Instituto Juan March
de Estudios e Investigaciones

18 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Mechanisms of Macrophage Activation

Organized by

C. Nathan and A. Celada

D. O. Adams
M. Aguet
C. Bogdan
A. Celada
J. R. David
A. Ding
R. M. Fauve
D. Gemsa
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J. A. M. Langermans
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MOLECULAR MECHANISMS OF MACROPHAGE ACTIVATION

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R.M. Fauve - Immunostimulatory Human Urinary Protein.

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INTRODUCTION

A. Celada

The idea of organizing the Workshop on "Molecular mechanisms of macrophage activation" in Madrid was to cover two objectives: first, to make the point of the recent discoveries in the field of macrophage activation. In this regard it is the first time that scientists working only on molecular biology were included in a meeting related to macrophage activation. The second objective was to meet, also for the first time, other Spanish collegues working in this field. Two people should take the major credit for the organization of this meeting. Andres Gonzalez: as usual he provided efficient management, which made my task easy and pleasant. The second person is the co-organizer, Carl F. Nathan, who helped me in contacting a selected list of brillant and active investigators in the field of macrophages. With his prestige, Carl supported this meeting, which, due to the small size and the good facilities of the Juan March Foundation, made for an easy interaction between participants. I am particularly grateful for the kindness of Carl F. Nathan, who was already involved in the organization of two other meetings for the present year in related fields. Without the effort of Andres and Carl this meeting would not have been possible. Finally, I should thank all the participants who spent some of their busy schedules coming to Madrid and making the successs of the meeting possible.

I would like to mention that Spanish research in the last 15 years has made a dramatic improvement. This is due, certainly, to the political and economical changes that have occured with the return of democracy to Spain. The increase in the budgets for research programmes resulted in a large number of publications appearing in scientific journals, produced by

groups working in Spain. Another important aspect of science is communication, and we are very glad that some private foundations such as Juan March decided to support with enthusiasm and organize these biology meetings that help the interactions between Spain and the rest of the Scientific community. One of the products of this effort is that many of the invited scientists to the workshop made their first visit to Spain, where they can learn through the presentations carried out during the meeting that, although Spanish science still needs to develop, puting our emphasis on the quality of the research, we are travelling in the good direction.

The small number of participants allowed good interaction during the presentations, the coffee breaks and lunches. Many cooperative projects will start from this meeting, helping all of us to do, in a better way, one of the things that we enjoy: research. Also, for young participants, it was a unique opportunity to meet one of the best groups of scientists working in the field of macrophages. I am quite sure that in the near future some of these participants will apply for training as post-docs in some of the labs that made the presentations.

Macrophages have an important role in the immune system. These cells use different mechanisms: a) directly, macrophages destroy bacteria, parasites, virus and tumor cells; b) indirectly, macrophages release several mediators (IL-1, TNF, etc) that can activate other cells and c) as accessory cells, macrophages are able to process antigens and to present digested peptides to T-lymphocytes. In order to become activated and to be able to develop functional activity, macrophages need to interact with molecules such as interferon gamma (IFN γ), Interleukin 4 (IL4), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), etc. These factors induce more than 100 different genes in macrophages. Some of the receptors for

the molecules that induce macrophage activation have been characterized. However, little is known about the events that follow the interaction of the ligands with the receptors, and which induce the expression of the different genes. At present, the study of the regulation of eukaryotic gene expression has focused on both the sequences required for gene expression and the cellular factors that interact specifically with these controlling elements. These regions have been defined as *cis*-acting elements and a series of proteins that bind these regions (*trans*-factors) have been identified and cloned. However, the mechanisms and modifications induced during the process of activation that allow these factors to initiate the transcription of genes are at present unknown. The concept of macrophage activation was defined in the 1960's, although with recent developments in the areas of cellular and molecular biology, this concept has had to be redefined 30 years later.

Although some molecules have been well characterized as macrophage activators, the list of such molecules is still growing. The recent techniques developed in biochemistry, cellular and molecular biology have allowed us to recognize, purify and clone new molecules that are able to induce, if not all, at least some of the functional aspects of macrophages.

In the study of molecules that activate macrophages, the next step is to define the interaction with the cells at the surface. Receptors are proteins in which, after the interaction with the ligands, an intracellular signal is produced that initiates the functional activity. Moreover, receptors usually internalize the ligands upon which they become degraded and the receptor again free of ligand can come back to the cell surface.

As a result of the ligand-receptor interaction a cascade of second signals is produced inside the cell. Some of these signals induce the expression in

minutes of some genes (early genes) that are required for the subsequent transcription of other genes directly related with the functional activity of the macrophages.

In a second step, after activation, some metabolic pathways are induced in macrophages. The products of these pathways are important elements in the defense against several microrganisms. However, under some conditions, these products could also be toxic for the macrophages.

The last step for the effects of the molecules that activate macrophages is the regulation of gene expression by transcription factors. In order to induce transcription, these factors, which are proteins, need to interact with the regulatory elements of the genes. In some cases, it has been shown that transcription factors required some modifications, such as phosphorylation to initiate gene expression.

One of the ways to understand the mechanism of macrophage activation is the use of inhibitors. Many of these inhibitors are molecules such as interleukins produced in the body during the immune response. In some instances, drugs have been shown to be potent inhibitors, and in some cases they can be used therapeutically.

Antonio Celada

1. Definition of Molecules that Induce Macrophage Activation

The Biology of Recombinant Migration Inhibitory Factor (MIF).

J.R. David, R. Titus, C. Shoemaker, M. Soares, C. Sassatti, I. Bosch, C. and W. Weiser

Migration inhibitory factor, identified by its ability to prevent the migration of macrophages out of capillary tubes, was the first lymphokine to be discovered. Using functional expression cloning in COS cells, a cDNA encoding a human MIF was isolated (1). Recombinant MIF is a 12.5 kD protein with an amino acid sequence different from any previously described protein or cytokine. It has two potential glycosylation sites, three cysteines and lacks a conventional leader sequence.

rMIF, when incubated with monocyte-derived macrophages, enhances or induces HLA-DR, IL-1b (2), IL-6, TNFα, and macrophage-to-macrophage adherence. It also induces nitric oxide synthase, nitric oxide (3) and hydrogen peroxide.

rMIF activates human and murine macrophages to kill *Leishmania* parasites (4), tumor cells, and to inhibit the growth of *Mycobacterium avium* (5).

Some of these properties suggest that rMIF might be useful in enhancing the immune response and be of use in developing vaccines. Indeed, rMIF when given to mice with BSA or HIV gp120 in incomplete Freund's (ICFA) adjuvant will elicit antigen-induced T cell proliferation similar to that elicited by complete Freund's adjuvant (2). Liposomes can be used instead of ICFA. rMIF also enhances antibody production.

In further studies to understand the mechanism of MIF, we found that rMIF will cause the proliferation of mouse spleen cells. Flow cytometry shows that the blasts are CD4 and CD8 T cells and B cells. Proliferation by rMIF is markedly enhanced by IL-2, IL-4, and IL-6, but is not affected by IL-1, IL-5, IFNγ, IFNα, and TNFα.

In preliminary experiments to determine whether rMIF can be used in developing vaccines, mice injected with rMIF and the recombinant leishmanial antigen gp63 (from McMaster) develop much smaller lesions to a subsequent challenge with live *L. major* than controls.

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Activation of Macrophages by Influenza A Virus Infection: Priming for Enhanced Cytokine Release and Death by Apoptosis

Diethard Gemsa, Institute of Immunology, Philipps University, Marburg, Germany.

Macrophages have been shown to be susceptible to influenza A virus, strain A/PR8. Although virus replication was rather restricted in macrophages, infection was accompanied by a marked TNF-α mRNA accumulation, an only low TNF-α protein release and subsequent cell death. TNF-α production was dependent on exposure to live virus, in contrast to IFN release that was also induced by UV-inactivated A/PR8. Most strikingly, low and by itself rather inefficient amounts of LPS (1-10 ng/ml) were capable of strongly potentiating TNF-α production from virus-infected macrophages. The potentiating effect of LPS was neither due to increased survival of macrophages nor to altered virus multiplication, enhanced TNF-α gene transcription, or shifts in the kinetics of TNF-α release. Thus, A/PR8 infection appeared to prime macrophages for high TNF-α production in response to secondary, triggering signals such as LPS. We found that virus induced accumulation of TNF-α mRNA was due to both transcriptional and post-transcriptional changes: an enhanced TNF-α gene transcription and a markedly prolonged half-life of TNF-α mRNA. A unique property of A/PR8-induced TNF-α gene transcription was the appearance of two types of mRNA, a typical 1.6 kb species which was also found after LPS stimulation, and a novel high molecular weight (hmw) TNF-α mRNA of 2.6 kb. Similar mRNA double-bands were also found with IL-1 and IL-6 gene transcripts. The hmw TNF-α mRNA did not contain intron structures as tested by intron-specific, PCR-generated cDNA probes, thus excluding the possibility that primary TNF-α gene transcripts in the nucleus were transported into the cytoplasm. A RACE analysis indicated that the hmw TNF-α mRNA was generated by addition of sequences located within the untranslation 5'region of an otherwise correctly spliced TNF-α gene transcript. A sequence analysis is presently in progress and may give a first hint whether the virus-induced hmw TNF-α mRNA codes for a biologically active product. Although influenza A virus infections of susceptible target cells are usually accompanied by a rapid cell lysis caused by membrane disruption of budding virus particles, macrophage killing was not due to cell necrosis but proceeded by programmed cell death, i.e. apoptosis. Apoptosis was documented by chromatin condensation, formation of membrane-bound apoptotic bodies and typical DNA fragmentation (DNA ladder) into multimers of 180 bp. These data show that influenza A virus represents an ultimately fatal macrophage activating factor which primes for a high cytokine release in response to secondary signals but simultaneously induces a program leading to controlled cell death.

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IMMUNOSTIMULATORY HUMAN URINARY PROTEIN.

Robert M. FAUVE, Elisabeth FONTAN, Hélène SAKLANI & Brigitte HEVIN, Unité d'Immunophysiologie cellulaire, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.

In earlier studies, we have shown that mice with talc-induced granuloma are more resistant against bacteria, parasites and malignant cells injected in a remote site. From the murine granuloma was extracted a 55 kDa protein pi:5 which was found to protect mice against a lethal inoculum of Listeria monocytogenes and to activate peritoneal cells against Lewis carcinoma cells. A polyclonal antibody prepared against this protein was shown to react with a human protein fraction isolated from the urine of healthy donors. From this fraction were isolated two glycoproteins : one of 43 kDa (HGP.43) and the other of 92 kDa (HGP.92). These two proteins once injected into mice fully protected them against a lethal inoculum of Listeria (250 μg/kg) and this protection was also found in scid mice. An increased bactericidal activity was found in resident macrophages and, with HGP.43 the increased resistance against Listeria was also found 8 hours after infection. Both proteins in nanomolar amount were able to activate resident and inflammatory macrophages in vitro against Lewis carcinoma cells. These effects were abolished with a monoclonal antibody. Furthermore, in HGP.43 treated mice injected with Lewis cells, no effect was found on the growth of the primary tumor but a reduction of lung metastasis was observed. Following N-glycanase treatment of HGP.92, the activity either in the Listeria system or in vitro against carcinoma was lost. Since HGP.43 is obtained from pronase treated HGP.92, we therefore better characterize this protein.

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MACROPHAGE ACTIVATION, THE CYTOKINE NETWORK AND ENHANCED ANTIMICROBIAL ACTIVITY

Jan A.M. Langermans, Mariëlle E.B. van Vuren - van der Hulst, Peter H. Nibbering, R. van Furth
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Macrophages are activated by a variety of factors, such as cytokines and microorganisms or their components. One of the major cytokines involved in macrophage activation is interferon-γ (IFN-γ) (1). Incubation of murine peritoneal macrophages with IFN-γ in vitro results in the secretion of TNF that in an autocrine fashion stimulates the cells to generate reactive nitrogen intermediates (RNI) (2). IL-1, which is also produced by IFN-γ-activated macrophages, is not involved in the generation of RNI by these cells (1,2).

The role of RNI in the microbicidal activities of activated macrophages is studied with the intracellular protozoa Toxoplasma gondii. This protozoa proliferates in nonactivated macrophages while in activated macrophages, e.g. by IFN-γ, this proliferation is inhibited. In vitro, two phases during macrophage activation are distinguished: the activation phase, i.e. the phase during the cells are incubated with cytokines, and the effector phase, i.e. the phase during which the enhanced microbicidal activities are effectuated. Induction of toxoplasmastatic activity correlates well with the generation of RNI by murine macrophages (3). Inhibition of the generation of TNF or RNI by macrophages during the activation phase abrogates the induction of toxoplasmastatic activity by the macrophages, whereas only a slight effect on the toxoplasmastatic effect was found when TNF and RNI production were inhibited during the effector phase (2). The RNI-generating compound nitroprusside (NP) did not affect the viability of T. gondii and did not inhibit the intracellular proliferation of T. gondii in nonactivated macrophages. In combination with a suboptimal concentration of IFN-γ, NP activated the macrophages to inhibit intracellular T. gondii proliferation. These data demonstrate that TNF and RNI are involved in the acquisition of the toxoplasmastatic activity of activated murine macrophages but indicate that these compounds are not toxic effector molecules for T. gondii.

Recently, we have found that NP increases tyrosine protein kinase (TPK) and G-protein activity in murine peritoneal macrophages. Inhibitors of G proteins and of TPK activity partly reduced the toxoplasmastatic activity of activated macrophages. Activation of soluble guanylatecyclase is not involved in the RNI-dependent toxoplasmastatic activity although NP also induces a transient increase in the cGMP concentration in murine macrophages.

The deactivating cytokine transforming growth factor β (TGF-β) inhibited the generation of RNI and the toxoplasmastatic activity of murine macrophages. TGF-β also inhibited the production of eicosanoids by activated murine macrophages, which raised the question whether these compounds are involved in macrophage activation. Inhibition of phospholipase A_2 (PLA_2) activity abrogated both RNI generation and toxoplasmastatic activity whereas inhibition of the cyclooxygenase and lipoxygenase pathways had no effect on these functions. These results indicate that arachidonic acid is an important intermediate in the RNI-generation and induction of toxoplasmastatic activity of murine macrophages.

Together, our results indicate that IFN-γ activates murine macrophages via induction of TNF that, in an autocrine fashion, activates PLA_2. Next, arachidonic acid stimulates the release of RNI that then stimulates various biochemical pathways which are involved in the enhanced antimicrobial activity of the cells.

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INTERLEUKIN 7 INDUCES MACROPHAGES TO KILLING OF INTRACELLULAR PARASITES.
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Recent work has shown that interleukin 7 (IL-7) is able to induce secretion of cytokines and tumoricidal activity by human monocytes (1). Here data will be discussed indicating that treatment of murine macrophages infected with Leishmania major with IL-7 reduces the percentage of infected cells in a dose dependent manner to a limited degree (45%) (2). Simultaneous treatment of macrophages with IL-7 and inferferon y resulted in an almost complete elimination of amastigotes. Production of nitric oxide was induced by IL-7. Addition of NMMA or anti TNFa reverted the leishmanicidal effects of IL-7. These data strongly suggest that IL-7, besides other functions, is important in mediating the host macrophages response to an intracellular parasitic infection, and complements the effects of factors such as IFNy, IL-4, TNFa and MIF (3, 4, 5)

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Regulation of macrophage activation by a new Th2 cytokine, IL-13

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The classification of helper T lymphocytes into functional subsets based on their cytokine secretion profiles (1) is of conceptual importance to the field of Mø research because cells of the monocyte/Mø lineage feature prominently among effector cells under the influence of these secreted cytokines. Cells of the Th1 subset are characterized by their production of lymphotoxin, IL-2 and interferon-γ, favouring cell-mediated immune events, with monocytes and Mø being attracted to sites of inflammation and activated for killing, notably by interferon-γ. The typical Th2 pattern, which promotes humoral immunity, is expression of IL-4, IL-5, IL-6, IL-10 and the induced gene P600, encoding the protein now known as IL-13. The changes these Th2 cytokines induce on Mø and the way these cells subsequently contribute to effector functions are less well defined. In common with IL-4, IL-13 inhibits proinflammatory cytokine production by Mø (2) and appears to be a member of the family of IL-4-related lymphokines (3) based on its close chromosomal linkage and coexpression with IL-4. We investigated the effects of murine rIL-13 on growth, phenotype and activation on bone marrow and peritoneal Mø populations. Significant proliferation of bone marrow and peritoneal macrophage populations was observed when cultured with IL-13. Expression of Mø mannose receptor, a receptor important in the binding and ingestion of mannosylated compounds such as glycoproteins and some microorganisms, and suggested as a marker of 'alternative' activation (4), was enhanced on elicited peritoneal Mø by IL-13, as was expression of MHC class II antigen. Killing capacity, as reflected by production of nitric oxide and superoxide anion was neither increased nor decreased. These activities closely mimic those of IL-4, but mediation of the effects by IL-4 was discounted by the use of a neutralizing MAb. Thus, IL-13 resembles IL-4 in its actions on Mø in providing a stimulus for both proliferation and differentiation, to the end of generating Mø committed to a non-inflammatory activation state distinct from classical activation induced by interferon-γ, and from the general deactivation induced by IL-10 (5).

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MACROPHAGE ACTIVATION OF THE SEAWATER TELEOST GILTHEAD SEABREAM (Sparus aurata L.) INDUCED IN VITRO BY CON A AND MAF-CONTAINING SUPERNATANT

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It is well known that mammalian respiratory burst activity can be increased by lymphokines such as γ-interferon. The biochemical basis of this process is the activation of an enzyme that catalyzes the one-electron reduction of melecular oxygen to superoxide (O_2^-) at expense of NADPH (Babior 1984). This radical can be detected by its capacity to reduce nitroblue tetrazolium (Rajagopalan and Handler 1964; Beauchamp and Fridovich 1971).

As in other vertebrates, fish phagocytes form a major defence against infection (MacArthur and Fletcher 1985; Olivier et al. 1986) however, yet little is known about the mechanims by which phagocytosed micro-organisms are killed in fish (Chung and Secombes 1988). There is no doubt that lower vertebrate phagocytes can undergo a respiratory burst (Plytycz and Bayne 1987; Chung and Secombes 1988). At present, data on the presence of soluble factors which could activate macrophages of seawater teleosts are not available. The aim of our work is the study of the ability of lectins or MAF (Macrophage Activating Factor)-containing supernatans from leucocytes of the seawater teleost gilthead seabream (Sparus aurata L.) to activate macrophages.

Head-kidney leucocyte cultures were pulsed for three hours with different concetrations of concanavalin A (Con A). After that, Con A was removed from the cultures by washing the cells with RPMI-1640 culture medium and the stimulated leucocytes were cultured 24 or 48 hours to produce MAF-containing supernatants. The supernatants were stored at -20°C until use and the leucocytes were procesed for their electron microscopic study. Both Con A and supernatants were tested to prove their capacities of activating macrophages. The reduction of nitroblue tetrazolium was performed to measure the production of superoxide anion (Pick et al. 1981; Rook et al. 1985) and the optical density was determined in a multiscan spectrophotometer at 620nm.

Our results indicate that Con A treated head-kidney macrophages appeared to have an irregular, dark cytoplasm with some pseudopodia, nevertheless the untreated control macrophages were more rounded showing a light cytoplasm. Furthermore, MAF-containing supernatants from Con A stimulated leucocyte cultures have the capacity of activating macrophage cultures by enhancing the O_2^- release by macrophages. The production of MAF-containing supernatants was generally optimal and significant when supernatants were obtained after 3 hours of stimulation with 10µg/ml Con A.

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2. Membrane Interaction (Receptors)

INTERFERON RECEPTORS AS PROBES INTO THE INTERFERON SYSTEM

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Two families of interferons (IFNs) can be distinguished: Type I IFNs (IFNs-α/β) are typically produced in the course of viral infections, type II IFN (IFN-γ) is produced upon activation of NK and T cells. The induction of IFN genes is subjected to tight regulation, and recently, some of the mechanisms that mediate this control have been elucidated. Type I and type II IFNs exert their effects through binding to distinct cell surface receptors. One component of the type I IFN receptor was cloned and shown to mediate both binding of and biological responsiveness to some subtypes of IFN-α, but additional elements seem to be required for full sensitivity to other type I IFNs[1, 2]. Likewise, the ligand-binding chain of the IFN-γ receptor is not sufficient to mediate responsiveness to IFN-γ[3-5]. An additional species-specific cofactor which is essential for signaling is currently being characterized in our laboratory (S. Hemmi, R. Böhni, & M. A., unpublished). Neither the type I nor the type II IFN receptors belong to any of the known receptor families. In the case of the type I IFN system, a signaling cascade from early postreceptor events to transcriptional activation of IFN-responsive genes has been described. Some elements of this cascade appear also to be involved in IFN-γ-mediated signaling.

In addition to their antiviral activity IFNs exert a variety of additional effects including immunomodulatory functions and inhibition of cell growth. To explore the physiological roles of IFNs *in vivo*, we generated mice that lack either type I (unpublished) or type II[6] IFN receptors. These mice were viable, anatomically normal and fertile. While the impact of these mutations on acquired immunity was unexpectedly minor, the phenotype of mice lacking IFN receptors dramatically revealed the primordial role of both IFN systems in innate immunity and starts to reveal their functional complementarity.

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Role of Interferon gamma internalization on macrophage activation

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After binding, interferon gamma (IFNγ) is internalized and degraded at a constant rate. The constant uptake of IFNγ by macrophages is due to the presence of an intracellular receptor pool (2/3 of the total receptor number) and to a mechanism of receptor recycling.

The functional relevance of the receptor is validated by the demonstration that receptor occupancy correlated with functional activities. By comparing the relationship between receptor occupancy and biologic response induction, two activation mechanisms became apparent. Induction of certain functions, such as H_2O_2 secretion or induction of Fc receptors, appeared to require only a single round of receptor occupancy. However, induction of more complex functions such as nonspecific tumoricidal activity appeared to require three to four rounds of receptor occupancy. Using saturating amounts of IFNγ , we found that between 0 and 30 min (time required by IFNγ to be internalized) of treatment there is a direct correlation between the length of treatment and the level of I-A expression. These results thus support the concept that internalization and receptor recycling are essential in the induction of nonspecific tumoricidal activity by macrophages.

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3. Second Signals and Early Genes

Macrophage Activation in 1993: External and Internal Signals, Transcription Factors, Genes, and Disease.

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We have previously reported that interferon-γ (IFN-γ), acting via ligation of its specific receptor and initiation of Na^+/H^+ ion fluxes, may induce transcription of class II MHC genes by enhancing the binding of a factor (NFY-γ) to the Y-box in the promoter of these genes. To test this hypothesis critically, we first examined in detail structural elements of the proximal promoter of the murine MHC class II *Ab* gene necessary for expression of the gene in macrophages. Each segment of the *Ab* promoter was specifically altered by site-directed mutagenesis; the mutant promoter was joined to the structural gene of human growth hormone (HGA); the resulting plasmid constructs were transfected into bone marrow-derived murine macrophages; and the expression of HGA RNA assayed. The results clearly show that induction by IFNγ requires four separate promoter elements: the W, X1, X2, and Y consensus sequences. Mutagenesis of the Y element demonstrated the only portion of that sequence necessary for gene expression is the inverted CCAAT box.

We had previously identified a protein, initially called NFY-γ, in macrophage nuclear extracts, which was induced by IFNγ to bind to the *Ab* Y element (but not to any other Y element). The recognition site for this protein determined by methylation interference is GAGGAA, which is adjacent to the inverted CCAAT box. This site has previously been described as the recognition sequence for the transcription factor PU.1 (a product of the *Spi-1* oncogene, present only in B lymphoid cells and macrophages). Recombinant PU.1 produced in *E. coli* binds the *Ab* Y element in the same manner as NFY-γ. Polyclonal antibody to PU.1 binds NFY-γ, indicating that this protein is induced in the PU.1 transcription factor. Mutagenesis of the PU.1 site in the *Ab* promoter did not, however, affect gene expression, indicating this transcription factor does not play a role in the response of the gene to IFNγ in macrophages.

To determine if PU.1 is active at all in murine-tissue macrophages, we introduced the PU.1 recognition site into a construct containing the TK promoter upstream of the transcribed region of the HGA gene. When this plasmid was transfected into macrophages, the level of expression was increased in response to LPS but not to IFNγ. We are currently investigating the molecular mechanism(s) of this induction.

The regulation of class II MHC genes as well as others in macrophages, such as those encoding nitric oxide synthetase, the recognition molecule B7, TNFα, MCP-1 and IL-1, is important to a wide variety of diseases. We have recently begun considering the role of macrophages and such gene regulation in atherogenesis. The current state of this field indicates that oxidatively altered low-density lipoproteins (LDL), formation of which can be promoted by smoking and by high-lipid diets, interact with mononuclear phagocytes to cause their adherence to the arterial endothelium, their accumulation and their development into foamy macrophages, which eventually evolve into fatty streaks and then into destructive atherosclerotic plaques. Persons with high levels of hostility are at higher risk for developing atherogenesis and also secrete high levels of catecholamines. Experimental animals given high-lipid diets and catecholamines develop macrophage-rich fatty plaques faster than animals given high-lipid diets only. Previous data from our laboratory have shown that acetylated/maleylated proteins,

clearing via scavenger receptors, induce an inflammatory phenotype in macrophages, characterized by activation for cytolysis of tumor cells, suppression of class II MHC genes, and expression of a number of pro-inflammatory genes such as TNFα and IL-1. To explain how high-lipid diets and stress may potentiate atherogenesis, we hypothesized that oxidized-LDL induces a macrophage phenotype which is the direct obverse of the above phenotype and whose formation is potentiated by catecholamines.

To begin testing this hypothesis critically, we manipulated murine mononuclear phagocytes in vitro. Oxidized-LDL suppress activation for cytolysis, release of reactive nitrogen intermediates, and expression of selected inflammatory genes such as MCP-1 and TNFα. These effects, for the most part, are mimicked by catecholamines or PGE_2 (both of which elevate intracellular levels of cAMP) or by dibutyryl cyclic AMP. Taken together, the data suggest that several behavioral patterns such as high-lipid diet, smoking and stress (acting via oxidized-LDL and catecholamines) induce a macrophage phenotype that would likely potentiate atherogenesis. This hypothesis is now being critically examined in humans and animals. Interestingly, this phenotype might promote the development of neoplasms and autoimmune diseases as well.

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Molecular structure and functional characterization of the early activation antigen AIM/CD69

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The genes that are induced during the early phase of cell activation are also known as immediate-early genes. To this group belongs several protooncogenes such as c-fos, c-jun or c-myc, and genes encoding molecules involved in signalling events including triggering molecules, growth factors, and some cytokine receptors.

The CD69 molecule, also designated as AIM, EA-1, Leu 23 or MLR-3 antigens, is a phosphorylated disulfide-linked 27/33 kDa homodimeric protein. This antigen seems to be the earliest inducible cell surface glycoprotein and is synthesized *de novo* and expressed by lymphocytes, NK cells and macrophage cell lines upon activation with a variety of stimuli. Other cell types including platelets and epidermal Langerhans cells also express the CD69 molecule.

The CD69 glycoprotein functions as a signal transmitting receptor in different cells. In T cells, the signals triggered by CD69 antibodies include rise of intracellular calcium concentration, expression of the IL-2, TNF-α and IFN-γ, and cell proliferation. Furthermore, the triggering of T cells through the CD69 activation pathway enhances the binding activity of transcription factor AP-1, which plays a key regulatory role in the initial steps of cell activation. Likewise, CD69 also acts as a triggering molecule on NK cells and platelets: CD69 antibodies are capable to induce target cell lysis by IL-2 activated NK cells, to trigger platelet aggregation, Ca^{2+} influx and hydrolysis of arachidonic acid.

We have isolated the cDNA coding for CD69 by a polymerase chain reaction (PCR)-based strategy using oligonucleotides deduced from peptide sequences of the purified protein. The isolated cDNA exhibited a single open reading frame (ORF) of 597 bp coding for CD69, and predicted an 199 amino acid protein of type II membrane topology, with extracellular (COOH-terminal), transmembrane and intracellular domains. The CD69 clone hybridized to a 1.7 kb mRNA species, which was rapidly induced and degraded after lymphocyte stimulation, consistent with the presence of rapid degradation signals at the 3'-untranslated region. Transient expression of the polypeptide encoded by CD69 cDNA in COS-7 cells, demonstrated that it presented properties comparable to native CD69 protein. The CD69 gene was regionally mapped to chromosome 12 p13-p12 by both somatic cell hybrid DNA analysis and fluorescence *in situ* hybridization coupled with GTG-banding (G-bands by tripsin using Giemsa). Protein sequence homology search revealed that CD69 is a new member of the Ca^{2+} -dependent (C-type) lectin superfamily of type II transmembrane receptors, that includes the human NKG2, the rat NKR-P1 families of NK cell-specific genes. CD69 also has structural homology with other type II lectin cell surface receptors as the T cell antigen Ly49, the low avidity IgE-receptor (CD23) and the hepatic asialoglycoprotein receptors. The CD69 protein also shares functional characteristics with most members of this superfamily that act as transmembrane signalling receptors in early phases of leukocyte activation.

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LPS Signaling: Taxol as a Tool

Aihao Ding

Lipopolysarccharide (LPS) exerts its profound effects on the host mainly by inducing numerous bioactive secretory products from macrophages. Despite the recent progress in identifying cell surface targets for LPS, it is clear that additional cellular component(s) exist that mediate transduction of LPS signal within the cell (1). While analyzing the regulation of TNFα receptors in macrophages, we discovered that taxol, a microtubule (MT) -binding diterpene, mimics the ability of LPS to induce TNFα release and down-regulate TNF receptor in a *Lps* gene-associated manner (2). Taxol also induces transcription of several LPS-inducible genes (3). That microtubules and their associated proteins (MAPs) might play a role in LPS signaling was supported by the fact that LPS bound specifically to β-tubulin in a cell-free system (4).

Recently we found that taxol, like LPS, rapidly induces (within 1 min) tyrosine phosphorylation of MAP-2 kinase (MAPK) and activates its kinase activity (5). Macrophages from C3H/HeJ mice, which carry a defective *Lps* gene, fail to activate MAPK in response to taxol or LPS, although they activate MAPK in response to insulin or interferon-γ. Tyrosine kinase inhibitors block MAPK activation as well as the ability of taxol and LPS to induce transcription of TNFα. These results suggest that a tyrosine kinase cascade participates in LPS signaling.The product of the *Lps* gene may be an "uptream activator" of MAPK.

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Mycobacterium tuberculosis lipoarabinomannan induction of JE, nitric oxide synthase, and IL-10 in _Lsh/Ity/Bcg_^{s} and _Lsh/Ity/Bcg_^{r} macrophages from congenic mice.

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Mycobacterium tuberculosis lipoarabinomannan (LAM) is a major cell-wall associated glycolipid, it is highly immunogenic and may be released by mycobacteria in copious quantities (1). Previous work identified structural differences in LAMs from the attenuated H37Ra strain compared with the virulent Erdman strain, arabinan motifs dominate the non-reducing end of H37Ra Ara-LAM, but these are extensively capped with mannosyl residues in Erdman Man-LAM (2). These structural differences affect LAM stimulation of macrophage TNF α secretion (3), and more recently the attenuated H37Ra Ara LAM, but not virulent Erdman Man-LAM was shown to stimulate expression of the macrophage early genes c-fos, KC, JE and TNF-α (4). Macrophage activation, in terms of induction of early (JE) and late (nitric oxide synthase, and IL-10) gene expression in response to LAM stimulation has now been examined. Man-LAM from Erdman, and a second virulent strain, H37Rv, were compared with H37Ra Ara-LAM, for their effectiveness in activation of macrophages from mice carrying resistant and susceptible alleles of the innate macrophage resistance gene *Lsh/Ity/Bcg*. The results indicate that relatively low dose (up to 2.5μg) of H37Rv or Erdman Man-LAM, failed to stimulate either JE, NOS or IL-10 expression in either *Lsh/Ity/Bcg*^{s} or *Lsh/Ity/Bcg*^{r} macrophages. In contrast, H37Ra Ara-LAM was able to induce JE, NOS and IL-10 expression in macrophages from both stains of mice, albeit Ara-LAM was not as potent a stimulus as *Salmonella typhimurium* LPS. The implications of these observations will be discussed.

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Monocyte Chemoattractant Protein-1 (MCP-1) Induction in the Lewis to Fisher Rat Cardiac Allograft Model of Transplant Arteriosclerosis

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Monocyte chemoattractant protein-1 is an immediate early gene encoding a chemotaxis factor specific for monocytes. It has been implicated in atherosclerosis by virture of it's identification in diseased vessels. To determine whether MCP-1 plays a role in chronic cardiac rejection, we studied time-dependent MCP-1 gene and protein expression in a heterotopic (Lewis to Fisher) rat transplantation model that allows chronic graft survival with staged development of arteriosclerotic lesions which mimic those seen in human tranplant vessels. Using a reverse-transcription polymerase chain reaction (PCR) analysis with ^{32}P-dCTP, we observed significant increases (8-12 fold) in MCP-1 gene transcript levels in rat cardiac allografts compared with paired host hearts (histologically normal but exposed to same circulation), syngrafts (Lewis to Lewis), and day-0 hearts (harvested but not transplanted) ($p<0.001$). Induction was early (day 7) and persistent (days 7, 14, 28, and 60) consistent with chronic or ongoing immune stimulation. The gene induction was specific to allogeneic tissue and localized to allografted tissue, in that, there was no induction in host hearts or spleens. Using immunostaining with anti-JE antibody, MCP-1 protein localized to mononuclear cells (found mostly in the interstitium but also in the vascular adventitia, lumen and neointima). 90% of MCP-1-positive cells double-stained with anti-macrophage antibody (ED-1). There were correlate significant increases in both MCP-1- and ED-1-positive cells (5-7% and 25-34%) in allografts compared with day-0 hearts. The early and persistent expression of MCP-1 in this subset of "activated" monocytes in allografts suggests that this inducible mediator may contribute to monocyte accumulation after cardiac transplantation by recruiting additional monocytes in a positive feedback mechanism.

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CROSSLINKING OF CD14 ON MONOCYTES ACTIVATES β2 INTEGRINS

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Binding of human monocytes onto nonactivated endothelial cells (EC) involves β2-integrins on the former cells and ICAM-1 molecules on the latter. When EC are activated by various kinds of cytokine (e.g. IL-1α, TNF-α, IFN-γ) the adherence of monocytes to these cells is increased. This increased binding is inhibited by monoclonal antibodies (mAb) against CD14 on monocytes. CD14 is the receptor for bacterial lipopolysaccharide (LPS) and/or LPS-LPS binding protein (LPS-LBP). Crosslinking of CD14 on monocytes with anti-CD14 mAb and F(ab')2-goat antimouse immuneglobulin resulted in adhesion of these monocytes to EC and in a transient increase of $[Ca^{++}]_i$. The adhesion of monocytes with crosslinked CD14 to activated EC is inhibited by staurosporine, an inhibitor of protein kinase activity, and by anti-β2-integrin mAb and anti-ICAM-1 mAb. These results have led to the following conclusion: at the initial phase of contact between monocytes and cytokine-activated EC CD14 molecules on monocytes interact with an unknown counterstructure on EC. Most likely this counterstructure is a lectin-like molecule since this binding is inhibited by the lectin weat germ agglutinin. The contact between monocytes and activated EC induces a cascade of intracellular signals in the monocytes which results in an increased avidity of their β2-integrin molecules for ICAM-1 on EC. Next transendothelial migration will occur, which is supported by in vivo studies with anti-β2-integrin and anti-ICAM-1 mAb.

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HOW DO *LEISHMANIA* PROMASTIGOTES EVADE KILLING MECHANISM OF HOST MACROPHAGES?

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Leishmania parasites are protozoa with a digenetic life cycle, including an extracellular flagellated promastigote in the midgut of the sandfly vector, and an obligate intracellular aflagellate amastigote in the macrophages of the vertebrate host. As an intracellular parasite of macrophages, this protozoa has been used frequently as a model to study Mø/pathogen interactions. Promastigotes differentiate in the sandfly midgut from non infective (procyclic) to infective forms (metacyclic). Using procyclic and metacyclic promastigotes obtained from axenic culture, we are exploring *in vitro* the mechanisms whereby macrophages are activated to kill procyclic promastigotes but not metacyclics. The work has examined oxygen-dependent killing mechanisms by correlating parasite fate with their ability to trigger the respiratory burst; and non oxygen-dependent mechanisms (cytokine production and L-arginine pathway) by a quantitative RT-PCR. Procyclic promastigotes triggered a very strong burst, as measured by NBT reduction, whereas a high proportion of metacyclic promastigotes triggered no burst at all. In contrast to their effects on oxidative metabolic pathways, procyclic and metacyclic promastigotes of *L. major* induced very low levels of NO synthase and cytokine gene expression (IL-1α, IL-1β, IL-6, TNFα, IL-10, IL-12, GM-CSF) by resident, thioglycolate elicited or bone marrow macrophages up to 4 hours infection. Of those cytokines for which transcripts could be detected, only IL-1α and IL-12 seemed to be more efficiently induced by procyclics.

4. Metabolic Pathways

Macrophage activation : some lessons from the *Leishmania* model.

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I. Murine macrophages activated *in vitro* by lymphokines *plus* a phagocytic stimulus acquire the capacity to synthesize reactive oxygen (ROI) and nitrogen (RNI) intermediates. Both types of compounds are toxic for *Leishmania* parasites. Toxicity of ROI is restricted however to the earlier phase of the macrophage-parasite encounter since, once the micoorganisms have reached their intracel-lular niche, stimulation of macrophage ROI production fails to kill them (1). Moreover, *Leishmania* is capable of inhibiting generation of ROI (2). Killing of the intracellular microorganisms then requires synthesis of RNI (3, 4). Both reactive intermediates are therefore produced under different sets of circum-stances. Release of ROI is a rapid-onset event. Conversely, production of RNI increases steadily over the course of at least 48 h. Increased HMPS activity in RNI-producing cells can be accounted for almost entirely by the need to provide NADPH for NO synthesis. Since NADPH and oxygen are required for the generation of both ROI and RNI, the enzyme systems producing both interm-ediates have to compete for these essential cofactors. Restriction of ROI production to the phagocytizing cell may provide a control mechanism whereby both pathways can be differently regulated.

II. Macrophages exposed to IFN-γ *alone* (1 - 10 U/ml) release little RNI. Synthesis of the latter compounds is induced upon phagocytosis of *Leishmania* parasites or other particles, and depends on the production of TNFα, which appears to function as a major physiological autocrine regulator of the activation process (5, 6). Macrophage activation by IFN-γ *plus Leishmania* is enhanced by PGE_2 (6); similarly, intracellular killing of *Leishmania* in macrophages exposed to IFN-γ *plus* TNFα is stimulated by PGE_2, concomitant with increased glucose oxidation through the HPMS pathway and nitrite production. Activation in the presence of arachidonate, a prostaglandin precursor, results in a similar enhancement of parasite killing and nitrite release. Conversely indomethacin, a cyclooxygenase inhibitor, reduces HMPS activity, nitrite release and parasite destruction ; all these parameters are fully reversed however by addition of PGE2, strongly suggesting that decreased killing and associated metabolic parameters are related to inhibition of prostaglandin production in the indomethacin-treated cultures. Finally, dibutyryl-cAMP, as well as theophylline (which blocks cAMP degradation by phosphodiesterase) and forskolin (an activator of adenylate cyclase) all stimulate intracellular killing and nitrite production by the activated cells, consistent with the hypothesis that enhanced activation induced by PGE2 may be related to its capacity to increase intracellular cAMP levels.

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Antiviral and Pro-Inflammatory Activities of Inducible Nitric Oxide Synthase: Potential Explanations for Widespread Inducibility of an Autotoxic Enzyme

Carl Nathan

It is a mystery why evolution has conferred on so many types of cells the capacity to respond to immune or inflammatory signals with the induction of an isoform of NO synthase that, once translated, can function for days to generate large amounts of a potentially toxic radical (1). We reasoned that NO may defend against a class of pathogens by which many cell types can be infected, and may be a toxin against which it is difficult for pathogens to evolve to a resistant state. Consistent with this view, Karupiah et al. (2) demonstrated that iNOS can inhibit ectromelia, herpes simplex and vaccinia virus replication. Indeed, induction of iNOS appears to be both necessary and sufficient to account for the antiviral action of interferon-γ in the systems studied (2). Antiviral effects of NO were demonstrable in vitro and in vivo (2).

If high-output NO production is so beneficial, why did evolution arrange matters so that the host must depend on its induction? In other words, why is the calcium-independent form of NO synthase not constitutive? The answer presumably has to do with its potential for autotoxicity. This was demonstrated in vivo by McCartney-Francis et al. (3). NO synthase was induced in rat synovial tissue by a single intraperitoneal injection of streptococcal cell wall fragments. The development of chronic erosive arthritis was blocked by adminstration of an NO synthase inhibitor. Thus, blockade of NO production can prevent tissue destruction in chronic inflammation.

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Differentiation and modulation of macrophages *in vitro* and *in vivo*

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Heterogeneity of macrophages in tissues reflects their differentiation and modulation by inflammatory and endocytic stimuli and cellular interactions in their local microenvironment. Their resultant properties reflect diverse homeostatic functions in tissue growth, repair and modelling as well as host defence against intra- and extracellular microorganisms. We utilise a range of membrane molecules which serve as antigenic markers to localise macrophages in different murine tissues (1), and have analysed their modulation in various cell culture systems. Examples to be discussed include a novel cell adhesion function for the endocytic macrophage scavenger receptor (2), the type 3 complement receptor (3), sialoadhesin, a haemopoietic cell interaction molecule (4), macrosialin, a heavily glycosylated endosomal antigen(5), and the lectin-like mannosyl receptor (6). Effects of cytokines on their expression include differential up- and downregulation by Th1 and Th2 lymphokines, and by M-CSF. Natural (7) and experimentally induced (8) cytokine deficiencies contribute to our analysis of macrophages within the host, in control and infected animals. The role of macrophages in HIV infection has been studied in a primary human cell culture model, that reveals interesting differences among cytokines in modulation of HIV entry and replication (9).

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Investigations into the Modulation of Macrophage Phagolysosomes by *Mycobacteria*

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The survival of the intramacrophage pathogen *Mycobacterium* depends on its ability to establish an inhabitable intracellular compartment. Formation of this vacuole is determined by the actions of both pathogen and macrophage. To assess the activity of the pathogen on modulating "normal" phagosome maturation, we have developed techniques facilitating isolation of intact *Mycobacterium*- containing vacuoles from infected macrophages. The macrophage proteins incorporated into these phagosomes are being analyzed by 2D-PAGE and compared to the protein profiles in phagosomes formed around carboxylated polystyrene beads derivatized with defined ligands.

In addition, immunoEM analysis has been employed on both isolated vacuoles and macrophages during particle internalization to study the relative rates of fusion with intracellular markers, i.e. LAMP's, M6PR, and lysosomal hydrolases. These results are compared to purified phagosome proteins analyzed by 2D-PAGE and Western blotting. Both procedures demonstrated that the mycobacterial phagosome is positive for LAMP-1. In contrast, the mycobacterial vacuole is markedly lacking in the 56kDa and 31kDa components of the vacuolar proton pump which is responsible for endosomal acidification. These subunits are abundant in both the *Leishmania* and IgG-latex bead phagosomes. Replicas from the cytoplasmic face of the isolated vacuoles revealing the proton pump assemblies on the *Leishmania* vacuoles and the smooth membrane of the *Mycobacterium* vacuole supports this contention, and correlates with previous observations concerning the vacuole's restricted acidification.

Finally, immunoEM with anti-LAM antibodies demonstrated that mycobacterial-derived material is actively trafficked out of the bacteria-containing compartment. This observation, coupled with the fact that the vacuole remains LAMP positive throughout extended infection periods (14 days), indicates that the vacuole must be fusing with some vesicular components of the macrophage's endosomal network, despite the vacuole's known isolation from freshly formed endosomes. These results have significance both towards understanding the survival mechanisms of *Mycobacterium* spp. and the differentiation of the vesicular network responsible for phagosomal/lysosomal maturation in macrophages.

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IDENTIFICATION AND EXPRESSION OF TWO FORMS OF THE HUMAN SURFACE ANTIGEN ENDOGLIN WITH DISTINCT CYTOPLASMIC REGIONS

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Endoglin is an homodimeric membrane antigen (1) with capacity to bind transforming growth factor-β (TGF-β) (2) and containing the tripeptide arginine-glycine-aspartic acid. Although endoglin is absent from peripheral blood monocytes, it is expressed by in vitro differentiated monocytes as determined by fluorescence flow cytometry and Northern blot analyses (3). Furthermore, endoglin has been found to be present in human tissue macrophages (3, 4). We have isolated full length cDNA clones from a λgt10 library, prepared from PMA-differentiated HL60 cells by screening with an endoglin specific cDNA probe from endothelial cells. Sequencing of the largest clone (3073 bp), revealed that the leader sequence contains 25 residues and that the 586 amino acids of the extracellular and transmembrane domains were identical to those described for endothelial endoglin. However, the cytoplasmic tail encoded by this cDNA clone contains only 14 aa as opposed to the 47 residues previously reported, suggesting the existence of two alternative endoglin variants. The expression of these isoforms was demonstrated by PCR analyses on endothelial cells, myelomonocytic cell lines HL-60 and U-937, and placenta. Independent cDNA constructs corresponding to both forms were transfected into mouse fibroblasts leading to the expression of two distinct endoglin molecules. Both forms were shown, when overexpressed in transfected mouse fibroblasts, to form disulfide linked homodimers, indicating that the cysteine residues present in the extracellular domain are responsible for the dimerization (5).

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ACTIVATION OF PERITONEAL MACROPHAGES DURING THE PREDIABETIC PHASE IN LOW-DOSE STREPTOZOTOCIN-TREATED MICE.

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Macrophages play a pivotal role in the immune attack against the pancreatic islets in the low-dose streptozotocin model of type I diabetes [1,2]. Recently, it has been been shown that islet cell lysis induced by *C. Parvum*-activated macrophages is mediated by NO since cell destruction can be counteracted by NO synthase inhibitors [3]. However, evidence concerning the activation state of macrophages and the nature of the effector molecules that mediate islet destruction in experimental immune diabetes is lacking.

Glucose metabolism and the production of $O_2^{\cdot}$ and $NO_2^{\cdot}$ have been studied in peritoneal macrophages from mice injected with 5 subdiabetogenic doses of streptozotocin. On day 12 after beginning of the treatment, peritoneal macrophages produced significantly higher amounts of lactate than macrophages from control mice. In addition, $NO_2^{\cdot}$ release and phorbol ester-induced $O_2^{\cdot}$ production were significantly augmented in macrophages from streptozotocin-treated mice. γ-interferon induced in a dose-dependent manner the activity of NO synthase only in macrophages from streptozotocin-treated mice. In conclusion, our data show for the first time that peritoneal macrophages from streptozotocin-treated mice are activated and produce effector molecules such as $O_2^{\cdot}$ and NO which could participate in the destruction of pancreatic islets. The actions of macrophages may be controlled by citokines such as IFN-γ released by lymphocytes in the vicinity of the β-cells in this model of type I diabetes.

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THE ROLE OF THE TRANSCRIPTION FACTOR PU.1 IN MACROPHAGE-SPECIFIC EXPRESSION OF THE C-FMS PROTO-ONCOGENE

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The *c-fms* protooncogene encodes a member of the protein tyrosine kinase family of cell surface receptors, and binds the lineage-specific growth factor macrophage colony-stimulating factor (CSF-1) (Sherr, 1990). Expression of full length *c-fms* mRNA is restricted to trophoblasts and various stages of differentiation in the monocuclear phagocyte cell lineage. The first exon of the gene is transcribed only in trophoblasts, whereas exon 2, the first coding exon 25kb downstream, is flanked by a macrophage-specific site of transcription initiation (Roberts *et al.*, 1991; Visvader & Verma, 1989). We have isolated and sequenced the region surrounding exon 2 in the mouse *c-fms* gene. As in the human gene, transcription of the mouse *c-fms* in mouse macrophages was shown to occur from multiple start sites by primer extension and by RNase protection analysis. These analyses revealed the existence of short transcripts in a wide range of non-macrophage cell lines, suggesting that regulation of gene expression occurs via transcription attenuation (Yue *et al.* 1993). Northern analysis with an exon 2 riboprobe revealed transcripts in non-macrophages of approximately the same size (ca. 3.5kb) as full length *c-fms*. On this basis, the site of transcription attenuation occurs at the end of intron 2 or the beginning of exon 3. Consistent with this conclusion, a luciferase reporter gene construct containing 3.5kb of 5' flanking sequence was constitutively active in transient transfection in all cell lines tested, whereas a construct in which the reporter gene was fused into the exon 3 generated macrophage-specific activity.

Although the promoter of *c-fms* is not functionally macrophage-specific, it does contain multiple binding sites for the macrophage and B cell restricted DNA binding protein PU.1 (Klemsz *et al.*, 1990), a member of the *ets* transcription factor family. We have examined the expression of PU.1 by electrophoretic mobility shift analysis and present evidence that nuclear DNA binding activity of PU.1 is a highly-specific macrophage marker, present at high levels in all primary macrophages and macrophage cell lines, barely detectable in B cells and absent from T cells and non-haematopoietic cells. Co-transfection of a PU.1 expression plasmid with *c-fms* promoter constructs in the MOPC31C B cell line *trans*-activated reporter gene expression when the 5' flanking sequence alone was included, but did not overcome the block induced by inclusion of the attenuator region. By contrast, over-expression of PU.1 in the macrophage line RAW264 had no effect on the activity of the *c-fms* promoter, but *trans* -repressed when the attenuator region was included. We suggest that PU.1 binds to the *c-fms* promoter and influences transcription elongation by interacting with another macrophage-specific nuclear protein. One candidate is a macrophage-specific nuclear protein, identified by EMSA, that interacts with a conserved CCAAT-like sequence in the *c-fms* promoter.

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REGULATION OF THE EXPRESSION OF LEUKOCYTE INTEGRINS.

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The integrins LFA1, Mac-1 and p150,95 are formed by the non-covalent association of a common ß subunit (ß2, CD18) to three distinct but related α subunits, αL (CD11a), αM (CD11b), and αX (CD11c). The ß2 integrins are exclusively expressed on leukocytes and mediate leukocyte adhesive interactions required for the proper induction and regulation of immune and inflammatory processes. Integrin expression is regulated during differentiation as well as by numerous growth factors and cytokines.

We have analyzed the changes in p150,95, VLA-4 integrin subunits mRNA levels that take place during the myeloid differentiation of HL60 and U937 cells, and compared them to other integrins (LFA-1, Mac-1, VLA-5) with similar functional activities (1). Northern blot analysis revealed that the monocytic differentiation of U937 and HL60 cells alters the p150,95α and VLA-4α mRNA steady-state levels in opposite ways: p150,95α mRNA is induced *de novo* while VLA4α mRNA decreases to undetectable levels. Both changes were dependent on the activity of Protein Kinase C and were also observed upon granulocytic differentiation of HL60 cells. Parallel analysis of other integrin subunits mRNA (ß1, VLA5α, ß7) demonstrated that the mRNA levels for the α subunits of the fibronectin receptors VLA-4 and VLA-5 are differentially regulated during the monocytic differentiation of myeloid cell lines, and suggested that myeloid cells express an heterodimer formed by the association of ß7 with an integrin α subunit distinct from VLA-4α. Nuclear transcription assays revealed that the transcription rate of the p150,95α gene is considerably elevated after PMA treatment of U937 cells, indicating that the regulation of the p150,95 integrin expression during myeloid differentiation is mainly exerted at the transcriptional level. Conversely, transcriptional and post-transcriptional mechanisms are responsible for the loss of VLA4 from the cell surface of PMA-differentiated U937 cells.

To understand the mechanisms that direct the constitutive and regulated leukocyte expression of p150,95 we have isolated and structurally characterized the CD11c promoter region and initiated its functional dissection (2). The CD11c promoter lacks TATA- and CCAAT-box, directs the synthesis of transcripts with heterogeneous 5' ends, and contains an initiator-like sequence at the major transcription initiation site. Several putative binding sequences for ubiquitous (Sp1, AP-1, AP-2, NF-kB) and leukocyte-specific (PU.1) transcription factors have been identified in the proximal region of the CD11c promoter which may participate in the regulation of the expression of p150,95. Transient expression of CD11c-based reporter gene constructs indicates that the CD11c promoter dictates the tissue-specific expression of p150,95 and that sequences contained within 160 bp 5' from the major transcriptional start site are involved in the tissue-specific and regulated expression of p150,95. DNase I protection analysis on the promoter region spanning from -160 to +40 revealed four regions of DNA-protein interactions (FPI-FPIV), two of which (FPIII and FPIV) correlate with the cell type-specific and the regulated expression of the CD11c gene.

Unlike p150,95, LFA-1 is the only integrin expressed on all leukocyte lineages. The expression of both subunits of LFA-1 is absolutely restricted to leukocytes and is regulated by cytokines and during cell activation and differentiation. To elucidate the molecular basis for the leukocyte-restricted expression of LFA-1, the promoter region of the CD11a gene has been isolated and functionally characterized (3). The 5' region of the CD11a gene exhibits a similar exon/intron organization as the CD11b, CD11c and VLA-2α genes, but is different from that of the genes encoding VLA-4α, VLA-5α and gpIIb. Primer extension and nuclease S1 assays have identified several tightly clustered transcription initiation sites on the CD11a gene, with the major site resembling the "Initiator" sequence. The sequence of a DNA fragment extending 880 bp 5' from the major transcription start site revealed the absence of TATA- and CAAT-box, as well as the presence of several potential binding sites for transcription factors (Sp1, PU.1, AP-2) and retinoic acid-responsive elements. Transient expression of CD11a promoter-based reporter gene constructs in both $LFA1^+$ and $LFA1^-$ cell lines demonstrated that the fragment spanning from -880 to +83 functions as a promoter and is involved in the tissue-specific expression of LFA-1. Functional analysis of different fragments within the -880/+83 fragment suggested the presence of negative regulatory elements between -880 and -226 and demonstrated that the proximal region of the CD11a gene promoter exhibits tissue-specific activity.

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GLUCOCORTICOIDS PREVENT THE INDUCTION OF NITRIC OXIDE SYNTHASE IN RAT MESANGIAL CELLS AT THE TRANSCRIPTIONAL LEVEL.

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Mesangial cells are modified vascular smooth muscle cells in the renal glomerulus which are able to synthesize nitric oxide (NO) when exposed to cytokines or lipopolysaccharide (LPS). We studied if this induction could be regulated by drugs with recognized antiinflammatory properties such as glucocorticoids. Rat mesangial cells (RMC) (primary culture) were treated or not with tumor necrosis factor-α (TNF-α) (100 ng/ml, 8 h) + LPS (10mg/ml, 8 h). Dexamethasone (Dx) was either added at the same time (10^{-6} M, 8 h) or 16 h before (10^{-6} M, 24 h). NO synthase activity was measured using a reporter cell-assay by which the capacity of NO to stimulate soluble guanylate cyclase in a fibroblast cell line (RFL-6) is exploited. RFL-6 cells were co-incubated with supernatants of RMC treated as described. Treatment of RMC with TNF-α + LPS significantly augmented RFL-6-associated cGMP (control: 016±0.08, TNF-α + LPS: 1.37±0.19 pmol/well, $p < 0.05$, n=2). Pretreatment with Dx significantly blunted TNF-α + LPS-induced NO synthase activity (0.46±0.01 pmol/well, $p<0.05$, n=2). However when Dx was simultaneously added with TNF-α + LPS to RMC this inhibition was not observed. Dx (10^{-6} M, 24 h) alone did not modify RMC basal NO synthase activity (0.28±0.07 pmol/well, n=2). We further explored mRNA expression of NOSi in rat mesangial cells using a murine macrophage NOSi cDNA probe for Northern analysis. The latter showed a visible 4.8 Kb transcript in induced RMC. Treatment with Dx significantly blunted the intensity of this message. Northern analysis of the same membrane using a housekeeping gene (rat β-tubulin) as a probe revealed no significant differences in the corresponding message of this gene among the different experimental conditions. We take these data to indicate that glucocorticoids regulate the induction of NO synthase in the glomerular mesangium at the transcriptional level probably by the interaction with a potential consensus sequence for the glucocorticoid receptor in the promoter region of the NOSi gene. Also, the potential preventive action and therapeutic relevance of glucocorticoids in NO-mediated pathophysiological phenomena within the mesangial cell is established.

5. Gene Expression and Transcription Factors

REGULATION OF MHC CLASS II GENE EXPRESSION IS CONTROLLED BY THE TRANSACTIVATOR GENE CIITA, WHICH IS MUTATED IN THE BARE LYMPHOCYTE SYNDROME

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Modulation of MHC class II gene expression on various cell types, and in particular induction by Interferon gamma, is a functionally important aspect of antigen presentation and of T lymphocytes activation. The cascade of events that result from receptor activation involves complex and largely unknown steps and numerous factors, including those that bind to MHC class II promoters. CIITA is a novel MHC class II transactivator gene shown to control the constitutive expression of class II genes on B lymphocytes. CIITA was also shown to be the gene responsible for the Bare Lymphocyte Syndrome, a disease of HLA class II gene regulation and was shown to be mutated in this disease. The CIITA gene is itself differentially expressed and highly regulated, with a pattern that corelates with that of MHC class II gene expression. More importantly, CIITA is also shown be an essential mediator of the induction of MHC class II genes by Interferon gamma in various cell types, including monocyte lines, and to be itself regulated by Interferon gamma. In addition to controlling constitutive MHC class II gene expression, CIITA is therefore also responsible for the mechanism of induced class II expression and for the physiological modulation of the level of MHC class II molecules in various cell types.

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"Tyrosine Kinases Mediate the Activation of Specific Interferon Stimulated Signal Transduction Pathways"

Central to the biological response of cytokines is the rapid induction of unique sets of genes. Analogous to a viral infection, it is the induction of immediate early genes (IEGs) that is likely to define the specificity of the response, whereas later genes may be less specific. Some of the proteins involved in carrying a signal, with high fidelity, from the cytoplasmic domain of the receptor into the nucleus, have recently been elucidated for IFN-α and IFN-γ. In both cases, latent cytoplasmic signalling proteins are rapidly activated by a tyrosine kinase(s) in response to ligand binding cognate receptor. The activated signalling proteins are subsequently rapidly translocated into the nucleus where they bind specific enhancer elements, culminating in the induction of the corresponding gene (1).

In response to IFN-α, three cytosolic proteins, p113, p91 and p84, are rapidly phosphorylated on a conserved tyrosine (2). This phosphorylation is dependent on the presence of the IFN-α receptor associated tyrosine kinase, tyk-2 (3). It is now known that only two of the cytoplasmic proteins are required for signalling. p84, which is structurally similar to p91 (i.e. it is missing the last 38 carboxy terminal amino acids) appears to be entirely redundant in the IFN-α pathway (4). After activation, p113 and p91 (or p84) associate in a complex (presumably a heterodimer), which is subsequently translocated to the nucleus, where it associates with p48 (the DNA binding component) to form a stable activation complex (i.e., Interferon-α Stimulated Gene Factor-3, orISGF-3) on the Interferon-α Stimulated Response Element (ISRE: ref. 1).

In response to IFN-γ only p91 is rapidly phosphorylated (on the same tyrosine) and subsequently translocated to the nucleus (5). This activation event is not dependent on tyk-2 (3). Once in the nucleus, p91 is believed to bind to the GAS element as a homodimer (known as the Gamma Activation Factor, or GAF) and stimulate transcription. Although p84 is activated by IFN-γ, and able to bind GAS elements, it is unable to activate transcription (4). Hence, p84 is not a positive activator in the IFN-γ pathway and may have a counter-regulatory role in some IFN-γ responses.

Although much progress has been made in understanding the basic outline in the signal transduction pathways stimulated by IFN-α and IFN-γ,

much work remains to be done in understanding of molecular details of this pathway. The kinases and receptor components involved in these responses have not been fully elucidated. However, the presence of SH-2 and a potential SH-3 domain in these signalling proteins provides a tantalizing clue as to the nature of some of these interactions (1). Also the ability of cells to differentially regulate the expression of these cytoplasmic signalling proteins, as is the case when U937 cells are induced to differentiate (6), provides an additional promising avenues for the investigation of how cells may control their response to IFNs (and perhaps other cytokines). Additionally, the preliminary identification of novel cytokine specific family members of the p91/p113 family suggests that the IFN signalling pathways will serve as a paradigm for other cytokines.

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Promoter of the Mouse Gene for an Inducible Nitric Oxide Synthase: Inducibility by Bacterial Lipopolysaccharide and Interferon-γ

Qiao-wen Xie

The inducible nitric oxide synthase (iNOS) tightly binds calmodulin and its activity is independent of an elevation of Ca^{2+} (1). We cloned the cDNA for iNOS from mouse macrophages and proved that iNOS is induced at the transcriptional level after exposure to bacterial lipopolysaccharide (LPS) and interferon(IFN)-γ (2). Cycloheximide almost completely blocked the induction of iNOS in mouse peritoneal macrophages treated with LPS and IFN-γ. Genistein, a tyrosine kinase inhibitor, completely blocked the induction of iNOS by IFN-γ and partially blocked induction by the combination of IFN-γ and LPS. We recently cloned and analyzed an 1.7 kb promoter region of the *iNOS* gene from mouse (3). It contains a TATA box upstream of the mRNA initiation site and at least 22 oligonucleotide elements homologous to consensus sequences for the binding of transcriptional factors involved in the inducibility of other genes by cytokines or bacterial products: 10 copies of IFN-γ response element (γ-IRE); 3 copies of γ-activated site (GAS); 2 copies each of nuclear factor-κB (NF-κB), IFN-α-stimulated response element (ISRE), activator protein-1 (AP-1), and tumor necrosis factor response element (TNF-RE); and one copy of X box. Using the system with a reporter gene encoding bacterial chloramphenicol acetyltransferase (CAT), we show that the downstream portion of the promoter region is sufficient to confer inducibility by LPS, while upstream portion contains enhancer region responsive to the synergistic action of IFN-γ with LPS.

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Isolation and characterization of the DNA binding proteins that regulate the expression of the MHC class II gene, I-Aβ. Richard Maki, Scott McKercher, and Antonio Celada.
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The MHC class II genes are expressed in macrophages after the macrophages have been treated with interferon gamma (IFN-γ). We have identified the cis-acting elements within the promoter of the MHC class II gene I-Aβ that are necessary for the expression of this gene in macrophages as well as B cells. The important cis-acting elements lie within 124 bp of the transcription start site. There are three elements that are important for the expression of the I-Aβ gene and these resemble the cis-acting elements found in the promoters of other MHC class II genes. The elements have been named the W, X, and Y boxes.

The Y box element contains the sequence motif CCAAT. The protein that binds to this sequence consists of two components, (34Kd and 42-46 Kd), both of which are required for the binding of the protein to DNA (1). For the I-Aβ gene the two components appear to be identical to the proteins NF-YA and NF-YB (2).

The X box is an important element for the expression of MHC class II genes and appears to be the binding site for two proteins. We have identified a protein (45Kd) that binds to the upstream side of the I-Aβ X box (X_1) and cloned the gene for this protein. The gene has been named IAX. The sequence of IAX shows no homology to previously isolated genes and the putative protein sequence does not resemble any of the known DNA binding proteins. We are currently examining the function of the IAX protein and its role as a transcriptional activator.

The third cis-acting element, called the W box, is important for the IFN-γ induced expression of the I-Aβ gene. A W box binding protein has been purified and has a molecular size of about 43 Kd. The characterization of this factor is currently underway.

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6. Inhibitors of Macrophage Activation

Suppression of Inducible Nitric Oxide Synthase by Transforming Growth Factor-β

Carl Nathan

The potential for cell, tissue and organ destruction by inducible NO synthase (iNOS) suggests that the regulation of iNOS function is not likely to reside solely in its inducibility. As for other toxic products of the activated macrophage, production of NO is likely to be subject to suppression by cytokines. The iNOS-suppressing cytokines may be just as important to the regulation of iNOS as those that induce it. We have identified 5 such cytokines: MDF (1), TGF-β1-3 (1), and IL-4 (2). I will focus here on the mechanism of action of TGF-β1 (3).

TGF-β reduced iNOS specific activity and protein in both cytosolic and particulate fractions of primary mouse peritoneal macrophages, suppressing nitrite release with a 50% inhibitory concentration of 5.6 ± 2 pM. TGF-β reduced iNOS mRNA without affecting the transcription of iNOS, by decreasing iNOS mRNA stability. Even after iNOS was already expressed, TGF-β reduced the amount of iNOS protein. This was due to reduction of iNOS mRNA translation and increased degradation of iNOS protein. Thus, the potency of TGF-β may reflect its ability to suppress iNOS expression by three distinct mechanisms-- decreased stability of iNOS mRNA, decreased translation of iNOS mRNA, and increased degradation of iNOS protein. This is the first evidence that iNOS is subject to post-transcriptional regulation.

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Repression of Major Histocompatibility Complex IA Expression by Glucocorticoids

Antonio Celada, Scott McKercher and Richard A. Maki

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Glucocorticoids are effective repressors of major histocompatibility complex (MHC) class II gene expression. The repression occurs in B cells, which constitutively express MHC class II, as well as in macrophages, which only express MHC class II after the cells are treated with interferon γ. Fot the MHC class II gene IAβ, this negative regulation has been linked to the X box DNA sequence, located with the IAβ promoter. The addition of the glucocorticoid receptor was shown to inhibit the DNA binding of the X box DNA binding protein to the X box. The DNA binding of two other DNA binding proteins that recognize elements within this promoter was unaffected by the addition of glucocorticoid receptor. It is likely that the repression of IAβ gene expression by glucocorticoids occurs because the X box DNA binding protein is prevented from binding to the DNA and activating transcription.

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DOWN-REGULATORY AND UP-REGULATORY CYTOKINE ACTIVITIES IN PARASITIC INFECTION: RELATION TO MACROPHAGE EFFECTOR FUNCTION.

Alan Sher, Laboratory of Parasitic Diseases, NIAID, Bethesda, MD. 20892, USA

Different parasitic infections stimulate different host cytokine responses. We have postulated that these response patterns reflect the adaptation of the particular parasite to the host immune system. Thus, helminths (e.g. *Schistosoma mansoni*) and protozoa which produce long-lived chronic infections induce the synthesis of cytokines that down-regulate cell-mediated immunity while highly virulent intracellular protozoa such as *Toxoplasma gondii* trigger strong cellular immune responses to limit their own numbers thus ensuring host survival. Since the macrophage is a crucial effector cell in the host protective response against parasites, much of this regulation is likely to occur at the level of macrophage activation and function. The major cytokines produced during parasitic infection which down-regulate macrophage effector function are the Th2 cytokines, IL-4 and IL-10 and TGF-β.The production of reactive nitrogen oxides (NO) is thought to be the major mechanism by which lymphokine activated macrophages kill both intracellular and extracellular parasite targets and each of the inhibitory cytokines have been shown to down-regulate NO production by these effector cells and can synergize in this suppression. In the case of IL-10, inhibition of NO production and parasite killing by IFN-γ activated inflammatory macrophages correlates with the suppression of endogenous TNF-α synthesis and is restored by the additon of exogenous TNF-α.

Intracellular parasites up-regulate macrophage effector function by a variety of mechanisms, one of which is the T-independent induction of IFN-γ synthesis by NK cells. This pathway has recently been shown to depend on the induction from macrophage accessory cells of IL-12 which acts in synergy with TNF-α. In the case of *Toxoplasma gondii,* IL-12 production is stimulated by both live tachyzoites and heat labile parasite molecules released in culture and is amplified by IFN-γ, the end product of the NK cell response. IL-10 down-regulates IL-12 production by macrophages and in turn IL-12, by its inhibition of Th2 cell differentiation, can block IL-10 responses in vivo. These observations suggest that IL-10 and IL-12 have antagonistic effects on cell-mediated immunity and that their preferential induction may dictate the outcome of infection.

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Suppression of Macrophage Cytokine Production and Expression of Inducible Nitric Oxide Synthase by Interleukin 10, Interleukin 4 and Lipopolysaccharide

Christian Bogdan

Macrophages play a central role during inflammatory processes. Due to their production of cytokines and reactive oxygen and nitrogen intermediates they exert antiparasitic and tumoricidal functions, but they are also potentially destructive for host tissues which necessitates a tight regulation of the production of these mediators *in vivo*. In so far, host-mediated suppression of cytokine, H_2O_2 and NO release is desirable and part of a normal immunoregulation. On the other hand, suppression of these macrophage functions by microbes or microbial products may serve as an evasion mechanism for the pathogen. In this presentation I will focus on IL-10 and IL-4 as well as on bacterial endotoxin as candidate suppressants.

COS cell-expressed IL-10 reduced the production of TNF-α, IL-1, H_2O_2 and NO by mouse peritoneal exudate macrophages. However, the concentrations of IL-10 required for 50% suppression were strikingly different for the 4 secretory pathways (0.04 , 0.35, 3.7 and ≥ 10.0 CSIF U/ml). IL-10 markedly suppressed TNF-α, IL-1α and IL-1β mRNA levels without suppressing transcription of the corresponding genes (1, 2).

IL-4 suppressed IFN-γ-induced NO production (IC 50: 45 ± 0.6 pM) while at the same time synergizing with IFN-γ for the secretion of TNF-α. When the macrophages were pretreated with IL-4 for 6-9 h, the suppression of IFN-γ-induced NO release was strongly increased (IC 50: 1.4 ± 0.3 pM) despite unaltered TNF-α production; under these conditions, IL-4 also blocked the induction of NO by the combination of IFN-γ and LPS (IC 50: 3 ± 0.4 pM). Suppression of NO production was paralleled by a reduction of inducible nitric oxide synthase (iNOS) enzyme activity, protein expression and mRNA levels (3).

Bacterial endotoxin (LPS) is known as a potent co-inducer of IFN-γ-stimulated iNOS activity, protein- and mRNA-expression in murine peritoneal macrophages. We found that small amounts of LPS (50-200 pg/ml) can also have an adverse effect if the macrophages are exposed to LPS for ≥ 6 h prior to activation by IFN-γ. The initial (5 h) expression of iNOS mRNA was unaffected, while by 24-48 h the iNOS mRNA levels were strongly suppressed, indicating that LPS pretreatment does not lead to a reduced IFN-γ responsiveness of the macrophages (4).

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List of Invited Speakers

Workshop on

MOLECULAR MECHANISMS OF MACROPHAGE ACTIVATION

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266 **Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects.**
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268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
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Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodriguez-Navarro, R. Serrano and R. G. Wyn Jones.

269 **Workshop on Neural Control of Movement in Vertebrates.**
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16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
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