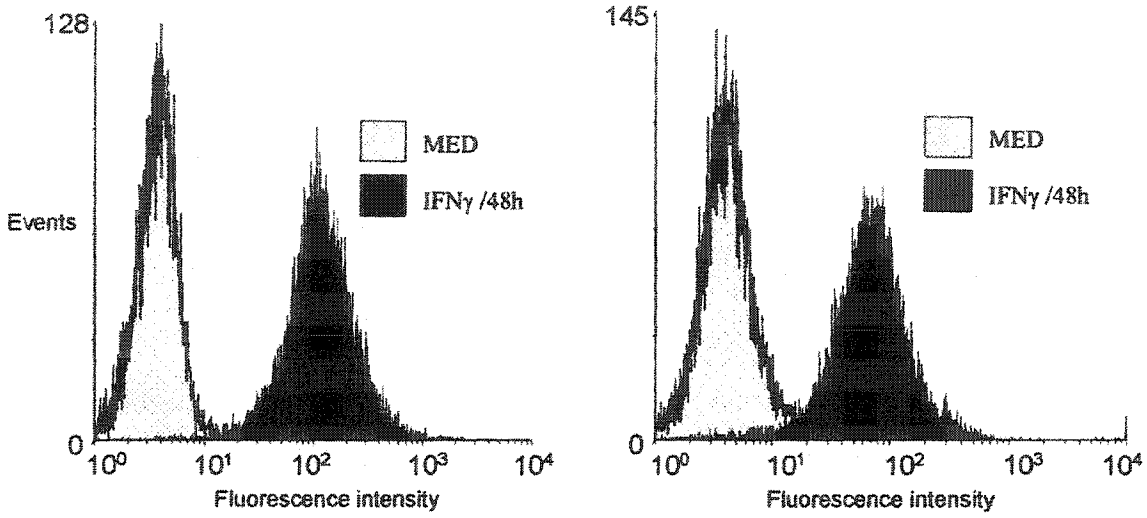

relative KIOD
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 MIN
5 MIN
15 MIN
30 MIN
129/Mϕ
129/Nramp1-KO

FIGURE 5. Ia expression in B10R and 129/Mϕ macrophages. *A*, FACS analysis of IFN-γ-induced expression of Ia Ag in B10R and 129/Mϕ macrophages. Macrophages were stimulated for 48 h with 10 U/ml IFN-γ. The cells were than labeled with anti-I-A^b (B10R) or with anti-I-A^b (129/Mϕ) Abs conjugated to FITC and analyzed by FACS. *B*, Northern blot analysis of I-A_β and CIITA mRNA in B10R and 129/Mϕ stimulated with 10 U/ml of IFN-γ for 24 h.

A


128
Events
0
MED
IFNγ /48h
10^0 10^1 10^2 10^3 10^4
Fluorescence intensity
145
0
MED
IFNγ /48h
10^0 10^1 10^2 10^3 10^4
Fluorescence intensity

B

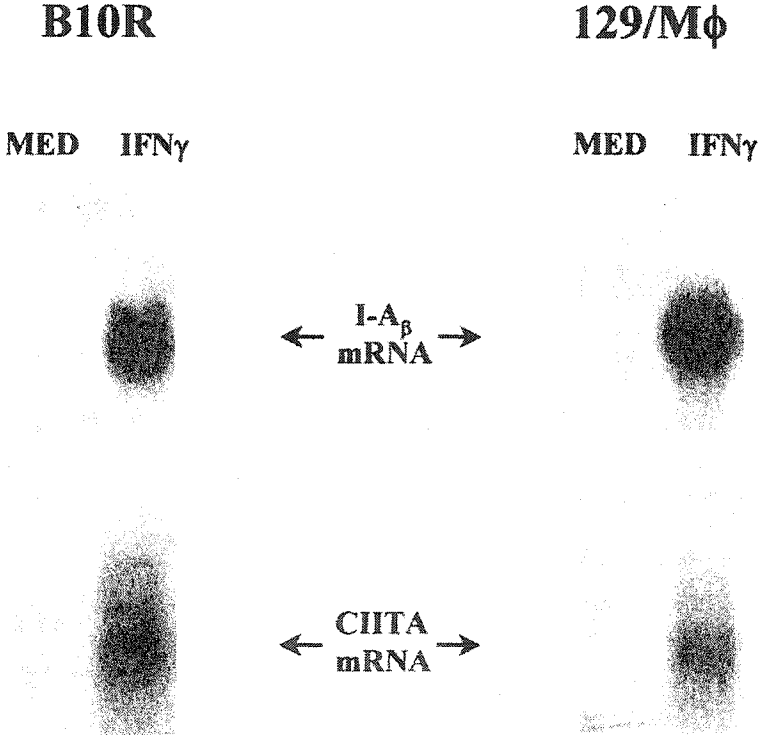
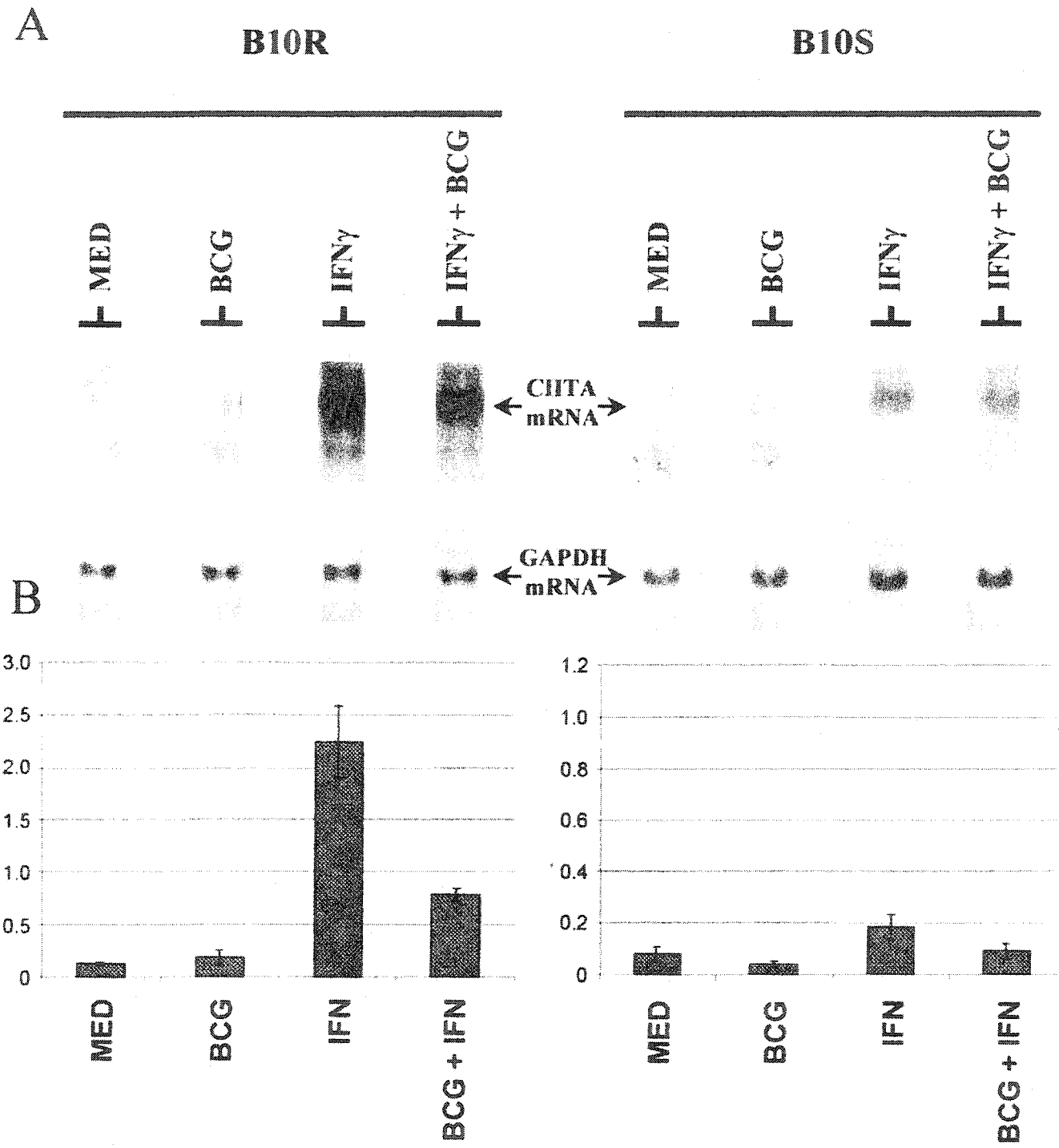

B10R
129/Mϕ
MED IFNγ
MED IFNγ
I-Aβ mRNA
CIITA mRNA

FIGURE 6. CIITA mRNA expression in noninfected and infected with *M. bovis* BCG B10R and B10S macrophages activated with IFN-γ. *A*, B10R and B10S macrophages were stimulated with 10 U/ml of IFN-γ either alone or in combination with *M. bovis* BCG at a ratio of 10:1 (bacteria to macrophage) for 24 h. The data shown are representative of four replicative experiments. *B*, Scanning densitometry of the autoradiographs shown in *A*. The density of the bands corresponding to CIITA mRNA expression was determined and normalized against GAPDH mRNA expression. There was statistical significant difference in the level of CIITA mRNA expression between *M. bovis* BCG-infected IFN-γ-treated, compared with uninfected IFN-γ-treated cells ($p < 0.0028$ for B10R cells and $p < 0.033$ for B10S cells, by paired two-tailed t test).


A
B10R
B10S
MED
BCG
IFNγ
IFNγ + BCG
MED
BCG
IFNγ
IFNγ + BCG
CIITA mRNA
GAPDH mRNA
B
3.0
2.5
2.0
1.5
1.0
0.5
0
MED
BCG
IFN
BCG + IFN
1.2
1.0
0.8
0.6
0.4
0.2
0
MED
BCG
IFN
BCG + IFN

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CHAPTER III

INHIBITION OF IFN-γ INDUCED MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II EXPRESSION BY LIPOPOLYSACCHARIDE AND *MYCOBACTERIUM BOVIS* BCG OCCURS IN A MyD88-DEPENDENT MANNER AND INVOLVES ATTENUATION OF CIITA mRNA EXPRESSION

PREFACE

In previous chapter we showed that *M. bovis* BCG is able to inhibit IFN-γ-stimulated expression of MHC-II in macrophages. We also demonstrated that down-regulation of MHC-II expression is correlated with similar inhibition of CIITA mRNA expression. In this chapter we present results of the subsequent studies that show that exposure of IFN-γ-simulated macrophages to lipopolysaccharides (LPS), the molecules derived from cell wall of Gram-negative bacteria, also leads to downregulation of MHC-II expression. The main goal of the studies presented in this chapter was to establish the molecular mechanism responsible for LPS-induced inhibition of MHC-II expression. We also wanted to compare this mechanism to the mechanism regulating MHC-II inhibition by *M. bovis* BCG.

SUMMARY

Macrophages play an important role in immunological defense both as phagocytes and antigen presenting cells. Many viruses and intracellular pathogens such as *Mycobacteria* have the ability to disrupt the antigen presentation thereby enabling their survival and successful propagation inside macrophages or other cells. One of the strategies to decrease efficiency of antigen presentation is by attenuating the expression of MHC antigens. In this study, we showed that both *Mycobacterium bovis* BCG and lipopolysaccharide (LPS) from *E. coli* inhibited IFN-γ-induced expression of MHC-II gene I-A_β in murine bone marrow-derived macrophages. We also demonstrated that down-regulation of MHC-II by LPS in macrophages was accompanied by attenuation of Class II Transactivator (CIITA) gene expression which has previously been reported by us for *Mycobacterium bovis* BCG. Although the effect of LPS on CIITA expression did not directly affect IFN-γ signaling pathway (as demonstrated by analysis of STAT1 activation and binding to the CIITA promoter IV), down-regulation of CIITA mRNA does require the expression of the Toll-like receptor 4 (TLR4). Finally, we demonstrated that the effect of LPS and *M. bovis* BCG on IFN-γ-induced MHC-II expression was dependent on the presence of the MyD88 adaptor protein.

INTRODUCTION

The importance of MHC-II (MHC-II) antigens in the development of an immune response against microbial infections is well known and intensively studied (1). The MHC-II molecules are used by antigen presenting cells (APCs) to activate specific T-cells during the early phase of development of adaptive immunity. The mechanism of assembly of the MHC-II/antigen complex as well as the nature of the interaction of the APC and T-cell is well studied and characterized. The process of antigen presentation requires the coordinated expression and processing of MHC molecules as well as a number of accessory proteins. Disruption of this process may lead to severe immunodeficiency or certain types of autoimmune diseases .

The MHC-II is constitutively expressed only by a limited number of cell types such as B-cells and dendritic cells. Other cell types, such as macrophages, endothelial cells, fibroblasts, glial cells, astrocytes and others express high levels of MHC-II antigens after stimulation with IFN-γ. The MHC-II molecule is a glycoprotein formed by non-covalent association of α and β chains. In humans there are three known different isotypes of MHC-II molecules (HLA-DR, -DQ and -DP), which are encoded by highly polymorphic A and B genes for α and β chain respectively, arranged in gene clusters with HLA-DM, TAP and LMP genes. Products of these genes together with the invariant chain (Ii) take part in the assembly and loading of the MHC-II/antigen complex. In mice, there are two MHC-II isotypes: IA and IE. All MHC-II genes are regulated at the transcriptional level, which involve both a conserved promoter region and a specific set of transcription factors. Several highly conserved *cis*-acting elements, including S, X, X2 and Y boxes are present in the 150 bp proximal region of the MHC-II promoter (1, 2). The presence of that region is both necessary and sufficient for expression of the MHC genes

although the optimal level of expression may require additional enhancer elements located further up-stream from promoter region (3). Furthermore, it appears that the presence and spacing between regulatory elements is important for interaction of transcription factors. The overall process of assembly of the transcriptional complex on the promoter is coordinated by the class II transactivator (CIITA) protein – the so called "master regulator" of MHC transcription. Both constitutive and inducible expression of MHC-II and in certain degree class I antigens are dependant on the presence of CIITA (4, 5). Abrogation of MHC-II genes expression due to a mutation in the CIITA gene leads to the development of the bare lymphocyte syndrome (BLS) . Four different promoters (designated as PI to PIV) have been described in the human CIITA gene. Unlike humans, only three promoters are present in the mouse gene (6). Depending on the cell type and the type of stimulus, three different CIITA mRNAs were isolated, which differed in the first exon as a result of utilization of different promoter. The activity of a particular CIITA promoter as well as its responsiveness to a particular stimulus (cytokines, bacterial cell products) is highly tissue specific (6). The PI promoter is constitutively active in B-cells, PIII is active in dendritic cells and PIV is primary responsible for IFN-γ induced expression in macrophages and other cells . Recently PIII was also shown to be under control of IFN-γ (7, 8). The transcript of the PII promoter has not yet been found. The efficient activation and interaction of STAT1 and USF-1 transcription factors were shown to be of particular importance for IFN-γ induced expression of CIITA (9). The CIITA does not directly interact with DNA but rather coordinates binding of a number of other transcription factors (RFX, X2BP, NF-Y, CREB) to an appropriate region in the MHC gene promoter (2).

MHC-II is a very attractive target for pathogens which survival in the host can be prolonged if the antigen presentation by the infected macrophage is less efficient. In fact, it was

observed that infection of cells with certain microorganisms, including *Mycobacterium* and viruses, leads to the down-regulation of both MHC-I and MHC-II expression (10-12).

There are three general mechanisms of blocking the function of MHC by pathogens: 1) direct disruption of assembly of MHC complex; 2) inhibition of signal transduction pathways such as IFN-γ which is essential for upregulation of MHC expression in phagocytes; and 3) upregulation of production of certain cytokines, such as IL-10 which has an inhibitory effect on MHC expression.

In our previous studies we were able to demonstrate the inhibitory effect of *M. bovis* BCG on IFN-γ stimulated expression of MHC- II in bone marrow derived murine macrophage cell lines (13). The effect of LPS on MHC-II expression is dependent on both the cell type and the stimulation protocol used. Therefore, the initial goal of the present study was to establish the effect of LPS on MHC-II and CIITA expression in IFN-γ-stimulated macrophages. In addition, we were interested in determining the role of Toll-like receptor 4 (TLR4) in LPS and *M. bovis* BCG induced attenuation of CIITA and MHC-II expression. Although it was shown that responses to the mycobacterial stimuli are mediated by TLR2 but not TLR4 the results published recently suggested that both of these receptors were involved in activation of dendritic cells by *Mycobacteria* (14). We also assessed the possible involvement of the MyD88 (myeloid differentiation protein) accessory protein in the modulation of MHC-II and CIITA genes expression since the alternative pathway for the TLR4 signaling was recently discovered (15, 16).

MATERIALS AND METHODS

Reagents and cells

Dulbecco's modified Eagle Medium (DMEM), penicillin/streptomycin and Dulbecco's Phosphate Buffered Salt (DPBS), the nick translation DNA labeling kit and the Trizol reagent were purchased from GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) characterized for low level of endotoxin was obtained from Hyclone (Logan, UT, USA). Recombinant murine interferon gamma (IFN-γ) was purchased from Amgen (Thousand Oaks, CA, USA). Goat anti-rabbit IgG and rabbit anti-mouse IgG polyclonal horseradish peroxidase conjugated antibody and lipopolysaccharide from *Escherichia coli* serotype 0111:B4 were purchased from SIGMA Chemicals (St.Louis, MO, USA). The phenol re-extracted LPS was prepared according to the protocol published previously (17). Rabbit anti-murine phospho-STAT1 (Tyr701) polyclonal Ab and T4 polynucleotide kinase were purchased from New England Biolabs (Mississauga, ON, Canada). Rabbit polyclonal Ab against mouse p84/p91 (STAT1) and USF-1 and goat anti-human/murine CD64 (Fc receptor type I) Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against murine Fc receptor type II and III (FcBlock), FITC anti mouse I-A^b and FITC anti mouse I-A^k Ab were obtained from Pharmingen Canada (Mississauga, ON, Canada). α^{32}P-dCTP and γ^{32}P-ATP were purchased from Amersham (Amersham, UK). The chemiluminescent reagent SuperSignal was obtained from PIERCE (Rockland, IL, USA). Poly dI:dC was obtained from Roche Diagnostic Corp. (Indianapolis, IN, USA). The Complete protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Germany). Macrophage cell lines were obtained as previously described (18, 19) by immortalization of the bone marrow derived macrophages from B10A.Bcg^r (B10R

cell line), C57BL/10ScCr (TLR4-Del cell line) and MyD88-/- (MyD88-KO cell line). Cell lines were cultured in DMEM supplemented with 6% heat inactivated fetal bovine serum and penicillin/streptomycin antibiotic mixture. The subconfluent cell cultures were used for all the experiments. *Mycobacterium bovis* BCG strain Montreal was propagated according to the procedure described previously (13) and stored frozen in 15% glycerol in -80°C.

FACS analysis of cell surface Ia expression

Macrophage cell lines were plated at a concentration of 0.5 – 1 million per ml and treated with appropriate stimuli for 48 h. Cells were stained using strain specific anti I-A^k (B10R) and I-A^b (MyD88-KO) antibodies directly labeled with FITC following the protocol described before (13). Stained cells were analyzed using a dual laser FACstar Plus flow cytometer (Becton Dickinson). A green fluorescence histogram of 1000 channel resolution was collected from 10,000 cells counted for each sample analyzed. The FACS data were analyzed and processed using WinMDI software.

Northern blot analysis

The analysis of steady-state levels of mRNA expression was performed as described previously (13). Briefly total RNA was isolated from 5-10x10^6 cells plated on 10 cm plastic Petri dish plates using Trizol reagent according to manufacturer's recommendations. 15μg of RNA was run on 1.2% formaldehyde gel and transferred to nylon membrane. After overnight hybridization with DNA probes labeled with ^{32}P using nick translation method, autoradiograms were scanned using PhosphoImager (Storm 860, Molecular Dynamics, Sunnyvale, CA) and

analyzed using ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA). Generation of specific I-A_β and CIITA probes was provided previously (13).

Analysis of STAT1 phosphorylation

Macrophages were plated at 1 million per ml concentration on 24-well plates stimulated with 10 U/ml of recombinant IFN-γ in the absence or presence of 100 ng/ml of LPS or *M. bovis* BCG at ration 10 bacilli per 1 macrophage, washed with DPBS and lysed directly with SDS-PAGE sample buffer. 15-20 μg of total protein cell lysate was resolved on 10% SDS-PAGE and transferred to PVDF membrane (Millipore). The non-specific background was blocked by overnight incubation at 4°C in 5% solution of non fat skimmed dry milk (Cornation) in PBS with addition of 0.1% of Tween-20 (PBS-T). The membranes were incubated for 1 h at room temperature first with anti-phospho STAT1 (1:2000 dilution in 5% milk in PBS-T) and then after washing in PBS-T with anti-rabbit horseradish peroxidase conjugated antibodies (1:5000 dilution in 5% milk in PBS-T). The signal was visualized using chemiluminescence SuperSignal reagent and exposed to X-ray film. To assess total amount of STAT1 protein the anti phospho-STAT1 antibodies were removed by 30 minutes incubation at 55°C in buffer containing 62.5 mM Tris-HCl pH 6.7, 2% SDS and 70 mM β-mercaptoethanol, and membrane was incubated with anti-STAT1 antibodies following the same protocol.

DNA binding assay

To obtain nuclear extracts 10^7 cells were harvested and washed two times in ice cold DPBS. After transferring to eppendorf tube the cells were lysed by incubation on ice for 5-10 minutes in 1 ml of solution A (10mM HEPES pH 7.4, 15mM KCl, 0.1mM EDTA, 2mM $MgCl_2$,

0.5% NP-40 supplemented with protease inhibitors mix, 1mM, 1mM Na_3VO_4 and 10mM NaF). After spinning down (1000 rpm, 5 minutes, 4°C) and removing the supernatant, the nuclei were washed with solution A without NP-40. The nuclear membranes were disrupted by gentle lysis with drop wise added 100 µl of lysis buffer (25mM HEPES pH 7.4, 1.8M KCl, 0.1mM EDTA, 2mM $MgCl_2$, 20% glycerol with addition of protease inhibitors mix, 1mM DTT, 1mM Na_3VO_4 and 10mM NaF) and 15 minutes incubation on ice. Next 200 µl of solution B (25mM HEPES pH 7.4, 0.1mM EDTA, 20% glycerol with addition of protease inhibitors mix, 1mM DTT, 1mM Na_3VO_4 and 10mM NaF) was added and lysates were incubated for 5 minutes on ice with occasional agitation. After centrifugation at maximum speed at 4°C for 20 minutes the protein concentration in the supernatant was estimated using Bradford method (Bio-Rad). Lysates were kept frozen at -80°C.

The DNA probes used in EMSA were obtained based on the sequence published by Muhlethaler-Mottet et al. (6). The GAS element containing probe (5'-AGC TTC TGA GAA AGC ACG T-3') and the IRF1/2 element containing probe (5'-ACA GAA AGT GAA AGG GGG-3') were generated by annealing synthetic oligonucleotides with complementary sequences. The probe spanning GAS, E-Box and IRF1/2 elements was obtained by PCR amplification of mouse genomic DNA extracted from B10R macrophage cell line using as primers GAS and IRF1/2 oligonucletides. The probes were end-labeled with ^{32}P using T4 polynucleotide kinase according to the vendor's recommendations.

Nuclear extracts were pre-incubated for 15 minutes at room temperature with poly dI:dC (1µg per sample) in binding buffer (25mM HEPES pH 7.5, 10% glycerol, 50mM NaCl, 0.05% NP-40 and 1mM DTT) and were subsequently incubated for 30 minutes at room temperature with 10,000-20,000 cpm of labeled DNA probe. The DNA-protein complexes were resolved on

4% non-denatureting polyacrylamide gel containing 0.01% NP-40, dried and exposed to X-ray film.

RESULTS

IFN-γ-induced expression of MHC-II protein is down-regulated by lipopolysaccharide from Gram-negative bacteria cell wall in macrophages

Our previous studies revealed that IFN-γ induced expression of MHC-II antigens is inhibited when macrophages are co-stimulated *in vitro* with *Mycobacterium bovis* BCG (13). We demonstrated that MHC-II expression is attenuated in macrophages infected with *M. bovis* BCG and this attenuation involves CIITA. The effect of endotoxins, such as LPS, derived from cell wall of Gram-negative bacteria, on the regulation of expression of MHC-II and antigen presentation seems to be cell type specific (20-25). In the present study we focused our analysis on the molecular mechanism responsible for the inhibition of IFN-γ-induced MHC-II expression by LPS. As shown in figure 1 LPS was able to inhibit MHC-II protein expression with a similar efficiency to what we previously reported for BCG (Fig.1 A and B). Since the LPS is a very potent inducer of nitric oxide (NO) production in macrophages, we wanted to test if the inhibition of iNOS activity and NO production would affect the ability of LPS to inhibit IFN-γ-induced MHC-II expression. As shown in figure 1C, the inhibitory effect of LPS was not dependent on the production of nitric oxide. These results were also corroborated using a macrophage cell line derived from iNOS knockout mice (in the absence of the nitric oxide

production we still observed the inhibitory effect of LPS on MHC-II expression; data not shown). Next, we tested the effect of LPS on IFN-γ-induced I-A_β and CIITA mRNA expression in macrophages. As shown in Fig. 1D, LPS was able to inhibit IFN-γ-induced expression of I-A_β and CIITA mRNA. We have previously reported that infection of macrophages with BCG inhibits IFN-γ-induced expression of MHC-II. This inhibition was associated with the down-regulation of CIITA mRNA expression. Since the CIITA is the so called master regulator of MHC-II genes transcription, its inhibition by BCG consequently leads to lower levels of MHC-II antigens. As shown in fig.1D, a similar phenomenon was observed when cells were activated with IFN-γ in the presence of LPS.

STAT1 protein phosphorylation is not affected by LPS or M. bovis BCG

The precise molecular mechanism responsible for the inhibition of CIITA by either *M. bovis* BCG or LPS is unknown. It has been previously shown that IL-1β, TNF-α and TGF-β can down-regulate expression of both CIITA and MHC-II (26-28). Both TNF-α and IL-1β as well as BCG and LPS are all able to induce NFκB activation in macrophages and utilize partially overlapping signal transduction pathways. To investigate the molecular mechanism involved in MHC- II inhibition we focused our study on the effect of LPS and *M. bovis* BCG on CIITA and MHC-II expression. First, we tested whether the IFN-γ signal transduction pathway is directly affected in macrophages by the treatment of the cells with LPS or *M. bovis* BCG. Since the activation of STAT1α by JAK1/2 kinases represents a crucial event in IFN-γ signaling, we decided to test if the tyrosine phosphorylation of STAT1 was impaired by either *M. bovis* BCG or LPS. We stimulated B10R macrophages for 10, 30 and 60 minutes with IFN-γ alone or with addition of LPS or BCG and the tyrosine phosphorylation levels of STAT1 protein were assayed

using a Western-blot analysis. As shown in figure 2 we found no impairment of STAT1α/β phophorylation by neither LPS nor *M. bovis* BCG.

Stimulation of macrophages with LPS or M. bovis BCG does not affect IFN-γ-induced binding of transcription factors to the CIITA promoter

Although IFN-γ-induced phosphorylation of STAT1 was not affected by LPS or BCG it was possible that the binding of nuclear factors to the CIITA promoter was impaired by treatment of cells with these stimuli. In macrophages, there is no constitutive expression of MHC-II antigens. IFN-γ up-regulates CIITA expression by induction of the CIITA promoter IV activity. Since it has been shown that LPS affects serine 723 phosphorylation of STAT1 which in turn may affect binding of STAT1 to the CIITA promoter, we decided to compare binding of STAT1 to the GAS element in IFN-γ inducible CIITA promoter IV in macrophages stimulated with IFN-γ alone or in combination with LPS or BCG. Figure 3A shows the kinetic of binding of nuclear proteins to STAT1 and IRF1/2 binding elements from the CIITA promoter IV. Binding to the GAS element is strongest at 2 h following IFN-γ stimulation. We therefore decided to focus on this time point for subsequent analysis. As presented in figure 3B, there was no significant difference in STAT1α binding to the GAS element in nuclear extracts from macrophages treated with IFN-γ in the presence of LPS or BCG as compared to cells stimulated only with IFN-γ. It was shown that commercially available preparations of LPS are able to induce biological effects in cells from C3H/HeJ mice which express mutated non-functional TLR4. The simple phenol re-extraction of LPS removes compounds which activate cells via receptors other than TLR4 (17). The exposure of macrophages to phenol purified LPS (LPS-PE)

had the same inhibitory effect on MHC-II expression as un-purified LPS preparation (data not shown). Therefore, we decided to compare the effect of both LPS preparations on DNA binding activity of STAT1. As shown in figure 3B both LPS preparations had no effect on IFN-γ-induced DNA binding activity of STAT1. The presence of STAT1α in the complexes was demonstrated using specific antibodies in a super shift assay.

However, it was still possible that cooperative binding of STAT1, USF-1 and IRF-1/2 (7, 29), all of which were previously shown to be necessary for efficient CIITA expression, might be affected by LPS or BCG. In order to test this hypothesis we generated a probe spanning the CIITA promoter IV region containing the GAS, E-box and IRF1/2 elements. As shown in figure 4 A and B neither STAT1α nor USF-1 binding was decreased in nuclear extract from macrophages stimulated with IFN-γ and LPS when compared to IFN-γ alone. Overall the analysis of binding of nuclear proteins to the full length sequence of CIITA promoter IV (data not shown) or to the smaller probe spanning GAS, E-box and IRF-1/2 elements did not reveal any significant differences in binding patterns between nuclear extracts from macrophages stimulated with IFN-γ alone or IFN-γ and LPS.

IFN-γ-induced expression of MHC class II and CIITA mRNA is inhibited by M. bovis BCG but not by LPS in TLR4-Del macrophages

Recently, there has been great interest in the understanding of the role of Toll-like receptors in the mechanism mediating interaction of the host cells with microorganisms. The receptors belonging to the family of toll like receptors were shown to be responsible for recognition of bacterial products by cells from variety of different species. In mammals, Toll-like receptor 4 has been shown to mediate responsiveness to LPS (30, 31). However the ability to

bind LPS has been reported also for other proteins like macrophage scavenger receptor and leukocyte CD11/CD18 beta 2 integrins (MAC-1) (32, 33). To test the role of Toll-like receptor 4 in the regulation of IFN-γ-induced expression of CIITA and I-A_β mRNA in macrophages treated with LPS or *M. bovis* BCG we used a macrophage cell line derived from the bone marrow of C57BL/10ScCr mice (TLR4-Del macrophage cell line), which have a null mutation (deletion) in the Toll-like receptor 4 gene. TLR4-Del macrophages were infected with *M. bovis* BCG at the ratio of ten bacteria per one macrophage cell or treated with 100 ng per ml of LPS in the presence of IFN-γ. As shown in figure 5, stimulation of TLR4-Del macrophages with 10 U per ml of IFN-γ for 18 h resulted in a substantial induction of both I-A_β and CIITA mRNA expression. Treatment of IFN-γ-stimulated TLR4-Del macrophages with *M. bovis* BCG, but not with LPS, resulted in a significant inhibition of both CIITA and I-A_β mRNA expression. These results suggest that LPS requires TLR4 for inhibition of I-A_β mRNA whereas the effect of *M. bovis* BCG on MHC-II expression is TLR4 independent and most likely uses Toll-like receptor 2 (TLR2). TLR2 was shown to be involved in the stimulatory effect of *Mycobacterium* on activation of NFκB and expression of TNF-α (34, 35).

Inhibitory effects of LPS and BCG on IFN-γ - induced expression of MHC class II are mediated through MyD88 protein

In the toll-like signal transduction pathway MyD88 protein plays an important role as an adaptor protein which couples particular receptor with common downstream components of the signaling pathways. It seems that several toll-like receptor signaling pathways merge at the level of MyD88 together with the IL-1 receptor signal. Recently two independent laboratories described a novel adaptor protein called MAL/TIRAP (15, 16), which mediates signal

transduction from TLR4 but not from TLR2 or TLR9. Discovery of the second adaptor protein involved in Toll-like system signaling could explain the previously observed phenomenon that LPS could induce the differentiation of dendritic cells as well as activation of NFκB in cells lacking a functional MyD88. We wanted to test whether the inhibitory signals from either LPS or/and BCG require MyD88 protein or utilize an alternative MAL/TIRAP based pathway. In order to address this question, we treated IFN-γ-stimulated macrophages derived from MyD88 knockout (MyD88 -/-) mice with LPS (100 ng/mL), *M. bovis* BCG (10:1 bacilli to macrophage ratio) for 48 h and then analyzed MHC-II surface expression by FACS using anti-Ia antibodies. As shown in figure 6, we did not observed any effect of LPS or BCG on the expression of MHC-II in MyD88 - KO cells. In addition, we analysed both CIITA and I-A_β mRNA expression in MyD88 deficient macrophages following overnight exposure to LPS or BCG either in the presence or absence of IFN-γ. As shown in figure 6D neither LPS nor BCG had an inhibitory effect on IFN-γ-stimulated expression of CIITA or MHC-II mRNA expression. Results presented in figure 6 allow us to conclude that MyD88 protein mediates the inhibitory signals of both LPS and *M. bovis* BCG in macrophages treated with IFN-γ. These findings demonstrated that MyD88 accessory protein is absolutely necessary for mediation of both LPS and BCG inhibitory signals. Overall, these data show that macrophage activation by IFN-γ can be regulated by the stimuli that utilize Toll-like signaling system via the MyD88 accessory molecule. Further studies are required to elucidate the exact molecular link between Toll-like receptor and IFN-γ pathway.

DISCUSSION

In recent years a great deal of research has focused on studying the role of the MHC system in the immunological processes. The importance of antigen processing and presentation in host defense, transplantation, autoimmunity and cancer development is now well known and understood. The MHC system plays a crucial role in the process of the immunological response and therefore the development of a successful treatment for health conditions in which the immunological response is involved requires broad knowledge of both the function and regulation of expression of MHC antigens. In this paper we report the results of our studies on the molecular mechanism of inhibition of IFN-γ-induced expression of MHC-II antigens and CIITA in macrophages exposed to *M. bovis* BCG and lipopolysaccharide (LPS) extracted from *E. coli*. In our previous studies we demonstrated that IFN-γ induced expression of I-A_β was attenuated when macrophages were infected with *M. bovis* BCG (13). The precise molecular mechanism of this effect remains to be established. Our studies aimed to address the mechanism of inhibitory effect of *M. bovis* BCG and Gram-negative bacteria endotoxin on IFN-γ-induced MHC-II expression in macrophages.

The effect of endotoxins, in particular lipopolysaccharide (LPS), a virulent factor and a structural component of Gram–negative bacteria cell wall, on expression of MHC-II was studied by many investigators. It was shown that injection of mice with LPS leads to the augmented expression of MHC-II antigens on peritoneal macrophages as well as on cells isolated from kidney, liver, heart, lung, and pancreas (20-22).

The *in vitro* effect of LPS on MHC-II expression differs depending on the cell type. For example exposure of B-cells to LPS up-regulated MHC-II expression (36), while in macrophages LPS down-regulated IFN-γ-stimulated expression of MHC-II (37, 38). However in human

endothelial cells LPS augmented class I MHC (HLA-A,B,C) expression but inhibited IFN-γ induced expression of class II MHC (HLA-DR) molecules (39).

The precise molecular mechanism of the adversary effect of LPS on MHC-II expression remains to be established. The data presented in this and our previous paper (13) suggests that down-regulation of MHC-II expression follows the inhibition of CIITA mRNA expression (Fig.1 and 5). An analogous phenomenon was observed in IFN-γ-stimulated primary murine renal tubular epithelial cells (F1K cells) where LPS treatment resulted in down-regulation of both MHC-II and CIITA genes (23). Since the expression of MHC-II directly depends on the presence of CIITA, we decided to more closely investigate the mechanism of inhibition of CIITA expression by LPS. It seems that CIITA expression is mainly regulated at the transcriptional level although mRNA stability has been also indicated as a mechanism of regulation of CIITA expression (26). IFN-γ is a very potent activator of CIITA transcription and therefore any alterations in the IFN-γ signaling pathway should affect CIITA expression. In fact, many pathogens target the IFN-γ signal transduction pathway directly as a part of their survival strategy in the host (40-42). For example in *M. avium* infected macrophages the down-regulation of IFN-γ receptor expression as well as STAT1 activation was correlated with decreased expression of I-E (MHC-II), IRF-1, Mg21 (encoding GTP-binding protein) and CIITA mRNA (43).

The IFN-γ induced expression of CIITA in macrophages was shown to be under the control of the CIITA promoter IV with a contribution from promoter III (7). The analysis of the sequence of the CIITA promoter IV revealed the importance of the region proximal to the transcription start site containing the STAT1 binding GAS element, the IRF1/2 binding element and the E-box which is constitutively occupied by the USF-1 transcription factor. The interaction

of STAT1 and USF1 was shown to be necessary for efficient induction of CIITA by IFN-γ (29). The importance of IRF-2 transcription factor was also demonstrated using IRF-2 knockout mice (44). Our results suggested that both LPS and *M. bovis* BCG do not affect STAT1 phosphorylation (Fig.2). Also, binding of STAT1 to the GAS element of CIITA promoter IV (Fig.3) as well as to the fragment of that promoter spanning through the regions containing IRF1/2, E-Box and GAS elements (Fig.4) does not seem to be affected by LPS or BCG treatment as shown using EMSA. In addition the IFN-γ-induced binding of the proteins to IRF1/2 element as well as USF-1 protein to E-box were not altered by treated with LPS or *M. bovis* BCG. These findings are supported by studies on the effect of IL-1β and TGF-β on the CIITA expression, which showed that IFN-γ signal transduction pathway was not affected when cells were treated with these two cytokines (27, 28). Similar studies on the effect of LPS on differentiation and the mechanism of CIITA silencing in dendritic cells (DC) showed that DNA binding activity of transcription factors to CIITA promoters I and III was not affected by LPS (45). In the same study it was suggested that general de-acetylation of chromatin in the CIITA promoter region might be responsible for the diminished transcriptional activity of this gene in differentiating dendritic cells. Whether a similar mechanism is responsible for the inhibitory effect of LPS on IFN-γ-induced expression of CIITA in macrophages remains to be established. Another general mechanism of down-regulation of gene expression may involve competition of signal transduction pathways for common factors which exist in limited quantities. Since both IFN-γ and LPS are very strong activators of transcription it is possible that this kind of competition leads to diminished efficiency of CIITA transcription. In fact CIITA itself competes with NF-AT for CBP/p300 cofactors which are required for efficient transcription of many genes. It was demonstrated that this kind of competition led to down-regulation of IL-4 expression (46).

The inhibition of IFN-γ-stimulated MHC-II and CIITA expression does not seem to be specific for LPS or *M. bovis* BCG. We observed that similarly to LPS and *M. bovis* BCG, TNF-α and IL-1β could also inhibit MHC-II expression (data not shown). The inhibitory effect of IL-10 as well as IL-4, TNF-α, IFN-α/β and TGF-β on IFN-γ induced expression of MHC-II and CIITA genes was observed in variety of cell types including murine microglial, human astroglioma, fibrosarcoma and fibroblast cells as well as macrophages (28, 47-51). TNF-α was also shown to lower the stability of CIITA mRNA and to inhibit MHC-II expression induced by IFN-γ in human fibrosarcoma cell line HT1080 (26). An inhibitory effect of LPS might therefore be attributed to the autocrine action of cytokines secreted by activated macrophages during their response to IFN-γ and LPS stimulation.

The inhibitory effects of LPS and TNF-α on MHC-II genes expression was previously correlated with their ability to induce NO production (52, 53). The demonstrated results of the inhibitory effect of NO on both CIITA and MHC-II expression using SNAP as an external donor of NO (54). The authors demonstrated that the inhibitory effect of NO could be observed only when SNAP was added to the cells at the same time as IFN-γ. Our results however demonstrated that the inhibition of MHC-II expression by LPS can be still decreased even when NO production was absent or inhibited by treating the cells with aminoguanidine. In our experimental model, the production of NO could be observed only after at least 12 h exposure to IFN-γ and LPS, at a time where it does not affect CIITA or MHC-II expression (54). In addition we observed a similar inhibition of MHC-II by both LPS and *M. bovis* BCG in macrophages derived from iNOS knockout mice (data not shown).

The possible involvement of tyrosine phosphatases in the process of down-regulation of IFN-γ signaling was suggested in studies on the effect of *M. tuberculosis* and *L. donovani* on the

infected macrophages' responsiveness to IFN-γ stimulation. It was shown that the *Src* Homology 2 containing Tyrosine Phosphatese 1 (SHP-1) activity is augmented in macrophages treated with lipoarabinomannan from *M. tuberculosis* (55) or in macrophages infected with *L. donovani* (40, 42) leading to dephosphorylation of JAK1/2 kinases and consequently to inhibition of IFN-γ signaling pathway. To test whether the inhibitory effect of LPS and *M. bovis* BCG on IFN-γ stimulated expression of CIITA or MHC-II depends on activation of SHP-1, we used a macrophage cell line derived from SHP-1 deficient mice. We found that the inhibitory effect of LPS or *M. bovis* BCG on IFN-γ-induced expression of both CIITA and MHC-II was intact in these cells (data not shown). These data suggest that SHP-1 activation does not play a major role in LPS or *M. bovis* BCG induced inhibition of MHC-II expression.

Detailed analysis of all events which lead to activation of LPS signal transduction pathway is required in order to fully understand its effect on MHC-II gene expression. In recent years great progress has been achieved in describing the mechanism of LPS action resulting from identification of the Toll like receptor 4 as the *Lps* gene (30, 31) and its characterization. The signal transduction pathway for LPS and other ligands which require Toll-like receptors was described and studied in detail (56, 57). It was shown that Toll-like receptors have a structure similar to IL-1β receptor especially in the cytoplasmic domain. Due to this similarity, the LPS signal transduction pathway uses the same downstream mediators as the IL-1β pathway. In addition, general toll/IL-1 and TNF-α signal transduction pathways partially overlap at the level of TRAF-6 protein and activate NFκB. This may explain the similar effect of all these stimuli on IFN-γ induced expression of CIITA and MHC-II genes. It was shown that TLR4 interacts mainly with gram-negative bacterial cell wall components whereas TLR2 is responsible for binding gram-positive as well as *Mycobacteria* derived ligands (56). We observed however that

commercially available preparation of LPS from gram-negative bacteria which contain other compounds, in addition to LPS, may still activate TLR4 deficient cells in the presence of a second activator like IFN-γ (data not shown). Therefore, we tested whether the inhibitory effect of LPS on IFN-γ stimulated expression of MHC-II and CIITA is TLR4 dependent. Using a macrophage cell line derived from TLR4 deletion mutant mice of the C57B10/ScCr strain we were able to demonstrate that LPS inhibitory effect requires TLR4 (Fig.5). On the other hand the *M. bovis* BCG inhibitory effect is independent of TLR4 (Fig.5). Both TLR4 and TLR2 interact with MyD88 adaptor protein whose presence was shown to be essential for activation of NFκB by bacterial products. It was also reported that DC from MyD88 knockout mice undergo differentiation upon LPS stimulation (58, 59) suggesting that LPS may also activate a MyD88 independent pathway. Recently two independent laboratories reported that TLR4 uses a second adaptor protein named MAL/TIRAP which enables MyD88 deficient cells to respond to LPS (15, 16). We demonstrated that the surface expression of MHC-II as well as mRNA expression of both MHC-II and CIITA genes were not affected by LPS or *M. bovis* BCG in macrophages derived from MyD88 deficient mice indicating an important role of MyD88 accessory protein in regulation of MHC-II genes expression in macrophages.

Overall, our results indicate that LPS is a very potent inhibitor of IFN-γ stimulated expression of MHC-II antigens. The mechanism of that inhibition involves most likely transcription of MHC-II genes since expression of CIITA mRNA is affected by LPS. The precise mechanism of that phenomenon has to be studied further, as such studies may help understand regulation of expression of MHC-II and eventually design better treatment strategies in infectious and autoimmune diseases.

FIGURE LEGENDS

FIGURE 1. LPS inhibits MHC-II expression. The surface expression of I-A_β antigen was analyzed by FACS in B10R macrophages which were stimulated for 48 h with IFN-γ [10 U/mL] and *M. bovis* BCG [ratio: 10 bacteria per 1 macrophage] (A), or IFN-γ and LPS [100 ng/mL] in the absence (B) or presence (C) of aminoguanidine [0.5 mM]. Panel D: the effect of LPS on IFN-γ-stimulated expression of I-A_β and CIITA mRNA in B10R macrophages stimulated for 18 h with 10 U/mL of IFN-γ with or without LPS [100 ng/mL].

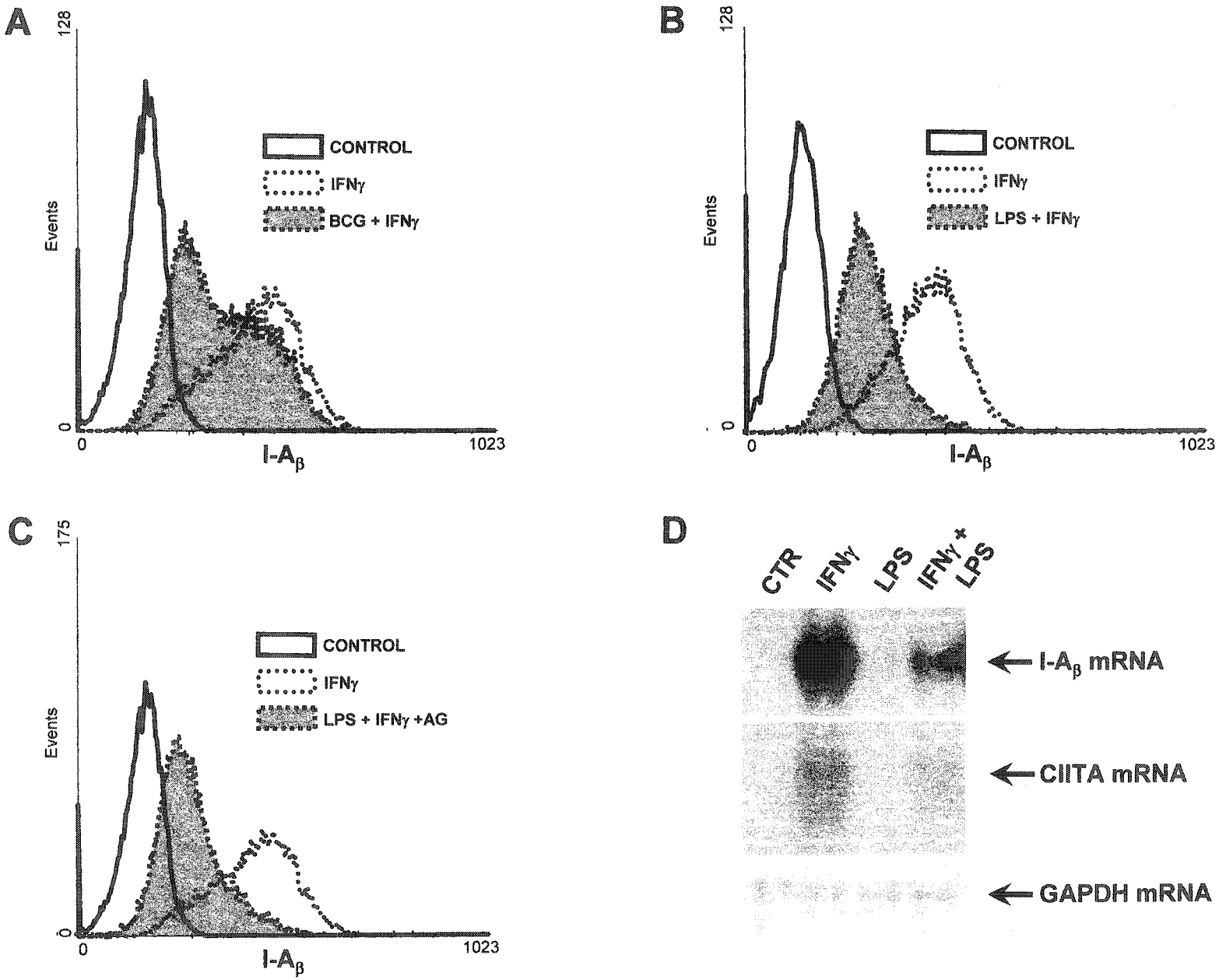

A
128
Events
0
0
1023
I-Aβ
CONTROL
IFNγ
BCG + IFNγ
B
128
Events
0
0
1023
I-Aβ
CONTROL
IFNγ
LPS + IFNγ
C
175
Events
0
0
1023
I-Aβ
CONTROL
IFNγ
LPS + IFNγ +AG
D
CTR
IFNγ
LPS
IFNγ + LPS
I-Aβ mRNA
CIITA mRNA
GAPDH mRNA

FIGURE 2. The effect of LPS and BCG on IFN-γ-stimulated tyrosine phosphorylation of STAT1 protein in macrophages. B10R macrophages were stimulated for the indicated amount of time with IFN-γ alone or in combination with LPS or *M. bovis* BCG. The levels of phosphorylation were analyzed by Western-blot using specific anti-phospho STAT1 antibodies (upper line). The total amount of STAT1 was subsequently analyzed using specific anti-STAT1 antibodies after stripping of the membrane (lower line).

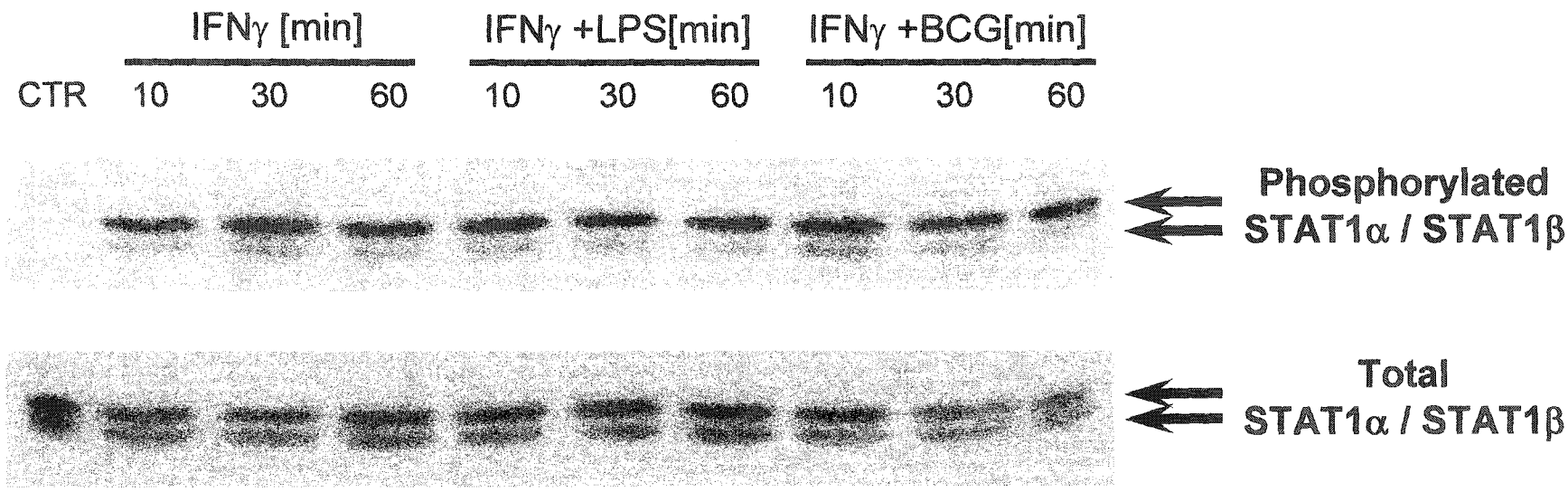

IFNγ [min]
IFNγ +LPS[min]
IFNγ +BCG[min]
CTR
10
30
60
10
30
60
10
30
60
Phosphorylated
STAT1α / STAT1β
Total
STAT1α / STAT1β

FIGURE 3. Binding of STAT1α to GAS element in CIITA promoter IV is not affected by LPS or BCG treatment. A. Time-dependent binding of nuclear proteins to radio-labeled DNA probes derived from GAS and IFR1/2 binding sites of CIITA promoter IV. Nuclear extracts were isolated from nuclei of B10R macrophage cell line stimulated with 10 U/mL of IFN-γ for the indicated amount of time. B. Neither LPS nor BCG affected IFN-γ-induced binding of STAT1α to GAS element derived from CIITA promoter IV. B10R macrophages were stimulated for 2 h with IFN-γ [10 U/mL] alone or in combination with 100 ng/mL of LPS, LPS re-extracted with phenol (LPS-PE) [100 ng/mL] or with *M. bovis* BCG [ratio: 10 bacteria to 1 macrophage]. The controls for LPS, LPS-PE and BCG alone were also included. The proteins which bound to GAS element from CIITA promoter IV was "super shifted" using specific anti-STAT1α Ab (right part of panel B). The arrows indicate the position of the probe/Stat1α complex on the gel alone (STAT1α) or the same complex further retarded by using specific anti-Stat1 antibodies (SS STAT1α). "FP" indicates lines were free probes were loaded.

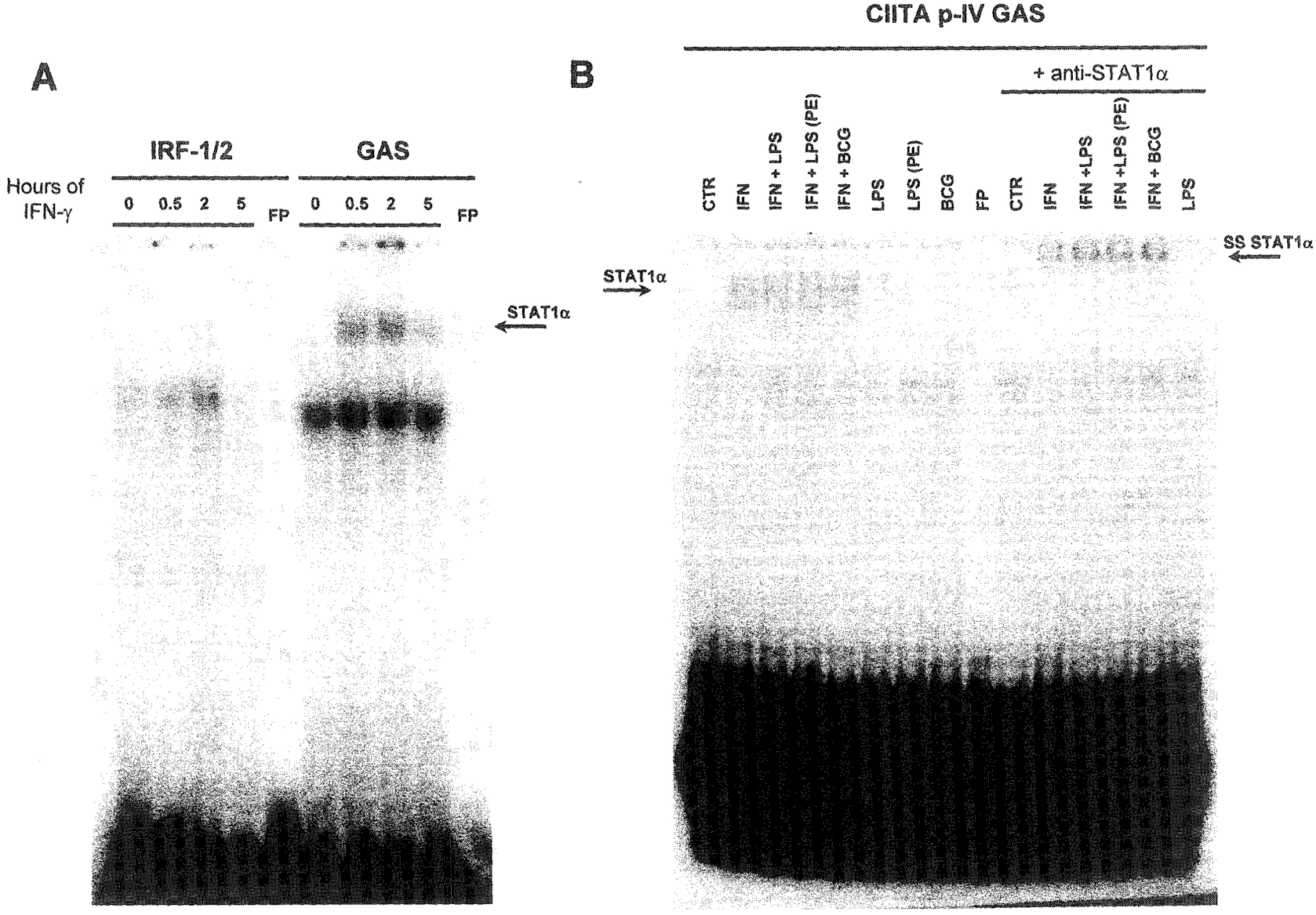
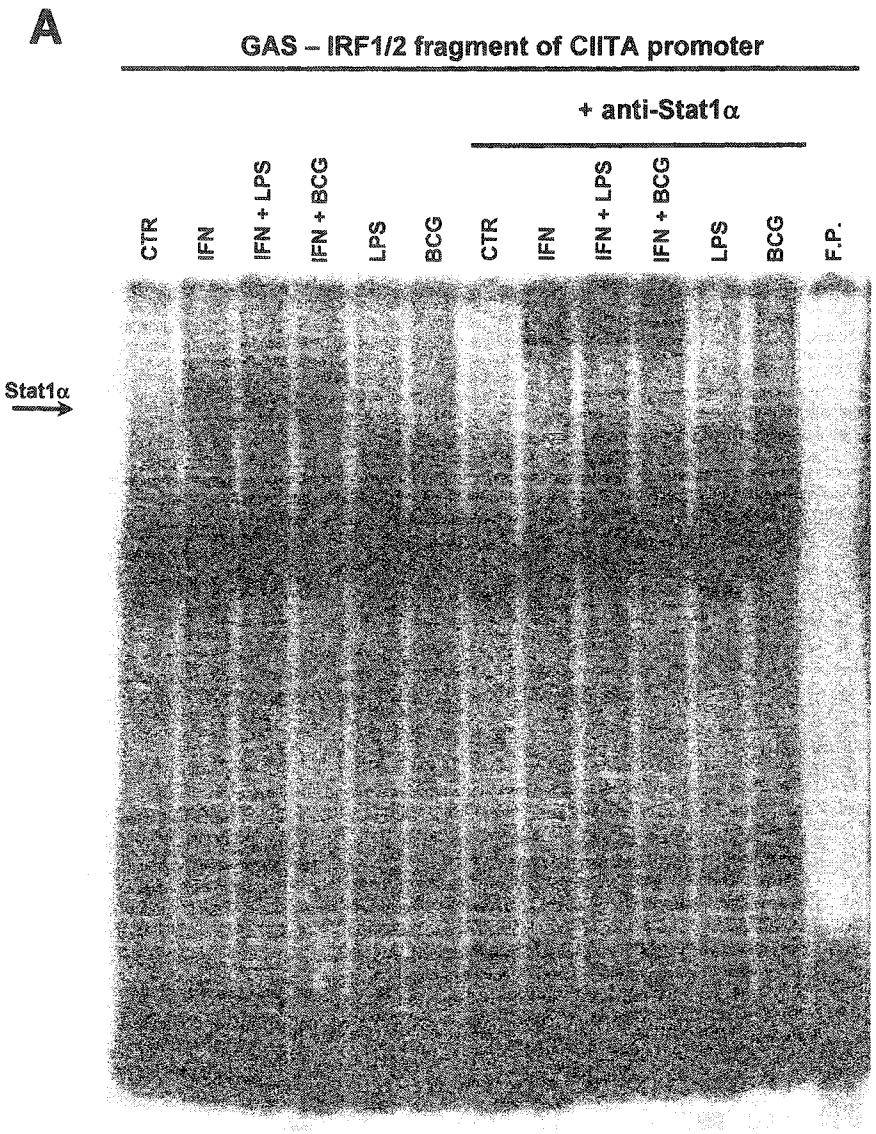

A
IRF-1/2
GAS
Hours of IFN-γ
0
0.5
2
5
FP
0
0.5
2
5
FP
STAT1α
B
CIITA p-IV GAS
+ anti-STAT1α
CTR
IFN
IFN + LPS
IFN + LPS (PE)
IFN + BCG
LPS
LPS (PE)
BCG
FP
CTR
IFN
IFN +LPS
IFN +LPS (PE)
IFN + BCG
LPS
SS STAT1α
STAT1α

FIGURE 4. Binding of STAT1α and USF-1 to the probe derived from the CIITA promoter IV containing GAS/E-box/IRF sites is not altered upon LPS or BCG treatment. A. Binding of STAT1α to large fragment of CIITA promoter IV is not affected by LPS or BCG. Nuclear extracts from B10R macrophages were incubated with probe derived from the fragment of CIITA promoter IV containing the GAS, E-Box and IRF1/2 binding elements. To identify complexes containing STAT1, a "super shift" assay was performed using anti-STAT1α Ab (right part of panel A). B. Binding of USF-1 to CIITA promoter IV is not affected by LPS or BCG. The assay was performed as in panel A except that an anti-USF-1 Ab was used to identify bands containing USF-1 bound to DNA probe. The arrows indicate the position of the probe/Stat1α complex on the gel alone (STAT1α) or the same complex further retarded by using specific anti-Stat1 antibodies (SS STAT1α). "FP" indicates lines where free probes were loaded.


A
GAS – IRF1/2 fragment of CIITA promoter
+ anti-Stat1α
CTR
IFN
IFN + LPS
IFN + BCG
LPS
BCG
CTR
IFN
IFN + LPS
IFN + BCG
LPS
BCG
F.P.
Stat1α

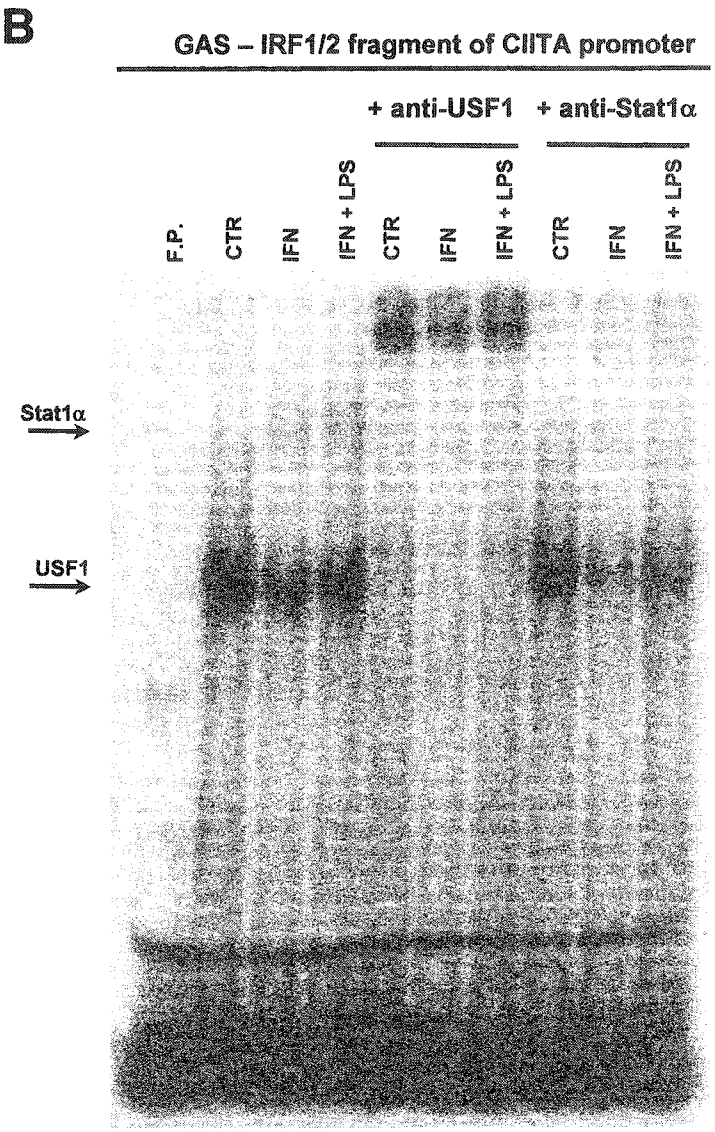

B
GAS – IRF1/2 fragment of CIITA promoter
+ anti-USF1
+ anti-Stat1α
F.P.
CTR
IFN
IFN + LPS
CTR
IFN
IFN + LPS
CTR
IFN
IFN + LPS
Stat1α
USF1

FIGURE 5. BCG but not LPS has an effect on I-A_β and CIITA mRNA expression in TLR4-Del macrophages treated with IFN-γ. TLR4-Del macrophage were incubated for 18 h with IFN-γ alone or together with LPS or *M. bovis* BCG and the isolated total RNA was analyzed by Northern blot using I-Aβ (MHC-II) and CIITA probes. 18s rRNA levels were used as a control for equal loading of the gel.

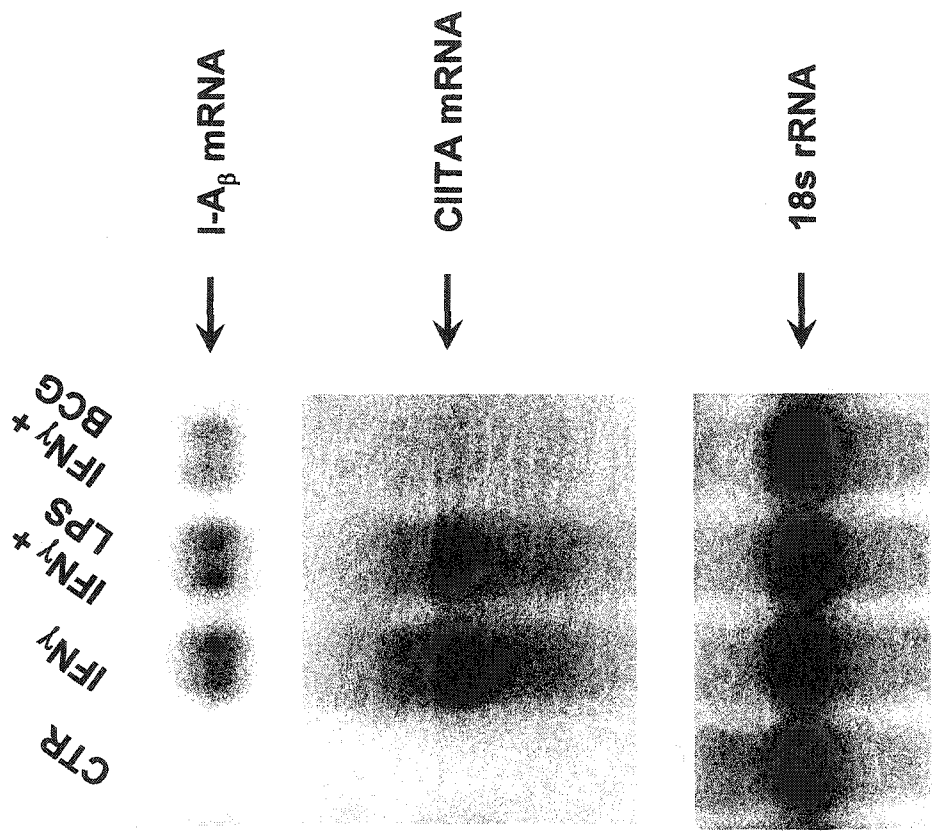
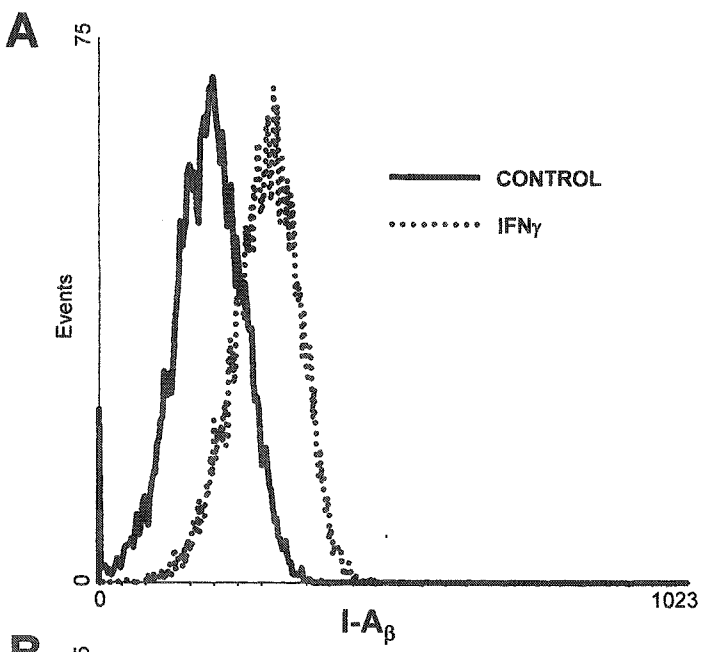
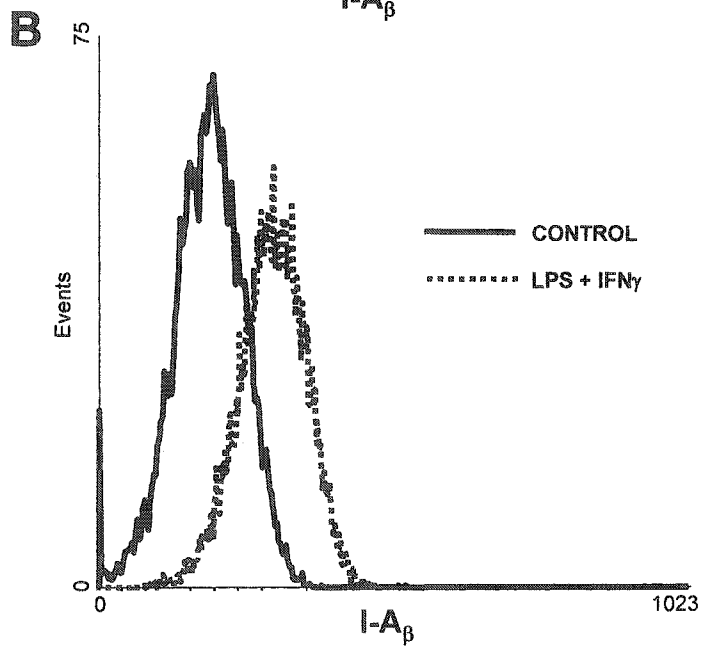
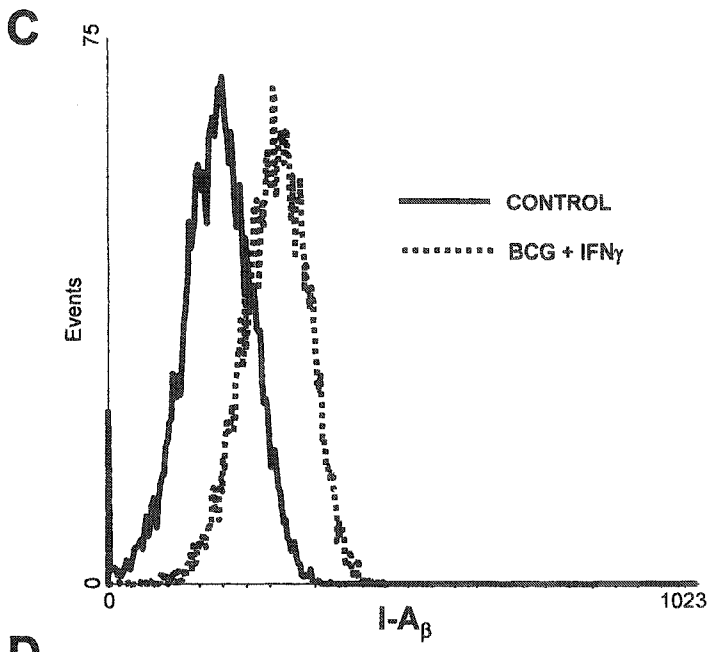
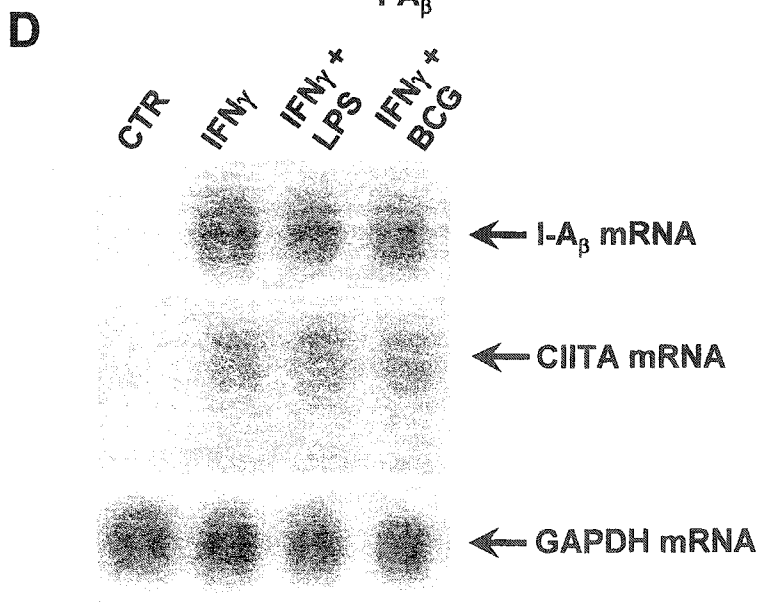

I-Aβ mRNA
CIITA mRNA
18s rRNA
CTR
IFNγ
IFNγ + LPS
IFNγ + BCG

FIGURE 6. LPS or BCG treatment does not affect MHC-II or CIITA expression in MyD88 deficient macrophages. FACS analysis of I-A_β surface expression on MyD88 deficient macrophages stimulated for 48 h with IFN-γ [10 U/mL] alone (A), or together with LPS [100 ng/mL] (B) and *M. bovis* BCG [ratio 10:1] (C). D. I-A_β (MHC-II) and CIITA mRNA expression in MyD88 deficient macrophages stimulated with IFN-γ alone or in combination with LPS or BCG.


A
75
Events
0
0
1023
I-Aβ
CONTROL
IFNγ


B
75
Events
0
0
1023
I-Aβ
CONTROL
LPS + IFNγ


C
75
Events
0
0
1023
I-Aβ
CONTROL
BCG + IFNγ


D
CTR
IFNγ
IFNγ + LPS
IFNγ + BCG
I-Aβ mRNA
CIITA mRNA
GAPDH mRNA

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CHAPTER IV

IFN-γ INDUCES PROLONGED TLR2 PROTEIN EXPRESSION IN TLR4 DEFICIENT BUT NOT IN WILD TYPE MACROPHAGES.

PREFACE

We showed in chapter III that TLR4 mediates the inhibitory effect of LPS on IFN-γ-induced expression of MHC-II. It was previously demonstrated that commercially available preparations of LPS contain fraction(s) which are apparently able to stimulate macrophages using receptors other than TLR4. Activation of TLR4-deficient macrophages by both unpurified LPS preparations and the active fraction (TRIL) isolated from it requires the presence of IFN-γ. We decided to investigate in more detail the nature of this fraction and postulated that Toll-like receptor 2 might be involved in the interaction with this fraction. We analyze the regulation of TLR2 expression by both IFN-γ and the active fraction present in the LPS, using both wild type and TLR4 macrophages.

SUMMARY

Interferon gamma (IFN-γ) is a potent and important activator of macrophages which is able to modulate responsiveness of cells to a variety of endogenous and external stimuli. Here we present results showing that IFN-γ is able to induce Toll-like receptor 2 protein expression in TLR4-deficient macrophages but not in macrophages expressing wild type TLR4. IFN-γ renders TLR4-deficient macrophages responsive to unfractionated LPS preparations but not to phenol re-extracted preparations. These results demonstrate that the TLR4 signal transduction pathway may be involved in regulation of TLR2. In addition, we partially characterize a heat-stable, protease-resistant, and phenol-soluble fraction which is present in commercially-available preparations of LPS and is able to stimulate macrophages using a receptor other than TLR4. Our data also demonstrate that this potential TLR2 ligand can be found in Gram-negative bacteria including *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Escherichia* coli, it has similar properties to the lipoproteins found in some species of *Mycobacteria*, *Mycoplasma*, and Gram-negative bacteria.

INTRODUCTION

Bacterial cell wall components, including lipopolysaccharides (LPS), play an important role in the activation of the immune system, by stimulating different types of cells to produce pro-inflammatory cytokines and other mediators of the immune response (1). A great deal of research has focused on immuno-regulatory and immuno-stimulatory properties of LPS as well as its involvement in the development of septic shock. However, the recent cloning and characterization of mammalian genes homologous to the *Drosophila* Toll system has prompted more rapid progress in the understanding of the molecular mechanism of LPS action (2-4). The key element of this system appears to be the family of so-called Toll-like receptors. So far, ten members of this highly-conserved family of proteins have been described and cloned (5). The ligand(s) for Toll-like receptors 2, 3, 4, 5, 7 and 9 are known. Also, the functions of TLR1 and TLR6 have been partially defined and described. TLR2 was shown to bind peptidoglycans and lipoproteins of Gram-positive bacteria and of mycobacterial origin, whereas TLR4 has been implicated in response to Gram-negative bacteria and their products, such as LPS and lipoteichoic acid (LTA) (6). Interestingly, TLR9 was shown to bind unmethylated bacterial CpG DNA, whereas TLR3 seems to mediate responsiveness to dsRNA (7). Although no specific ligands for TLR1 and TLR6 have yet to be identified, it was shown that they may form heterodimers with other Toll-like receptors. TLR2 and TLR6 apparently cooperate in the response to peptidoglycans and phenol-soluble modulin (PSM) from *Staphylococcus epidermidis*; however, TLR2 activation by bacterial lipoproteins does not require TLR6. TLR1, on the other hand, interferes with the TLR2-TLR6 heterodimer formation thereby inhibiting responsiveness to PSM (8, 9). Finally, flagellin from both Gram-positive and Gram-negative

bacteria were recently described as the ligand for TLR5 (10). Furthermore, the imidazoquinoline compounds like imiquimod and R-848, which are powerful activators of macrophages (11), have recently been shown to bind TLR7 (12). Structurally, Toll-like receptors exhibit striking similarity to the cytoplasmic domain of the IL-1 receptor. This part of the Toll-like receptors contains the Toll/IL-1 receptor (TIR) domain. The TIR domain was shown to be responsible for the specificity of the response; it was also shown to be responsible for the interaction with TIR domains of other Toll-like receptors as well as interaction with adaptor proteins and kinases involved in the regulation of the signal transduction pathway (5, 13). The extracellular portion of a TLR contains characteristic leucine-rich repeats responsible for ligand-binding specificity. The Toll-like linked signal transduction pathway is composed of the adaptor protein MyD88 and the recently cloned MAL/TIRAP. Both MyD88 and MAL/TIRAP were shown to facilitate activation of IL-1 receptor-associated kinases (IRAKs), which participate in the activation of downstream elements of the pathway, including TRAF6, IKK and NFκB transcription factor (14, 15). The activation of AP-1 is achieved through an alternative pathway involving Jun kinase (16). The specificity of the response is achieved on the level of formation of homo- and heterodimeric receptors followed by the recruitment of specific adaptor proteins and accessory proteins, some of which have already been characterized.

The specific role of TLR4 versus TLR2 in the activation of the LPS signal transduction pathway has been a subject of controversy (17). It was shown that commercially available preparations of LPS commonly-used by many researchers are not homogenous; they contain various bacterial cell wall components capable of interacting with different cellular receptors, including Toll-like receptors (18, 19). The responsiveness of macrophages depends on the presence of different ligands in the LPS preparation and therefore accounts for the variability in

the results obtained by various investigators. Hirschfeld et al showed that simple phenol re-extraction completely removes TLR4-independent activity from the original LPS preparation. In the present study, we investigated the mechanism of macrophage activation in response to bacterial cell wall components, in the context of the Toll-like receptor system, and the role of IFN-γ in that process. Using macrophages derived from TLR4 and TLR2 deficient mice respectively, as well as macrophages derived from two other control strains of mice expressing TLR2 and TLR4 genes, we were able to show that the phenol-purified fraction of the commercially-available LPS preparation was unable to activate macrophages that do not express functional TLR4. We also demonstrated that both IFN-γ and LPS upregulate expression of TLR2. This receptor might be responsible for mediating responsiveness of cells to Gram-negative bacterial cell wall components other than LPS. Finally, we showed that TLR2 protein expression is differentially regulated in wild-type versus TLR4-deficient macrophages.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin and Dulbecco's Phosphate Buffered Salt (DPBS), the nick translation DNA labeling kit and the Trizol reagent were purchased from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS), characterized for low level of endotoxin, was obtained from Hyclone (Logan, UT). Recombinant murine interferon gamma (IFN-γ) was purchased from Amgen (Thousand Oaks, CA).

Lipopolysaccharides from *Escherichia coli* serotype 0111:B4, *Pseudomonas aeruginosa* serotype 10, *Salmonella typhimurium* as well as goat anti-rabbit IgG polyclonal horseradish peroxidase-conjugated Ab and rabbit anti-actin polyclonal Ab were all obtained from Sigma Chemicals (St.Louis, MO). Rabbit anti-mouse Toll-like receptor 2 Ab was obtained from eBioscience. Ammonium hydroxide, silver nitrate, formaldehyde and acetic acid were purchased from Fisher Scientific (Ontario). Periodic acid was obtained from Aldrich Chemicals (Wisconsin). α^{32}P-dCTP was purchased from Amersham (Amersham, UK). The chemiluminescent reagent SuperSignal was obtained from PIERCE (Rockland, IL, USA).

Cell lines

Macrophage cell lines were generated as previously described (20, 21) by immortalization of bone marrow-derived macrophages from B10A.*Bcg*^{r} (B10R cell line), C57BL/10ScCr (TLR4-Del cell line) and TLR2 knockout (TLR2-KO) mice. The RAW 264.7 macrophage cell line was purchased from ATCC.

Phenol extraction of LPS preparations

LPS preparations obtained from Sigma Chemicals (called throughout these studies "unpurified LPS" or "unLPS-E", "unLPS-P" and "unLPS-S" depending on whether they were obtained from *Escherichia coli*, *Pseudomonas aeruginosa* or *Salmonella typhimurium*, respectively) were re-extracted with phenol according to the previously described method (18, 19). The unLPS was dissolved in PBS (containing 0.5% sodium deoxycholate) at a concentration of 5 mg/ml. A total of 250 μg of LPS in 0.5 ml of buffer was extracted with 0.5 ml of water-saturated phenol by vortexing at room temperature for 5 minutes. The phases were left to

separate for 5 minutes at room temperature and then were sedimented by centrifugation at 10,000 RPM for 2 minuter. The aqueous phase was transferred to a new tube and the organic phase was mixed with 0.5 ml of PBS; the extraction process was then repeated. The aqueous phases were pooled and precipitated with 70% ethanol in the presence of 10mM sodium acetate (final concentration) for 1 h at -20°C. The pellet containing purified LPS (called throughout these studies "LPS-E", "LPS-P", and "LPS-S", depending on the bacterial species from which they were extracted) was lyophilized (after spinning down at 15,000 RPM for 15 minutes at 4°C) and resuspended in PBS to a final concentration of 1mg/ml, assuming 75% recovery efficiency. Alternatively, the solution containing purified LPS was not precipitated with ethanol but was concentrated using centrifugal filter units with a cut-off of 5,000 kDa (Millipore). To recover fractions trapped in a phenol/buffer inter-phase, the inter-phase and phenol-phase obtained following extraction of LPS were diluted with PBS to the 1% final concentration of the phenol. Next, centrifugal filter units with a cut-off of 5,000 kDa were used to concentrate the recovered material to the original volume of phenol/inter-phase dissolved in PBS. Both the phenol-recovered fraction and the fractions present in unLPS, responsible for the activation of TLR4-Del cells, are collectively termed throughout the text; "Toll-like Receptor Independent Ligand" "TRIL–E", "TRIL-P" or "TRIL-S" (depending on the bacterial origin).

Proteinase K digestion of LPS preparation

50 μg of unLPSs were incubated for 12 h at 55°C with 100 μg/ml of Proteinase K. After inactivating the protease at 100°C for 15 minutes, the solution was diluted with PBS and then used to stimulate the cells.

Silver staining of LPS samples

5μg of LPS samples (unpurified, phenol extracted and unpurified treated with Proteinase K) were resolved on 10% SDS-PAGE and stained using the protocol adapted from the method previously published by Fomsgaard (22). Briefly, the gel was incubated for 20 minutes at room temperature with a solution containing 0.7% periodic acid, 40% ethanol and 5% acetic acid. Next, the gel was washed with 5 changes of deionized water over a period of 30 minutes. After washing, the gel was stained with a solution prepared as follows; 143 ml of deionized water, 0.28 ml of 10 N sodium hydroxide, 2 ml of concentrated ammonium hydroxide and 5 ml of 20% silver nitrate. After washing with 5 changes of water over 20 minutes, the gel was developed in 200 ml solution containing 10 mg of citric acid and 130 μl 40% formaldehyde. The reaction was stopped by transferring the gel to 5% acetic acid solution. The gel was then washed 3 times for 5 minutes each with water, and dried.

Measurement of nitric oxide production

Nitric oxide production was measured using the Greiss reaction as previously described (11). Briefly, $5x10^5$ cells were plated in 1 ml of DMEM on 24-well plates and were stimulated with the appropriate stimuli for 24 h. After that, the cell-free supernatants were collected and mixed with an equal volume of Greiss reagent. The absorbance was measured at 543 nm and nitric oxide concentration was established using the standard curve obtained using sodium nitrite. The values were expressed as μM of nitrite per $5x10^5$ cells in 1 ml.

Northern blot

The total RNA was extracted from 5-10 million cells plated on 10 cm Petri dishes, using Trizol reagent from Gibco. 15 μg of RNA was separated on 1.2% formaldehyde gel and transferred overnight to a nylon membrane using the capillary method. After overnight hybridization with DNA probes labeled with ^{32}P, using the nick translation method, the blots were exposed to X-ray film or scanned using the PhosphoImager (Storm 860, Molecular Dynamics, Sunnyvale, CA) and analyzed using ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA). The TLR2 probe was obtained by PCR amplification of TLR2 cDNA from B10R macrophages using a pair of primers (5'-CCT CAG CGA AAA TCT GAT GG-3'and 5'- GCA GGA GCA AAA CAA AGT GGT-3') encompassing the sequence between positions 1413 and 1932 of the TLR2 cDNA sequence (accession number 6760422). The DNA fragment containing the TLR2 probe sequence was sub-cloned into pGEM-T Easy Vector (Promega, Wisconsin, USA) according to the manufacturer's protocol and re-amplified using the same primers.

Western blot analysis

Macrophages were plated at a concentration of $5x10^5$ cells per ml on 24-well plates. Cells were stimulated with 10 U/ml or 100 U/ml of recombinant IFN-γ in the presence or absence of 100ng/ml of unLPS-E or phenol-extracted LPS-E. They were then washed with DPBS and lysed directly with the SDS-PAGE sample buffer. 15-20 μg of the total protein cell lysate was resolved on 10% SDS-PAGE NuPAGE pre-cast gels (Invitrogen) and transferred to a PVDF membrane (Millipore). The non-specific background was blocked by overnight incubation at 4°C in a 5% solution of non-fat skimmed dry milk (Carnation, Nestle) in PBS with the addition of 0.1%

Tween-20 (PBS-T). The membranes were incubated for 1 h at room temperature with anti-TLR2 (1:1,000 dilution in 5% skimmed milk in PBS-T). After washing, they were incubated in PBS-T with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution in 5% skimmed milk in PBS-T). The signal was visualized using the chemiluminescence SuperSignal reagent and exposed to X-ray film. The actin levels were used as a loading control.

RESULTS

Unpurified LPS (unLPS) activates macrophages derived from TLR4-Del mice

In our studies on the mechanism of macrophage activation, we observed that macrophages derived from the bone marrow of C57B10/SnCr mice did not, as expected, respond to stimulation with Gram-negative bacteria lipopolysaccharides (unLPS-E). However, when cells were simultaneously incubated with unLPS-E and IFN-γ, a substantial amount of nitric oxide and TNF-α could be detected. It was reported (18, 19) that simple re-extraction of the unLPS preparation with phenol removes the so-called contamination which interacts with receptors other than TLR4. We used the Greiss reaction assay to measure nitric oxide production, as a quick and simple method to evaluate macrophage activation. Figure 1 shows the levels of NO produced by wild type B10R macrophages (Fig.1A), TLR4-Del (Fig.1B) and TLR2-KO (Fig.1C) macrophages. Cells were stimulated with commercially-available preparations of LPS from *E. coli* serotype 0111:B4 (unLPS-E), the same LPS re-extracted with phenol (LPS-E) or with TRIL-E (Toll-like Receptor 4 Independent Ligand) recovered from the inter-phase after re-extraction of unLPS-E with phenol. These different samples of LPS were used alone or together with 10 U per

mL of recombinant IFN-γ. As shown in Fig. 1, using our wild type B10R and TLR4-deficient cell lines we first confirmed a previously published observation made using primary macrophages from TLR4-deficient mice (18, 19). Re-extraction of the unLPS preparation with phenol, renders TLR4-defective cells unresponsive to LPS-E stimulation (Fig. 1A, 1B). Both unLPS and phenol extracted LPS-E failed to stimulate TLR4-Del macrophages; however, in the presence of IFN-γ, the unLPS, but not the phenol-extracted LPS-E, could activate these macrophages (Fig. 1B). The LPS-E preparations were still active in cells with normal expression of TLR4 and in TLR2-defective cells (Fig.1B, 1C). The synergistic effect between IFN-γ and unLPS-E on NO production was readily observed in B10R and TLR2-KO macrophages even at very low doses of unLPS-E (1-10ng/ml). No synergistic effect between IFN-γ and phenol-extracted LPS-E could be observed in TLR4-Del macrophages, even when 1 μg per ml of LPS-E was used to stimulate the cells. The putative ligand recovered from the phenol/buffer inter-phase, TRIL-E, when used together with IFN-γ, was also able to stimulate NO production in all three cell lines tested.

Phenol re-extracted LPS from P. aeruginosa and S. typhimurium also fail to activate TLR4-Del macrophages

To verify if the observed phenomenon is unique to the LPS from *E. coli*, we also tested the effect of phenol re-extraction on LPS derived from *Pseudomonas aeruginosa* serotype 10 and *Salmonella typhimurium*. Phenol extraction of all three LPSs removed TLR4-dependent activity and TLR4-Del cells were no longer responsive to stimulation with re-purified LPSs (Fig. 2B). In contrast B10R macrophages expressing functional TLR4 were fully activated in the same conditions (Fig. 2A). The use of several other control macrophage cell lines, including B10S and

RAW 264.7 cells showed that all these 'wild type' cell lines fully responded to the phenol-extracted LPS (data not shown).

The macrophage-activating fraction interacting with receptors other than TLR4 can be inactivated by ethanol precipitation

Analysis of the commercially-purified and phenol-extracted LPS preparations by SDS-PAGE and silver staining did not reveal any obvious differences in their composition (Fig.3). Thus we decided to learn more about the chemical properties of TRIL. This would eventually be very helpful in further characterization and purification of the fraction. The results obtained using phenol re-extracted LPS suggested that this fraction might be a lipoprotein. To test this possibility we performed proteolytic digestion of the original commercially purified (SIGMA) preparation of LPS with proteolytic enzymes. As shown in figure 4, digestion of unLPS preparation with 1 μg per ml of Proteinase K at 55°C for 12 h reduced its activity in TLR4-Del cells (by about 45%; Fig.4B, line 3) however failed to completely remove TRIL activity. Similar results were obtained using pronase (data not shown). Neither overnight heating at 55°C nor incubation at 100°C for 15 minutes (Fig.4, lines 4 and 5) altered the ability of unLPS preparations to activate either B10R or TLR4-Del macrophages.

Interestingly precipitation of unLPS-E with ethanol abrogated its activity in TLR4-Del cells. Efficiency of ethanol-induced inactivation depends on the concentration of unLPS-E in 70% ethanol. When we used 10, 25, 100 and 250 μg of unLPS-E for precipitation with 1mL of 70% ethanol, we observed a concentration-dependent reduction in TRIL-E activity (99.6, 96.5, 91.2 and 66.4%, respectively, Fig. 5B lines 5 to 8). Using spin-down filter units with a 5 kDa cut-off filter membrane, we were unable to recover any activity from the supernatant obtained

after precipitation of LPS. Therefore, unlike phenol extraction, ethanol precipitation did not fractionate the LPS preparation into TLR4-dependent and independent fractions. We concluded that ethanol most likely had a direct effect on TRIL present in unLPS and modified its chemical properties. In the previously described (19) method of LPS re-purification, ethanol was used to precipitate purified LPS at the end of the procedure. Therefore, we tested the relative contribution of ethanol precipitation in the process of removal (or inactivation) of the TLR4 active fraction (TRIL) from the unLPS preparation. We concluded that the removal of TRIL from the unLPS preparation was not solely due to ethanol precipitation. In addition, we also observed that acetone precipitation of unLPS did not affect its activity in TLR4-Del cells (data not shown).

Both IFN-γ and LPS activate expression of TLR2 in macrophage cell lines

Northern-blot analysis was performed to investigate whether the responsiveness of TLR4-Del cells to unLPS-E in the presence of IFN-γ is due to the upregulation of TLR2 expression or not. As shown in figure 6, in wild type macrophages, TLR2 mRNA expression was upregulated by both IFN-γ and unLPS-E. However, the effect of IFN-γ was very weak in comparison to unLPS alone and maximum expression was delayed. Interestingly, the steady state levels of TLR2 mRNA were quickly upregulated with unLPS-E and they reached the peak of expression at 3 h post-stimulation. This expression was diminished over time and could barely be detected 18 h following exposure of the cells to unLPS-E. The kinetics of IFN-γ-induced expression of TLR2 was delayed compared to unLPS-induced expression, with the peak of expression observed between 8 and 11 h following stimulation. Similarly to unLPS-E, the IFN-γ-induced expression of TLR2 mRNA did not persist and could not be detected at 18 h post-

exposure. In TLR4-Del cells, the expression of TLR2 mRNA was also upregulated in response to IFN-γ and unLPS (Fig. 7). Two hours after stimulation with unLPS-E, high levels of TLR2 mRNA could be detected in TLR4-Del macrophages, similarly to in B10R cells. IFN-γ did not have any effect on TLR2 mRNA expression. At the 9-h time point, unLPS-induced TLR2 expression decayed in a fashion similar to that observed in wild-type cells. Treatment of macrophages with IFN-γ for 9 h induced TLR2 mRNA expression in both cell lines; however TLR2 expression was stronger in TLR4-Del cells than in B10R cells. At this time point, the level of TLR2 mRNA induced by IFN-γ in TLR4-Del macrophages was higher than the levels induced by unLPS-E. In contrast, in B10R cells, the levels of TLR2 mRNA induced by unLPS-E were higher than the levels of TLR2 mRNA induced by IFNγ. In TLR4-Del cells, the levels of TLR2 mRNA induced at 9 h of stimulation with unLPS-E alone was very similar to the levels obtained in cells treated with both IFN-γ and unLPS-E. The level of TLR2 mRNA induced following the 9-hour treatment of B10R cells with both IFN-γ and unLPS-E or LPS-E were slighty lower than for unLPS or LPS-E alone. The patterns of IFN-γ and unLPS-E or LPS-E-induced TLR2 mRNA expression in the RAW 264.7 macrophage cell line were very similar to those observed using B10R cells (data not shown). As expected, LPS-E alone was unable to stimulate TLR2 mRNA expression in TLR4-Del macrophages.

As shown in figure 8, which illustrates the results of the Western blot analysis, 12 h of stimulation with unLPS-E or LPS-E augmented TLR2 protein expression in both the B10R and RAW 264.7 macrophage cell lines but not in TLR4-Del cells (Fig. 8). Interestingly, when the TLR4-Del cells were treated with IFN-γ, a very significant augmentation of TLR2 protein expression could be observed (Fig. 8). No significant augmentation of TLR2 protein expression by IFN-γ stimulation was observed either in B10R or RAW 264.7 cells.

The induction of the TLR2 gene expression in response to unLPS-E or LPS-E occurred at both the mRNA and the protein level in B10R and RAW 264.7 macrophages. In TLR4-Del cells no induction of TLR2 mRNA espression or TLR2 protein could be detected in response to LPS-E. Although unLPS-E treatment of TLR4-Del cells induced high levels of TLR2 mRNA (Fig.7), the level of TLR2 protein induced was barely detectable (Fig.8). Treatment with IFN-γ resulted in a small increase at the level of TLR2 mRNA, both in B10R and TLR4-Del cells. A dramatic increase in the amount of TLR2 protein production occurred in TLR4-Del cells treated with IFN-γ but not in wild type macrophages (B10R and RAW 264.7). In TLR4-Del cells this increase at the level of TLR2 protein production seems to be regulated, at least in part, by post-transcriptional mechanisms.

These data strongly suggest the existence of cross-talk between IFN-γ-induced signaling and Toll-like receptor gene expression regulation. Further studies are required to fully elucidate the precise molecular interactions between the IFN-γ and LPS pathways involved in macrophage activation.

DISCUSSION

It has been previously demonstrated that both TLR2 and TLR4 may be the essential components of the LPS signal transduction pathway (17, 23-26). It was also established that commercially-available preparations of LPS (unLPS) were not homogenous and contained components which were able to activate cells through receptors other than TLR4 (18, 19). Previous reports demonstrated that phenol re-extracted LPS was no longer able to activate

TLR4-deficient cells, even in the presence of IFN-γ. It was also reported that the accessory protein MD-2, together with CD14 and TLR4, is an essential part of the LPS recognition complex (27, 28). Interestingly when MD-2 was overexpressed, it formed a complex with TLR2 that rendered TLR4-deficient cells responsive to LPS (29).

We used a macrophage cell line, TLR4-Del, derived from the bone marrow of C57BL10/SnCr mice, to study the activating properties of phenol-extracted and unpurified, commercially available preparations of LPS. The C57BL10/SnCr strain of mice was shown to be resistant to endotoxin-induced septic shock due to the null mutation in Toll-like receptor 4 gene. We partially characterized the fraction (TRIL) of the bacterial cell wall present in commercial preparations of LPS that was able to activate TLR4-Del cells in the presence of IFN-γ. TRIL can be recovered from phenol or inter-phase and is at least partially resistant to proteolytic digestion and high temperature. A similar characteristic was previously described for phenol-soluble modulin (PSM) from *Staphylococcus epidermidis* and lipoproteins of mycobacterial origin (8, 9, 30). Interestingly, both PSM and mycobacterial lipoproteins were described as TLR2 ligands. We also showed that ethanol precipitation of the unLPS preparation leads to removal or inactivation of TRIL. Our results, using commercially-available preparations of lipopolysaccharides from *E. coli*, *P. aeruginosa* and *S. typhimurium* suggest that these Gram-negative, in addition to published previously Gram-positive bacterial species, may express a ligand that binds to TLR2 or other receptors (8, 9).

In wild type B10R or RAW 264.7 macrophages, both unLPS and LPS-E (unLPS after phenol purification) were able to upregulate the expression of TLR2 mRNA and protein. In these cells IFN-γ alone had a very modest effect on TLR2 mRNA expression. In contrast, in TLR4-deficient cells, the effect of IFN-γ is more persistent and leads to a prolonged and strong

expression of both TLR2 mRNA and protein. Since upregulation of TLR2 mRNA expression in TLR4-Del cells stimulated with unLPS did not lead to upregulation of protein levels, it appears that post-transcriptional mechanisms are involved, at least in part, in the regulation of TLR2 gene expression. The process of translation may directly influence the stability of mRNA of many genes. Most known examples of this include cytoplasmic, secreted, and nuclear regulatory proteins such as β-tubulin, cytokines, histones, c-fos and c-myc respectively (31-34). The expression of all these genes can be quickly adjusted in response to endogenous and exogenous changes. In contrast, regulation of expression of constitutive genes usually does not involve changes in mRNA stability. The regulation of translation-dependent mRNA stability of proteins with fast turnover may depend both on sequences present in the mRNA (for example AU rich elements in the 3'UTR) and on the process of translation itself and the proteins produced. Regulation of TLR2 expression may involve translation-dependent downregulation of mRNA stability since the expression of TLR2 mRNA stimulated with unLPS decays quickly once the protein is produced.

It seems that TLR4-dependent repression of TLR2 translation may exist. In the wild type (B10R and RAW 264.7) macrophages, LPS, both unpurified and phenol extracted, can stimulate TLR2 mRNA and protein expression. IFN-γ, on the other hand, is rather ineffective in inducing the TLR2 expression of in these cells. In contrast, in TLR4-defcient cells, IFN-γ stimulation leads to a dramatic upregulation of TLR2 protein expression although mRNA expression is induced to similar levels as in the control wild type cells. It may suggest that the TLR4-mediated signal which is independent from LPS-stimulation blocks TLR2 translation. This signal may be provided by host proteins that are able to bind constitutively to TLR4. It was demonstrated that TLR4 can bind the host heat-shock protein hsp60 as well as fibronectin (35-37). If this kind of

signal exists, it would not be able to repress TLR2 translation in TLR4-deficient cells, allowing them to express TLR2 protein in response to IFN-γ. TLR4, however, must also be responsible for the activation of TLR2 protein expression, since stimulation of the control wild type cells with a highly purified TLR4 ligand (LPS-E) leads to strong upregulation of TLR2 protein expression. At the same time, unpurified LPS is unable to stimulate TLR4-Del cells to express TLR2 protein, although it is able to upregualte TLR2 mRNA expression. This suggests that; 1) translation of TLR2 requires a signal originating from TLR4; 2) the repression of TLR2 translation provided by an alternative signal from TLR4 can be overridden by binding of LPS; 3) IFN-γ can very efficiently stimulate TLR2 protein expression in the absence of TLR4-mediated repression of translation. The ability of TLR4 to activate at least two independent signaling pathways was demonstrated during studies of MyD88-independent and MAL/TIRAP-mediated responses (14, 15).

The full elucidation of mechanisms regulating TLR2 expression in macrophages will require more detailed analyses of both mRNA and protein expression including studies on mRNA stability and protein turnover. Furthermore it would also be essential, for understanding the molecular mechanism of regulation of TLR2 expression, to analyze the IFN-γ- and LPS-dependent expression of TLR2 in macrophages deficient in particular components of the Toll-like signaling pathway. In addition, the possible role of IL-12 in the process of TLR2 expression regulation should be evaluated, since it was recently demonstrated that C57BL10/SnCr mice (bone marrow macrophages from these mice were used to generate the TLR4-Del cell line) are unresponsive to IL-12 due to the mutation in the IL-12 receptor beta gene (38).

Interestingly, we have shown that IFN-γ, whose presence is required for activation of TLR4-deficient cells with unLPS, is able to induce higher levels of TLR2 expression in TLR4-

Del cells compared to wild type macrophages. The activation of TLR4-Del cells with IFN-γ might allow these cells to respond better to ligands binding to TLR2. If the unLPS preparation contains compounds which are able to interact with TLR2, then upregulation of this receptor by IFN-γ would provide an explanation for TLR4-deficient cells' ability to respond to commercial, unpurified LPS stimulation in the presence of IFN-γ (but not to unLPS in the absence of IFN-γ). The fraction recovered from the buffer/phenol interphase (TRIL) may not be homogenous and may contain more than one compound, including lipopolysaccharides which may interact with different receptors expressed by macrophages, including TLR4. To assess the role of TLR2 in the responsiveness of TLR4-Del cells to unLPS in the presence of IFN-γ, we would need to use double knockouts (TLR2 and TLR4) and/or identify the fraction interacting with TLR2. Alternatively, the activation of TLR4-Del macrophages with unpurified LPS involves a receptor(s) other than TLR2. In this case the precise characterization of the compounds present in the commercial LPS preparation that are responsible for the activation of TLR4-Del cells and its signaling pathway would require further studies using highly purified material and a panel of cell lines deficient in different Toll-like receptors (work in progress).

A better understanding of the precise molecular mechanism of interaction between various Toll-like (and other) receptors and their signal transduction pathways will help us to find better pharmacological ways of intervention to enhance protective functions of macrophages against bacterial infections.

FIGURE LEGENDS

FIGURE 1. Phenol re-extraction removes TLR4-independent activity from unLPS. TLR4-Del, B10R and TLR2-KO macrophages were plated at a density of $5x10^5$ cells per mL on 24-well plates. They were stimulated overnight with either 100ng/mL of unLPS from *E. coli* (unLPS-E), 100 ng/ml of phenol re-extracted LPS from *E. coli* (LPS-E) or with the fraction recovered from the phenol/buffer inter-phase following LPS re-extraction (TRIL for Toll-like Receptor 4 Independent Ligand). These LPS fractions were administrated alone or in combination with 10 U/mL of recombinant IFN-γ. The bars represent the mean value of the concentration of nitrite measured using the Greiss reaction method. The concentration of nitric oxide was measured in the supernatant collected from the culture of $5x10^5$ cells per 1 ml of media. The figure illustrates one of three independent experiments done in triplicate.

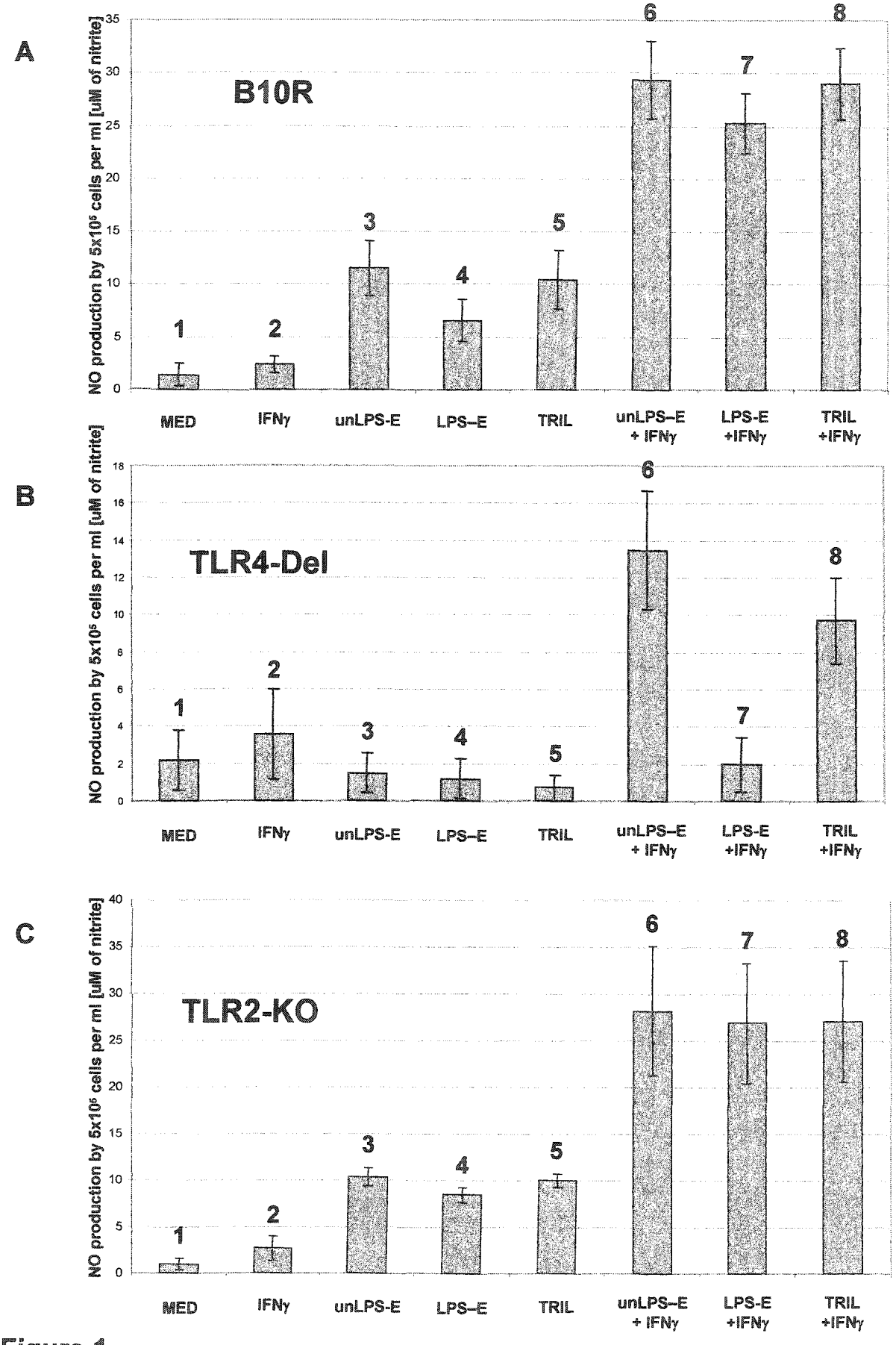

A
B10R
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
1
2
3
4
5
6
7
8
MED
IFNγ
unLPS-E
LPS-E
TRIL
unLPS-E + IFNγ
LPS-E +IFNγ
TRIL +IFNγ
B
TLR4-Del
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
2
4
6
8
10
12
14
16
18
1
2
3
4
5
6
7
8
MED
IFNγ
unLPS-E
LPS-E
TRIL
unLPS-E + IFNγ
LPS-E +IFNγ
TRIL +IFNγ
C
TLR2-KO
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
MED
IFNγ
unLPS-E
LPS-E
TRIL
unLPS-E + IFNγ
LPS-E +IFNγ
TRIL +IFNγ

Figure 1

FIGURE 2. Comparison of the effect of phenol re-extraction on unLPS from *E. coli* (unLPS-E and LPS-E), *P. aeruginosa* (unLPS-P and LPS-P) and *S. typhimurium* (unLPS-S and LPS-S). Panels A and B represent results from B10R and TLR4-Del macrophages respectively. Cells were stimulated overnight with 100ng/mL of unpurified LPSs (unLPS-E, unLPS-P and unLPS-S), or phenol re-extracted LPS (LPS-E, LPS-P and LPS-S) alone or in combination with 10 U/mL of recombinant IFN-γ. The bars represent the mean value of the concentration of nitrite measured using the Greiss reaction method. The concentration of nitric oxide was measured in the supernatant collected from the culture of $5x10^5$ cells per 1 ml of media. The figure illustrates one of three independent experiments done in triplicate.

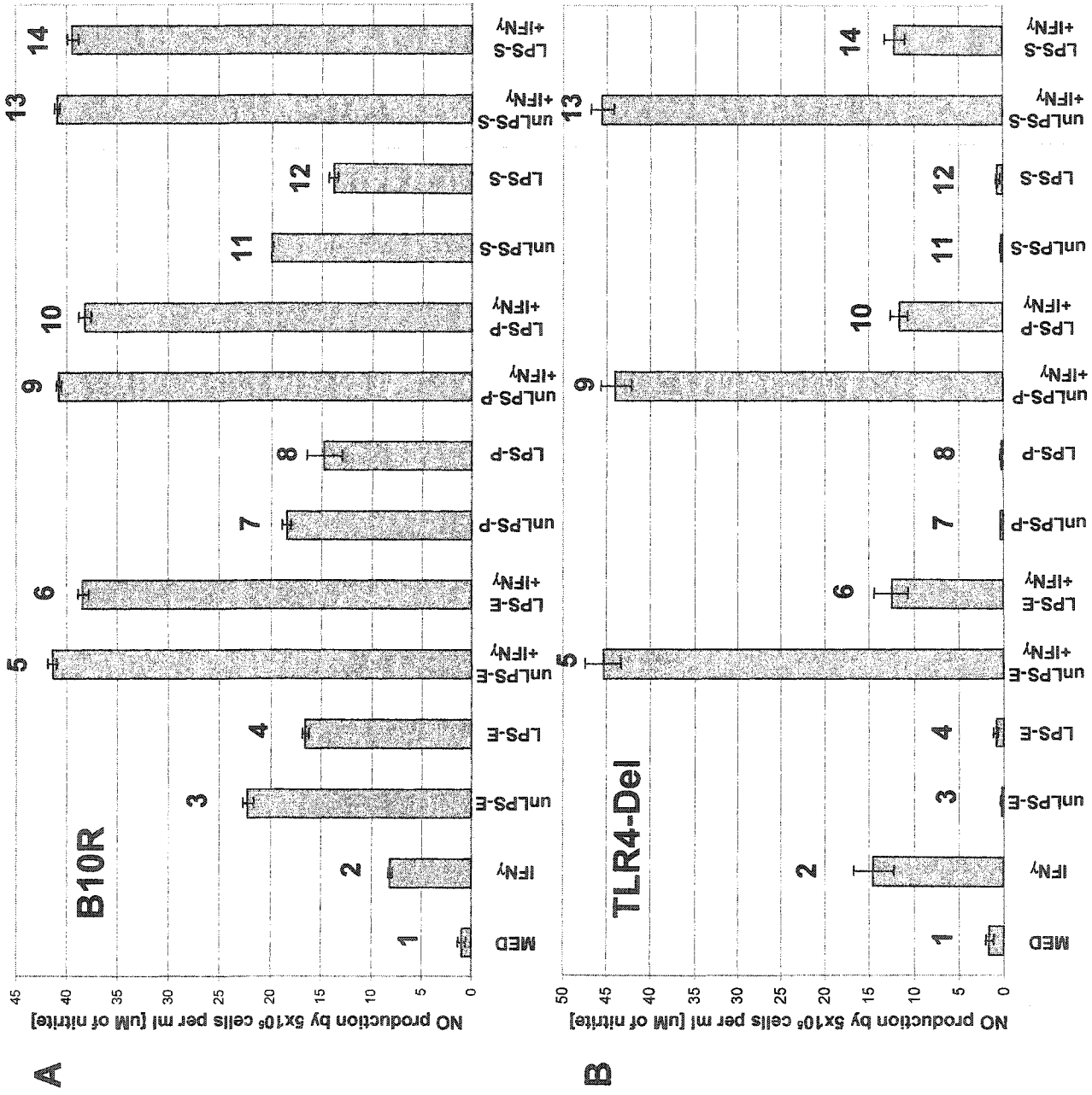

A
B10R
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
40
45
1
2
3
4
5
6
7
8
9
10
11
12
13
14
MED
IFNγ
unLPS-E
LPS-E
unLPS-E +IFNγ
LPS-E +IFNγ
unLPS-P
LPS-P
unLPS-P +IFNγ
LPS-P +IFNγ
unLPS-S
LPS-S
unLPS-S +IFNγ
LPS-S +IFNγ
B
TLR4-Del
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
8
9
10
11
12
13
14
MED
IFNγ
unLPS-E
LPS-E
unLPS-E +IFNγ
LPS-E +IFNγ
unLPS-P
LPS-P
unLPS-P +IFNγ
LPS-P +IFNγ
unLPS-S
LPS-S
unLPS-S +IFNγ
LPS-S +IFNγ

FIGURE 3. Silver staining of original preparations (unLPS), phenol re-extracted (LPS) and pronase-treated (LPS-Pr.T.) LPSs from *E. coli, P. aeruginosa* and *S. typhimurium*. 10 μg of each preparation was loaded onto a 10% SDS-PAGE gel. The gel was then stained with silver nitrate to visualize lipopolysaccharides, and dried.

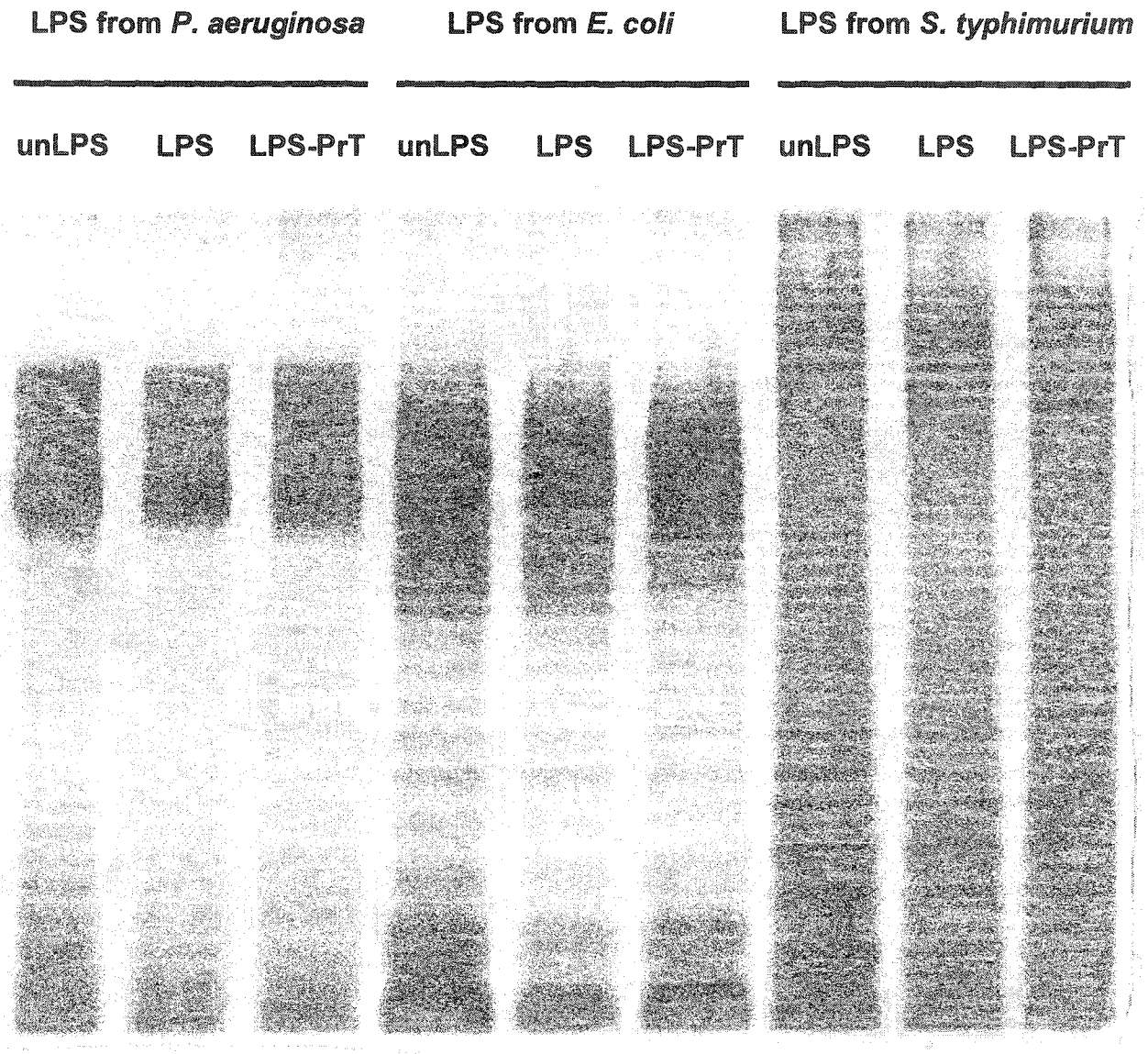

LPS from *P. aeruginosa*
LPS from *E. coli*
LPS from *S. typhimurium*
unLPS
LPS
LPS-PrT
unLPS
LPS
LPS-PrT
unLPS
LPS
LPS-PrT

FIGURE 4. Nitric oxide production by B10R and TLR4-Del macrophages activated with unLPS digested with Proteinase K in the presence of IFN-γ. Cells at a concentration of $5x10^5$ per ml were either treated with 10 U/mL of IFN-γ alone or in combination with 100ng/ml of different samples of LPS; unLPS(PK) - LPS from *E. coli* incubated with Proteinase K, unLPS(55) - incubated for 12 h at 55°C, unLPS(100) - unLPS incubated for 15 minutes at 100°C, unLPS-E – control, untreated LPS or LPS-E - phenol extracted unLPS-E. Panel A and B represent results obtained using wild type B10R and TLR4-deficient macrophages respectively. The bars represent the mean value of the concentration of nitrite measured using the Greiss reaction method. The concentration of nitric oxide was measured in the supernatant collected from the culture of $5x10^5$ cells per 1 ml of media. The figure illustrates one of three independent experiments done in triplicate.

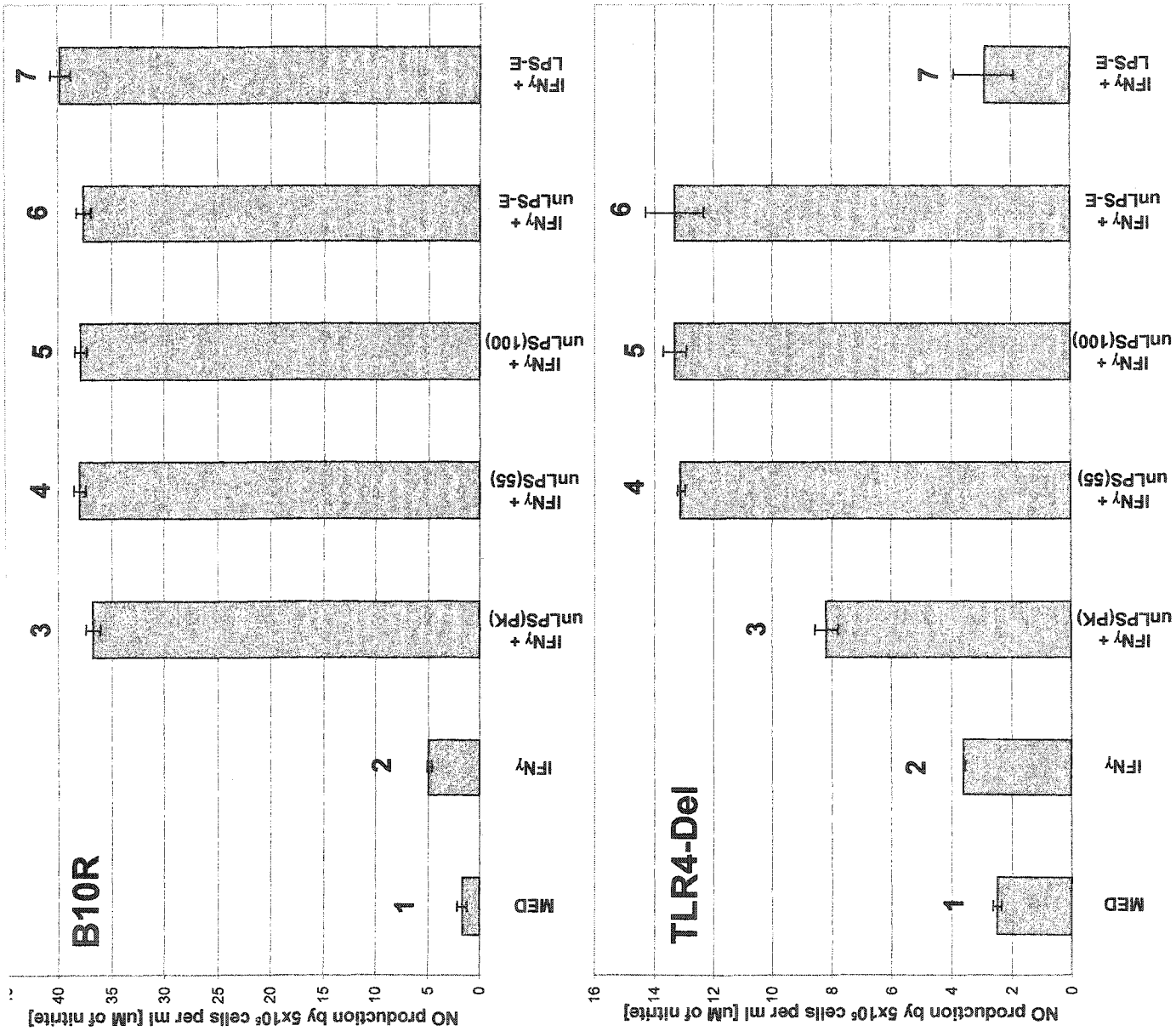

A
B10R
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
MED
IFNγ
IFNγ + unLPS(PK)
IFNγ + unLPS(55)
IFNγ + unLPS(100)
IFNγ + unLPS-E
IFNγ + LPS-E
B
TLR4-Del
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
2
4
6
8
10
12
14
16
1
2
3
4
5
6
7
MED
IFNγ
IFNγ + unLPS(PK)
IFNγ + unLPS(55)
IFNγ + unLPS(100)
IFNγ + unLPS-E
IFNγ + LPS-E

FIGURE 5. Nitric oxide production by B10R and TLR4-Del ethanol precipitation inhibits unLPS-E activity. Cells at a concentration of $5x10^5$ cells per ml were either treated with 10 U/mL of IFN-γ alone or in combination with 100ng/ml of different samples of unLPS-E precipitated with ethanol. Numbers indicate the amount of micrograms of unLPS-E precipitated with 1 mL of 70% ethanol. The samples obtained using control preparations of unpurified and phenol-purified LPS [unLPS-E and LPS-E, respectively] were also used. Panels A and B represent results obtained using wild type B10R and TLR4-deficient macrophages respectively. The bars represent the mean value of the concentration of nitrite measured using the Greiss reaction method. The concentration of nitric oxide was measured in the supernatant collected from the culture of $5x10^5$ cells per 1 ml of media. The figure illustrates one of three independent experiments done in triplicate.

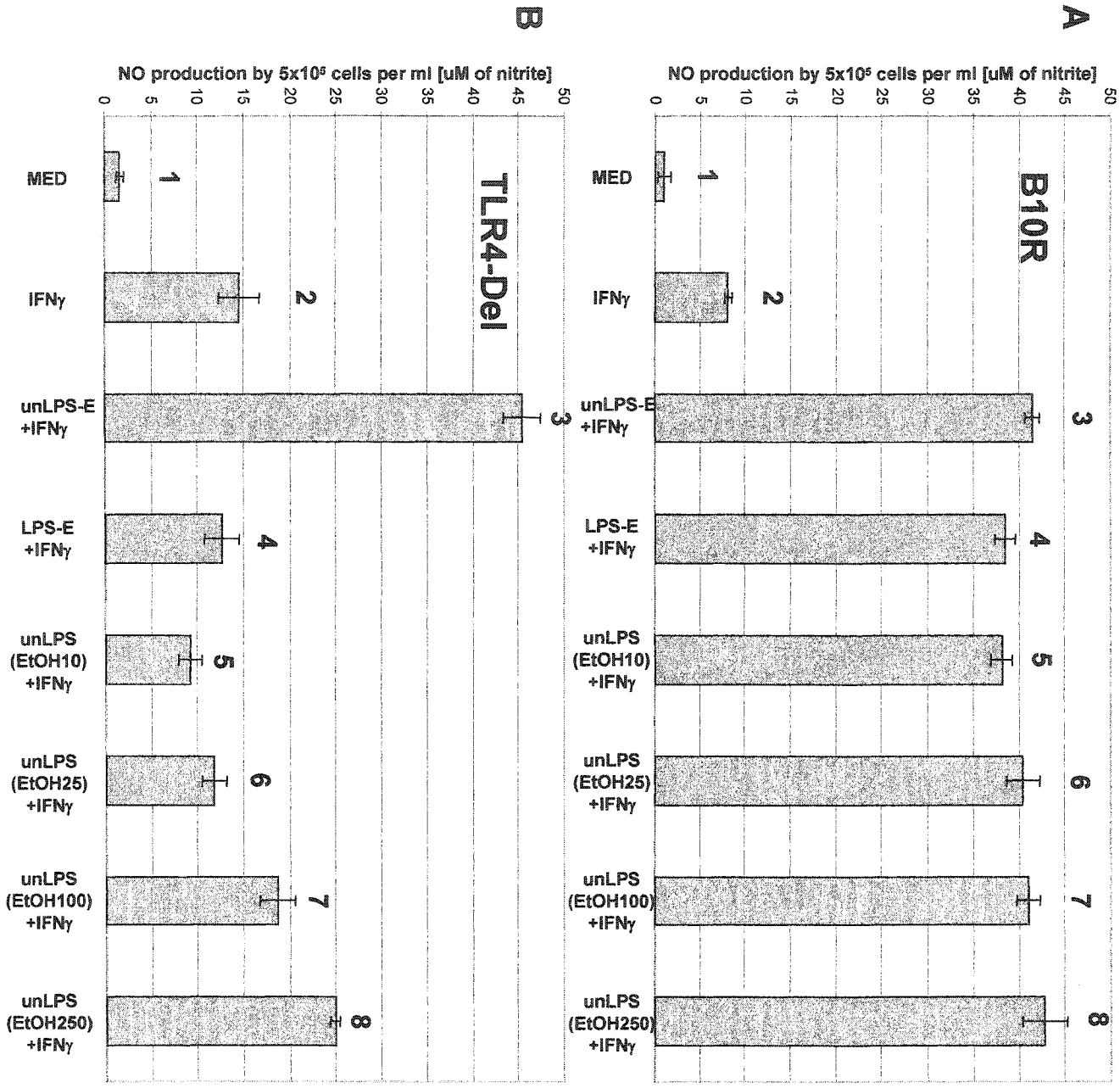

A
B10R
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
40
45
50
MED
IFNγ
unLPS-E +IFNγ
LPS-E +IFNγ
unLPS (EtOH10) +IFNγ
unLPS (EtOH25) +IFNγ
unLPS (EtOH100) +IFNγ
unLPS (EtOH250) +IFNγ
1
2
3
4
5
6
7
8
B
TLR4-Del
NO production by 5x10⁵ cells per ml [uM of nitrite]
0
5
10
15
20
25
30
35
40
45
50
MED
IFNγ
unLPS-E +IFNγ
LPS-E +IFNγ
unLPS (EtOH10) +IFNγ
unLPS (EtOH25) +IFNγ
unLPS (EtOH100) +IFNγ
unLPS (EtOH250) +IFNγ
1
2
3
4
5
6
7
8

FIGURE 6. Northern blot analysis of the kinetics of IFN-γ- and unLPS-dependent expression of TLR2 mRNA in the wild-type B10R macrophage cell line. B10R cells were treated for indicated periods of time with 10 U per ml of IFN-γ, 100ng per ml of unLPS or both of these stimuli simultaneously. Results illustrated in the figure are representative of three independent experiments.

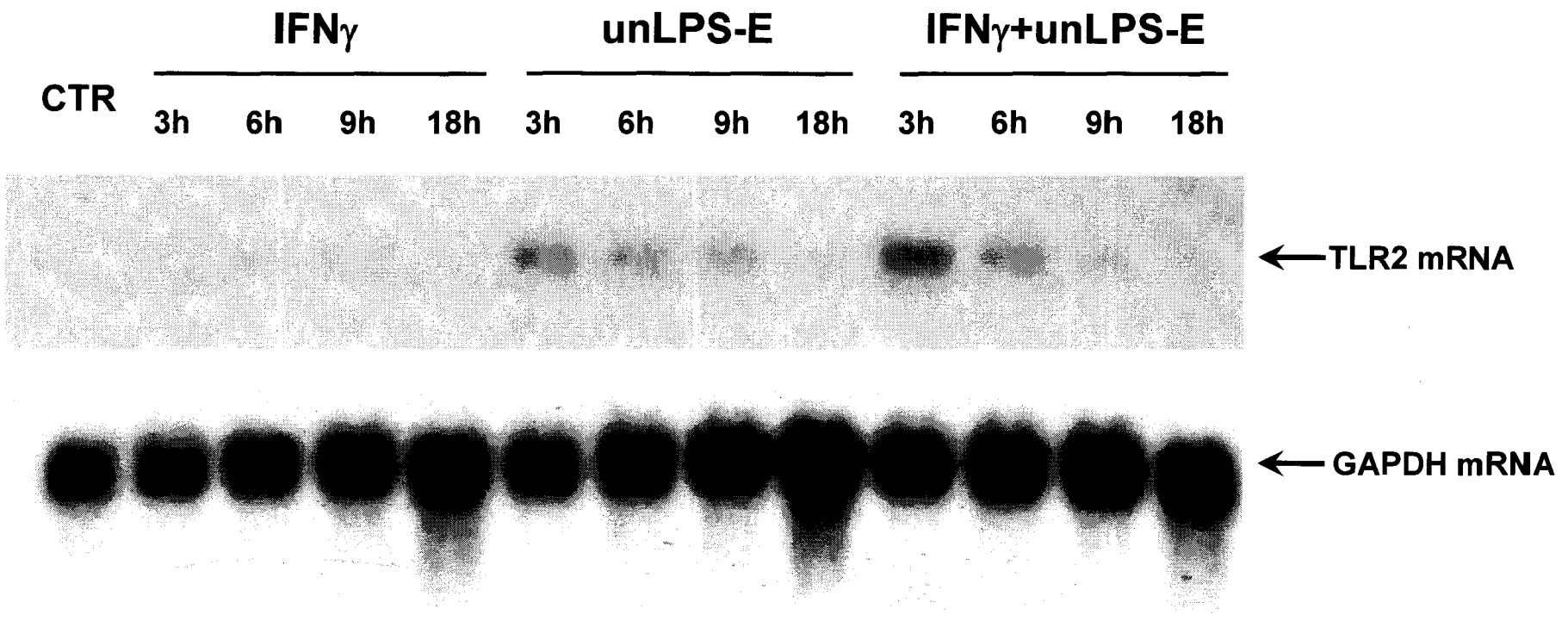
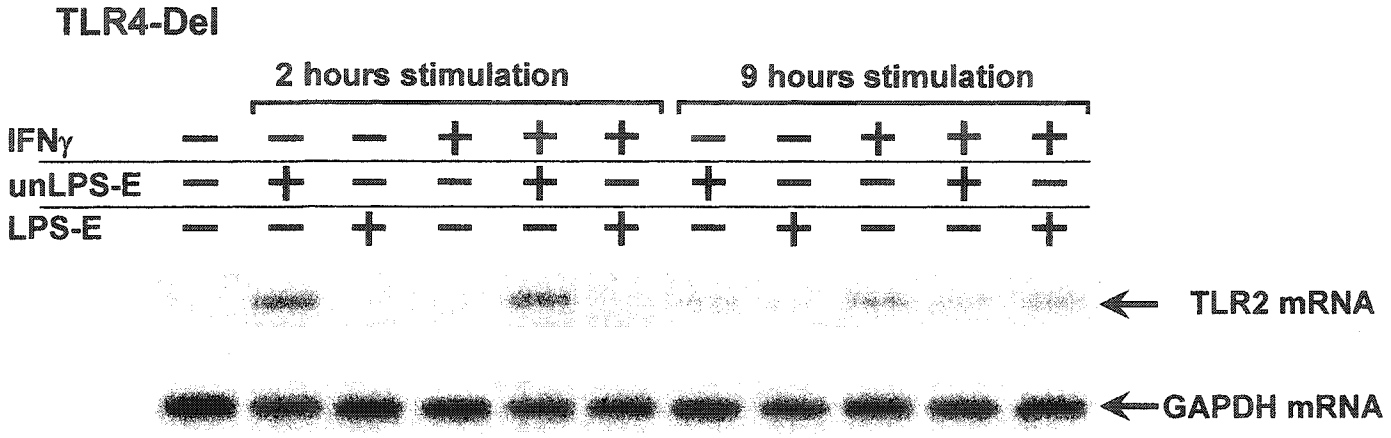

IFNγ
unLPS-E
IFNγ+unLPS-E
CTR
3h
6h
9h
18h
3h
6h
9h
18h
3h
6h
9h
18h
TLR2 mRNA
GAPDH mRNA

FIGURE 7. Northern slot blot analysis of IFN-γ, unLPS-E and LPS-E-dependent expression of TLR2 mRNA in TLR4-deficient and control B10R macrophage cell lines. Macrophages were treated with the indicated stimulus for 2 or 9 h and analysis of TLR2 mRNA expression was performed. Recombinant IFN-γ was used at a concentration of 10 U per ml and both unpurified [unLPS-E] and phenol extracted [LPS-E] LPSs were used at a concentration of 100ng per ml. Results illustrated in the figure are representative of three independent experiments.


TLR4-Del
2 hours stimulation
9 hours stimulation
IFNγ
unLPS-E
LPS-E
TLR2 mRNA
GAPDH mRNA

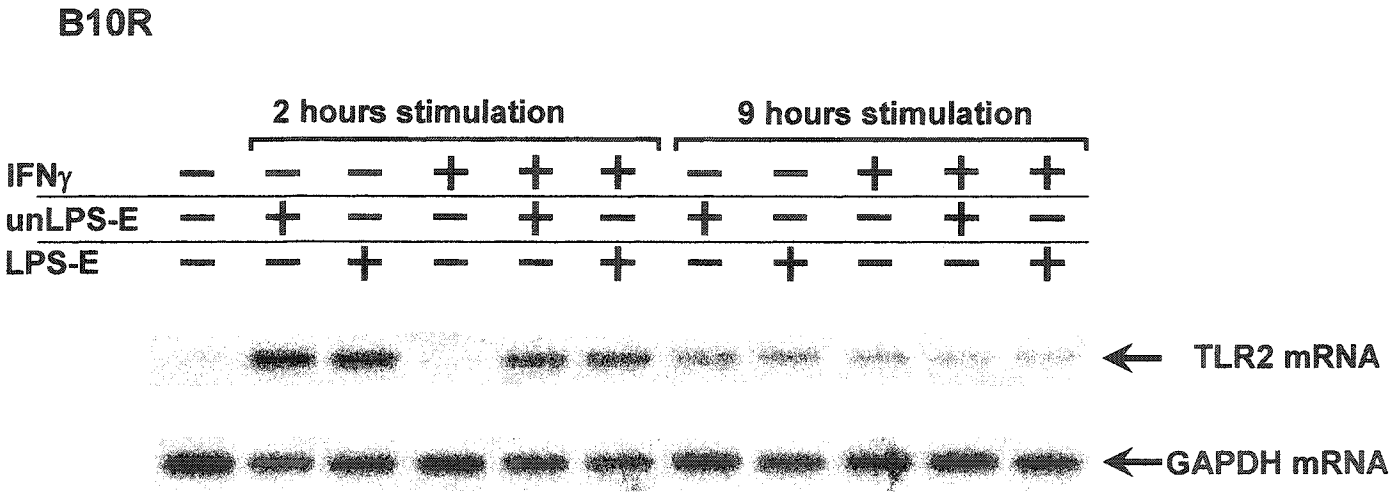

B10R
2 hours stimulation
9 hours stimulation
IFNγ
unLPS-E
LPS-E
TLR2 mRNA
GAPDH mRNA

Figure 7

FIGURE 8. IFN-γ and LPS effects on TLR2 expression. A Western-blot analysis of TLR2 protein expression in wild type B10R cells, TLR4-deficient TLR4-Del and control RAW 264.7 cells stimulated for 12 h with IFN-γ, unpurified LPS [unLPS-E], phenol extracted unLPS-E [LPS-E] or with a combination of both stimuli. The IFN-γ was used at a concentration of 10 or 100 units per ml. Both unpurified and phenol extracted LPS-E were used at a concentration of 100 ng per ml. Results illustrated in the figure are representative of three independent experiments.

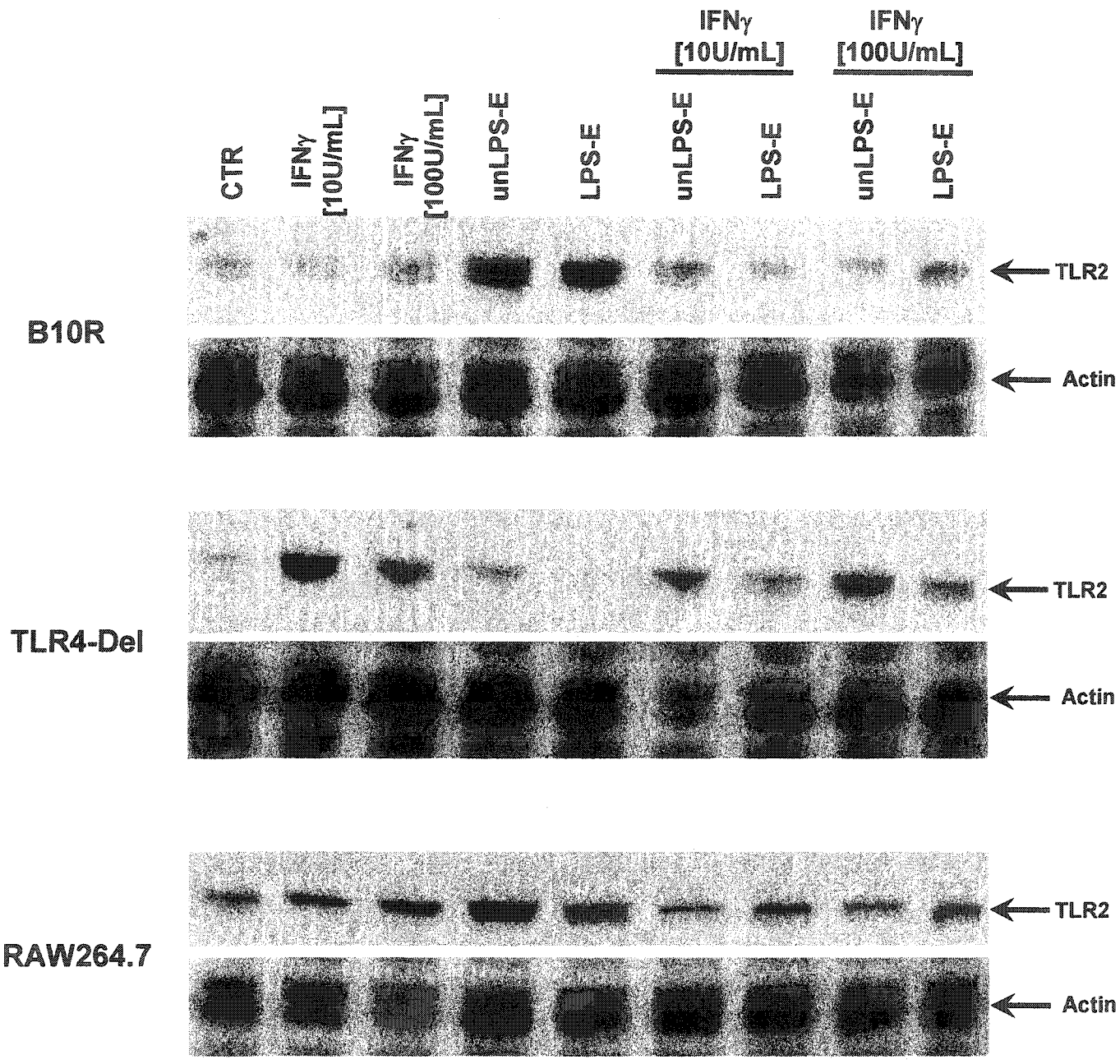

IFNγ [10U/mL]
IFNγ [100U/mL]
CTR
IFNγ [10U/mL]
IFNγ [100U/mL]
unLPS-E
LPS-E
unLPS-E
LPS-E
unLPS-E
LPS-E
B10R
TLR2
Actin
TLR4-Del
TLR2
Actin
RAW264.7
TLR2
Actin

Figure 8

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CHAPTER V

GENERAL DISCUSSION

The defense mechanisms protecting higher organisms against infections from microorganisms have evolved in mammals into highly complex and adaptive processes commonly known as the immunological response. This response cannot be easily described since different specific mechanisms are involved, depending on the nature of the infecting agent. The specificity of the immunological response results from the cooperative action of many different types of cells that have highly specialized and usually well-defined functions in the immunological response. Thus, active defense of the host against the infecting agent is usually described as a chain of events developing in a time-dependent fashion. This process involves the sequential activation of various cell types exhibiting specialized functions, resulting in the development of an efficient effector mechanism that enables elimination of the infection.

Overall, immunological defense can be divided into non-adaptive (or innate) and adaptive immunity (1). Innate immunity provides a very fast and effective mechanism for clearing the infection without, however, generating immunological memory. Adaptive immunity, on the other hand, requires more time to develop but provides the host with immunological memory that facilitates host response to re-infection, resulting in a faster and more specific reaction. The cell types that are involved in the development of innate immunity include granulocytes, macrophages, and dendritic cells which are able to recognize, immobilize and kill infectious agents in a way that is independent of other cells. All the above functions are greatly enhanced by the presence of IFN-γ. The two main sources of IFN-γ are γδT-cells and NK cells (ref....). Most local infections can be controlled by innate immunity. The most important cells in adaptive immunity are B- and T-lymphocytes. In contrast to the cells involved in innate immunity, lymphocytes cannot act independently; they require very specific interactions with other cells. Due to the highly-specialized functions of the lymphocytes, it is very rare that cells which first

encounter infecting microorganisms are also effector cells responsible for its elimination. The involvement of other cell types, like epithelial cells, fibroblasts and hepatocytes, in both types of immunological response is now well documented. Different cell types communicate with each other using soluble mediators as well as cell-to-cell interactions. Both processes are in many cases difficult to separate and simultaneous involvement of certain cell types in innate and adaptive immunity is necessary for their normal development. For example, antigen-presenting cells, like dendritic cells or macrophages, which are normally involved in the development of innate immunity, are necessary for the activation of specific T-lymphocytes and subsequent development of adaptive immunity. On the other hand, lymphocytes produce mediators that are very potent and important activators of innate immunity.

As described in chapter I, macrophages are cells which are strongly involved in both types of immunological response (2). In order to be functionally efficient, macrophages have to be activated. Usually, an encounter with a microorganism is sufficient to activate macrophages; however, macrophages are very rarely exposed to IFN-γ at the same time as this encounter takes place. *In vitro* experiments provide evidence that bacterial cell wall products (for example, lipopolysaccharides from Gram-negative bacteria) synergize with IFN-γ during the process of macrophage activation (3). IFN-γ was shown to be a very effective activator of macrophage functions such as phagocytosis, antigen presentation and oxidative burst, relevant for their participation in the immunological response (4). Therefore, studies on internal and external factors influencing IFN-γ-dependent activation of macrophages are very important in order to fully understand processes regulating host/microorganism interactions.

One of the best characterized genetic factors which affects IFN-γ responsiveness, specific for macrophages, is the *Nramp1* gene (5, 6). Nramp1 has been characterized as a transport

protein which determines the resistance or the susceptibility of mice to infections with certain intracellular pathogens. Interestingly, the susceptibility of mice to a limited number of intracellular microorganisms is Nramp1-dependent. The precise function of Nramp1 and the mechanism through which it regulates innate immunity is still not established. It was shown that macrophages which express the *Nramp1* allele, which confers resistance to infection with *M. bovis* BCG (*Nramp1*^{r}, with Gly at position 169 of the amino acid sequence), are more responsive to IFN-γ stimulation then macrophages carrying the form of the *Nramp1* gene responsible for susceptibility to infection (*Nramp1*^{s}, with Asp at position 169 of the amino acid sequence) (7, 8). This phenomenon can be demonstrated in vitro using macrophage cell lines derived from the bone marrow of resistant and susceptible strains of mice. In this model, macrophages from susceptible mice express less mRNA for inducible nitric oxide synthase (iNOS), MHC-II, class II trans-activator (CIITA) and KC, and produce less reactive oxygen intermediates, nitric oxide and TNF-α than resistant mice (9-14). Similar results were obtained using macrophages derived from *Nramp1* knockout mice. We were able to show that in the macrophage cell line B10S (carrying Nramp1^{s}), phosphorylation of the STAT1 protein was less efficient upon IFN-γ stimulation when compared with B10R (carrying Nramp1^{r}) cells. STAT1 phosphorylation is an early event involved in the IFN-γ signal transduction pathway. This phosphorylation is absolutely required for normal IFN-γ signaling, as it facilitates dimerization of STAT1 and its translocation to the nucleus. Therefore, we correlated low levels of MHC-II expression in B10S with insufficient induction of CIITA, a factor necessary for MHC transcription. CIITA expression is upregulated by IFN-γ in macrophages and normal function of STAT1 has been shown to be required for this process. The data established up to date concerning Nramp1 structure and function has not yet provided an explanation of its involvement in IFN-γ signaling.

Nramp1 may function as a transporter affecting the availability of certain microelements, which would influence the activity of crucial enzymes involved in generating signal transduction (6). However, this possibility would most likely affect other signal transduction pathways also. No general defect in the signal transduction of macrophages expressing the susceptible form of Nramp1 has been reported. Therefore, another more specific mechanism of Nramp1-dependent regulation of IFN-γ-signaling might involve interaction of Nramp1 with components of the JAK/STAT system. Such an interaction could be mediated by the potential SH3 domain-binding region present in the N-terminal part of Nramp1. This part of the protein contains multiple proline residues that, although they are not organized into a typical SH3 binding domain, may however exhibit some binding activity (15). Many proteins, like kinases belonging to the *src* family, phospholipase C γ (PLCγ), phosphatidylinositol 3-kinase (PI3K), protein tyrosine phosphatases (PHP) and STAT proteins, contain SH3 domains (16-18). The binding of Nramp1 to STAT1 or other proteins containing an SH3 domain could be important for the assembly of the IFN-γ receptor complex, which permits the subsequent signal transduction initiation. Although this model seems to be very attractive, it is difficult to reconcile the fact that Nramp1 localizes in the membrane of the endosome and not in the cellular membrane where the IFN-γ receptor is assembled (19-21). Other genetic factors also affect the efficiency of IFN-γ signaling. We have observed a large heterogeneity in the magnitude of macrophage responses to IFN-γ if they are derived from genetically different strains of mice. In fact, we believe that the effect of the *Nramp1* gene can only be accurately analyzed using macrophages with the same or very similar genetic backgrounds that only differ from each other at the *Nramp1* locus, carrying either a susceptible, null, or resistant allele.

Macrophage activation is very efficiently achieved by exposure of the cells to a broad variety of compounds present mostly on the surface of the microorganism's cell. The best characterized activators of macrophages and other eukaryotic cells are lipopolysaccharides (LPS) produced by Gram-negative bacteria (3). The potency of the activating ability of LPS is demonstrated in a severe and very often fatal condition known as septic shock. It is characterized by an uncontrolled release of TNF-α and IL-1β as well as other mediators from activated macrophages (22). When tested *in vitro*, macrophages stimulated with LPS in the presence of IFN-γ produce large quantities of TNF-α, nitric oxide and other toxic nitrogen and oxygen intermediates. The nature of the observed synergy between IFN-γ and LPS in the mechanism of macrophage activation is not fully understood, but may be partially explained by the additive effect of activation of specific transcription factors for both stimuli. In addition, it was demonstrated that IFN-γ signaling is directly affected by LPS. In fact, LPS was shown to promote full activation of STAT1 by phosphorylation of S_{723} (23). On the other hand, IFN-γ activates dsRNA-activated protein kinase (PKR), which is implicated in the activation of NFκB transcription factor in response to LPS (24-26).

IFN-γ and LPS (or other compounds derived from bacteria, yeasts or parasites) usually augment each other's effects, although there are also multiple exceptions from this rule. Data presented in chapters II and III indicate that both *M. bovis* BCG and LPS from *E. coli* have a negative effect on IFN-γ-stimulated expression of MHC-II molecules. This effect seems to be mediated through the inhibition of CIITA mRNA expression. The effect of endotoxin from Gram–negative bacteria on the expression of MHC-II was extensively studied over the past few years. It was shown that LPS, when injected into mice, has a positive effect on MHC-II expression (27-30). However, when used *in vitro*, LPS may inhibit IFN-γ-stimulated expression

of MHC-II in a variety of cell types (31-33). For example, studies on IFN-γ-stimulated primary murine renal tubular epithelial cells (F1K cells) revealed that LPS treatment results in downregulation of both MHC-II and CIITA genes (34). In human endothelial cells, LPS augmented class I MHC (HLA-A,B,C) expression but inhibited IFN-γ-induced expression of class II MHC (HLA-DR) molecules (30). Sicher showed that LPS is able to inhibit IFN-γ-stimulated MHC-II expression in macrophages when the cells are exposed to both stimuli simultaneously, but not when the cells were treated with IFN-γ prior to the LPS addition. The inhibitory effect of LPS on MHC-II protein production was also shown in peritoneal and P388D1 cell line macrophages stimulated with IFN-γ (27, 31). The observed inhibitory effect was attributed to LPS-induced secretion of prostaglandin E2 by macrophages. In contrast, Ziegler demonstrated that the expression of MHC II on peritoneal macrophages from mice injected with *Salmonella minnesota*-derived lipopolysaccharides (LPS) or live *Listeria monocytogenes* was augmented (29, 32). Jephthah-Ochola presented *in vivo* results which show that administration of LPS to the mice augments expression of both MHC II and MHC I antigens on a variety of cell types including the kidney, the liver, the heart, the lung, and the pancreas. By using neutralizing antibodies, it was shown that IFN-γ is required for LPS-induced upregulation of MHC antigens. This suggests that upregulation of MHC-II expression observed *in vivo* is mediated by LPS-induced production of IFN-γ by lymphocytes. Interestingly, the upregulation of MHC II (but not MHC I) occurred only after repeated injection of LPS, which may suggest that developed tolerance to endotoxin leads to a decreased production of IFN-γ (28). In addition, human monocytes stimulated with IL-4 did not express high levels of MHC II when co-stimulated with LPS. A similar effect was not observed in monocytes stimulated with IL-4 in addition to TNF-α or GM-CSF. Also, it was shown that IL-10 did not contribute to the inhibitory effect of LPS (35).

It has been well-established that some cytokines such as IL-1β, TNF-α and TGF-β are also able to inhibit IFN-γ-induced CIITA mRNA expression (33, 36-41). Since TNF-α and IL-1β are produced by activated macrophages, it is not clear whether the negative effect of bacterial products on MHC-II expression is direct or is mediated by those cytokines. Since both signaling and biological effects (at the cellular level) of LPS, TNF-α and IL-1β are very similar, one can speculate that all of these stimuli contribute to the inhibition of MHC-II expression. An interesting additional question is whether the inhibition of MHC-II by microorganism-derived compounds is an adaptation to modify the immunological response or if it is an opportunistic exploitation of the regulatory mechanism developed by the host. The lack of MHC-II antigen expression, for example, in the rare condition known as bare lymphocyte syndrome (BSL), leads to reoccurring and fatal infections (42). Therefore, inhibition of antigen presentation by downregulation of MHC-II expression would provide a survival advantage for the infecting agent. On the other hand there are mechanisms negatively controlling multiple signaling pathways, involving SOCS, protein tyrosine phosphatases or PIAS proteins (43). These mechanisms are often activated by IFN-γ and may be accelerated by LPS or other mediators negatively influencing MHC-II expression. Expression of MHC-II genes by macrophages *in vitro* requires at least 18 h (9), which is much longer than the time necessary for upregulation of iNOS or TNF-α genes, whose expression is positively regulated by both IFN-γ and LPS. In fact, steady state levels of expression of these genes are reduced at time points longer than 16 h (12, 44). It suggests that, 12 to 16 h post-stimulation, the effects of IFN-γ and LPS are already being downregulated. It is possible that transcription of MHC-II requires positive signals to be present for a relatively long time to be able to activate all required factors involved in the process. This

may explain why the IFN-γ transcription activating signal accelerated by LPS inhibition affects expression of MHC-II genes but not iNOS or TNF-α genes.

The above speculations generate questions concerning the importance of LPS and *M. bovis* BCG in the regulation of MHC-II expression *in vivo*. Although there is no direct evidence that antigen presentation plays any role in eliminating infection of *M. bovis* BCG in mice, there is a correlation between less efficient i*n vitro* expression of MHC-II by macrophages expressing Nramp1^s and the susceptibility of mice carrying the *Nramp1*^{s} allele to infection with *M. bovis* BCG. On the other hand, it was shown that peritoneal macrophages from mice injected with LPS exhibit augmented expression of MHC-II (29). This would suggest that MHC-II is differentially regulated *in vitro* and *in vivo*. The augmented MHC-II expression was also observed in B-lymphocytes and dendritic cells isolated from animals injected with LPS (45).

The relevance of MHC inhibition for the development of infection is also supported by observations that many infectious agents develop specific mechanisms for interfering with the normal process of antigen presentation. For example, *Mycobacterium tuberculosis* was shown to partially inhibit both expression of MHC II and antigen presentation in murine bone marrow-derived macrophages (46). In addition, Wadee and colleagues showed that a 25 kDa glycoprotein encoded by *M. tuberculosis* was able to inhibit MHC-II expression in monocytes (47, 48). In the case of *M. avium*-infected macrophages, the downregulation of the expression of the IFN-γ receptor and STAT1 activation were correlated with decreased expression of I-E (MHC-II), IRF-1, Mg21 and CIITA mRNA (49). By using two murine macrophage cell lines; J774A.1 and P388D1, it was shown that infection of cells with another intracellular pathogen infecting macrophages, *Listeria monocytogenes*, (not with the non-pathogenic species of *L. innocua*) leads to the inhibition of MHC-II genes expression (50). Also, it was shown that outer membrane

vesicles of *Porphyromonas gingivalis*, when used in vitro on IFN-γ-stimulated human vascular endothelial cells, have a very potent inhibitory effect on MHC-II expression, regardless of whether the cells were stimulated before, at the same time, or after addition of IFN-γ (51). Both MHC-I and MHC-II expression were shown to be downregulated in bovine umbilical endothelial cells (BUEC) and bovine brain endothelial cells (BBEC) upon infection of these cells with *Cowdria ruminantium* (52). Also, treatment of mouse resident peritoneal macrophages with high molecular weight material (MDHM), purified from *Mycoplasma fermentans*, had an inhibitory effect on IFN-γ-induced MHC-II expression after its initial upregulation (53). It was also observed that mice infected with the fungus *Paracoccidioides brasiliensis* showed decreased levels of Ia expression in the spleen (54).

Evidence for the possible mechanisms utilized by microorganisms to target the expression of MHC-II antigens comes from the studies on human fibroblasts and epithelial cell lines infected with *Chlamydia trachomatis*. It was shown that infection of cells with chlamydia leads to the downregulation of both MHC-II and MHC-I antigens through a mechanism involving degradation of two transcription factors: USF-1and RFX-5 respectively (55, 56). The identification of a lactacystine-sensitive proteosome-like activity, which could degrade both USF-1 and RFX5 in a cytosolic fraction of chlamydia-infected cells, was shown to be of chlamydia origin rather than of host origin. Also, many viruses are able to directly inhibit the IFN-γ signal transduction pathways, or to downregulate the expression of the components of the IFN-γ signal transduction pathways, downregulating expression of both MHC-I and MHC-II antigens (57). For example, the varicella-zoster virus (VZV) infection of IFN-γ-stimulated human fibroblasts leads to downregulation of the expression of STAT1 and JAK2 proteins which, consequently leads to the decreased expression of IRF-1 and CIITA (58). The Ebola virus

affects the expression of genes regulated by IFNs, such as MHC I , 2'-5' oligoadenylate synthetase [2'-5'(A)N], and IFN regulatory factor 1 (IRF-1) (59). Using IFN-γ-stimulated U373 MG astrocytoma cells, it was shown that the human cytomegalovirus is able to downregulate the expression of CIITA, which leads to suppression of HLA-DR gene expression (60). Interestingly, peripheral human monocytes infected with the measles virus (MV) expressed 10-fold higher levels of MHC-II than uninfected monocytes. However, IFN-γ-stimulated expression of MHC II in the monocytic cell line THP-1 was strongly inhibited by MV infection (61). Some other strategies employed by viruses involve disruption of the normal process of MHC class I complex formation. For example, during infection with the herpes simplex virus (types 1 and 2) the human transporter associated with antigen presentation (TAP) is very efficiently inhibited by the ICP47 protein expressed by HSV-1 and HSV-2, which leads to the blocking of MHC-I antigen presentation to CD8+ T-cells (62, 63).These numerous examples of disruption of the normal process of antigen presentation provide important clues regarding the mechanism of antigen presentation in infected cells.

Pathogens may also use host natural regulatory mechanisms to protect themselves from recognition and elimination. As mentioned before, many cytokines modulate expression of both MHC-II and CIITA when tested *in vitro*. Infection of macrophages with murine cytomegalovirus (MCMV) leads to early induction of high levels of the IL-10 cytokine, which results in downregulation of expression of MHC-II genes, thus allowing the virus to escape immunological surveillance (64-66). The negative effect of IL-10 as well as that of IL-4 and TGF-β on IFN-γ-induced expression of MHC-II and CIITA genes was observed in the murine microglial cell line EOC 20. The inhibitory effect of IL-10 and TGF-β but not that of IL-4 was also seen in primary murine microglia (41). TGF-β appears to be the main negative regulator of CIITA transcription

and its effect was demonstrated not only in macrophages, but also in IFN-γ-stimulated human astroglioma, fibrosarcoma and fibroblast cell lines (38-40). TGF-β is produced by macrophages when stimulated with LPS in the presence of IFN-γ; therefore, it could contribute to the inhibition of MHC-II *in vitro* (67). This cytokine, however, has very broad and generally negative effects on macrophages, manifested by the inhibition of proliferation and the blockage of the activated state of macrophages. The negative effect of LPS and *M. bovis* BCG on IFN-γ-induced expression of MHC-II and CIITA seemed to be rather specific and did not restrict the expression of other genes. Interleukin-1β is another cytokine which has the ability to inhibit MHC-II expression. Using human astroglioma cell lines, it was shown that IL-1β had an inhibitory effect on IFN-γ–stimulated transcription of the CIITA gene, but it had no effect on STAT1 phosphorylation, nuclear translocation or binding to the GAS element in the CIITA promoter IV region (36). This characteristic is similar to that we observed when the inhibitory effect of LPS was tested. This suggests that mechanisms of LPS and IL-1β-induced repression of MHC-II expression may overlap, which is understandable since both of these stimuli utilize almost identical signal transduction pathways. On the other hand, both IL-1β and TNF-α were able to augment IFN-γ-induced expression of MHC-II on human monocytes and on the monocytic cell line THP-1. The augmented MHC-II expression was shown to result from increased responsiveness of those cells to IFN-γ due to the IL-1β- and TNF-α-induced upregulation of the IFN-γ receptor expression (68). Interestingly, it was shown that IL-1β expression is inhibited in macrophages by IFN-γ in STAT1 dependent-manner (69, 70). This means that IFN-γ may positively influence MHC-II expression not only by stimulating its transcription but also by eliminating negative factors.

In addition to TGF-β and IL-1β, the inhibitory effect of IFN-α/β on IFN-γ-stimulated expression of Ia was also shown in macrophages (71, 72). IFN-β has an antagonistic effect on IFN-γ-induced expression of CIITA. The IFN-stimulated gene factor 3gamma (ISGF3) was shown to be essential for IFN-β to mediate inhibition of MHC-II induction (73). This adds an interesting aspect to the molecular mechanism of inhibition of CIITA and MHC-II expression. Macrophages are able to produce IFN-β when activated with IFN-γ and LPS (74, 75) and it was demonstrated that low basal levels of IFN-β are necessary for efficient formation of the IFN-γ receptor complex and its signal transduction. Therefore, the observed inhibitory effect of IFN-β on MHC-II expression would suggest that the concentration of stimuli, the sequence of the exposure to stimuli, and the way cells are stimulated with respect to the timing and relative order of stimuli may determine the nature of its effect. This is also true for TNF-α, which can either augment or inhibit IFN-γ-stimulated expression of MHC-II, depending on the cell type and its developmental stage (68, 76-78). It was reported that incubation of the murine myelomonocytic cell line WEHI-3 with both IFN-γ and TNF-α, leads to upregulation of I-A gene expression (79). Also, human islet beta-cells expressed MHC-II when stimulated with IFN-γ and TNF-α (80). In contrast, Collins showed, using human endothelial cells (HEC) and human dermal fibroblasts (HDF), showed that TNF-α is not effective in the induction of MHC-II expression, although it did stimulate expression of MHC-I molecules (81). TNF-α was also shown to lower the stability of CIITA mRNA, thereby affecting MHC-II expression induced by IFN-γ in the human fibrosarcoma cell line HT1080 (37). The above examples of the complexity of the effects that different cytokines may have on MHC-II expression make the relative contribution of all possible factors affecting it difficult to assess. For example, concentrations of cytokines endogenously produced by macrophages in response to stimulation with LPS increase slowly and

may have a different effect than the same stimulus added once at high concentration. Gradual increase in the concentration of a particular cytokine leads to different dynamics of the mechanism regulating the feed-back response compared to the feed-back mechanism induced by a high dose of the same cytokine. On the other hand, complete removal of a certain factor, using neutralizing antibodies or cells derived from knockout animals, may sometimes lead to unexpected results that are difficult to interpret. For example, removal of IFN-β may lead to impaired IFN-γ signaling and not to lack of inhibition of MHC-II, as anticipated. Therefore, the study of the basic molecular mechanism of LPS-induced inhibition of MHC-II expression is important and may allow identification of factors triggering this mechanism.

Based on the results presented in chapters II and III, the inhibition of IFN-γ-stimulated expression of MHC-II appears to be correlated with inhibition of CIITA. As described in chapter I, the so-called master regulator of MHC-II gene expression is believed to be absolutely necessary for both constitutive and inducible expression of these genes. In macrophages, both CIITA and MHC-II are expressed only in an inducible manner. IFN-γ is one of the best characterized and apparently one of the most biologically important inducers of both CIITA and MHC-II expression. So far, very little is known about post-transcriptional regulation of CIITA expression and studies are mainly focused on transcriptional control of this gene. In macrophages, IFN-γ inducible expression of CIITA is regulated by promoter IV (PIV), although the influence of PIII in this process was reported as well (82, 83). Nevertheless, it was shown many times that the presence of the proximal region of promoter IV is necessary for IFN-γ-dependent expression of CIITA. This part of the promoter contains the GAS element binding the STAT1 homodimer and the IRF1/2 binding site, which are both separated by a USF-1 (up-stream stimulating factor 1) binding region called the E-Box (84-87). All three elements and the

presence of factors which bind to them are required for optimal expression. Interestingly, it was shown, using IRF-2 knockout cell lines, that the IRF-2 transcription factor is also crucial for IFN-γ-stimulated expression of CIITA. This is a rare example of an enhancing effect of IRF-1 and IRF-2 on transcription (88, 89). As shown in chapter III, IFN-γ-induced binding of STAT1 and USF-1 to the proximal region of CIITA PIV is not altered by LPS in magnitude, which would explain the dramatic decrease in CIITA transcriptional activity. Since the stability of CIITA mRNA was not affected by LPS treatment (data not shown), it is possible that more distal parts of PIV are responsible for LPS and *M. bovis* BCG-induced inhibition of CIITA transcription. This region of PIV contains potential AP-1 sites and an NF-GMα element that are still not yet fully characterized (82, 87). In experiments where binding of nuclear proteins to the full length sequence of PIV was evaluated, we did not observe any obvious differences between samples from cells stimulated with IFN-γ and with IFN-γ in the presence of LPS. This suggests that other mechanisms are involved in the inhibition of CIITA transcription, like, for example, competition of IFN-γ and LPS signaling pathways for common transcription factors or accessory proteins which are expressed in limited quantities. Alternatively, testing of more broad kinetics of PIV binding by nuclear proteins, as well as the analysis of promoter IV deletion mutants in a reporter gene assay, would be required to exclude inhibition at the level of promoter.

An interesting possible way of regulation of the expression of MHC-II is the natural mechanism of CIITA expression silencing used by human fetal trophoblasts. It involves methylation of the CIITA promoter region, thus preventing its transcription (90, 91). Similar blockage or silencing of CIITA gene transcription takes place in maturating dendritic cells, although the mechanism in this case is completely different. During LPS-induced maturation of dendritic cells (after the initial increase in MHC-II transcription), general deacetylation of

histones in the entire regulatory region of the CIITA gene occurs (92). The increase in MHC-II expression is believed to be due to the increased stability of the mRNA but not due to upregulated transcription. CIITA is downregulated very quickly and affects promoters I and III, which regulate constitutive transcription of this gene in dendritic cells. Whether deacetylation of histones of the promoter IV in macrophages is responsible for LPS-induced inhibition of CIITA remains to be established.

Recent advances in our understanding of the LPS signal transduction pathway thanks to the cloning and the characterization of the Toll-like receptor system allows us to appreciate the complexity and importance of mechanisms involved in recognizing microorganisms (3). The type of molecules bound by Toll-like receptors range from lipopolysaccharides derived from Gram-negative bacteria (TLR4), through to lipoarabinomannan, peptydoglycans, small lipoproteins (TLR2) to double-stranded RNA and unmethylated CpG DNA sequences (93). The structures recognized by TLRs are very distinct and characteristic of the pathogens. Usually, a compound that is recognized by a TLR is important if not essential for the microorganism to survive. Therefore, it should usually not be subjected to random mutation, which would change its structure and its functionality. This provides a perfect target for the receptor and provides a high degree of specificity. Thanks to this specific recognition, innate immunity is usually much less likely to develop autoimmune reactions than adaptive immunity. Thus macrophages, for example, are "equipped" with a repertoire of receptors which allow them to specifically detect both Gram- positive and Gram-negative bacteria, mycobacteria, fungi and protozoa. In addition to high specificity, the ability of the Toll-like system to simultaneously recognize multiple structures from the same organism provides a very strong activating signal, especially since it is linked to the powerful transcription factor NFκB (94).

Although it seems that all TLRs use the same general signal transduction pathway, there are certain differences in the way particular receptors trigger biological effects. For example, signals originating from TLR4 use at least two separate adaptor proteins: Myd88 and TIRAP/MAL (95, 96). Using cells from MyD88 knockout mice, it was shown that both pathways trigger activation of different genes. On the other hand, TLR2 and TLR9 use the MyD88-dependent pathway only, which allows us to introduce a precise mechanism of regulation for both types of signal transduction. It seems that differences in TLR expression in the cell determines the cell's ability to recognize microorganisms. The constitutively-expressed TLR4 gene allows quick response to infections and microbial products (97). In contrast, TLR2 expression is induced in macrophages. For example, induction can happen in response to LPS and most likely also to by other bacterial products, including products derived from *Mycobacteria*. This mode of expression imposes some delay in response but also provides a mechanism to specifically react to an increasing number and concentration of stimuli. Combination of such independently-developed signals may lead to the generation of specialized responses depending on the type of infection, its dynamics and even its localization in the body. Although not yet fully tested, the repertoire of expressed TLRs may differ depending on cell type, or even depending on subpopulations of macrophages. As pointed out in chapter I, the fact that the binding of certain ligands require formation of specific heterodimers provides a very powerful mechanism of regulating responsiveness to that ligand. In addition, certain TLRs may compete for the formation of the receptor heterodimer with other TLRs and, by that, would generate nonfunctional complexes or complexes with altered specificity.

The results presented in chapter IV suggest that TLRs may regulate each other's expression. We observed that in cells which do not express TLR4, IFN-γ had a strong

upregulating effect on TLR2 expression. These results also demonstrate that even in the absence of TLR4, macrophages are able to detect Gram-negative bacteria. In fact, commercially available, unpurified preparations of LPS are able to stimulate TLR2 mRNA quite efficiently on their own, although efficient translation of the mRNA seems to require IFN-γ. Whether TLR2 is in fact responsible for activating TLR4-deficient macrophages remains to be established. Results using TLR2 knockout macrophages indicate that this receptor is not necessary for normal response to LPS alone or in combination with IFN-γ.

The interaction between TLR pathways is limited and, for example, tolerance to subsequent stimulation induced by one ligand affects responsiveness to another ligand even when it is using a different receptor. Pretreatment of mouse peritoneal macrophages with macrophage-activating lipoprotein 2 kDa (MALP-2), derived from mycoplasma ligand for TLR2, leads to subsequent hyporesponsivnes of the cells to stimulation with LPS (which binds TLR4). This tolerance was not due to the downregulation of TLR4 expression (98, 99). This suggests that so-called cross-tolerance is the result of disregulation of the components of TLR signal transduction pathways downstream from the receptors. The cross-tolerance can also be induced by TLR4 ligands, namely LPS, and impair responsiveness to TLR2 ligands (100). In addition, it has been shown that soluble paracrine mediators are not responsible for induction of hyporesponsiveness to both TLR2 and TLR4 ligands. The induction of tolerance to endotoxin represents an important mechanism for silencing the signal induced by LPS. This phenomenon adds another level of regulation to macrophage activation.

The biological effects mediated by Toll-like receptors seem to be regulated in a complex fashion despite the fact that almost all the pathways utilize the same components. To understand and define these interlocked dependencies, it is important to characterize ligands stimulating

particular pathways. In addition, as proven by the discovery of so-called "contamination" in commercially-available preparations of LPS, it is also very important to use highly purified homogenous ligands. As described in chapter IV, re-purification of commercial preparations of LPS with phenol is believed to generate a pure fraction able to activate macrophages only in the presence of functional TLR4. However, the existence of fractions with suppressive ability in this preparation cannot be excluded. In fact, we were able to observe a very weak negative effect of LPS re-purified with phenol on NO production as well as on TLR2-protein expression. The TLR4-Del cells stimulated with phenol-extracted unLPS (LPS-E) only produced levels of nitric oxide lower than basal levels. These results, although statistically not significant (mainly due to the very low values measured), were observed very consistently (data not shown). In addition, down-regulation of IFN-γ-induced expression of TLR2 protein by pure LPS-E could be observed (chapter IV) in TLR4-deficient macrophages. Less pronounced inhibition of IFN-γ-induced TLR2 expression by unpurified unLPS could be explained by the fact that this preparation of LPS has the ability to induce TLR2 expression in TLR4-deficient cells. At the same time, LPS-induced expression of TLR2 in wild-type macrophages (B10R, RAW264.7) seems to be inhibited by IFN-γ. This phenomenon, however, may result from a very strong initial stimulation of TLR2 expression by IFN-γ and LPS. The analysis of TLR2 mRNA expression revealed that stimulation of macrophages with LPS in the presence of IFN-γ leads to a quick and very strong upregulation of this gene. However, negative feedback mechanisms in response to LPS are also induced more efficiently in the presence of IFN-γ, leading to an acceleration of downregulation compared to with the effect of LPS alone.

In conclusion, the process of macrophage activation appears to be tightly regulated and depends on endogenous genetic factors and on the context of external stimuli. The regulation of

expression of genes affected during macrophage activation involves products secreted by activated macrophages. The involvement of cytokines and other factors (like prostaglandins) in macrophage function is also apparent. To propagate primary cells *in vitro*, they have to be exposed to growth factors; therefore, the results obtained *in vitro* should always be interpreted with caution. Similarly, any results obtained using immortalized cell lines should be verified with freshly isolated cells. However, the primary macrophages are heterogenous and selection of the appropriate source of fresh macrophages might be sometimes difficult. For example, freshly isolated bone marrow macrophages must be constantly cultured in the presence of growth factors such as CSF-1 or GM-CSF, that were shown to cause macrophages to differentiate into slightly different phenotypes. The bone marrow macrophages cultured in the presence of CSF-1 produce higher levels of IFN-α/β compared to macrophages stimulated with GM-CSF, making them more resistant to infection with vesicular stomatitis virus (VSV) (101). In contrast, macrophages exposed to GM-CSF expressed higher levels of MHC-II and were much better able to activate specific T-lymphocytes than macrophages incubated in the presence of CSF-1. On the other hand, CSF-1-stimulated macrophages expressed higher level of MHC-II in response to IFN-γ (102). The above observations might suggest that the phenotype of freshly isolated macrophages might vary depending on culture conditions. Since the macrophage cell lines used in the presented studies were developed from the bone marrow macrophages initially cultured in the presence of GM-CSF their phenotype was most likely determined prior to immortalization.

The ultimate goal of studies on macrophage function is to modify this function to better control infection or cancer disease or to prevent the development of such fatal conditions as sepsis. The knowledge of regulatory mechanisms employed by pathogens can also help develop better treatments for autoimmune diseases.

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CHAPTER VI

CLAIMS TO ORIGINALITY

1. The phenotypic differences between macrophages from *M. bovis* BCG resistant and susceptible mice are manifested by their different responsiveness to IFNγ. We showed that in macrophages derived from *M. bovis* BCG susceptible mice the IFNγ-induced phosphorylation of STAT1 protein is less efficient that in their resistant counterparts.
2. It was previously published that macrophages from *M. bovis* BCG susceptible mice express lower levels of MHC class II mRNA and protein in respond to IFNγ stimulation when compared to macrophages from *M. bovis* BCG resistant mice. We demonstrated that Class II Transactivator (CIITA) mRNA levels are also lower in these macrophages upon stimulation with IFNγ.
3. CIITA was shown to be required for both inducible and constitutive expression of MHC class II. We demonstrated that IFNγ-induced expression of MHC class II and CIITA was inhibited in *M. bovis* BCG infected macrophages.
4. The CIITA expression in IFNγ-stimulated macrophages can be inhibited by exposure of the cells to lipopolysaccharide (LPS). We demonstrated that the negative effect of LPS on CIITA transcription does not result from impaired binding of STAT1 protein to the CIITA promoter IV.
5. The inhibitory effect of lipopolysaccharide but not that of *M. bovis* BCG on IFNγ-induced expression of CIITA and MHC class II expression is dependent on Toll-like receptor 4.

6. The Toll-like receptors-mediated pathways include the adaptor protein MyD88 which participates in transmitting signals originating from a specific ligand binding to a Toll-like receptor. For TLR4 however, there exists an alternative pathway which depends on another adaptor protein called TIRAP/MAL. We showed that the inhibitory effect of *M. bovis* BCG infection and lipopolysaccharide stimulation on IFNγ-induced expression of CIITA and MHC class II expression is MyD88-dependent.
7. The phenol soluble fraction present in commercial preparation of LPS from Gram-negative bacteria was shown to be able to activate macrophages in a TLR4 independent manner. We have partially characterized this fraction and demonstrated that this fraction (called by us TRIL) is phenol-soluble, heat-stable, protease-resistant and ethanol-labile.
8. The expression of Toll-like receptor 2 in macrophages was shown to be upregulated by a variety of cytokines as well as bacterial cell wall products. We demonstrated that IFNγ-induced TLR2 expression is dependent on the expression of functional TLR4 in murine macrophages.
9. We demonstrated that post-transcriptional mechanisms might be involved in the regulation of expression of Toll-like receptor 2.