NO, Immunosuppression and Tumor Immmunotherapy

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NO·
Immunosuppression
and
Tumor Immunotherapy

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To
Maria
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CONTENTS

SUMMARY 6

ORIGINAL PAPERS 7

ABBREVIATIONS 8

INTRODUCTION 9

NO IN IMMUNITY AND TUMOR GROWTH 14
- NO Synthesis 15
- Biology of Nitric Oxide 20
- NO and Effector Functions In Immunity 23
- Immune Regulatory Functions of NO 27
- Tumor Promoting Effects of NO 35

TUMOR IMMUNOSUPPRESSION 38
- Tumor Derived Immunosuppressive Factors 38
- Indirect Suppression 41
- Gliomas 48

THE PRESENT STUDY 50
- Aims 50
- Glioma immunosuppression (Paper I) 50
- Colon carcinoma immunosuppression (Paper II) 53
- Expression of iNOS in Immunized rats (Paper III) 56
- Inhibition of iNOS in Immunized Rats (Paper IV) 58
- Concluding Remarks and Future Perspectives 61

POPULÄRVETENSKAPLIG SAMMANFATTNING 66

ACKNOWLEDGEMENTS 69

REFERENCES 71

APPENDIX (Papers I-IV) 107

SUMMARY

This study aims at clarifying the role of NO in the immunosuppression induced by *in vivo* tumor growth and by tumor immunotherapy, and determining whether the inhibition of NO production can be used as an adjuvant in tumor immunotherapy. We have shown previously that tumor cells, glioma (N32) and colon carcinoma (H1D2), when genetically engineered to express such immune stimulatory cytokines as IFN-γ and IL-18, induce strong anti-tumor immune response, in immunized tumor-free rats, whereas only a limited therapeutic effect is achieved in rats in which a tumor has already become established. This led us to our studying whether immmunosuppression induced in rats by a subcutaneously growing malignant glioma and in rats with an intrahepatic growing colon carcinaoma would attenuate anti-tumor immune responses. Anti-tumor cytolytic responses, proliferative responses and cytokine production were found to all be strongly suppressed in the spleen cells of these tumor-bearing rats. The suppression was also shown to be partially dependent on the production of nitric oxide (NO) by the suppressor cells found in the plastic adherent fraction of the spleen cells. Since during inflammatory responses the major part of NO is derived from the IFN-γ induced expression of inducible nitric oxide synthase (iNOS), it was of interest to investigate whether the expression of iNOS is induced after immunization by IFN-γ-secreting glioma cells (N32 IFN-γ) and whether the inhibition of NO generated by iNOS in immunized rats would enhance anti-tumor immune responses. The expression of iNOS was found to be elevated, both at the immunization site and in the brain tumors. The selective inhibition of iNOS was shown to enhance anti-tumor immune responses. In conclusion, our results demonstrate that in immunized rats both growing tumors and immunotherapeutic intervention by use of IFN-γ secreting tumor cells tends to induce NO-dependent suppressor cell activity that inhibits anti-tumor immune responses. It appear then that the selective inhibition of iNOS can be used as an adjuvant for enhancing type 1 anti tumor T-cell responses during anti-tumor immunotherapy.

ORIGINAL PAPERS

The thesis is based on the following papers.

I. Hegardt, P., Widegren, B., and Sjögren H.O. Nitric Oxide dependent systemic immunosuppression in animals with progressively growing malignant gliomas. *Cellular Immunology*, 200, 116-127 (2000)

II. Hegardt, P., Widegren, B., Li, L., Sjögren, B., Kjellman, C., Sur, I., and Sjögren, H.O. Nitric oxide synthase inhibitor and IL-18 enhance the anti-tumor immune response of rats carrying an intrahepatic coloncarcinoma.*Cancer Immunology Immunotherapy*, 50, 491-501 (2001)

III. Johansson, A.C., Hegardt, P., Janelidze, S., Visse, E., Widegren, B., and Siesjö, P. Enhanced expression of iNOS intra tumorally and at the immunization site after immunization with IFN-γ secreting rat glioma cells. *Journal of Neuroimmunology*, In press

IV. Hegardt, P., Esbjörnsson, M., Salford, L.G., and Siesjö, P. Selective inhibition of inducible nitric oxide synthase enhances anti-tumor immune responses in rats immunized with IFN-γ secreting glioma cells. Submitted

ABBREVIATIONS

APC	Antigen Presenting Cell
cNOS	constitutive NOS
CD	Cluster of Differentiation
COX	Cyclo Oxygenase
CTL	Cytotoxic T Lymphocyte
eNOS	endothelial NOS
GM-CSF	Granulocyte and Macrophage Colony Stimulating Factor
GSH	Glutathione
IFN	Interferon
iNOS	inducible NOS
IRF	Interferon Regulatory Factor
JAK	Janus Kinase
LAK	Lymphokine Activated Killer cell
L-NAME	N-nitro-L-arginine methyl ester
L-NIL	L-N^6-(1-Iminoethyl)-L-lysine
LPS	Lipopolysaccharide
IL	Interleukin
MHC	Major Histocompatibility Complex
NF	Nuclear Factor
NK	Natural Killer cell
NOS	Nitric Oxide Synthase
NO	Nitric Oxide
nNOS	neuronal NOS
PBL	Peripheral Blood Lymphocytes
PGE	Prostaglandin E
PHA	Phytohaemagglutinin
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
ROI	Reactive Oxygen Intermediates
SEA	Staphylococcal Enterotoxin A
STAT	Signal Transducer and Activator of Transcription
TB	Tumor Bearing
TF	Tumor Free
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper cell
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

An outline of tumor immunology

The idea that the immune system might possibly be involved in the control of cancer was first brought up more than 100 years ago by physicians who had observed that cancers sometimes diminished after bacterial infections or after the administration of extracts of soluble toxins (438). During the course of the century that has since intervened, various non-specific immunotherapeutic approaches to treating cancer, including the use of microbiological agents and recombinant inflammatory cytokines. Some of these approaches, and particular that of administering Bacillus Calmette-Guérin, have shown clinical benefits (108). There are obvious drawbacks to non-specific methods of this sort, however, such as toxicity toward the host and the absence of immune memory, since these approaches primarily activate the innate immune system. It would be more advantageous to instead induce tumor specific immune responses. For much of the last century, however, immune reactions were thought to only be induced against foreign substances and not against autologous cells. Since cancer cells arise from host cells, immunologists assumed that they did not express antigens sufficiently different to elicit an immune reaction against the tumor. When inbred mouse strains became available it was possible to perform tumor transplantation studies involving use of syngeneic tumors, demonstrating that immune responses to tumors could be induced without toxicity to normal tissue (30, 123, 216). This gave rise to the concept of tumor-specific antigens.

The concept of tumor-specific antigens was challenged, however, due to its being found in animal studies that spontaneously arising tumors, in contrast to chemically-induced or virus-related ones, were largely non-immunogenic. This led to the assumption that tumors do not differ sufficiently from normal tissue to activate immunity against them (167). Later, animal experiments demonstrated the lack of tumor-immunogenicity to be due to the inability of tumors to activate immune response, rather than to the absence of tumor antigens (426).

Effector cells against tumors

Cancer vaccines designed to activate specific immune reactivity against tumor antigens have been studied widely due to such responses, mediated by T- and B-cells, being expected to induce long-lasting anti-tumor immune memory with a low probability of toxicity to normal tissue. Historically, antibodies were thought to be the best mediators of specific anti-tumor immunity. However, although tumor-reactive antibodies appear frequently in cancer patients, their occurence does not correlate with disease state (99). Moreover, vaccination by tumors can induce tumor-specific humoral responses that are non-protective (345). Humoral tumor

antigens appear in most cases, therefore, to be poor as tumor-rejection antigens. In some cases, nevertheless, therapy by use of monoclonal antibodies has been shown to exert therapeutic benefits, both in experimental models and in cancer patients (166).

Results for animal models as well as improved understanding of the underlying immunological mechanisms suggest antigen-specific tumor rejection to primarily be a function of the cellular arm of adaptive immunity, the T-cells. The processing of antigens by way of the major histocompatibility complex (MHC) class I pathway enables cytotoxic T-lymphocytes (CTL) to recognize subtle changes in the protein repertoire of most somatic cells. Lymphocyte depletion and the adoptive transfer of T-cells to animals have demonstrated that $CD8^+$ CTLs, either alone or in combination with $CD4^+$ T-cells, represent the most important anti-tumor effector arm of the adaptive immune response. It is also becoming increasingly clear that responses of $CD4^+$ T-helper (Th) cells play an important role in the rejection of tumors. The primary role of the Th cells, or specifically of the subset of Th1 cells, is to enhance the induction and maintenance of CTL responses *in vivo*. However, $CD4^+$ T-cells have also been found to elicit direct effector functions (324). The effector mechanisms of the innate immune system, such as macrophages, natural killer cells (NK-cells), and granulocytes, together with the complement system, can also be thought to in a coordinated way promote the action of the T-cells.

Tumor antigens

The first description and isolation of a naturally occurring human tumor antigen shown to elicit a CTL response was the melanoma antigen MAGE-1 (422). To date, a large number of T-cell defined tumor antigens have been characterized and been shown to induce clinically meaningful responses (350). Tumor antigens recognized by T-cells are either tumor-specific or tumor-associated. Tumor-specific antigens are not expressed in normal tissue and can either be unique to each patient or be shared by several cancer patients. Such antigens are derived from mutations of the tumor cells, from mutated or translocated oncogenes (p53 and ras), or from viral antigens in cancers of viral etiology (e.g., the Epstein Barr virus (EBV) and the human papilloma virus (HPV)). Tumor-associated antigens are non-mutated antigens expressed by genes that, in most normal tissues, with the exception of testis, are completely silent but are reactivated in tumors (such as the prototypic melanoma families MAGE, GAGE, and BAGE) or are differentiation antigens corresponding to such normal tissue-specific gene-products as the melanocyte antigen MART-1/Melan A (reviewed in (144)). To date, the majority of the antigens that have been characterized are tumor-associated ones.

Cancer vaccines

Various approaches to delivering tumor antigens in cancer vaccines have been utilized. One approach is to deliver defined antigens as pre-synthesized peptides or in the form of either DNA or RNA vectors. Another approach is to use autologous whole tumor cells, tumor cell lysates, or heat chock proteins purified from tumor cells, as sources of tumor antigens in tumor vaccines. An advantage of this latter strategy is that, because of the genetic diversity which is otherwise difficult to detect and produce, a whole array of different tumor antigens can be presented, including tumor-specific antigens unique to the patient. A possible problem with use of synthetic peptides is that tumor escape variants may be generated. Nevertheless, the availability and concentration of relevant tumor antigens can be much higher, and the risk of inducing autoimmunity lower, when pre-synthesized tumor specific peptides are employed. Regarding the latter, few reports of autoimmunity induced by tumor vaccination have appeared (reviewed in (144)).

Most of the vaccines, synthetic peptides and naturally processed peptides need to be administrated in the context of adjuvants in order to be maximally effective. Historically, microbial and chemical adjuvants have been widely used without knowledge of the mechanisms that control the immune responses. With the gradual clarification of the molecular processes underlying the control of immune responses, it has been possible to utilize increasingly precise strategies. In general, the activation of T-cell responses requires a second (co-stimulatory) signal from the activated antigen-presenting cells (APC). Cytokines have been used in a variety of settings to optimize these responses. It is important to note, however, that several of these pro-inflammatory cytokines, including IL-2, IFN-γ, IL-12 and IL-18, also activate the production of such factors as nitric oxide and oxygen radicals that in turn suppress the T-cell responses. The second signal to the T-cells can also be delivered by tumor cells that are genetically engineered to express costimulatory molecules, such as CD40. Another strategy of interest is to use ex vivo activated autologous dendritic cells as adjuvants. These cells can be pulsed by tumor peptides that are fused to tumor cells or admixed with whole tumor cells and are then given back as tumor vaccine.

Tumor immune escape

A paradox in tumor immunology is the ability of immunogenic tumors to grow in immunocompetent individuals and thus escape immune destruction. Tumor cells are under selective pressure to escape the growth-restricting mechanisms acting on the organism, and most tumors also develop strategies to evade attacks by the immune system. A variety of mechanisms have been proposed to account for tumor-immune escape. In principle, tumor-immune escape mechanisms can act either on the tumor or on the immune system (see figure 1).

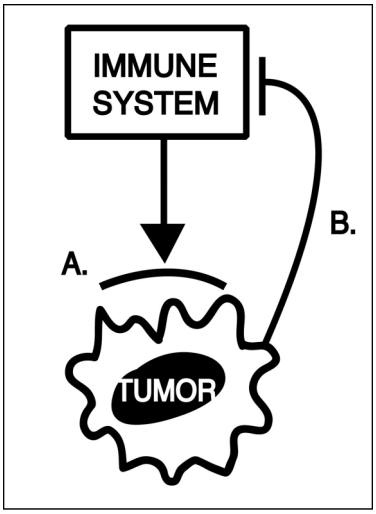

Figure 1. Tumor immune escape. Several mechanisms have been proposed to account for tumor immune escape. In principal the escape mechanisms could act on A) the tumor and B) the immune system.

Several mechanisms that act on the tumor make it less susceptible to immune recognition and destruction. Malignant transformation and tumor progression in cancer patients are frequently associated with the down-regulated expression of MHC class I or the altered function of it (reviewed in (171)). The molecular basis for this may well be either mutations of the MHC class I genes, together with abnormal regulation of their expression, or defects in the antigen presenting machinery such as loss of TAP or tapasin, or defects of the proteasome. The loss of MHC class I expression can render tumor cells resistant to CTL killing, but can also make them more susceptible to NK-cell mediated lysis. However, several tumors have been shown to be resistant to NK cell cytotoxicity, and MHC class I negative tumor cells can exist in patients without their being susceptibile to NK-cell mediated attack (332). Antigen presentation can also be defective without an overall loss in MHC class I expression, such as in the case of proteasome defects. There are also examples of mechanisms that make tumor cells resistant to anti-tumor CTL effector mechanisms, such as the expression of receptors that neutralize the FAS-ligand (340).

The progression of tumors in animals and in cancer patients is often associated with dysfunctional cellular immune responses. Mechanisms that act on the immune system may be a direct effect of immunosuppressive factors produced by the tumor cells or be an indirect effect through activating the suppressor cells. Various tumor-derived factors have been shown to suppress the effector functions of macrophages and of CTL- and NK-cells. Moreover, these tumor-derived factors can alter or suppress the functions of antigen presenting cells and of T-helper cells, thus preventing the activation of anti-tumor immune responses. Tumor growth has been shown to be associated with an increase in the number of natural

suppressor cells that inhibit T- and NK-cell responses. The major factors responsible for this natural suppressor activity appear to be PGE-2, reactive oxygen intermediates (ROI), and nitric oxide (NO). NO- and ROI-mediated suppressor cell activity can also be induced by immunotherapeutic intervention. NO and ROI also mediate macrophage anti-tumor cytotoxicity, however. The existence of well-established tumors *in vivo* indicates that tumors circumvent this paradox, presumably through the differential regulation of NO and ROI at tumor-proximal and tumor-distal sites. The enhanced production of NO at the site of the tumor can also have the effect of promoting tumor growth, for example by the enhancement of angiogenesis and the shunting of L-arginine (the substrate required for NO synthesis)-metabolism in favor of the synthesis of polyamine tumor-growth factors. Thus, the suppression of cellular anti-tumor-immune responses can be induced both by tumor growth and by immunotherapeutic intervention. The present study aimed at investigating the development of anti-tumor-immune responses in tumor-bearing rats undergoing immunotherapeutic treatment, as well as the involvement of NO in tumor-induced immunosuppression and the regulation of anti-tumor immune responses in immunized rats. General aspects of tumor-induced immunosuppression and the complex role of NO in both immune functions and tumor growth are reviewed.

NO IN IMMUNITY AND TUMOR GROWTH

A report by Prout in 1818 indicated large amounts of nitrite to have been found in the urine of a febrile patient (343). Today, the level of nitrite in the urine and the plasma, as well as in culture superanatants, is routinely used to provide an indirect estimate of NO production both *in vivo* and *in vitro*. Thus, the report by Prout can be assumed to be the first to implicate the nitric oxide (NO) pathway as being involved in immunity. It took almost 190 years, however, to specifically identify NO as a mediator of physiological and immunological responses *in vivo*. In 1986 Robert Furchgott and Louis Ignarro suggested that the endothelium-derived relaxing factor (EDRF) was NO, and in 1987 results reported by Palmer et al. and Ignarro at al. demonstrated that EDRF was actually NO (185, 327). The study of the role of NO within medical biology grew very rapidly and in an issue of Science in 1992 NO was deemed the molecule of the year (86). In 1998 the Nobel Prize in medicine was awarded for having identifying“ NO as a signaling molecule in the cardiovascular system”. However, shortly before Ignarro and Palmer reported their findings, the pathway of NO in the killing of tumor-cells was implicated by Hibbs et al., as an analogue to L-arginine (NG-monomethyl-L-arginine) being able to block the nitrite production and the anti-tumor cytolytic activity of activated macrophages (168). This pathway was implicated also by the antimicrobial activity of activated macrophages (295, 393). In 1989, NO was shown to be the molecule responsible for the L-arginine-dependent cytolytic activity of macrophages (394). Although the basic conception of NO’s role in immunity and of its being a tumoricidal and antimicrobial molecule generated by activated macrophages is still true, findings during the last decade indicate the functions of NO in immunity to be far more diverse. Not only does NO function as a toxic molecule that combats infectious agents and tumors, but it is also involved in the regulation of innate and adaptive immune responses (46). The multiple functions of NO in immunity can be attributed to a number of factors: 1) that a large number of immune cells (in addition to macrophages) produce and respond to NO, 2) that not only iNOS but also constitutive NOS (eNOS and nNOS) operates within the immune system, 3) that the activity of NO is not restricted to its production site, and 4) that cell signaling is not limited to a single defined receptor, NO reacting with other inorganic molecules as well, and with DNA structures, prosthetic groups and proteins. Apart from its tumoricidal activity, NO can also promote tumor growth by the inhibition of anti-tumor immune responses and the enhancement of angiogenesis and of tumor blood flow.

NO Synthesis

NO synthesis and nomenclature

NO is synthesized from a family of enzymes, the nitric oxide synthases (NOS), by conversion of the amino acid L-arginine and oxygen (O_2) to L-citrulline and NO (see Figure 2). The reaction requires NADPH, FAD, FMN, tetrathydrobiopterin (BH_4), and a thiol donor as cosubstrates and cofactors. Currently, there are three major NOS isoforms known, encoded by three distinct genes (71, 213, 280, 453). There are three different nomenclatures in use, based respectively on the cell-type from which they were first cloned, the major characteristics of their regulation, and the historical order of cloning. Neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) usually exist in the cell as constitutively expressed proteins (thus collectively named constitutive NOS (cNOS)), regulated primarily by Ca^{2+} influx and by the subsequent binding of calmodulin. In contrast, macrophage NOS (macNOS or NOS2) is not found in resting cells and its production is strongly induced by cytokines and other immunological stimuli (thus being named inducible NOS (iNOS)).

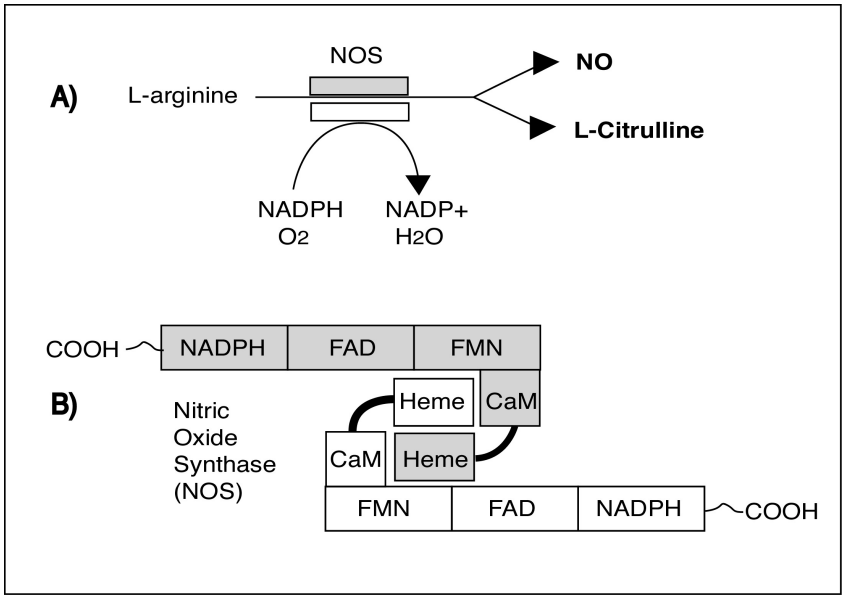

Figure 4. A) Biochemical pathway of nitric oxide (NO) production in mammalian cells and B) domain arrangement of nitric oxide synthase (NOS).

Each isoform is highly conserved across the mammalian species, with an amino acid sequence homology of approximately 90%, whereas the homology of the different isoforms to one another is around 50% (125, 314). The NOS in mammalian species are large and complex proteins that require 17 binding reactions to assemble the active homodimers, comprised of 130-133 kDa (eNOS, and nNOS) or 160 kDa (iNOS) (125, 267). Each

subunit consists of two halves, a C-terminal electron generating a reductase region and an N-terminal oxidase region.

NOS activity

The enzyme activation of cNOS (eNOS and nNOS) occurs rapidly and transiently, in accordance with the kinetics of the calcium signal as induced, by the arrival of an action potential at a nerve ending, or by the acethylcholine activation of the endothelial cell receptors, for example. The equally transient production of NO provides a rapid pulse-like signal to the responding cells (299). In contrast, iNOS is regulated at a transcriptional and posttranscriptional level, a number of different signal transduction pathways and molecules being involved. The time that mRNA and protein synthesis require results in a lag phase of several hours between cell activation and the appearance of the NO that is generated from iNOS (314, 392, 393). In contrast to cNOS, iNOS generates high concentrations of NO, this level of synthesis being sustained as long as the enzyme is present and the substrate and the cofactors are available (hours, days or even longer). Generally, the low-output pathway that cNOS sustains supports physiological functions in the healthy host, whereas the high-output pathway in which iNOS activation occurs plays an important role during inflammation and infection. There are several important exceptions to this, however.

Calmodulin (CaM) is of central importance in the regulation of NOS activity and in creating functional distinctions between the different NOS isoforms. All three NOS isoforms require CaM for activity. However, nNOS and eNOS only bind CaM at elevated levels of Ca^{2+}, whereas iNOS binds tightly to CaM even at the lowest Ca^{2+} levels a cell can sustain (53, 77, 126, 361, 428). When Ca^{2+} is added, CaM attaches, electrons beginning instantly to move from the flavins to heme, thus activating the NOS (1). This explains the pulsative, Ca^{2+}-dependent regulation of cNOS activity, in contrast to the continuous Ca^{2+}-independent activity of iNOS.

There are a number of examples of tissues or cells that constitutively express iNOS, such as airway epithelium (constantly exposed to potentially inductive stimuli), and rat ovarian cells (during certain cell cycles) (106, 221, 427). However, most cells express iNOS only after exposure to an inductive stimulus. Of the genuine cells of the immune system known to express iNOS (macrophages, dendritic cells, NK-cells, neutrophils, eosinophils, mast cells, microglia, and Kuppffer cells), macrophages are probably the most prominent (45, 267). It is questionable whether T- and B-cells express iNOS (34, 411, 416).

iNOS inductive stimuli

The expression of iNOS is mainly induced by immunological stimuli, such as cytokines, microbial and viral products, and cell-cell contact (see Figure 3). Although novel molecules continue to be discovered as being

positive regulators of iNOS, IFNγ, the prototypic example (along with LPS), is (still) believed to be one of the major inducers of iNOS expression (66, 89, 178, 194, 453). The central role of IFN-γ is evident from the fact that iNOS is hardly expressed in mice that are deficient in the expression of IFN-γ or of its receptor (89, 178). When tested alone, IFN-γ has also been found to be the only agent able to effectively induce iNOS in peritoneal mouse macrophages (267). Several cytokines, such as IL-12 and IL-18, have also been shown to act through an indirect mechanism mediated by the autocrine or paracrine production of IFN-γ (146, 220, 443, 473). Other examples of cytokines involved in the positive regulation of iNOS, alone or in synergistic pairs, are TNF-α (39, 224, 367), IL-1β (39, 142, 159), and IL-2 (78, 202, 458). Growth factors such as the vascular endothelial growth factor (VEGF), for example have been shown to enhance the expression of iNOS in human endothelial cells (HUVEC) (230).

Crosslinking of the cell-surface receptor CD23 (Fcγ receptor IIb) or CD8 (α- or β-chains) induces the expression of iNOS in human monocytes and rat macrophages, respectively (46). Cell-cell contact through CD40-CD40L interaction has also been found to drive the induction of iNOS in a graft-versus-leukemia reaction (306).

There are several examples of microbial and viral products that have been shown to enhance the expression of iNOS in macrophages such as the HTLV-1 transactivator Tax, the 19-kD lipoprotein of Mycobacterium tuberculosis (via TLR-2), bacterial DNA, and DNA from various protozoan parasites (138, 304, 382, 413).

iNOS bimodal stimuli

IL-4 and IL-10 are mainly classified as inhibitory cytokines (10, 17, 47, 48), but have also been shown to exert stimulatory effects on the production of NO *in vivo* by means of IFN-γ-dependent mechanisms (399, 454) (see Figure 3). In this context, IL-4 was shown to stimulate the production of NO through the expansion of IFN-γ-producing dendritc cells (454). IFN-α/β, has also been shown to exert a bimodal effect on the production of NO (98, 137, 286). In addition, PGE-2 has been shown to both enhance and suppress the expression of iNOS (73, 254, 281, 412). It has been proposed too that PGE-2 and NO cross talk by the transcriptional regulation of their respective enzymes COX (1 and 2) and iNOS (79, 279, 341).

Transcriptional regulation of iNOS

The activation of iNOS by cytokines, microbial products, and other stimuli is initiated at the transcriptional level (see Figure 3). There have been found to be numerous of binding sites for transcription factors within the iNOS promotor. After the binding of cytokines and microbial products to cell surface receptors different upstream signaling pathways that are

dependent on the stimuli are initiated, such as those of Janus kinases (JAK), p38 mitogen-activated protein kinases (MAPK), extracellular signal regulated kinase (ERK 1/2), and protein tyrosine kinases (66, 203, 217, 229). This leads to the activation of specific transcription factors such as NF-κB, interferon regulatory factor-1 (IRF-1), AP-1, the nuclear factor interleukin-6 (NF-IL-6), the signal transducer and activator of transcription 1α (STAT-1α) (66, 101, 136, 217). One should be bear in mind that considerable species and cell-type differences exist. The induction of murine and human iNOS expression by IFN-γ and LPS has been shown to involve the JAK 2-STAT1 and NF-κB pathways (136). However, the binding elements for STAT and NF-κB have been found to be much further upstream in the human than in the murine iNOS promoter. This might serve to explain the relative ease of iNOS induction in murine as compared to human cells.

Interestingly NO itself has a biphasic effect on the transcription of iNOS. Low levels of NO activate NF-κB and thus the expression of iNOS, whereas high concentrations of it suppress the expression of iNOS (82, 419). Thus, NO can support the production of NO during the onset of an immune response (positive feedback) but can also prevent the potentially harmful overproduction of NO (negative feedback) from occuring.

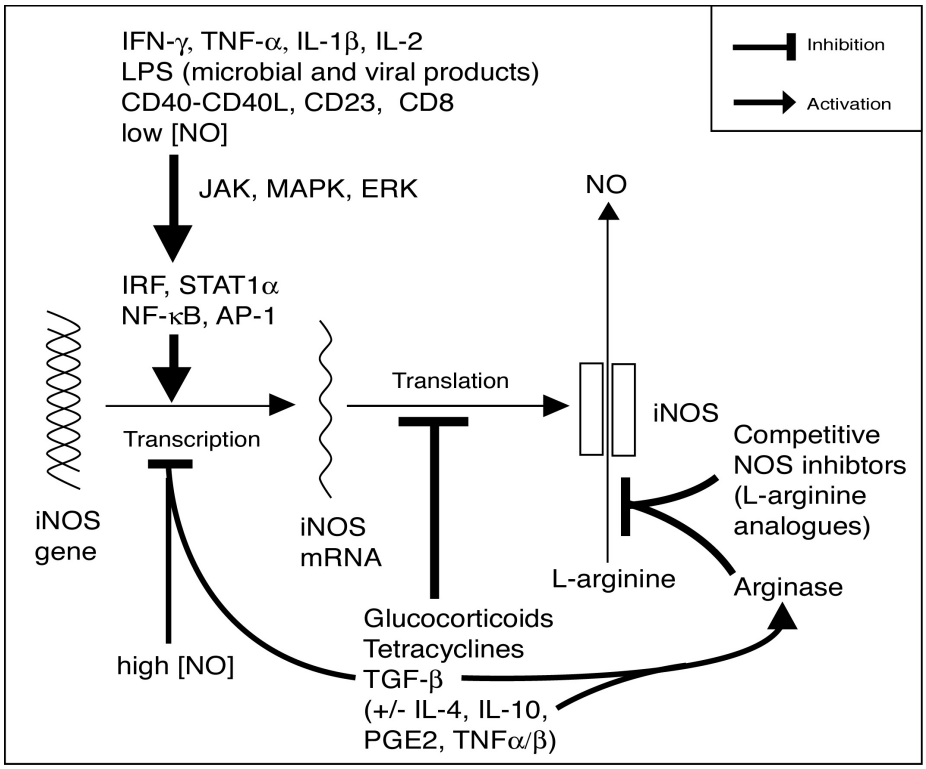

Figure 3. Regulation of iNOS biosynthesis.

Posttranscriptional and transcriptional inhibition of iNOS

TGFβ is a potent inhibitor of iNOS, all three isoforms (1-3) having been shown to suppresses the production of the NO stemming from macrophages (97) (see Figure 3). Results of studies examining the mechanisms involved suggest it is mainly at the posttranscriptional level that TGF-β affects the expression of iNOS, through destabilizing iNOS mRNA and increasing the rate of iNOS protein degradation (431). However, studies of other cells have shown that both the transcriptional and the posttranscriptional levels can be affected by TGF-β (119, 336). TGF-β down-regulates the cytokine-induced high-mobility group protein (HMG-I(Y)), a transcription factor that has been shown to bind to the iNOS promoter and thus to facilitate the expression of iNOS (333). The physiological relevance of TGF-β in iNOS inhibition has been shown by the spontaneous expression of iNOS in TGF-β1-deficient mice (432). Pharmacological levels of glucocorticoids involve a combination of transcriptional and posttranscriptional effects that inhibit the induction of iNOS(238). Tetracyclines have also been shown to regulate the expression of iNOS at the post-transcriptional level through inducing mRNA degradation (21, 331).

Another level of post-translational regulation concerns the availability of the NOS substrate L-arginine. The high output production of NO by macrophages, for example, is dependent on extracellular L-arginine pools (67). The extracellular concentration of L-arginine is modulated by arginase, which can be released into the extracellular space (449). Th2 cytokines (IL-4, IL-10, and IL-13) TGFβ, LPS and dexamethasone have been shown to increase the levels of arginase in macrophages and in dendritic cells, and to thus prevent by means of substrate depletion the production IFN-γ-induced NO production from occurring (152, 311, 362). The posttranslational regulation of NOS on the basis of the availability of the co-substrates heme and BH_4 has also been suggested to occur (11, 418). The majority of the NOS inhibitors utilized in experimental settings are analogues of L-arginine and thus regulate the activity of NOS at the post-transcriptional level.

Biology of Nitric Oxide

Biochemistry

NO is a small (30 Da) lipid- and water-soluble inorganic radical gas with one unpaired electron ($NO^{\cdot}$). In an aqueous solution, NO is soluble at concentrations of up to 2 mM. Since under both physiological and pathophysilogical conditions concentrations of NO remain within the micro-molar range, in biological systems NO should not be regarded as a gas (235). Despite its being a radical, the reactivity of NO in biological

systems is relatively weak and it basically interacts only with oxygen, other free radicals, and transition metals (36). The relatively low reactivity of NO together with its small size and lipophilicity, allows it to easily diffuse across membranes and to thus function as a messenger (446). The flux of NO and of the chemical microenvironment surrounding it determine the chemistry of NO, strongly affecting the bilological responses that occur under a given set of conditions.

It is convenient to categorize the chemical reactions of NO in terms of direct and indirect effects (see Figure 4). The direct effects involve reactions in which NO interacts directly with biological molecules such as transition metals and free radicals, whereas the indirect effects result from reactions with either oxygen or superoxide radicals (O_2^-), producing reactive nitrogen species (RNS). Generally, the direct effects of NO, which occur when the production of NO is of low level or is brief, primarily support protective and signaling functions, whereas the indirect effects, which occur mainly during sustained and hogh-level production of NO, result primarily in toxic effects.

Direct effects (NO)

There are three major types of reactions of NO with metal complexes: 1) direct reactions with the metal at the center of a protein, 2) redox reactions with metal-oxygen complexes (hemoglobin) and with high-valence metals, and 3) binding to iron-sulfur clusters in proteins. The most relevant reactions in biological systems are those with the iron in proteins that contain heme. These reactions which yield a stable nitrosyl complex by displacement of the iron, can result in quite differing effects, depending on the protein affected, such as the activation of guanulyl cyclase, the activation (at low concentrations of NO) or inhibition (at high concentrations of NO) of cyclooxygenase (COX), the enzymatic inhibition of cytochrome P-450, the inhibition of NO synthases (cNOS being much more sensitive than iNOS), or the inhibition of catalase (at high concentrations of NO) (114, 156, 208, 391, 446). Redox reactions with metal-oxygen complexes (oxyhemoglobin) and high-valent metals (Fe^{4+}) result in NO-scavenging and the reduction of oxidative stress, respectively (149, 157). The binding of NO to iron-sulfur centers in proteins yields the iron-sulfur nitrosyl complex and activates certain enzymes, such as the iron-regulatory factor (104, 328).

NO also reacts directly with other radical species. Its reaction with the tyrosyl radical leads to an inhibition of ribonucleotide reductase and of DNA synthesis, which could be a factor in the cytostatic properties of NO (244). Reactions with carbon-centered radicals can also stabilize radiation-induced lesions and lead to the radiosensitization of the cells (294). In addition, NO can protect cells against peroxide-induced cytotoxicity by scavenging of lipid hydroxyl radicals (161).

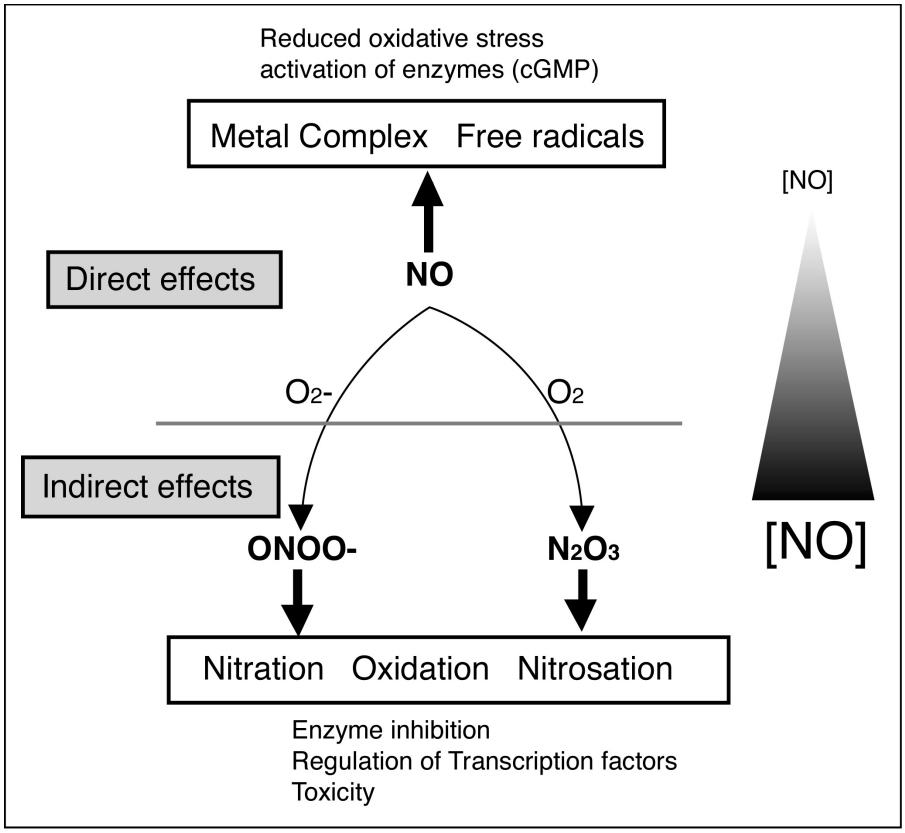

Figure 4. The chemical biology of nitric oxide (NO). Separation between direct and indirect effects of NO.

Indirect effects (RNS)

Direct reactions between NO and thiols or other bioorganic molecules are far too slow to be significant in a biological setting. Such reactions are indirect effects mediated by the reactive nitrogen species (RNS) derived from reactions between NO and either oxygen or the superoxide radical (O_2^-) (447).

At high concentrations and under aerobic conditions NO is unstable and rapidly undergoes auto-oxidation (see Figure 5). The resultant intermediate, both in the gas phase and in aqueous solutions is dinitrogen trioxide (N_2O_3). "Free" NO_2, in contrast, is only generated in the gas phase and not in aqueous solutions (446). Thus, in biological systems N_2O_3 is the predominant RNS that is formed from the auto-oxidation of NO. The half-life of NO depends mainly on the concentration of NO (124). Thus, high concentrations of NO reduce the half-life and increase the rate of the NO auto-oxidation reaction, whereas at low concentrations NO diffuses away from its site of origin. During high flux of NO, therefore, the indirect effects mediated by NO-oxygen reactions prevail, whereas at low NO concentrations the direct effects dominate. The rate of the NO auto-oxidation reaction is much higher in hydrophobic milieus, such as in the cell membrane (256).

$2\ NO^{\cdot} + O_2 \longrightarrow \mathbf{2\ NO^{\cdot}_2}$
$2\ NO^{\cdot}_2 \longleftrightarrow \mathbf{N_2O_4} \longrightarrow NO_2^- + NO_3^-$
$NO^{\cdot} + NO^{\cdot}_2 \longleftrightarrow \mathbf{N_2O_3} \longrightarrow 2\ NO_2^-$

$NO^{\cdot} + O_2^- \longrightarrow \mathbf{ONOO^-}$

Figure 5. Reaction products of NO with oxygen (O_2) and the superoxid radical (O_2^-). Unstable and highly reactive nitrogen species (RNS) are shown in bold letters.

The primary reaction of N_2O_3 is that of the nitrosation of thiols and amines, which gives rise to nitrosamines and S-nitrosothiols. Because of the relative stability of S-nitrosothiols, with half-lives ranging from minutes to hours, and their ability to donate NO, they act as a major storage and carrier system for NO (63). The S-nitrosylation of proteins also plays a significant role in the regulation of biological processes, comparable to that of phosphorylation (57). A large number of molecular targets important for biological responses have been identified, such as receptors, ion-channels, metabolic proteins, signal amplification systems, and transcription factors. The S-nitrosylation of glucocorticoid receptors decreases the binding of steroids (135). NO-dependent vascular relaxation is partly due to the activation of calcium-dependent potassium channels in the smooth-muscle cells. S-nitrosylation also alters the kinase activity of protein kinase C (PKC), and thus inhibits the PKC-dependent signaling cascade (148). Several transcription factors are also regulated by S-nitrosylation, such as the inhibition or activation of NFκB activity, the modulation of AP-1 activity, and inhibiting the binding of c-jun and c-myb to DNA (54, 214, 334, 401). In addition, both the transcription factors and DNA repair enzymes that use zinc finger motifs for their DNA binding, such as Sp1 and EGR-1, are inhibited by the S-nitrosylation of zinc thiolate centers, which leads to Zn^{2+} ejection (38, 232, 445). Another important effect of S-nitrosylation is the depletion of reduced gluathione (GSH), enhancing cellular susceptibility to nitrosative and oxidative damage, and thus amplifying the effects of inflammation-induced cytotoxic activity (141, 434). The differential effects of high versus low concentrations of NO is underlined by the fact that low concentrations of NO are beneficial to maintenance of the intracellular GSH level, NO functioning as an antioxidant and as a cytoprotective molecule (298).

NO reacts with the superoxide radical (O_2^-) rapidly near the diffusion-limited rate to form the powerful and highly reactive oxidant, peroxynitrite ($ONOO^-$) (228). However, NO competes for O_2^- with the super oxide dismutase (SOD). Since NO reacts with O_2^- three times as rapidly as SOD reacts with it, NO is the only known biomolecule capable of outclassing

SOD in reacting with O_2^-. However, because of the concentrations of SOD being high, it is only when NO is present in high concentrations and is located close to the source of O_2^- that it can compete with SOD by reacting with O_2^- so as to produce peroxynitrite (446). The half-life of peroxynitrite is about 1 second. Peroxynitrite has primarily deleterious and toxic effects, such as of oxidizing lipids, thiols, proteins and nucleic acids, and is involved in numerous of pathophysiological conditions (253). Peroxynitrite has also been shown to nitrate tyrosine residues. However, this is not an exclusive effect of peroxynitrite formation, but rather is associated with various processes involving nitrosative stress (186). Tyrosine nitration affects both the structure and the function of proteins, such as by enzyme inhibition, producing disorganization of the cell architecture, and inducing dopamine deficiency (25, 85, 268). The nitration of tyrosine kinase substrates has also been shown to inhibit the phosphorylation of tyrosine kinases, and thus tyrosine-kinase-dependent signaling as well(225).

NO and Effector Functions in Immunity

Anti-microbial and related activity

The anti-microbial activity of NO against bacteria and its destruction of parasites and fungi are well documented, the important role of iNOS in the control of these infectious agents having been clearly demonstrated in transgenic mice with deficient iNOS expression (46). In addition, deterioration in the control of pathogenic agents in models of iNOS deficient mice is due to more than simply deficiencies in the effector mechanisms, since the models also show that NO generated from iNOS is required for the regulation and functioning of innate immune responses during the early phases of an infection (93, 94). Some pathogens inhibit the expression of iNOS in macrophages, and can thus facilitate their initial survival in the host (76, 313). Results obtained from some infection models indicate, however, that the expression of iNOS can be dispensable or even be counterprotective in the control of some pathogens.

Although NO is an important anti-microbial effector molecule, the enhanced production of it following infection has also been shown to induce the suppression of lymphocyte reactions in the infected host. Immunosuppression due to the enhanced production of NO has been reported in connection with various infection models, including salmonella typhimurium (10, 176, 265, 374), Listeria monocytogenes (264), Paracoccidioides brasiliensis (43), and plasmodium (4).

Several microbial products are known to induce the expression of iNOS. Microbial lipoproteins, including proteotypically LPS, activate the iNOS in macrophages by toll-receptor signaling (55). Exotoxins are another group of microbial products that induce the expression of iNOS in

macrophages (and in other cell types). In some cases, their action has been shown to be dependent on the presence of IFN-γ (and LPS), and in some cases the activation of iNOS has been found to be deleterious rather than protective (52, 122).

Pathogenic bacteria have developed mechanisms that mediate the resistance to nitrosative effector molecules. Some of the genes responsible for these mechanisms have been identified. The production of the flavohemoglobin, which detoxifies NO to NO_3^-, is induced by NO through the inactivation of an iron-dependent repressor. Two genes newly isolated from Mycobacterium Tuberculosis (not expressed in nonpathogenic or opportunistic Mycobacterium) have also been found to protect against RNI (and ROI), although the mechanisms have not yet been identified.

Antiviral activity

For some viruses, most typically the DNA viruses (poxvirus, and herpes viruses) and the RNA viruses (e.g., coxsackievirus) (205, 266, 470), the antiviral effects of NO are known. Recent reports also show, however, that there are several viruses, such as vaccinia virus, coronavirus, and lymphocyte choriomeningitis virus (LCMV), which against NO has no antiviral effect (6, 33, 204, 421, 450). Antiviral responses against the influenza and Sendai viruses in mice treated with NOS inhibitors, or in transgenic mice with deficiencies in the expression of iNOS, have been found to not be influenced at all or to be enhanced (6, 204). The inhibition or lack of NO production reduces the pathological consequences of several virus infections *in vivo* (6, 7, 9, 204). In mice infected by the influenza virus, the inhibition of NO production and the scavenging of oxygen radicals have been found to ameliorate the pathological condition of the lungs and to improve the survival rate of the mice. These results also suggest that the NO-dependent formation of peroxynitrite can be an important factor in influenza-induced pneumonia. Thus, NO is not entirely an antiviral molecule. Although it can contribute to the direct antiviral effects of innate immunity, it may also impair T-cell-dependent antiviral responses by suppressing T-cell functions.

Since Th1 responses are important for the recovery from many viral infections, the induction of iNOS can be attributed in many viral infections to the production of IFN-γ (7, 274). In some viral infections, viral replication or the presence of viral components induces the expression of iNOS, of the HIV-1 envelope-glycoprotein and of the paramyxovirus, independent of the presence of pro-inflammatory cytokines (2, 417).

Anti-tumor effects

The first function of NO in the immune system to be discovered was that of macrophages killing tumor cells (168, 169, 394). More recent reports have demonstrated that NO is generated by macrophages, as well as by

NK-cells, Kupffer-cells, and endothelial cells, to participate in the killing of several different types of tumor cells (87, 118, 194, 201, 218, 239, 251, 354, 458). Interestingly enough, the induction of iNOS within the tumor cells after exposure to TNF-α or to IFN-γ released from lymphokine-activated killer cells can also result in the death of tumor cells (243).

To date several different mechanisms have been described for the NO derived from macrophages in killing of tumor cells or inhibiting their growth, such mechanisms involve the inhibition of enzymes essential for tumor growth, cell-cycle arrest through down-regulation of cyclin D1, induction of apoptosis by activation of the caspases and the accumulation of p53, and sensitization of the tumor cells to TNF-α-induced apoptosis (35, 116, 239, 337, 378, 451). NO can also reduce intracellular stores of GSH, making tumor cells more susceptible to other toxic mechanisms (261, 338). Not all tumor cells are sensitive to NO toxicity, however (267). This diversity of tumor cell responses to NO *in vitro* is mirrored by equally diverse results obtained *in vivo*. Results obtained for various tumor models show that production of NO *in vivo* is important for inhibiting tumor growth and can prevent tumor metastasis and angiogenesis (115, 339, 452, 457). However, results for other models show that production of NO can promote tumor growth by way of several different mechanisms (see further discussion below), including enhanced vascularization (193).

One reason for this variability in the susceptibility of tumor cells to NO toxicity is that tumors have evolved strategies for evading NO-mediated toxicity. Some tumors express factors such as TGF-β, the macrophage deactivation factor, and phosphatidyl serine that suppress the expression of iNOS (64, 97). The fact that tumor-infiltrating macrophages in mice are much lower in the expression of iNOS and in cytoxic activity than more distally located macrophages suggests that such mechanisms are active *in vivo* (95). Tumor cells exposed to NO also enhance the DNA-PKCs required for the repair of double-stranded DNA breaks, thus protecting the cells from the toxic effects of NO (and also from radiation and chemo-therapy) (455). Similarily the accumulation of mutant and wild-type P53 protein protects the glioma cells from the toxicity of NO (353).

The iNOS and arginase pathway by which macrophages metabolise L-arginine affects tumor growth differentially. iNOS uses L-arginine to produce NO, whereas arginase uses L-arginine to synthesize polyamines, an essential nutrient for the proliferation of mammalian cells. *In vitro* results indicate that the induction of arginase in macrophages promotes growth of tumor cells by providing the tumors with polyamines and suppressing the toxicity toward the tumors by reducing the production of NO (68).

Some tumors have been shown to produce a factor designated as the tumor-derived recognition factor (TDRF). This factor activates macrophages to produce NO in synergy with IFN-γ, thus mediating cytotoxicity of the same target (194-196).

Results for different immunotherapeutic models based on use of immune-stimulatory cytokines show that the inhibition of tumor growth is associated with enhanced production of NO (31, 92, 182, 184, 364, 399, 458). Several studies also indicate that activated T-cells are essential for inducingNO production (181, 182, 364).

As already discussed, the direct effects of NO are primarily protective, supporting physiological functions, whereas the indirect effects, through the formation of peroxynitrite ($ONOO^-$) and of dinitrogen trioxide (N_2O_3), mediate oxidative (peroxynitrite) and nitrosative stress, which are basicaly cytotoxic. Thus, in addressing the issue of the involvement of NO in inhibiting the promotion of tumor growth, one needs to also consider whether it is the direct or the indirect effects of NO that can be expected to prevail. Macrophages in direct contact with tumors can be expected to produce about 5 µM NO and to thus be able to mediate indirect effects (247), whereas more distally located tumor cells should experience a lower degree of NO flux, so that the direct effects of NO may dominate. The NOS isoform expressed by tumor cells or the infiltrating leucocytes also need to be considered, since they correlate with the amount of NO produced.

Autoimmune and inflammatory diseases

There is considerable evidence that during inflammatory and autoimmune diseases NO participates in tissue damage. The expression of iNOS in human autoimmune diseases has been described as correlating with disease activity, such as in rheumatoid arthritis (RA), multiple sclerosis (MS), and Sjögren's syndrome (233). The positive staining of nitrotyrosines, indicative of nitrosative stress, has also been found in MS patients (175). In experimental models, the expression of iNOS has been detected in macrophage islet infiltrates during the early stages of autoimmune diabetes (215). iNOS has also been detected in the inflamed tissue of experimental RA and experimental allergic encephalomyelitis (EAE) (223). The ability of NO generated from activated macrophages or from NO-donors to damage target cells has been confirmed *in vitro*, and rat islets have been shown to be extremely prone to NO-induced cell death (231, 234). Further evidence for NO's contribution to tissue destruction comes from findings for several experimental models that either the administration of iNOS inhibitors or the genetic inactivation of iNOS tends to delay or suppress the autoimmune diseases (223).

Recent findings show the enhanced production of NO in inflammatory and autoimmune diseases to be associated not only with tissue destruction but also with impaired functioning of the lymphocytes. Both lowered production of NO when NOS inhibitors are administered and genetic inactivation of the iNOS gene have been shown for several disease models to restore lymphocyte functioning (84, 130, 380, 406). Results for several experimental models also show that neither treatment by NOS

inhibitors nor genetic inactivation of iNOS is always protective, and in some models, such as experimental cases of allergic encephalomyelitis (EAE), myasthenia gravis, and autoimmune interstitial nephritis, treatment led to exacerbation of the disease (84, 130, 147, 223, 380). Thus, in some autoimmune diseases NO appears to have a protective function through its silencing autoreactive T-cells.

The administration of NOS inhibitors to arthritic rats in which the proliferative responses of the spleen cells were completely suppressed was found to restore proliferative responses of the spleen cells and reduce paw swelling (406). In an experimental model of myasthenia gravis, iNOS-deficient mice were found to develop an exacerbated form of the disease in which there was also enhanced T-cell reactivity.

Expression of iNOS has also been found to correlate (both in humans and in experimental models) with the activity of chronically inflammatory diseases of the airways, the blood vessels, the bowels, the kidney, the heart, and the skin (223, 233). Treatment of experimental models by NOS inhibitors has been variously shown to suppress the disease, to have no effect on it, and even to lead to deterioration (223). Thus, the role of NO in chronic inflammatory diseases is as complex as it is in autoimmune diseases. Since there is considerable evidence that NO suppresses mast cell activation, both *in vivo* and *in vitro*, it appears to have a protective function in mast-cell-dependent inflammatory diseases such as asthma (107, 212). In asthma, NO can also have a beneficial effect through its ability to dilate airway smooth muscle.

Immune Regulatory Functions of NO

NO has been shown to affect numerous immune functions, such as T- and B-cell proliferation; leukocyte recruitment; production of cytokines, chemokines, and growth factors; T and NK cell cytotoxicity; APC functions; and T-cell deviation. Although the effect of NO is usually inhibitory, it can also either alter the immune response not alter it at all or have a stimulatory effect on it. Several factors are likely to determine the immune regulatory functions of NO, above all the concentration of NO, the cell type (primary or established, mammalian or human, and in the case of immune cells innate or adaptive), the stimulatory conditions (mitogen- or antigen-specific), the composition of the intra-and extracellular milieu (NO or $NO+O_2^-$), and the source of NO (exogenous or endogenous).

NO signaling in immunity

The signaling between immune cells for which NO is responsible is largely indirect through NO being generated from iNOS and reactive nitrogen species (RNS) subsequently being formed (see Figure 6).

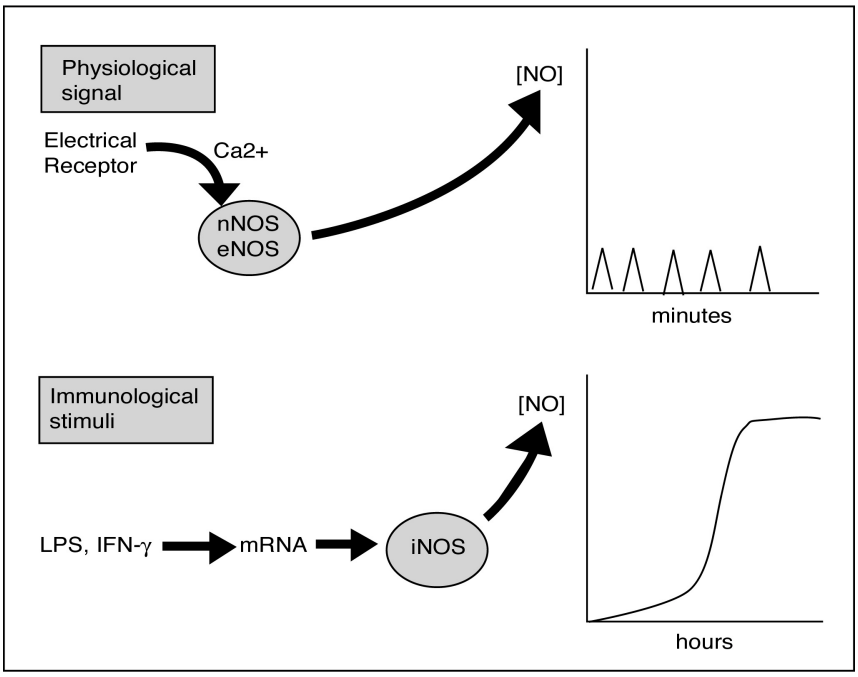

Figure 6. NO signaling. Induction of NO production from constitutive and inducible NOS.

NO generated from constitutive NOS (cNOS) has been shown to be involved in immune regulation as well, although a number of facts suggest direct signaling by NO to not be likely. First, cNOS is activated in response to physiological stimuli, whereas the expression of iNOS is induced by immunological stimuli in the inflammatory cells. Second, the production of NO from cNOS is rapid, transient and at a low level, wheras the production of NO from iNOS is slow (due to mRNA and protein synthesis) and is sustained and at a high level. Third, the direct actions of NO (at low concentration) lead to the production of cGMP and a rapid transient cellular response, whereas the indirect actions of NO (at high concentrations) through the generation of RNS lead to the inhibition of enzymes and of transcription factors (often through nitrosation of the thiol groups in the proteins) and to long-term cellular effects.

Immunosuppression and Suppressor cells

The first immune regulatory function that could be assigned to NO was its ability to inhibit lymphocyte proliferation (173). This suppressive effect of NO is one of the key mechanisms for so-called “suppressor macrophages” impairing the proliferative responses of the T-cells to antigens and to mitogens. Prior to the discovery that NO is a product of activated macrophages, studies of the mechanisms for macrophage-mediated suppression pointed to prostaglandines, reactive oxygen intermediates, various cytokines such as TGF-β, or still other, unidentified

factors as being possibilities. Since then, the NO-dependent suppressor activity of macrophages has been confirmed in numerous *in vitro* studies (13, 173, 423). The suppression of T-cell-proliferative responses that has often been observed in animals and in patients with infections, immunopathologies, growing tumors, and after transplantations or after treatment with immune stimulatory cytokines can often be attributed to enhanced production of NO by "suppressor macrophages" (10, 22, 198, 220, 241, 246, 283, 293, 296, 349, 380, 405, 409, 464).

Although macrophages that produce NO can be characterized functionally as suppressor cells, distinct lineages of suppressor macrophages are poorly defined. In several studies, however, suppressor cells derived from tumor-bearing hosts have been identified as granulocyte-macrophage progenitor cells (464). In mice treated by cyclophasphamide, the suppressor cells responsible for the NO-dependent inhibition of splenic T-cell proliferation were shown to be immature myeloid cells defined as $CD11b^{+}Ly\text{-}6(Gr\text{-}1)^{+}CD31(ER\text{-}MP12)^{+}$ (22). In tumor bearing mice, an increase in the number of $Gr\text{-}1^{+}$ myeloid cells, in the bone marrow or the spleen, was found to be responsible for NO- and peroxynitrite-dependent immunosuppression (241). NO-dependent suppressor cells in the spleens of bone-marrow-transplanted mice were characterized in a further study as being $Mac\text{-}1^{+}/SCA\text{-}1^{+}$ cells (198).

NK-cells can also be involved in NO-mediated immunosuppression. NO-dependent immunosuppression in mice infected with Salmonella typhimurium was found to be mediated by NK cells and by their production of IFN-γ (375).

The production of NO from dendritic cells has been shown to inhibit lymphocyte responses, primarily by the induction of apoptosis in thymocytes (as discussed below) and in T-cells (5, 49, 258). Results obtained from IL-4-treated rats with experimental allergic encephalomyelitis (EAE) showed dendritic cells that secreted high levels of NO to promote the apoptosis of autoreactive T-cells (454).

T-cell deviation and cytokine production

Although the immunosuppressive effects of NO are widely accepted, controversies exist regarding the involvement of NO in the regulation of cytokines and in deviations of the T-cells. Direct evidence for the critical function of iNOS-derived-NO in the development of Th1 responses has been obtained from mice with disrupted iNOS genes. Compared with wild-type mice, iNOS deficient mice developed enhanced Th1 responses and, after infections and antigenic stimulation, produced more IFN-γ and less IL-4 (266, 288, 439). The treatment of rats by inhibitors of iNOS (and of cNOS) was also found to enhance Th1 responses, as recorded in terms of enhanced expression of IFN-γ (307).

Varying results have been obtained for experiments *in vitro.* The results point nevertheless to several parameters that appear to determine the

effects of NO on T-cell differentiation and functioning, in particular the concentration of NO, the NO donors, the differences in cell type, the composition of the micro-environment, and the conditions for stimulation. The significance of the NO concentration was emphasized by results showing low concentrations of NO to selectively enhance Th1 cell response, but to have little or no effect on primary Th2 cell responses, high concentrations of NO in contrast suppressing the differentiation of Th1 cell responses and the IFN-γ production by these cells (318). However, NO only had a pronounced effect on cytokine production during the inductive phase of the Th1 cells and had little or no effect on the committed or cloned T-cells (318). This shows that the naïve or primary T-cells are more susceptible to the differentiation signals emanating from NO than the cloned or fully committed T-cells are. This might explain some of the conflicting results obtained for cloned or activated T-cells *in vitro*, which have varyingly shown NO to have a selective inhibitory effect on Th1-cell differentiation and on cytokine production (in addition to an enhancement of Th2 cytokine secretion), an equal suppression of the cytokine secretion of Th1 and of Th2 cells, a selective inhibition of the production of Th2 cytokine, and no effect at all on the production of cytokines from either Th1 or Th2 cells (34, 69, 321, 410, 411, 424). NO has also been shown to inhibit the proliferation of cloned T-cells or activated spleen lymphocytes, without any reduction in cytokine production. (183, 424). This could imply that NO regulates T-cell proliferation and cytokine production differentially.

The regulatory function of NO on Th1-cell development may also be an indirect process dependent on other cells or factors in the intra- and extracellular milieu. Thus, it has been shown that NO, through selective inhibition of IL-12 production by macrophages, can prevent amplification of the Th1 cells (177). Antigen presentation to Th1 but not to Th2 cells was found to result in NO production by macrophages and thus a selective inhibition of Th1 cells (425).

There are controversies regarding the level of NO expressed by the T-cells. It has been reported that cloned murine Th1- but not Th2-cells produce large amounts of NO that are able to selectively inhibit the secretion of IL-2 and IFN-γ by the Th1 cells (409, 411). Human leukemic T-cell lines and leukemic cells from adult patients were also found in one study to express iNOS, although no function for this was suggested (304). Others, in contrast, were unable to detect any significant production of NO by the T-cells involved in the differential regulation of Th1 and Th2 responses (such as by activated Th1- or Th2-cell clones, by T-cells from mice infected with Leihsmania major, or by activated human T-cells) (34, 416).

Disruption of intracellular signaling in T-cells

Various molecular targets for the immunosuppressive activity of NO have been identified in T-cells. NO abrogates the DNA-binding activity of the zink-finger transcription factors SP1 and EGR-1, known to be involved in the dominant regulation of IL-2 gene expression (38). This effect is selective since the DNA-binding activity of NF-AT, another regulator of IL-2 gene expression, is not affected by NO. NO also markedly reduces the tyrosine phosphorylation of the intracellular signaling molecules JAK3 and STAT5 in the T-cells, involved in the control of T-cell proliferation (40). NO (as well as other thiol oxidants) also inhibits the autokinase activity of JAK2 (105). Peroxynitrite ($ONOO^-$) has been shown to inhibit activation-induced tyrosine phosphorylation through nitration of the tyrosine residues (56). This primes the T-cells to undergo apoptotic death after activation by anti-CD3 or phorbol esters.

T-cell apoptosis

Apoptosis is one of the mechanisms by which NO inhibits the proliferation and activation of T-cells during infections and immunopathology (88, 407, 471). One of the mechanism by which NO induces apoptosis in the T-cells is through the down-modulated expression of Bcl-2 (18, 407). Endogenous NO generated by nNOS in T-cell-receptor-stimulated cells has been shown to enhance the expression of FAS-L (CD95L) and thus the apoptotic death of T-cells (444). NO has also been found to induce apoptosis in the T-cells without any modulation of FAS/FASL expression (282). Peroxynitrite ($ONOO^-$) was found to prime T-cells into undergoing apoptotic death after activation by anti-CD3 and PHA, through nitration of tyrosine residues that inhibited activation-induced tyrosine phosphorylation (56). NO has also been shown to up-regulate IFN-γ receptors 1 and 2 on the surface of the T-cells, making the T-cells susceptible to apoptosis in the presence of IFN-γ (18).

NO also plays a role in the selection and development of T-cells in the thymus. TCR-activated double-positive thymocytes are highly sensitive to NO induced apoptosis, whereas single positive cells remain viable after exposure to NO (5, 56, 117, 305, 405). Epithelial cells and dendritic cells in the thymus constitutively express iNOS, which is further up-regulated after exposure to self-antigens, alloantigens or thymocytes activated by TCR stimulation (5, 305, 405). This indicates NO to be one of the factors in the thymus that mediates the deletion of double-positive thymocytes.

The anti-apoptotic functions of NO have also been observed in the T-cells. The endogenous production of NO, generated by iNOS and eNOS in the T-cells, was shown to protect the T-cells from apoptosis (277, 376). NO was also shown to inhibit the activation of caspase-3 by S-nitrosylation, thus blocking apoptosis by helping to maintain the proenzyme in its inactive form (277, 358). NO generated by iNOS in macrophages was found to protect gamma delta T-cells from undergoing apoptosis-induced Mycobacterium tuberculosis (377). Since

gamma delta T-cells are recruited into mycobacterial lesions, early macrophages can determine the life span and function of the lymphocytes at the site of infection.

NK-and LAK-cells

The functions of the NK cells in the rat and the mouse, such as those of cytolytic activity and the production of IFN-γ, are usually enhanced by the production of NO and are dependent upon it. Expression of the cytotoxic molecule perforin in mouse NK cells relies on the normal expression of iNOS (62). In mouse NK cells, NO generated by iNOS has been shown to be essential for IL-12 induced IFN-γ production and for cytotoxic activity (93, 94). This is due to the inability of IL-12 to activate Tyk2 and STAT4 (the central signal transducers for IL-12 in NK-cells) in the absence of iNOS (although not in the T-cells) (94). NO generated by eNOS has also been shown to protect human NK cells from activation-induced apoptosis through the down-regulation of TNF-α expression (possibly by suppressing NF-AT activation) (129). It has also been repeatedly demonstrated that the lytic activity of rat NK-cells and production of IFN-γ by them that are stimulated by IL-2 are partly dependent on the NO generated by iNOS (78, 201, 202).

However, the effects of NO are not uniform and they appear to be dependent on the concentration of the NO, the differentiation stage achieved and the species. Low concentrations of NO (stemming from macrophages or from NO donors) have been shown to promote IL-2-induced spleenocyte growth and cytolysis in the rat, higher amounts being suppressive (28). The modulatory (enhancing or suppressing) effects of NO on LAK cells have been found to be restricted to the precursor cells, whereas the lytic activity of LAK cells that had already been activated being only minimally affected (200, 368). In contrast to rat and mose, the lytic activity of IL-12-(and TNF-α) activated human NK cells and of IL-2-activated human LAK cells was not found to be dependent on NO (200, 367). Rather, the inhibition of iNOS enhanced the lytic potential of both the NK- and the LAK-cells of humans. This was found to correlate with enhanced expression of IFN-γ and granzyme B in the NK-cells (367).

Macrophages

Results obtained in different experimental settings demonstrate that NO generated from iNOS can inhibit or enhance the production of IL-12 or leave it unaltered (93, 177, 307, 360). The production of IL-12 in iNOS-deficient mice infected by Leishmania Major is reduced during the first few days after infection, whereas at later stages of infection, the expression of IL-12 in these mice is elevated in comparision to that for wild-type mice (93). Since the inhibition of IL-12 (like that of IFN-γ) presumably requires a certain level of NO concentration, these results are not inconsistent with those obtained by others, showing there to be a significant enhancement of

IL-12 production by the macrophages of iNOS-deficient mice (177). The results point rather to the effects of NO on immune responses differing between the early and the late phase of infection. During the innate phase (just after infection), low production of iNOS enhances immune activation and the production of IL-12, whereas during the later phase when iNOS is further up-regulated by T-cells that produces IFN-γ, NO prevents an excessive amplification of Th1 cells through inhibiting IL-12 production.

In contrast to IL-12, NO has been consistently shown to enhance the production of TNF-α, even when IL-12 is suppressed (177, 245, 273, 289). The enhanced expression of TNF-α may possibly be due to the NO-dependent inactivation of the transcription factor SP-1, since this factor has been shown to function as a suppressor of TNF-α (437). However, NO has been shown to attenuate TNF-α synthesis in the macrophage cell line RAW 264.7. This difference compared with other studies may be a function of the differentiation stage of this specific cell line.

NO has been shown to inhibit caspase-1 activity in both human monocytes and mouse macrophages. Since, the proinflammatory cytokines IL-1β and IL-18 require a proteolytic enzyme, caspase-1 (he IL-β-converting enzyme (ICE)), for cleavage and secretion of its active molecule (96, 143, 158, 344), this results in the processing of IL-1β and IL-18 being suppressed (121, 211).

APC functions

NO inhibits the IFN-γ-induced expression of MHC class II in mouse macrophages (209, 383, 384). The inhibition has been shown to be due to the NO-dependent suppression of the Class II TransActivator (CIITA), which regulates the expression of MHC class II (209). The inhibition has been suggested to be selective since NO has been found to not decrease the expression of TNF-α (209).

The functional maturation of pulmonary dendritic cells, which is accompanied by an increase in the expression of MHC class II, is inhibited *in vitro* by the NO generated by the pulmonary alveolar macrophages (174). Exogenous NO released by NO-donors or by bystander phagocytes inhibits the TNF-α-induced maturation and down-regulation of the endocytic capacity of human monocyte-derived dendritic cells (329). Thus, NO can prolong the ability of human dendritic cells to internalize antigens at the site of infection although it also inhibits or delays antigen-specific immune responses. In addition, NO suppresses the functions of dendritic cells by inducing apoptosis. Both the endogenous production of NO induced by CD40 ligation and exogenous NO production can induce apoptosis in the dendritic cell (258). NO-dependent aptoptois has been shown to be mediated by a down-regulation of the cellular inhibitors of apoptosis proteins (cIAPs) and an up-regulation of the activity of the effector caspases 3 and 6 (387).

Leukocyte migration and extravasation

The recruitment of leukocytes to inflammatory sites involves a sequence of interactions between leukocytes and endothelial cells, such as the rolling of leukocytes along the endothelium, the detection of chemoattractants, activation and adhesion of the leukocytes, and their emigration through the vascular wall. Many inflammatory mediators are involved in the regulation of these processes, NO appearing to be capable too of regulating these events. The inhibition of constitutively expressed NOS by L-NAME increases the rolling and the number of adherent leukocytes in the mesenteric microvasculature (90, 237). Thus, the continuous production of NO by the endothelial cells has an anti-inflammatory effect, inhibiting the rolling of leukocytes and their adhesion to the endothelium. Similar observations from a wide range of tissues, including liver, lung, heart, and skeletal muscle, indicate this to be a universal phenomenon (8, 263, 287, 319). NO generated by constitutively expressed NOS attenuates leukocyte recruitment during inflammatory responses and protects against LPS-induced hepatic microcirculatory dysfunction during endotoxemia (319). Different mechanisms to explain these effects have been identified. NO directly inhibits the expression of adhesion molecule such as P-selectin (90). Treatment by NOS inhibitors induces oxidative stress in skeletal muscle and endothelial cells (320, 397), which in turn up-regulates the expression of adhesion molecules (199, 330)

A wide range of cell types normally resident in the microvasculature are capable of expressing iNOS during inflammatory responses, such as intra- and extravascular leukocytes, endothelial cells and smooth muscle cells. Results of both *in vitro* and *in vivo* experiments indicate that iNOS-derived NO inhibits the rolling of leukocytes, their adhesion to the endothelial cells and their recruitment to the inflammatory site (170, 236, 335). The mechanisms by which NO mediates these effects are those of inhibition of the adhesion molecules on the leukocytes and the endothelial cells, the suppression of leukocyte motility, the suppression of inflammatory cytokines expressed by the endothelial cells, and oxidative stress in the endothelial cells (41, 91, 207, 371). However, the effects that NO generated from iNOS has on leukocyte recruitment during inflammatory responses is not always inhibitory, but varies with the inflammatory mechanism, the cellular location and the temporal expression of iNOS (81, 145, 170).

NO Tumor-Promoting Effects

NOS correlation to tumor growth

In contrast to the tumoricidal effects just described, NO has also been proposed as being a mediator of tumor growth. Both constitutive and inducible NOS has been detected in tissue sections of human tumors, such as breast tumors (415), cervical tumors (414), brain tumors (including gliomas) (29, 80, 259), tumors of the colon (19, 193, 222), prostate carcinoma (219), and head and neck cancers (357). However, the expression of NOS is not restricted to tumor cells but is often situated in other cells likewise, resident in the tumor area, such as endothelial cells and infiltrating leukocytes. Thus, in several of the tumors that have been analyzed, such as gliomas, breast cancer, and prostate carcinoma, the expression of iNOS has been found to correlate with the infiltrating macrophages rather than with the tumor cells (29, 219, 259, 415). In some cases, however, the expression of iNOS has been reported to be restricted to the tumor cells (19, 357). In some tumor tissues too, the expression of eNOS and nNOS has been detected in the tumor endothelial cells as well (80, 259). Interestingly enough, in several of tumors a positive correlation between tumor grade and the expression of NOS has been observed. In invasive ductal carcinoma, NOS activity was found to be significantly higher for grade III than for grade II (415). In actrocytoma, the expression of eNOS has been found to be correlated with the histological grade and the proliferative potential of the tumor vessels (189). The expression of iNOS is enhanced in high-grade human mammary tumors, which tend to be more invasive (193). In human prostate carcinoma, malignant epithelial cells and cells with the morphology of macrophages were found to stain positive for iNOS, whereas benign tissue stained negative for it (219). The data involved indicate that NO can play a critical role in the growth and spread of tumors.

Carcinogenesis and neoplastic transformation

Reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) and N_2O_3 derived from NO during a high level of NO flux have been suggested to be genetoxic and thus to be involved in carcinogenesis *in vivo*. There are three major mechanisms by which NO can mediate DNA lesions. First, nitrosative stress mediated by N_2O_3 under conditions of chronic inflammation can lead to the formation of carcinogenic nitrosamines (255). Secondly, direct modification of the DNA by oxidative stress steming from peroxynitrite can lead to DNA strand breaks and to mutations (366). Thirdly, recent data indicate that NO can act indirectly by affecting the activity of the DNA repair enzymes, such as thiol-containing alkyl transferases, zink-finger motif containing glycosylases, and DNA ligase (154, 248, 445). Since the genotoxic events involving NO concern the

indirect chemistry of NO (RNS), it is reasonable to expect that sites of potential carcinogenic risks have had prolonged exposure to NO derived from iNOS, such as in the case of chronic inflammation. In fact, autoimmune inflammatory diseases such as rheumatoid arthritis have been associated with an increased risk of cancer (292). A linkage between inflammation, NO production and carcinogenesis has also been found in other studies. Infection of the gastric mucosa by H. pylori leads to a sustained expression of iNOS and the production of RNS, which has been suggested to contribute to the carcinogenesis of gastric cancer (151). The spontaneous mutation frequency of a mouse cell line was found to be elevated due to *in vivo* growth and to be correlated with the number of infiltrating leucocytes and the expression of iNOS (369). Endogenously produced NO was also shown to contribute to the neoplastic transformation of mouse fribroblasts (301).

Tumor-angiogenesis and vascularization

Evidence of NO being a pro-angiogenic factor comes from several sources. The vascular endothelial growth factor (VEGF) is believed to be the primary mediator of angiogenesis in tumors. The exposure of glioblastoma and hepatocellular carcinoma cell lines to NO donors was found to increase the expression of VEGF (75). This appears to primarily be an effect of mRNA stabilization. The regulatory role of NO in VEGF induced-angiogenesis could be demonstrated by the administration of an NOS inhibitor to rabbits, being found to block VEGF-induced angigonesis (472). NO has also been shown to function as a vascular permeability factor and to contribute to the enhanced vascular permeability often observed in tumors (270). This enhanced vascular permeability can facilitate angiogenesis and enable albumin and other macromolecules to extravasate from blood vessels. The evidence for a critical role of NO in the vascularization and growth of tumors is also emphasized by the suppression of vascular permeability and tumor growth which occurs after NOS inhibitors are administered to tumor bearers (102, 193, 270). The inhibition of NO production in a murine breast cancer model was found to significantly reduce tumor-induced neovascularization (191). The stimulatory effect of NO on the vacularization and growth of tumors was also demonstrated in a colon tumor line genetically engineered to express NOS, which resulted in tumors being more fully vascularized and more rapid-growing (193).

There are also studies demonstrating that NO has a stimulatory effect on the proliferation of endothelial cells. The proliferation of coronary postcapillary endothelial cells was found to be enhanced after treatment by an NO donor (300), the proliferative *in vivo* potential of the tumor vessels in the astrocytoma being found to be correlated with the expression of eNOS (189).

Immunosuppression

In considering the use of NO in the therapeutic treatment of cancer, much attention has been directed at its tumoricidal effects and the possibilities of enhancing its production. The role of NO in the immunotherapy of cancer is complex, however, since the production of NO from activated macrophages can also inhibit the activation of cytolytic T-cell responses. In many tumors the expression of iNOS is elevated and is correlated to infiltrating leucocytes such as macrophages and neutrophils, as was discussed earlier. Often, the expression of iNOS correlates with the grade of the tumor and thus with a poor prognosis. Accordingly, except for the tumor-promoting effects mentioned earlier, the enhanced production of NO intra-tumorally may be a contributory factor to tumor immune escape through the suppression of T-cell functions. The suppression of T-cell functions is often observed to accompany tumor growth. Possible mechanisms for tumor-induced immunosuppression, including that of NO will be discussed.

TUMOR IMMUNOSUPPRESSION

Immune responses to tumors have been shown to be defective in tumor bearing animals and in cancer patients. During the early stages of experimental tumor growth, a local antigen-specific T-cell tolerance may develop, although host-immune responses to non-specific antigens appears to be normal (389, 442). In contrast, during the later stages of tumor growth, systemic and non-specific suppression of immune responses has been shown to develop (210). Although the degree of immunosuppression is more pronounced in certain types of tumors, it is becoming increasingly clear that in their advanced stages the majority of solid tumors induce immonosuppression in their host. In patients with different types of hematological and solid tumors, DTH reactions have been shown to correlate inversely with the progression of the disease (210). In patients with a melanoma CTL responses to influenza viruses are also suppressed (372). Several mechanisms for tumor-induced immune suppression have been proposed. Schematically, the defective immune responses in tumor bearers can be induced directly by immunosuppressive factors produced by the tumor itself and indirectly by the tumor-induced suppressor cells (see Figure 7). It is often difficult, however, to distinguish between tumor-derived and suppressor-cell-derived factors.

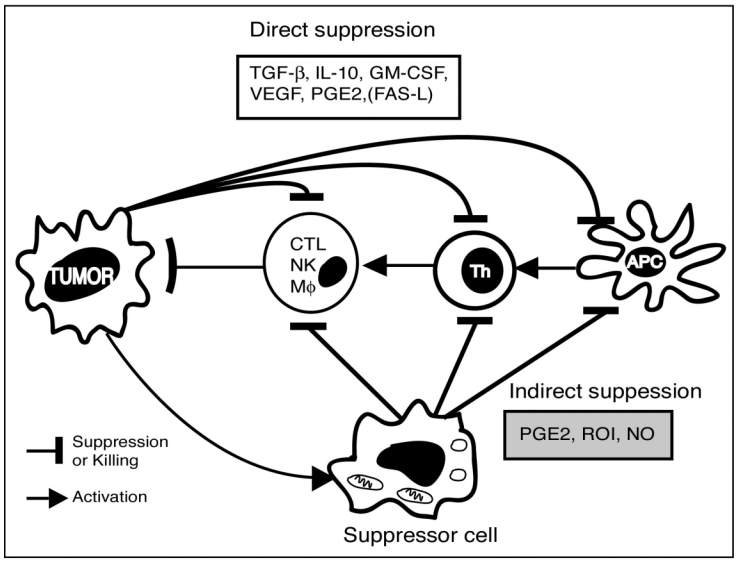

Figure 7. Immunosuppression exerted by tumor derived (direct suppression) and suppressor cell (indirect suppression) derived factors.

Tumor-Derived Immunosuppressive Factors

One reason for the impaired cellular immune responses often observed in tumor bearers may be the increased production of immunosuppressive factors.

TGF-β

The immunosuppressive capacity of TGF-β has been carefully delineated. It affects the activation and differentiation of both innate and adaptive immunity, these including the suppression of proliferative responses, inhibition of the production of immunoregulatory cytokines, the suppression of iNOS expression, and the inhibition of CTL differentiation. Elevated levels of TGF-β have been observed *in vivo* in cancer patients and in tumor-bearing animals. Several human and experimental tumors have been demonstrated to spontaneously produce TGF-β. Evidence for the involvement of TGF-β in tumor-induced immunosuppression has been obtained both *in vitro* and *in vivo.* This includes the suppression of T-cell proliferation, the suppression of macrophage tumoricidal activity (the inhibition of NO production), the suppression of pro-inflammatory cytokines (IFN-γ, IL-2, and TNF-α), the induction of immunosuppressive cytokines, and a shift in the balance of Th1/Th2 type responses (16, 190, 252, 269, 271, 272, 403, 440, 456). A shift toward Th2 responses has been shown to be both a direct effect through the inhibition of Th1 responses and an indirect effect through the up-regulation of IL-10 production by the macrophages (269, 271).

The inhibition of TGF-β production *in vivo* has been shown to reduce tumor-induced immunosuppression, to enhance tumor immunogenicity and to improve the efficacy of immunotherapy. Immunizing glioma-bearing rats with parental glioma cells genetically modified to express anti-sense TGF-β was found to enhance the tumor cytotoxicity of lymph node cells and to increased survival as compared with control rats (113). Reducing TGF-β production in rats with a growing hepatoma through bleomycin treatment resulted in a restoring splenocyte IL-2 production, IFN-γ production, and macrophage NO cytotoxicity, and in the regression of tumor growth (466, 467). Low-dose treatment by the anticancer drug melphalan reduced the tumor cell production of TGF-β, restored the CTL activity of immunosuppressed rats and enabled the tumor to be eradicated (440). Recently, strong evidence for TGF-β being an important suppressor of anti-tumor immune responses was demonstrated in transgenic mice with T-cells that were made insensitive to TGF−β signaling (150). These mice were shown to resist challenges by two different tumor cell lines. The therapeutic effect was likewise seen after the adoptive transfer of T-cells insensitive to TGF-β-signaling in mice with an established (3 day) tumor.

IL-10

IL-10 spontaneously produced by a variety of human tumors, most commonly by colon carcinoma, has been shown to affect immune responses in several ways (140, 172). Decreasing the expression of the MHC class I by local secretion of IL-10 can render tumor cells totally

insensitive to CTL-lysis (285). IL-10 produced by melanoma cells was found to inhibit the production of TNF-α in a mixed lymphocyte reaction (74). Both macrophage MHC class II expression and the expression of IFN-γ and TNF-α have also been found to be inhibited by glioma-derived IL-10 (172).

GM-CSF

Several human tumor lines produce GM-CSF spontaneously (58). In mice, the chronic production of GM-CSF from a growing tumor was found to suppress antigen-specific T-cell responses (58). The impairment of T-cell functioning was due to the unopposed production of GM-CSF, which disrupted the maturation of fully functional dendritic cells and gave rise instead to inhibitory Mac-1/Gr-1 double-positive cells. In contrast, irradiated tumor cells that are genetically modified to produce GM-CSF have been shown to stimulate anti-tumor immune responses, partly by promoting the maturation and growth of dendritic cells (103). Recombinant GM-CSF (in combination with IL-4) administered ex vivo has been found to induce the differentiation of dendritic cells (58). The chronic production of GM-CSF *in vivo* probably disrupts the balance of cytokines needed for functional dendritic cells to mature.

VEGF

The vascular endothelial growth factor (VEGF) expressed by nearly all tumors plays an important role in tumor angiogenesis. VEGF has been reported to also have immune regulatory properties and to thus affect the development of anti-tumor-immune responses.

The production of VEGF by human tumor-cell lines inhibits the functional maturation of dendritic cells from myeloid progenitor cells *in vitro* (132). NF-κB appears to be the molecular target for this inhibition, since through binding to the Flt-1 receptor on the hemopoietic progenitor cells VEGF significantly inhibits NF-κB-dependent activation (325). Since the binding sites (Flt-1) available for VEGF also decrease during the maturation of DC, the mature dendritic cells are less sensitive to being inhibited by VEGF.

The *in vivo* infusion of VEGF inhibits the functional maturation of DC and leads to an expansion of immature GR-1^+ myeloid cells in the spleen and to a less profound though significant increase in the lymph nodes as well (131). These effects occur at levels of VEGF such as those observed in tumor-bearing mice and in cancer patients.

PGE-2

Systemic levels of PGE-2 have been shown to increase during tumor growth, culture supernatants of many tumor cell types containing high levels of PGE-2 (260, 356, 460, 463). Several reports show that the

inhibition of PGE-2 production increases the cellular immune responses. The inhibition of PGE-2 production by tumor cells has been shown to enhance the proliferative responses of T-cells *in vitro.* Administering a PGE-2 inhibitor, indometacin, to tumor-bearing mice was also found to reduce the level of PGE-2 and to enhance spleen-cell anti-tumor cytoxicity (388, 460). The inhibition of PGE-2 production in cancer patients by the administration of indometacin was found to reduce the immunosuppression that the growing tumor exerted (70).

FAS-L (CD95L) counterattack ?

FAS (CD95) and its ligand FAS-L are molecules of the tumor necrosis factor family (396). FasL is an effector molecule used by cytoxic T-cells to induce apoptosis in Fas- bearing cells (257). However, monocytes, neutrophils and activated T-cells likewise express Fas, making them susceptible to FasL-delivered death signals (188, 262). It has been proposed that FasL can be expressed in certain tissues, this representing a counterattack on immune system. FAS-L expression in the eye has been suggested to contribute to immune privilege in the eye (155). It has also been claimed that Fas-L is expressed in different types of human cancers, where it can contribute to immune privilege (435). FasL expression has been claimed to be negatively correlated with prognosis in cancer of the breast, ovaries, and liver (187, 310, 348), and to be up-regulated in liver metastases, as compared with primary tumors in colon cancers, facilitating the metastatic colonization of the liver by colon cancer cells (276, 459).

It is important to note, however, that serious criticism has been directed against the FasL- counterattack theory (reviewed in (352)). Several of the early papers that appeared have been withdrawn or been refuted. One of the earliest description of FasL mediated immune privilege was withdrawn when the authours found that inflammation rather than immunosuppression took place. Melanoma cells were initially described as expressing FasL, but later studies could not confirm this. The transfection of these melanoma cells by FasL was found to result not in immune escape, but in rapid tumor rejection. There appear to have been problems connected with the antibodies used in earlier studies. One of the antibodies (from Santa Cruz Biotechnologies) was not highly specific and led to false positive results being reported.

Indirect Suppression

The immunosuppression that growing tumors exert can also evolve indirectly by activation of the suppressor cells. Some of these immunosuppressive mechanisms apparently evolve to protect the host from

immune pathology during immune responses, and can thus be induced through immunotherapeutic intervention (see Figure 8).

Natural suppressor cells

Natural suppressor cells have been characterized as having the ability to non-specifically suppress T-, B-, and NK-cell responses. Natural suppressor cells appear to be heterogeneous, various phenotypes and immunosuppressive factors involved having been described. In a normal host, natural suppressor cells of myeloid origin are typically found in the bone marrow (BM) and at sites of intense hematopoiesis or inflammatory response (23, 275, 373, 398). Immature myeloid cells with natural suppressor activity have been shown to increase in number in the BM and in the peripheral lymphoid organs of tumor-bearing mice and of cancer patients (240, 395, 462, 465). The natural suppressor-cell activity in tumor bearers has been associated with the expression of certain cell-surface markers. In mice suppressor activity has been found to correlate with the presence of Mac-1 and Gr-1 single-positive or double-positive myeloid cells (58, 192, 241, 323). Results obtained by others have shown tumor growth to induce shifts in the cell-surface markers expressed by the peritoneal macrophages Mac-3^+ to Mac-2^+ and by the spleenic macrophages Mac-1^- to Mac-1^+, the macrophages becoming more homogenous and smaller in size (reviewed in (109)). The natural suppressor (and cytotoxic) activity of the spleen and of peritoneal macrophages has been associated with MHC class II^- macrophages (15, 51, 139, 316, 436). These MHC class II^- cells, thought to be the main constitutive suppressor (and cytotoxic) macrophage subpopulation in the spleen, have been shown to increase in number during tumor growth (15, 139, 315, 436, 468, 469). Although the existence of distinct subsets of natural suppressor cells is controversial, the results indicate that tumor growth can alter macrophage development and differentiation and can thus induce the development of suppressor macrophages of an immature phenotype.

Natural suppressor mechanisms

Although defective T-cell functioning in tumor bearers has been associated with increased activity of these natural suppressor cells, the suppression mechanism is not always clear. However, several reports have shown that suppression is contact-dependent. In one report, Mac-1^+ myeloid cells were shown to meditate the suppression of CTL- and T-cell-proliferative responses by means of a contact-dependent mechanism (192). The direct interaction of T-cells with Mac-1^+ and Gr-1^+ positive myeloid cells of mice with growing tumors was shown to result in a decreased expression of CD3ζ or in apoptosis (58, 323). Evidence that macrophages suppress tumor immune responses was reported more than 25 years ago. Macrophages have been shown to produce several factors that are suppressive of T- and NK- cell functions, e.g., PGE-2, NO, H_2O_2, IL-10,

ROI and TGF-β. However, in tumor-bearing hosts macrophage-mediated suppression is primarily dependent on the production of PGE-2, ROI, and NO (reviewed in (109)).

Regulation of cytotoxic suppressor molecules

PGE-2 is a non-cytotoxic suppressor molecule, whereas NO and ROI represent macrophages that have both anti-tumor cytotoxicity and immune-suppressor activity. Thus, tumor-induced production of these cytotoxic molecules appears to be a contradictory event in the tumor-bearing hosts. However, present knowledge indicates that the net effect of these molecules is immune suppression rather than tumoricidal activity. Many tumors have mechanisms to resist macrophage-derived cytotoxic molecules and they recruit macrophages through the production of such chemotactic factors as TGF-β and macrophage chemotactic protein 1 (MCP-1) (278, 433). The presence of macrophages in tumors has also been correlated with tumor growth and metastasis. Without macrophages to stimulate the growth and development of blood vessels, tumors would rapidly die (249, 400). Several tumors produce factors such as PGE-2, IL-10, TGF-β, M-CSF and GM-CSF that can suppress the expression of macrophage cytotoxic molecules (TNF-α, NO, and ROI) in the tumor area. This was demonstrated in a mouse tumor model in which the NO-dependent tumoricidal and suppressor activity of peripheral macrophages was found to increase parallel to the progression of tumor growth (16). Through the production of TGF-β, IL-10, and PGE-2, these tumor cells also suppressed macrophage NO-production and cytotoxicity. Mediated by NO, growing tumors can thus induce distal macrophage-suppressor activity, and concomitantly, at the site of the tumor, suppress the tumoricidal activity of the macrophages (see Figure 8).

It has been shown that TGF-β, M-CSF, GM-CSF and other tumor derived cytokines regulate the production of NO, and ROI (and TNF-α) differentially in resting and in activated macrophages (reviewed in (109)). Resting peripheral macrophages can be primed by circulating tumor-derived cytokines such as TGF-β, M-CSF and GM-CSF to produce cytotoxic and suppressor molecules, whereas the cytotoxicity of the activated macrophages (activated by the tumor antigens and the extracellular matrix proteins) at the tumor site can be suppressed. In conclusion, tumors can escape macrophage-mediated immune destruction at the tumor site while maintaining macrophage suppressor activity at tumor-distal sites.

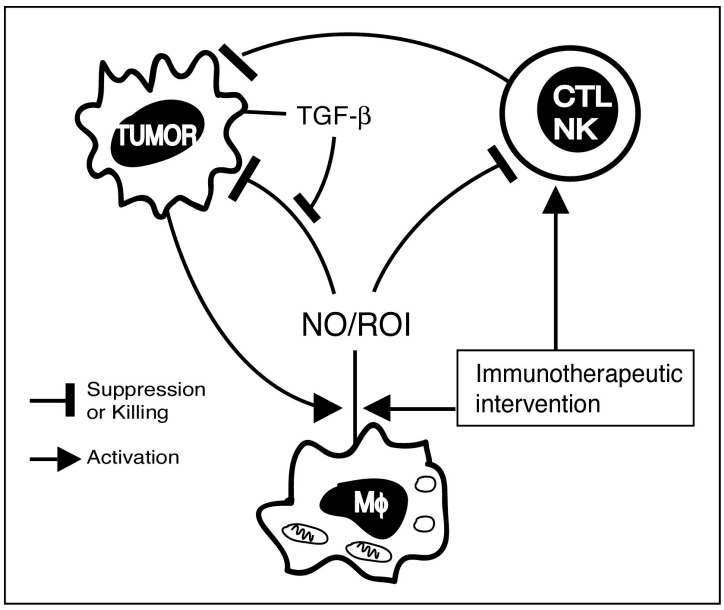

Figure 8. Induction of cytotoxic suppressor molecules through tumor growth and immunotherapeutic intervention.

MHC class II^- macrophages from tumor-beraing hosts become more suppressive in the presence of GM-CSF, whereas in normal hosts GM-CSF partially reverses the suppression that is mediated by these cells (reviewed in (109)). This in line with results demonstrating that GM-CSF and VEGF produced by tumor cells inhibits the maturation of hemopoietic progenitor cells and increases the production of immature $Gr\text{-}1^+$, $Mac\text{-}1^+$/ $Gr\text{-}1^+$ myeloid cells (58, 131, 132, 464). The GM-CSF-induced suppressor cells not only inhibit T-cell functions but also facilitate the metastasis and establishment of tumors (326). Interestingly enough, the vitamin D_3 treatment of mice with a growing GM-CSF secreting tumor was found to reduce tumor metastasis formation, tumor-induced myelopoietic stimulation, and immunosuppressive activity, and to restore T-cell functions (461). Combined vitamin D_3 and IL-12 treatment of tumor-bearing mice was found to augment the anti-tumor cytolytic activity of the regional lymph node cells synergistically (342).

Oxidative stress

Tumor-derived macrophages from mice and humans produce reactive oxygen intermediates (ROI) that suppress antigen-specific T-cell responses and NK-cell-mediated cytotoxicity. This ROI-mediated suppression has been correlated with down-regulated expression of the signal-transducing molecules CD3ζ and CD16ζ (227, 323). Administration of anti-oxidant compounds (catalase and N-acetylcysteine) to *in vitro* cultures was found to prevent this macrophage-mediated immunosuppression and the down-regulation of CD3ζ and CD16ζ from occurring. Several studies have shown that the unresponsiveness of tumor-infiltrating and peripheral T- and

NK-cells in cancer patients and in tumor-bearing mice is often associated with a decreased expression of the CD3ζ chain, which also appears to correlate with the progression of the disease (24, 83, 120, 160, 163, 226, 284, 297, 312, 351). In several forms of cancer, T- and NK-cells are subjected to oxidative stress, presumably mediated by macrophages. The down-regulated expression of the TCR zeta chain has been shown to correlate with the survival of cancer patients (242, 347).

NO dependent suppression

Deficient cellular immune responses in animals with growing tumors have been found to be associated with the enhanced production of NO by tumor-infiltrating and peripheral cells, commonly macrophages (14, 16, 17, 241, 250, 349). GR-1^+ and Mac-1^+ myeloid suppressor cells from the spleen and bone marrow of mice with a growing colon carcinoma were shown to suppress the proliferative responses of naïve but not pre-activated T-cells through their generation of reactive nitrogen intermediates, presumably peroxynitrite particular (241).

As already discussed, however, the function of NO in immunity is multifaceted. The ambivalent role of NO in regulating T-cell functioning in tumor bearing rats was revealed by the increased production of NO there suppressing the proliferative responses of tumor-infiltrating lymphocytes but at the same time increasing the life span of these T-cells (349).

As already discussed, several possible mechanisms for explaining how suppressor cell activity can be induced by growing tumors have been presented. The production of NO has been shown to be induced following cell contact with whole tumor cells or with cell debris (own unpublished observation and (109)). Thus, circulating tumor cell-membrane debris amy also activate peripheral macrophages to produce NO. The production of high levels of GM-CSF by a mouse tumor was shown to result in myelopoietic stimulation and in an increase in the number of natural suppressor cells, recognized as being GM-progenitor cells, in the bone marrow, spleen, lymph nodes and the tumor (464). These cells were found to suppress the proliferative responses of T-cells through producing NO and TGF-β. Low-dose treatment of these tumor bearing mice by IFN-γ and TNF-α was found to stimulate the differentiation of suppressive GM-progenitor cells and to thus decrease the frequency of suppressor cells producing NO and TGF-β.

Taxol is a potent anti-tumor chemotherapeutic agent that has immune modulatory effects. Treatment of suppressor macrophages from tumor bearing mice with taxol results in a reduced production of NO and in partially reconstituted T-cell reactivity (308). It also enhances macrophage antitumor toxicity (309). Thus, treatment of TB mice by taxol can alleviate the macrophage NO-mediated suppression of T-cell proileferative

responses in distal compartments and still promote sufficient NO-mediated toxicity against the tumor.

IL-10 treatment *in vitro* has been found to reverse the tumor-NO-mediated macrophage-induced suppression of T-cell proliferative responses (14, 17).

Immunotherapeutic intervention

Several lines of evidence demonstrate that tumor growth can induce an immature macrophage phenotype which, partly through enhanced production of NO and of ROI, can mediate the suppression of cellular-immune responses. However, NO- and ROI-mediated immunosuppression is not restricted to distinct subsets of suppressor macrophages but can also be an outcome of macrophage activation in response to immune challenge. The suppression of cellular-immune responses after various immunotherapeutic interventions has been observed and has been reported to be associated with enhanced production of NO and ROI by activated macrophages (see Figure 8). It can be speculated that peripheral macrophages from tumor bearing hosts are primed and are more sensitive to immune challenge than macrophages from tumor-free hosts.

Activated macrophages and monocytes have been shown to reduce zeta-chain expression in T and NK cells through production of ROI (24, 162). Immunotherapeutic intervention through administration of inflammatory cytokines such as IL-2 enhances the anti-tumor cytotoxicity of the LAK-cells, but also triggers the production of ROI by macrophages, which in turn inhibits the anti-tumor responses of the LAK-cells (165). Histamine, demonstrated to inhibit the generation of ROI by macrophages, has been found to significantly potentiate the IL-2 induced delay of tumor growth in rats with an established prostate adenocarcinoma (164, 197).

Immunotherapeutic treatment of mice with recombinant IL-12 was found to induce a profound suppression of cellular immune responses through the IFN-γ-dependent activation of macrophage NO production (220). However, when an NOS inhibitor was administered in combination with IL-12 during vaccination by tumor cells, suppression was averted and the ability of IL-12 to enhance protective anti-tumor immunity was revealed. Administration of an NOS inhibitor (N^G-monomethyl-L-arginine) to preimmunized mice was found to increase anti-tumor CTL responses (291). Since the production of NO from macrophages can mediate both the defense of the host (against tumors and infections) and immunosuppression, the authors in question argued that suppression is an outcome of macrophage activation generally, rather than of the activation of distinct subsets of suppressor macrophages. In another study, administration of the NOS inhibitor L-NAME to tumor-bearing (mammary adenocarcinoma) mice in combination with IL-2 treatment was found to enhance anti-tumor cytolytic responses of spleen cells against the parental

tumor and, to a lesser degree, against the NK-sensitive cell line YAC as well (322).

APC mediated suppression

Deficient dendritic cell functioning (adverse or ineffective antigen presentation) in tumor-bearing mice and in cancer patients has been reported by several groups (37, 72, 133, 134, 317, 408). Such defective cells have been found both intratumorally and in peritumoral areas. It has also been found that inadequate costimulatory signals from antigen-presenting cells can induce tolerance in tumor-specific T-cells (386). In mice with a growing B-cell lymphoma, bone-marrow-derived antigen-presenting cells, rather than the tumor cells, were found to induce tumor-antigen-specific T-cell tolerance (385). Thus, "suppressor" APC can capture antigens at the tumor site and then migrate to lymphoid organs, where they present antigens to the T-cells. Because of defective costimulatory signals, this induces T-cell tolerance then rather than priming. A similar mechanism is active in a normal host, in which bone-marrow-derived cells can induce peripheral tolerance to self-antigens (3, 390). Although the mechanisms of tumor-induced dendritic-cell dysfunction are not fully clarified, factors produced by tumor cells such as VEGF and GM-CSF have been identified as inhibiting the functional maturation of myeloid progenitor cells, as was discussed earlier (58, 132).

The manner in which cells die, by apoptosis or by necrosis, is another important factor determining whether APC (dendritic cells and macrophages) mature into activators or suppressors of immune responses. It has been suggested that, in the absence of necrosis or inflammation, immature dendritic cells, that are capable of phagocytosing apoptotic cells but are poor stimulators of lymphocytes, fail to receive maturation stimuli and thus induce a tolerance for self-antigens (390). In therapeutic intervention, the induction of apoptosis by irradiation of the tumor cells may be neccessary for the processing and presentation of antigens by dendritic cells (12). Macrophages have also been shown to distinguish between tumor cells dying of apoptosis and of necrosis. Tumor cells dying from necrosis (non-apoptosis) activate macrophages to produce inflammatory cytokines such as TNF-α, and IL-1β, whereas tumor apoptosis induces macrophages to produce immunosuppressive cytokines such as TGF-β, and IL-10 (153).

Regulatory T-cells

CD4-positive regulatory T-cells have been shown to be broadly involved in immune regulation (reviewed in (346)). These regulatory T-cells are enriched within the population of peripheral T-cells that constituently express CD25. They have been shown, for example, to inhibit autoimmune diseases, suppress the expansion of other T-cells both *in vitro*

and *in vivo*, and to impede the development of anti-tumor immunity. Elimination of CD25 CD4 positive T-cells in naïve mice has been found to elicite anti-tumor-immune response *in vivo* to a syngeneic tumor and the later rejection of the tumor (381). These responses were found to be mediated by tumor-specific CTLs and non-tumor-specific NK cells. CD25 CD4 positive regulatory T-cells are found to functionally suppress T-cell responses in a cytokine-independent but contact-dependent manner. In addition to the naturally occurring regulatory T-cells involved here there are also induced populations of regulatory T-cells, denoted as Th3, Tr1, and anergic T-cells. Tr1 cells produce IL-10, whereas Th3 produce TGF-β, the production of these cytokines being required for the functioning of these regulatory cells. Tumor-specific CD8-positive regulatory T-cells have also been shown to develop in tumor-bearing mice and in cancer patients (355). These cells specifically recognize the oncofetal antigen (OFA) expressed by the carcinoma in breast cancer patients and by the lymphoma in mice, and suppress IFN-γ production and cytotoxic activity by CD8-positive effector T-cells through their production of IL-10. In a lung tumor-model, T-cell-derived IL-10, found to be the predominant source of IL-10, led to impaired anti-tumor immune responses and to enhanced tumor growth (379).

Gliomas

Although gliomas remain and develop within the brain, rarely metastasizing, they are able to cause broad peripheral immunosuppression in their host. Studies *in vitro* have shown that peripheral blood lymphocytes from patients with gliomas proliferate poorly in response to T-cell mitogens and to antigenic stimulation, the same time as B-cell-proliferative responses to T-cell-independent mitogens appear to be normal (26, 110, 111, 290, 303). This suppression of T-cell responses has not been observed in patients with other types of brain tumors (26, 60, 359).

Several studies have demonstrated that the unresponsiveness of T-cells obtained from patients with gliomas is associated with T-cell-signaling defects. For example, tyrosine phophorylation was found to be reduced after stimulation by PHA or by anti-CD3 in comparison to control T-cells (26, 303). Both PLCγ1 and $p56^{lck}$ protein levels also tend to be reduced in the T-cells of patients with gliomas, the ability to mobilize Ca^{2+} also being impaired relative to T-cells from healthy donors (303).

Observations have shown cytokine production in glioma patients to be dysregulated. T-cells obtained from glioma-bearing patients have been found to produce less IL-2 than these from non-tumor bearers (27, 111), the expression of functional high-affinity IL-2 receptors on the T-cells from patients with gliomas also being below the normal levels (26, 112). Notably,

this alteration in IL-2-receptor expression appears to be unique for malignant tumors of astrocytic origin its not being observed in T-cells from patients with meningiomas or oligodendriogliomas (26). In PBL derived from glioma patients the production of IFN-γ is reduced, presumably due to the $CD4^+$ T-cells from glioma patients being less abundant and also less responsive to mitogens than normal $CD4^+$ T-cells are (110, 420).

The degree of immunosuppression in tumor patients has been shown to correlate with the size but not with the location of the tumor (303). Surgical excision results in a partial restoration of *in vitro* T-cell functioning and in an increase in the MCH class II expression by blood monocytes up to near its normal levels (59, 448). Although several lines of evidence show gliomas to produce factors capable of inhibiting immune functions such as TGF-β(1, 2, and 3) (44, 370, 404, 441), PG2-2 (42, 65), IL-10 (172, 179, 180), and gangliosides (127, 206), there is little evidence for their having the *in vivo* role of being suppressors. However, not all factors of an immunosuppressive nature expressed by gliomas have been fully characterized, the factors inhibiting T-cell responses also not appearing to be the same as those altering monocyte cytokine secretion patterns (474).

A decrease in the number of T-cells has also been observed in glioma patients (61). Glioma cells have been reported to express both Fas (CD95) and its counterpart Fas-ligand (CD95L), which may be involved in the FAS-L mediated apoptosis of Fas-expressing T-cells in the brain (363, 402). It should be noted, however, that T-cell infiltration into glioma tumors is generally low during tumor progression (100). An increase in apoptotic peripheral-blood T-cells in glioma patients has also been observed (302). Several lines of evidence suggest that both soluble factors and cytokines generated by peripheral-blood-derived monocytes can influence T-cell functioning and induce T-cell apoptosis *in vivo*. There are results indicating that monocytes from glioma patients are chronically activated, $CD14^+$ monocytes showing a decreased expression of MHC class II (32, 128, 448). The expression of MHC class II is also reduced as compared with monocytes obtained from patients with low-grade astrocytoma (448). As already discussed, the suppressor activity of macrophages is associated with MHC class II^- macrophages, indicating that gliomas are capable of inducing natural suppressor cell activity. Exposure to culture supernatants of glioma cells has been shown to induce the production of soluble factors by monocytes, resulting in the apoptosis of normal T-cells by anti-CD3. (302). Monocytes may thus contribute to the immune dysfunctions observed in patients with gliomas.

THE PRESENT STUDY

Aims

The aim of the present study was to clarify the role of NO in immunosuppression induced by *in vivo* tumor growth or by tumor immunotherapy, and to investigate whether the inhibition of NO production can be used as an adjuvant in tumor immunotherapy.

Specific aims:

- Evaluation of peripheral anti-tumor-immune functions in two different rat tumor models: subcutaneously growing malignant glioma and intrahepatic growing colon carcinoma (papers I and II).
- Elucidating the role of peripheral suppressor cells and their production of NO in tumor-induced immunosuppression (papers I and II).
- Clarifying the involvement of iNOS in anti tumor immune responses occurring locally at the site of immunization in lymphoid organs and in the brain tumors of rats immunized by IFN-γ secreting glioma cells (paper III and IV).
- Investigate whether selective or non-selective inhibition of iNOS should best be used as an adjuvant in the treatment of rats immunized by IFN-γ secreting glioma cells (paper IV).

Glioma immunosuppression (Paper I)

Immunosuppression

We have demonstrated earlier that peripheral immunization by IFN-γ -secreting N32 glioma cells induces complete tumor regression in more than 40% of rats with pre-established intracranial gliomas (430). Late-stage tumor growth in cancer patients and in tumor-bearing animals is often associated with defective lymphocyte functioning (reviewed in (210)). Since this could attenuate the curative effect of anti-tumor immunotherapy, it appeared important to study whether peripheral immunosuppressive mechanisms were induced in rats with a growing N32 glioma.

Three weeks after the subcutaneous isografting of N32 glioma cells, rats were immunized intraperitoneally by irradiated N32 glioma cells genetically engineered to express IFN-γ (N32 IFN-γ) (see Figure 9). One week after immunization, spleen cells (depleted by plastic adherence) from tumor-bearing (TB) and tumor-free rats (TF) were tested *in vitro* for immune reactivity toward tumor antigens and toward the polyclonal activators anti-CD3 and SEA. The cytolytic activity of the T cells toward N32 tumor cells was found to be strongly suppressed, as was the cytolytic

activity of the NK cells toward the NK sensitive cell line YAC. Spleen cell proliferation and the production of IFN-γ and IL-10 in response to tumor cells was suppressed as well. These results indicated a growing N32 glioma to induce immunosuppressive mechanisms that inhibit the development of a strong anti-tumor immune response. Spleen cell proliferation and cytokine production in response to SEA and anti-CD3 were also found to be suppressed. The proliferative response to anti-CD3 was found to not be permanently suppressed, however, but to recover *in vitro* after 4 days. This recovery of spleen cell proliferation after treatment by anti-CD3 indicated the immunosuppressive factors to be either eliminated or reduced *in vitro*.

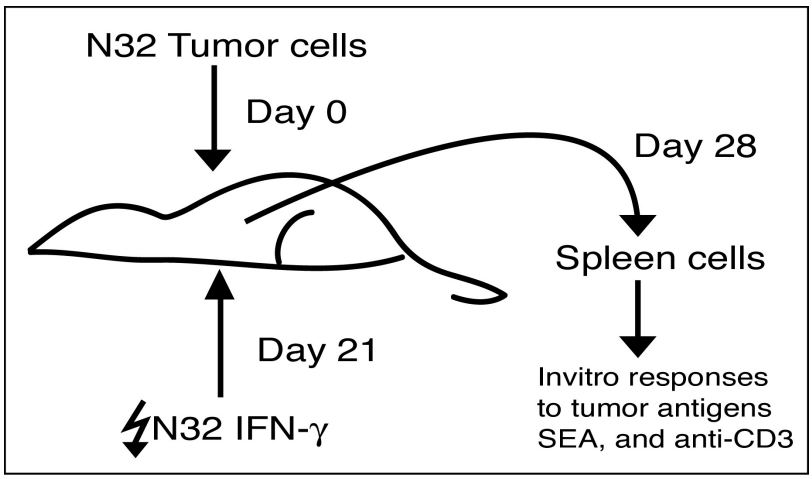

Figure 9. Experimental model for evaluating of tumor induced immunosuppression in rats with a subcutaneously growing malignant N32 glioma.

Suppressor cells

There were at least three good reasons to search for tumor-induced suppressor cells in the plastic adherent spleen cell fraction. First, previous studies had shown that natural suppressor-cell activity increases in the spleen of tumor-bearing hosts (reviewed in (109)). Second, plastic adherent spleen cells from rats, consisting mainly of macrophages, are known to possess natural suppressor activity. Third, as described earlier, proliferative responses of plastic-adherence-depleted spleen cells obtained from tumor bearing rats were found to recover *in vitro* in response to anti-CD3. This indicated suppressor cell activity to be present in the plastic-adherent fraction of the spleen cells.

This hypothesis was tested as follows: Spleen cells obtained from tumor-bearing (TB) and tumor-free (TF) rats were allowed to adhere to the plastic of a 96-well plate. Non-adherent spleen cells were discarded by thorough washing of the plates. Non-adherent spleen cells from TF rats were added to the plastic adherent spleen cells from TB or TF rats. SEA

was added, the suppressor cell activity in the TB fraction being compared then with that in the TF fraction (see Figure 10).

In the cultures containing TB-adherent cells, both the proliferative response and the production of IFN-γ and IL-10 were found to be significantly suppressed. Thus, the N32 glioma growing *in vivo* induced natural suppressor cell activity in the plastic adherent spleen cell fraction.

NO dependent immunosuppression

Although several lines of evidence show gliomas to produce factors capable of inhibiting immune functioning such as TGF-β (1, 2, and 3) (44, 370, 404, 441), PG2-2 (42, 65), IL-10 (172, 179, 180), and gangliosides (127, 206), there is little evidence for their role as suppressors *in vivo*. Some observations suggest indirect suppressor mechanisms to be active in glioma patients. Monocytes from glioma patients have been shown to be chronically activated and their expression of MHC class II to have decreased (32, 128, 448). Deficient MHC class II expression has been reported by others to be associated with macrophage suppressor activity (reviewed in (109)). Immunosuppression has been found to be associated too with the enhanced production of NO by suppressor cells (14, 16, 17, 241, 250, 349), which in turn has been shown to down-regulate MHC class II expression on macrophages (384). Since such an immunosuppression mechanism in animals with a progressively growing glioma had not been reported earlier, we wanted to investigate whether this immunopsuppression mechanism was to be found in our rat glioma model.

Nitrite, a product of the reaction between NO and O_2, accumulates in culture supernatants of NO- producing cells and could thus be used to provide an indirect estimate of the level of NO production. In order to evaluate the level of NO production, nitrite contained in the culture supernatants of TB- and TF- adherent spleen cells was analyzed. The production of nitrite was found to be significantly higher in cultures with adherent spleen cells that had been obtained from TB- than in those which had been obtained from TF-rats. The addition of an NOS inhibitor, L-NAME (N-nitro-L-arginine methyl ester), to the culture was found to significantly enhance the spleen-cell proliferation and the production of IFN-γ and IL-10 in response to the administration of SEA. Linear regression analysis revealed that the production of NO (nitrite) was inversely proportional to the proliferative response of the spleen cells. The anti-tumor cytolytic activity of the spleen cells from the TB rats was found to be slightly enhanced after *in vitro* treatment of the cells by L-NAME. This indicate the systemic immunosuppression that developed in the rats with subcutaneously growing N32 glioma to be partly dependent on the enhanced production of NO by the adherent spleen cells.

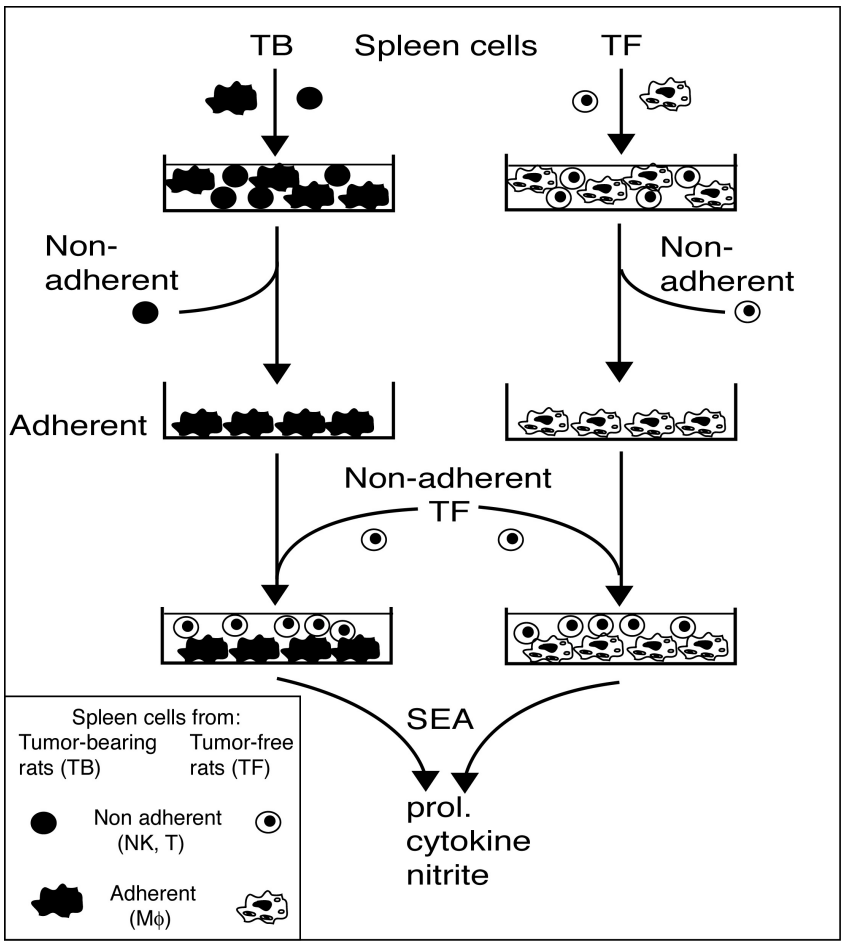

Figure 10. Analysis of NO dependent suppressor cell activity in tumor bearing rats. Adherent spleen cells from tumor bearing (TB) and tumor free (TF) rats were separated from non-adherent spleen cells by plastic adherence to wells of a 96-well plate. Non-adherent spleen cells from TF rats were added to plastic adherent cells from TB and TF rats. Spleen cells were grown together with the super antigen SEA, after which proliferative responses, cytokine production and the production of nitrite was analyzed.

Colon carcinoma immunosuppression (Paper II)

We have shown previously that the intraperitoneal immunization of rats by colon carcinoma cells (H1D2) genetically modified to express IL-18 (H1D2-IL-18) induces an isograft rejection response. In contrast to results for the rat glioma model, however, immunization by IL-18 producing tumor cells failed to induce a significant therapeutic effect in rats carrying a pre-established intrahepatic H1D2 tumor. This led to our investigating whether in tumor-bearing rats the anti-tumor immune responses are suppressed and whether this occurs as an early event during progression of the tumor. We wanted also to determine whether the NO-dependent immunosuppression recently discovered in our rat glioma model was active

in this tumor model and whether IL-18-producing gloma cells in combination with NOS inhibitors could be used to enhance anti-tumor immune responses in spleen cells of tumor-bearing rats.

The experimental setting was similar to that for the glioma model, except that the rats were immunized after either one or two weeks of intrahepatic tumor growth (see Figure 11). A week later, spleen cell responses, were evaluated *in vitro.* Results of these *in vitro* tests showed spleen cell responses both to tumor antigens and to the polyclonal stimulators anti-CD3 and SEA, to be strongly suppressed, already after two weeks of tumor growth (the rats having been immunized after one week of tumor growth). The suppression of spleen cell proliferation, cytokine production, and the cytolytic responses of the CTL- and NK-cells was also found to be still more pronounced in the rats bearing a tumor for three weeks than in those bearing it for only two, showing that in rats with a growing intrahepatic colon carcinoma the induction of systemic immunosuppression is an early event, the suppression increasing with the progression of the tumor. This is also in line with results from cancer patients showing the suppression of T-cell responsiveness and the decrease in the expression of CD3-ζ to be correlated with the stage of the disease (284).

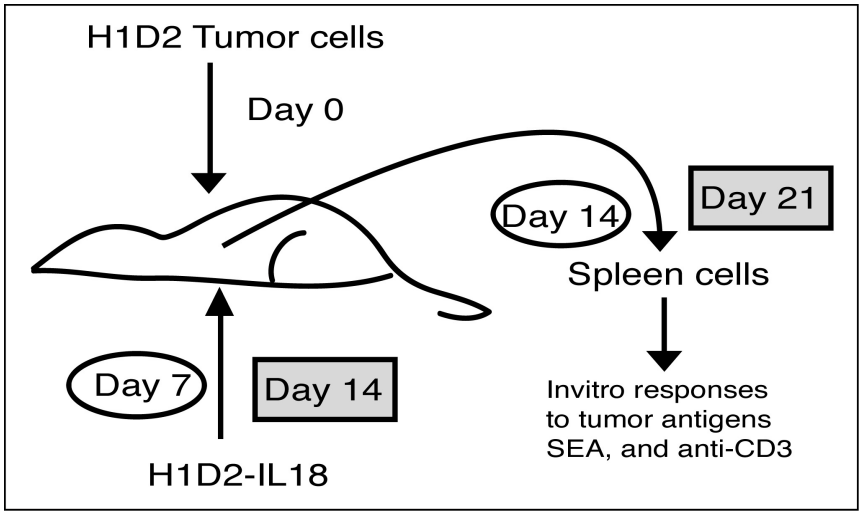

Figure 11. Experimental model for the evaluation of tumor induced immunosuppression in rats with an intrahepatic growing H1D2 colon carcinoma.

Our results show that spleen cells from tumor-free immunized rats display a strong proliferative response to IL-18-producing H1D2-tumor cells, whereas the proliferative response of spleen cells from the 3-week-tumor-bearing rats is almost completely suppressed. Results obtained in a previous study of ours of rats with a progressively growing malignant glioma showed tumor-induced immunosuppression to be associated with enhanced production of NO by the spleen cells. The IL-18 used to bring

about a strong cytotoxic responses in the T-cells may also activate the macrophages to produce NO and thus contribute to NO-dependent immunosuppression. In order to evaluate the involvement of NO in suppression here, spleen cells from tumor-bearing and tumor-free rats were grown *in vitro* together with IL-18-producing H1D2-cells and the NOS inhibitor L-NAME. The inhibtion of NO production was found to significantly enhance spleen-cell-proliferative responses to the IL-18 producing H1D2-cells, suggesting that the reduction in NO production achieved by means of the NOS inhibitors, in combination with the administration of immune-stimulatory cytokines such as IL-18 and IFN-γ could be useful for improving the immunotherapeutic treatment of tumors. However use of L-NAME for the treatment of spleen cells obtained from tumor free rats and depleted of plastic adherence cells was found not to enhance but rather to reduce the proliferative response. Others have demonstrated that a certain minimum level of NO is required for optimal T-cell activation to be achieved (318). Thus, depletion of plastic adherence cells, when occuring in combination with L-NAME treatment, may reduce the production of NO to a level below the optimal one. This illustrates the fact that the concentration of NO, and of possible other as yet unidentified factors, tends to determine the adjuvant effects of the NOS inhibition achieved in immunotherapeutic interventions. Taking this into account is important, therefore, in order to ensure that optimal immunotherapeutic effect can be achieved.

Treatment by NOS inhibitors was found to only partially enhance the proliferative responses of spleen cells obtained from tumor-bearing rats as compared with those from tumor-free rats. This could be due either to additional immunosuppressive factors produced in the spleen cell cultures of the tumor-bearing rats or to dysfunctional T-cells obtained from the tumor-bearing rats. To distinguish these effects, non-adherent spleen cells from tumor-free rats were admixed with adherent spleen cells from both tumor-bearing and tumor-free rats and were grown together with SEA and L-NAME. The results obtained show spleen-cell-proliferative responses to be suppressed in the presence of adherent spleen cells from tumor-bearing rats and this to be correlated with enhanced production of NO. The addition of L-NAME to adherent spleen cells from tumor-bearing rats decreased the production of NO and at the same time enhanced the spleen-cell-proliferative response so that almost normal levels were achieved. These findings indicate that the major part of the tumor-induced adherent spleen-cell-suppressor activity is dependent on the enhanced production of NO.

Expression of iNOS in Immunized rats (paper III)

NO generated from iNOS has been shown to perform multiple functions during inflammatory responses, including cytotoxic effector functions and the regulation of cellular immune functions (reviewed in (46, 267)). Since IFN-γ is one of the best characterized and most potent inducers of iNOS, we wished to investigate whether the expression of iNOS was expressed differently after immunization by N32 wild-type tumor cells and by N32 tumor cells genetically engineered to express IFN-γ (N32- IFN-γ).

Immunization site and lymphoid organs

In order to delineate the expression of iNOS at the site of immunization and in the lymphoid organs, Fisher rats were given intradermal injections of irradiated tumor cells (N32, N32-plxsn, and N23 IFN-γ) (see Figure 12). Immunohistochemical staining was used to examine the expression of iNOS in tissue sections of skin biopsies at the immunization site, in draining lymph nodes and in the spleen 1, 3, 7 and 10 days after immunization. The staining was analyzed by computerized image analysis.

Immunization of the rats by N32 IFN-γ tumor cells was found to significantly enhance the expression of iNOS at the site of immunization as compared with results obtained for rats immunized by N32 wild-type and N32 empty-vector (plxsn) tumor cells. Expression was observed already on day 1 and peaked on about day 7. In the spleen and in the draining lymph nodes, in contrast, the expression of iNOS remained low in all three groups.

Our results thus imply that in rats immunized by IFN-γ-secreting N32 tumor cells, NO is involved in the local regulation of the anti-tumor immune response. The regulatory effects of NO on immune functions are highly dependent on the local concentration of NO. Results obtained by others show the production of IFN-γ *in vitro* by naïve T-cells to be enhanced by low concentrations of NO and suppressed by high concentrations of it (318). It has also been shown that the early innate response of NK cells during infections is dependent upon the production of NO from iNOS, whereas the later adaptive response mediated by the T-cells is not (94). Findings have also been presented suggesting that a high level of NO production during inflammatory responses functions as a negative feedback mechanism through inhibiting IL-12 production by activated macrophages, thus indirectly inhibiting the induction of Th1 responses (177). Accordingly, it can be speculated that the relatively low level of expression of NO locally during the first few days after immunization by N32 IFN-γ is sufficient to support activation of the innate immune response but low enough to avoid suppressor activity, whereas the

elevated expression of iNOS during the later phase of the immune response inhibits the development of a strong Th1 response.

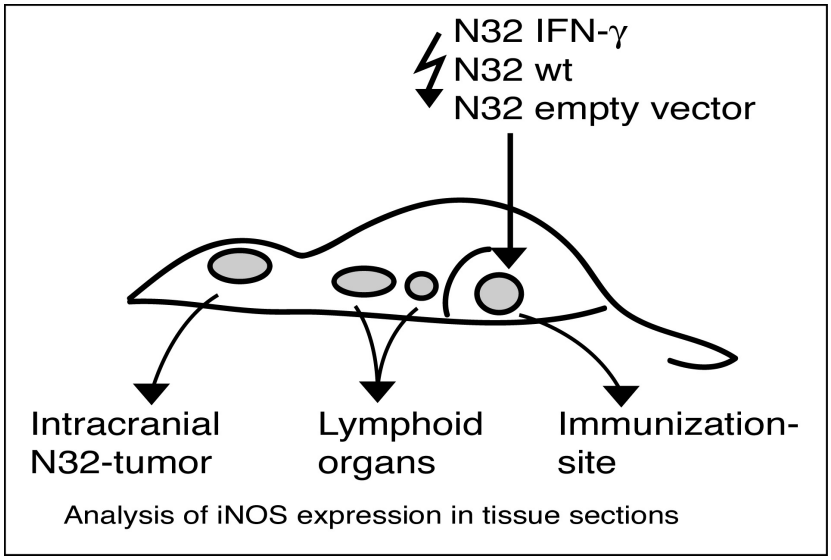

Figure 12. Evaluation of iNOS expression in the brain tumor, the lymphoid organs (spleen and lymph nodes), and at the immunization site after immunization of rats by N32 tumor cells (N32 IFN-γ, N32 wild type and N32 empty vector (only lymph nodes and immunization site)).

The phagocytosis of the tumor cells by iNOS-expressing cells at the site of immunization was assessed. Tumor cells labeled with green fluorescent dye were injected intradermally, the iNOS expression being analyzed in skin biopsies by means of immunohistochemical staining carried out 1 and 7 days, respectively, after immunization. On day 1, the iNOS-producing cells were found to be associated with intact or phagocytosized cells, whereas on day 7 the cells that had engulfed the tumor cells were basicaly separated from the iNOS expressing cells. Findings reported by others show NO to regulate both phagocytosis and APC functions. NO prevents maturation and prolongs the endocytic ability of dendritic cells, and down-regulates their antigen-presenting cell functions (174, 329). NO also down-regulates MHC class II expression by macrophages (384). We propose that the separation between iNOS expression and APC activity is necessary to prevent APC induced T-cell suppression (deletion), and to enable maturation of APC cells. Thymic dendritic cells have been shown, in fact, to delete T-cells in the thymus by producing NO (5, 117, 305, 405).

Tumor site

The involvement of NO in brain-tumor rejection was assessed by comparing iNOS expression in the brain tumors of rats immunized by N32-IFN-γ and by N32-wt, respectively. Immunohistochemical staining

demonstrated that in brain tumors of rats immunized by N32-IFN-γ cells the iNOS positive cells were distributed over the entire tumor area whereas only very few cells expressed iNOS in the brain tumors of rats immunized by N32-wt cells. The most plausible explanation of this enhancement of iNOS expression is that activated tumor-infiltrating T-cells produce IFN-γ, which induces the expression of iNOS. This is supported by earlier findings showing the numbers of tumor infiltrating T- and NK-cells to be significantly higher in rats immunized by N32 IFN-γ than in these immunized by N32-wt (429).

Immuno-fluorescence double-staining showed macrophages to express the major portion of the iNOS that was detected, both at the site of immunization and in the brain tumors. The role of this enhancement of iNOS expression in brain-tumor-associated macrophages is not clear. Findings by others show that NO production by intra-tumoral macrophages is able to suppress anti-tumor immune responses, and also to mediate anti-tumor effector functions directly. It was found recently that tumor-associated macrophages are highly proapoptotic and are able to mediate the apoptosis of T-cells through enhanced production of NO (365). The increase in the level of NO production is dependent upon IFN-γ production by the activated T-cells. The proapoptotic capacity of the tumor-associated macrophages, in turn, is dependent upon the expression of TNF receptors on their surface, since the binding of TNF to these receptors is necessary in order for the production of apoptotic levels of NO to be induced. In contrast to tumor-associated macrophages, peritoneal macrophages have been found to not express TNF receptors and to thus be non-proapoptotic (365). The expression of TNF in the present rat brain tumor model suggests the emergence of proapoptotic macrophages. Several studies, however, have shown activated T-cells to be less sensitive to NO-mediated suppresion than naïve T-cells are (318), implying that activated tumor-infiltrating T-cells may be less sensitive to NO-induced apoptosis. Recently, the rejection of tumors in preimmunized rats was found to be mediated by tumor-infiltrating macrophages and to be dependent upon their production of NO (50). The tumoricidal effector function of these macrophages was assumed to be induced by the adjacent lymphocytes. Obviously, the total net effect of NO production in the vicinity of the tumor is in need of further clarification.

Inhibition of iNOS in Immunized Rats (paper IV)

In earlier papers we were able to show that a major part of the tumor-induced suppressor-cell activity that occurs is dependent upon the production of NO. The immunization of rats by IFN-γ secreting glioma cells (N32 IFN-γ) was found to enhance the *in vivo* expression of iNOS. It

has been reported earlier that immunotherapeutic intervention that leads to the activation of macrophage-NO production suppresses cellular immune responses and inhibits protective anti-tumor immunity (220). Therefore, there is evidence that, by increasing the production of NO, both growing tumors and immunological stimuli suppress the anti-tumor immune response. This led us to investigate whether the enhanced expression of iNOS in rats immunized by IFN-γ-secreting glioma cells suppresses the induction of a strong anti-tumor immune response.

In the initial studies (papers I and II) the non-selective NOS inhibitor L-NAME was used *in vitro* to inhibit the production of NO by iNOS expressed in spleen cell cultures. Since NO generated by constitutively expressed NOS supports physiological functions *in vivo*, such as the regulation of vascular tone and of blood pressure, there may be disadvantage to using a non-selective inhibitor of NOS *in vivo*. In addition, given that the immunosuppressive action of NO is largely dependent upon iNOS activity, a selective inhibitor could be thought to be more effective. Thus, in the studies reported here we used a selective inhibitor of iNOS, namely L-NIL (L-N^6-(1-Iminoethyl)-L-lysine), in parallel with L-NAME.

In vitro studies showed L-NIL to be much more effective than L-NAME in reducing the production of NO by *in vitro* activated spleen cells. The reduction in NO production was found to correlate with an enhanced proliferation and production of IFN-γ, an enhancement found to be much more pronounced in spleen cell cultures containing L-NIL than in those containing L-NAME. The optimal dose of L-NAME corresponded to a sub-optimal dose of L-NIL.

The role of NO generated from iNOS in the development of an anti-tumor immune response in rats immunized by N32 IFN-γ was evaluated through the delivery of L-NIL and L-NAME to immunized rats by intraperitoneally placed mini-osmotic pumps at doses of 0, 0.1, 1, and 10 mg/day (see figure 13). A week later, the immune responses of the spleen cells to the tumor antigens SEA and anti-CD3 were analyzed *in vitro*. The results obtained revealed that 1 mg L-NIL/ day significantly enhanced the proliferation of spleen cells and the production of IFN-γ, but that at the higher dose of 10 mg/day this was not the case. Thus, a decrease in the production of NO by the selective inhibition of iNOS enhanced the development of anti-tumor immune responses in the immunized rats. There was also found to be an optimal level for the generation of NO by iNOS as regards the development of Th1 responses *in vivo*. This is also in line with *in vitro* results and the results of others, bothe of which have shown that low concentrations of NO enhance the production of IFN-γ by naïve T-cells whereas high concentrations of NO suppress the *in vitro* production of it (318). In contrast to treatment by L-NIL, L-NAME treatment did not enhance spleen-cell responses at any of the doses tested. Rather, the highest dose of L-NAME given significantly suppressed cellular immune responses

in the spleen. Although the underlying mechanisms for this suppression are not clear the results show that the selectivity of the NOS inhibitor is crucial for its regulatory effect on immune responses.

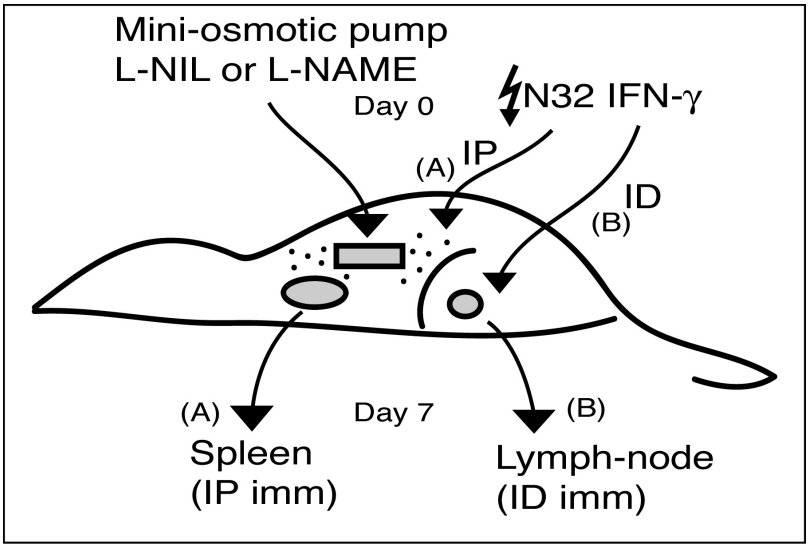

Figure 13. Administration of NOS inhibitors by intraperitoneally placed miniosmotic pumps to rats immunized with N32 IFN-γ tumor cells. Rats were immunized intraperitoneally (IP) or intra dermally (ID) and one week later spleen or lymph node cell responses were analyzed *in vitro*.

The effects of selective iNOS inhibition were also assessed in draining lymph nodes of rats immunized intra-dermally. It was found that both proliferative responses and the production of IFN-γ were enhanced in the draining lymph nodes of the rats treated by L-NIL. This could be due either to the T-cell activation being affected directly or to the antigen-presenting capacity of the dendritic cells being affected, since NO has been shown to inhibit the maturation of dendritic cells (174, 329). Since in our study mini-osmotic pumps were placed intraperitoneally at the time of the immunization, it obviously took some time before L-NIL reached a steady state level at the site of immunization (the right thigh). This delay may have been beneficial, since deficient NO production by iNOS during the early innate phase of an immune response has been shown to inhibit NK-cell activation, which can result in a suppressed or delayed T-cell response (94).

Concluding Remarks and Future Perspective

In conclusion, our results show that both tumor growth and immunotherapeutic intervention induce peripheral suppressor activity that inhibits anti-tumor immune responses through enhancing the production of NO.

Specifically we show the following:

- That tumor-induced systemic immunosuppression in two different rat-tumor models, viz. rats that had a subcutaneously growing malignant glioma and rats with an intrahepatic growing colon-carcinoma, is partly dependent on enhanced production of NO by adherent spleen cells (Paper I and II).
- That anti-tumor immune responses are enhanced both *in vitro* and *in vivo* by the inhibition of iNOS (Papers I, II, and IV).
- That enhanced expression of iNOS in rats that have been immunized by IFN-γ secreting N32 glioma cells prevents the induction of a strong peripheral type 1 T-cell response but can contribute to the tumoricidal effector functions at the site of the tumor (Paper III and IV).
- That anti-tumor immune responses are enhanced after combined treatment with NOS inhibitors and IL-18 or IFN-γ secreting tumor cells (Paper II and IV)
- That the adjuvant effect of NOS inhibitors *in vivo* is strongly dependent upon their concentration and isoform selectivity (paper IV).

We propose that selective inhibition of iNOS in immunized tumor-bearing rats could enhance the anti-tumor immunotherapeutic effect by reducing the systemic immunosuppression induced by the growing tumor and by the immunotherapy itself. NOS treatment *in vivo* is a complicated matter, however, its effect on immunity and on tumor growth dependending upon a) the isoform selectivity and, b) the local concentration of NO, as well as c) the site-specificity and d) the timing of the treatment. Since NO has also been shown to regulate anti-tumor immunity and tumor growth at different levels, various strategies for NOS inhibition could thus be utilized for promoting tumor immunity and reducing tumor growth.

Studies for clarifying the development of immunosuppression in rats with intracranialy growing N32 glioma are in progress. The results obtained thus far show that the production of IFN-γ by peripheral blood lymphocytes (as analyzed by ELISPOT) decreases with progression of the tumor, whereas the production of IL-4 remains principally intact. Studies

for clarifying the *in vivo* involvement here of NO are ongoing. Recently, we initiated studies for evaluating the therapeutic effects of selective iNOS inhibition in rats with an intracranially growing N32 glioma that had been immunized by IFN-γ-secreting N32 glioma cells.

Several studies have shown immunosuppression induced by a growing tumor or by immunotherapy to be associated with oxidative stress exerted by activated macrophages or suppressor cells. Findings obtained for various animal models have shown either that therapy-induced suppressor activity is dependent upon NO but not on reactive oxygen intermediates (ROI) or vice versa. These differential results may be due to that NO-mediated suppression being dominant in treatment involving lower doses of cytokines, whereas at higher doses ROI-mediated suppressor activity is dominant. It would be interesting, of course, to evaluate and compare inhibitors or scavengers of ROI with inhibitors of iNOS in tumor-bearing hosts and also in animals treated with increasing doses of cytokines. PGE-2 has also been implicated in suppressor-cell activity. There are studies as well that implicate NO in regulating the activity of Cox-2. Treatment with certain NOS inhibitors has also been shown to enhance the production of PGE-2. Thus the effects of Cox-2 inhibitors, both alone and in combination with NOS inhibitors should be evaluated as well.

The results of our studies show that the *in vivo* effects of NOS inhibitors are highly dependent upon the NOS isoform involved. Thus, other highly specific iNOS inhibitors should be evaluated in future studies. Since most experimental NOS inhibitors are L-arginine analogues, complete selective can scarcely be achieved. Also, since the activity of iNOS, in contrast to constitutive NOS, is regulated at the transcriptional and translational level, it should be possible to develop highly specific inhibitors of iNOS that act either on gene transcription or on mRNA translation. Interestingly enough, tetracyclines and chemically modified tetracylines have been shown to inhibit iNOS expression by the augmented degradation of iNOS mRNA (20, 21). Since these products are readily tolerated by humans, they should be evaluated along with other iNOS inhibitors and be compared with them. The Inhibition of iNOS at the translational level results in reduced expression of iNOS and make it possible, terefore to study the effects of iNOS inhibition *in vivo,* for example by means of immunhistochemistry.

The results obtained for iNOS-deficient mice indicate there to be a risk that selective inhibition of iNOS may lead to the deterioration of pathogen control. At the same time, one should note that NO, which is a phylogenetically old molecule, appears to be redundant in many responses. The expression of iNOS has been shown to be dispensable or even counter-protective in the control of various pathogens. The inhibition of iNOS in combination with the immuntherapeutic treatment of cancer will probably in most clinical cases be used for only limited periods of time.

Are tumor-induced and therapy-induced NO-dependent suppressor cell activity identical? This is not fully clarified. Data reported by several groups indicates that tumor growth can increase the number of immature myeloid cells for which enhanced suppressor activity can be mediated by NO, ROI or PGE-2. In contrast, results obtained from therapy-induced suppressor-cell activity shows the suppression to be dependent on ROI and on NO (but not on PGE-2) and to be a probable consequence of normal immune activation of macrophages rather than of the induction of specific subsets of suppressor cells. A comparison of NO, ROI and PGE-2 production by tumor-induced suppressor cells and by therapy-induced suppressor cells may indicate whether these represent different subsets of suppressor cells. Tumor- and therapy-induced suppressor effects may well be of overlapping charachter. Also, immunotherapeutic intervention by means of pro-inflammatory cytokines may well have an additive effect on tumor-induced suppressor cell activity. This could be evaluated by comparing the level of NO production in immunized and non-immunized tumor-bearing and tumor-free rats.

Possible effects of site specific NOS inhibition

The site and duration of iNOS administration appear to be important factors in determining the immunotherapeutic effects. In contrary to most other groups, which have administered NOS inhibitors by injections or by means of drinking water, we have utilized mini-osmotic pumps with continous and controlled delivery. In order to make a site-specific, continuous delivery of iNOS inhibitors possible, other technical solutions, providing slow and prolonged release, are needed.

The inhibition of constitutively expressed NOS locally, at the site of the tumor can be pro-inflammatory through up-regulation of the adhesion molecules on the endothelial cells enhancing leukocyte extravasation and leukocyte infiltration of the tumor. The inhibition of constitutive NOS can reduce tumor growth through the suppression of tumor angiogenesis. If activated tumor-infiltrating T-cells, as indicated by results of others, are less sensitive to NO-mediated suppression than naïve peripheral T-cells are, selective inhibition of iNOS at the site of the tumor should be avoided so as to enable activated macrophages to participate in combatting the tumor. However, since the results of others show that tumor-associated macrophages are proapoptotic, due to their enhanced production of NO, these macrophages may suppress the anti-tumor immune responses through induction of T-cell apoptosis at the tumor site. The enigma of the opposing effects of the NO that is generated by tumor-associated macrophages can only be solved by analysis of the in situ production of NO and by the site-specific inhibition of NO production.

The inhibition of iNOS in lymphoid organs appears to enhance the development of type 1 anti-tumor T-cell responses. Naïve T cells in lymphoid organs could be thought to be more sensitive to NO-mediated

suppression than activated T-cell are. The adjuvant effects of iNOS inhibition are dependent upon the concentration and the selectivity of the NOS inhibitor.

The complete inhibition of NO production at the site of immunization during the first few days after immunization could reduce or delay the immune response through inhibiting NK-cell activation. Since NO also controls the maturation of dendritic cells, the inhibition of NO can increase the maturation of the cells and at the same time reduce their phagocytic capacity. Ideally, the inhibition of iNOS should be exerted during the adaptive phase of the immune response (approximately after day 3) so as to promote the development of a strong T-cell response.

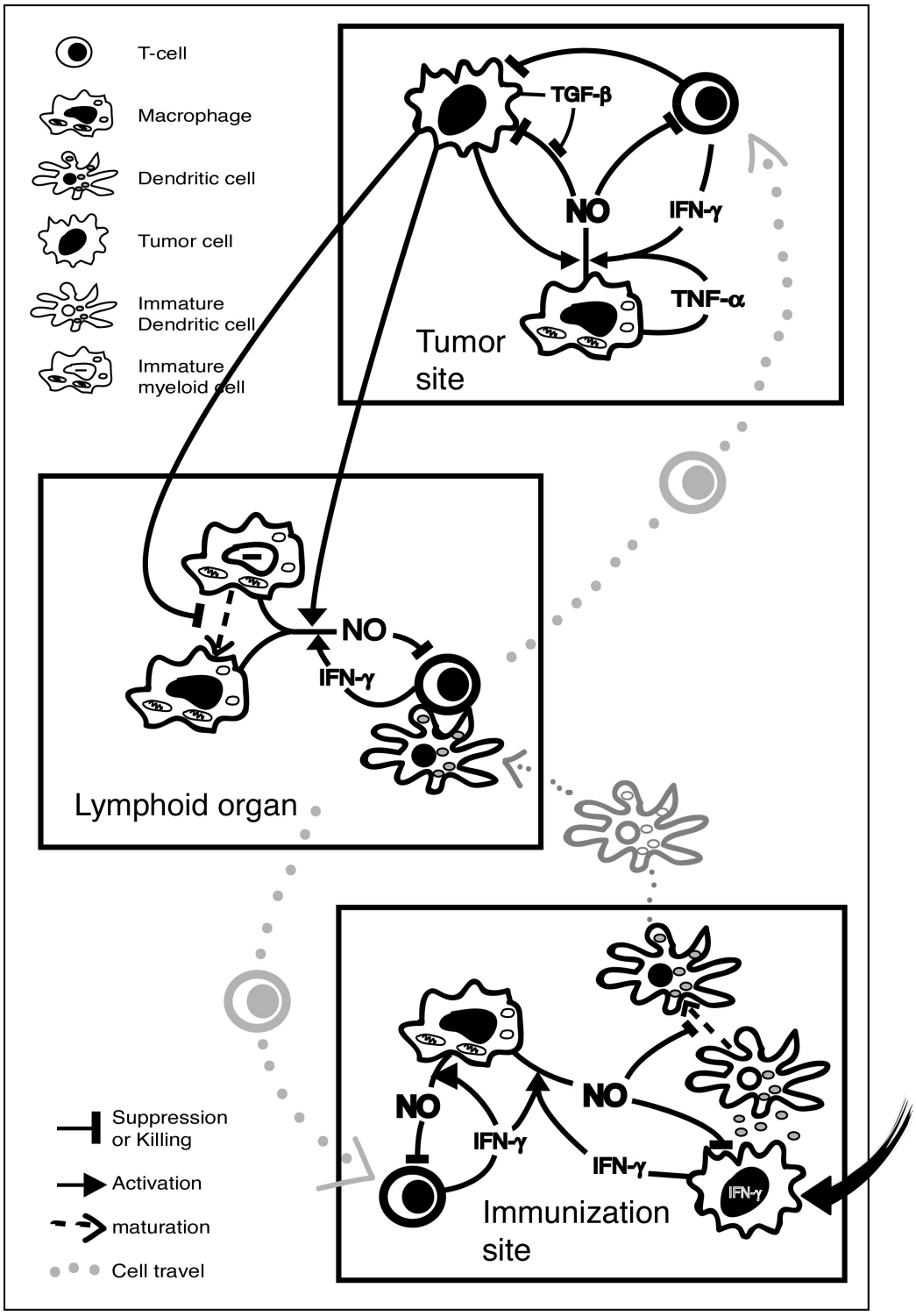

Figure 14. Proposed immune regulatory functions of NO and its production in tumor bearing immunized rats.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tumörimmunologisk grundkurs

En tumörcell har en gång i tiden varit en vanlig laglydig cell i någon av kroppens vävnader men på grund av arv eller dålig uppväxtmiljö så har denna cell upphört att följa de tillväxt begränsande regler som normala celler rättar sig efter. Förutom att kontrollen av celldelningen upphört så kan tumörcellen uppvisa ett mer eller mindre asocialt beteende. De tumörer som fortfarande är lyhörda för en del av kroppens tillväxt begränsande regler och signaler och därför inte invaderar omgivande vävnad brukar benämnas benign tumör. Medan den värre sorten som är helt okänslig för vad omgivningen tycker ger sig på att invadera omgivande vävnad. Denna tumörform är alltså malign och brukar benämnas cancer. När cancer invaderar annan vävnad kallas detta för metastasering.

Immunförsvaret liknas ofta vid vårt militära försvar och i grova drag så kan tre olika försvarslinjer urskiljas. Första försvarslinjen består av anatomiska, fysiologiska och kemiska barriärer dvs. hud, pH, temperatur och kemiska substanser. Den andra försvarslinjen består av så kallade fagocyterande celler (monocyter, makrofager, och neutrofiler) som ”patrullerar blod och vävnader” för att snabbt vara på plats för att döda och äta upp hela eller delar av invaderande mikroorgamismer. De fagocyterande cellerna och andra celler i det ospecifika immunförsvaret, som t.ex. NK celler, håller fienden stången till dess att den tredje försvarslinjen bestående av det specifika immunförsvaret alarmerats och aktiverats. Det specifika immunförsvaret består av högt specialiserade B och T lymphocyter där varje enskild cell är utbildad till att känna igen en specifik molekylstruktur eller antigen på ytan av till exempel infekterade celler eller ”fria” främmande agens i kroppsvätskor och vävnader. Samtidigt med denna specialisering så är detta immunförsvar oerhört diversifierat och kan aktiveras till att känna igen praktiskt taget vilken främmande molekyl struktur som helst. Dessa celler har också lärt sig att skilja kroppsegna ämnen från främmande. En annan viktig egenskap är att det specifika immunförsvaret har ett immunologiskt minne, vilket gör att immunförsvaret vid en andra infektionen av identiskt virus eller bakterie reagerar mycket snabbt och därigenom undviker att individen insjuknar i samma infektion en gång till. Man är immun.

Redan för mer än hundra år sedan så började en del läkare ana att immunförsvaret kunde aktiveras till att bekämpa tumörer. Man hade nämligen sett att en del tumörer minskade då patienter insjuknade i vissa bakterie infektioner. Under en stor del av 1900 talet så debatterades huruvida immunreaktioner kunde aktiveras mot tumörceller som uppstått från kroppens egna celler. Det dröjde till senare delen av 1900-talet innan man i djurmodeller lyckades visa att immunförsvaret utan att skada annan vävnad kunde känna igen och avstöta kroppsegna tumörer. Under det

senaste decenniet så har ett flertal tumörspecifika proteiner identifierats. Paradoxen inom tumörimmunologi är att tumörer, trots att immunreaktioner kan aktiveras till att stöta bort dessa, kan växa ut hos individer med väl fungerande immunfunktioner. Tumörer har alltså utvecklat strategier för att undfly immunförsvarets attacker. Många tumörer undviker att upptäckas av immunförsvaret genom att inte visa upp sina tumörspecifika proteiner på cellytan. Ett annan strategi är att aktivt ge sig på immunförsvaret och neutralisera dess förmåga till att attackera tumören. Hos patienter med cancer så har det observerats att immunförsvarsreaktioner försämras i takt med att tumören växer till.

Många olika strategier har använts för att aktivera immunförsvarsreaktioner mot tumörer, vid så kallad tumör vaccinering. En av de viktigaste cellerna i immunförsvarets attack mot tumörer anses av många vara de cytolytiska T-cellerna, men även andra inflammatoriska celler såsom NK-celler och makrofager kan understödja i attacken mot tumören. I våra vaccinationer så använder vi oss därför av tumörceller som har manipulerats (genetiskt) till att uttrycka molekyler som skall förstärka dessa immunreaktioner mot tumören.

Denna studie

I denna studie så har vi arbetat med två olika rått-tumörmodeller, en gliom och en koloncancer. Hos råttor med etablerad hjärntumör så har vi tidigare visat att vaccinering med gliom-celler som manipulerats till att producera den immunstimulerande molekylen IFN-γ leder till tumöravstötning och långtidsöverlevnad hos över 40% av råttorna. I kolon-tumörmodellen så har vi vaccinerat med tumörceller som producerar IL-18. Vaccinering med den senare leder till skydd mot nyetablering av tumör men inte till avstötning av redan etablerad tumör. En av förklaringarna till utebliven bot i dessa båda modeller (60% i gliom och 100% i kolon) kan vara att tumören lyckats avväpna eller passivisera immunförsvaret. För att ta reda på om detta var fallet i vår tumörmodell så undersöktes immunfunktionen hos immunceller från tumörbärande djur. Det visade sig då att immunceller från djur med växande gliom eller kolontumör hade kraftigt försämrad förmåga till att attackera tumörceller. Dessutom så kunde vi se att funktionen hos dessa immunceller försämrades i takt med att tumörerna växte.

Tumörer kan neutralisera immunförsvaret genom att utsöndra faktorer som direkt neutraliserar immunförsvaret eller genom att aktivera andra celler i immunförsvaret till att producera immunhämmande faktorer. En överreaktion eller okontrollerad immunreaktion är direkt livsfarlig för den enskilde individen (jämför med en armé som vänder sig mot den egna staten), därför är immunförsvaret konstruerat så att en immunaktivering automatiskt skall stängas av då faran är över. En del av de faktorer som produceras av aktiverade immunceller bidrar till att dämpa eller nedreglera immunreaktioner. I många fall så tycks det som om tumörer lyckats med

konststycket att aktivera immunceller till att producera immmunhämmande substanser. Detta undersöktes därför i våra tumörmodeller och vi kunde konstatera att i mjälten på våra råttor så fanns det immunceller som verkade hämmade på immunreaktionerna mot tumören. Dessa celler visade sig också producera onormalt höga nivåer av kväveoxid.

Det har varit känt i knappt 15 år att kväveoxid är en viktig signalsubstans för reglering av blodflöde. Denna upptäckt belönades också 1998 med nobelpriset i medicin, och har bland annat lett till utvecklandet av den kända medicinen Viagra. Intresset för kväveoxidernas funktion inom medicinsk biologi har därefter vuxit explosionsartat och kväveoxider har tillskrivits allt fler fysiologiska funktioner. För cirka 10 år sedan så kunde man visa att kväveoxider produceras från aktiverade immunceller och framförallt ifrån makrofager. Denna kväveoxid-produktion utnyttjas av immunförsvaret till att ta kål på tumörceller eller invaderande mikroorganismer men kan också leda till att immunceller och framförallt de viktiga T-cellerna inaktiveras. I vår studie så kunde vi visa att den ökade kväveoxid-produktionen var en av de bidragande orsakerna till de försämrade immunfunktionerna hos tumörbärande råttor.

Kväveoxid-produktion från aktiverade immunceller kan alltså påverka immunförsvarets attacker mot tumörer. Därför ville vi undersöka om vaccinering med våra IFN-γ producerande tumörceller påverkade kväveoxid-produktionen hos våra råttor. Resultat från denna studie visar att vaccinering av råttor med dessa IFN-γ producerande tumörceller leder till ökad kväveoxid produktion från makrofager såväl vid immuniseringsstället som i den växande hjärntumören. För att ta reda på vilken roll denna ökade kväveoxid-produktion kunde ha för immunförsvarsfunktionerna så behandlades vaccinerade råttor med en substans som hämmar kväveoxid-produktionen. Det visade sig att behandling med kväveoxid-hämmare ledde till förstärkta immunreaktioner mot tumören.

Våran slutsats är att behandling med kväveoxid-hämmare i kombination med tumör-vaccinering skulle kunna användas för att förbättra immunoterapi-behandling av cancer. Försök har inletts för att studera detta.

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APPENDIX