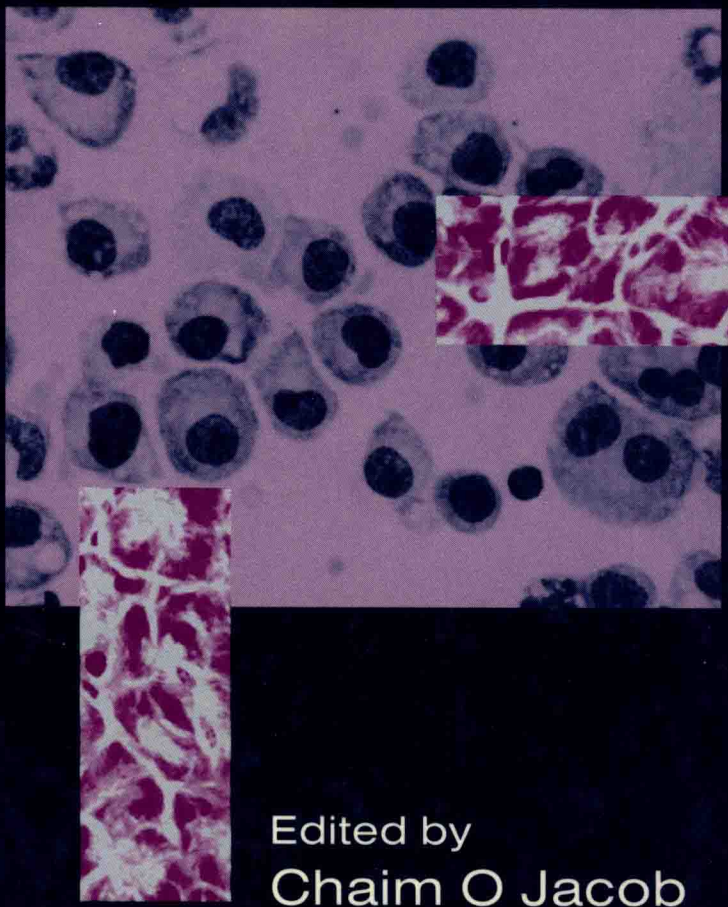
Overexpression and Knockout of Cytokines in Transgenic Mice



Edited by
Chaim O Jacob

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University of Southern California School of Medicine,
Los Angeles, CA, USA

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List of contributors

A.K. ABBAS Immunology Research Division, Department of Pathology, Brigham & Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA

J. ALLISON The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

H.J. BURSTEIN Immunology Research Division, Department of Pathology, Brigham & Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

F.F. CSAIKL National Cancer Institute, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Building 560, Room 31–45, Frederick, MD 21702–1202, USA

U.M. CSAIKL National Cancer Institute, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Building 560, Room 31–45, Frederick, MD 21702–1202, USA

R.J. DIEBOLD Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0524, USA

T. DOETSCHMAN Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0524, USA

S.K. DURUM National Cancer Institute, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Building 560, Room 31–45, Frederick, MD 21702–1202, USA

W.R. HEATH The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

T. HIRANO Division of Molecular Oncology, Biomedical Research Center, Osaka University Medical School, 2–2 Yamada-oka, Suita, Osaka 565, Japan

T. HÜNIG Institute for Virology and Immunobiology, University of Würzburg, Versbacherstrasse 7, D97078 Würzburg, Germany

C.O. JACOB Division of Rheumatology and Immunology, Department of Medicine, University of Southern California School of Medicine, Los Angeles, CA 90033, USA

A.B. KIER Division of Comparative Pathology, Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0524, USA

D. METCALF The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

J.F.A.P. MILLER The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

R. PALACIOS Department of Immunology, Box 178, University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

J. SAMARIDIS Basel Institute for Immunology, Grenzacherstrasse 487, Basel CH-4005, Switzerland

C.J. SANDERSON Western Australian Research Institute for Child Health, Princess Margaret Hospital, GPO Box D184, Perth, WA 6001, Australia

A. SCHIMPL Institute for Virology and Immunobiology, University of Würzburg, Versbacherstrasse 7, D97078 Würzburg, Germany

M.M. SHULL Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0524, USA

G.L. SEMENZA Center for Medical Genetics and Departments of Pediatrics and Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

C.L. STEWART Department of Cell and Developmental Biology, Roche Institute of Molecular Biology, 340 Kingland Street, Nutley, NJ 07110, USA

M. STRATH National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

R.I. TEPPER MGH Cancer Center, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02114, USA

M. YIN Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0524, USA

Introduction

Cytokines are a steadily growing family of molecules which are responsible for intercellular communication. Despite the ability of these molecules to mediate a wide variety of functions and to exert remarkably diverse effects, their precise biological relevance to the homeostasis of the immune and other biological systems *in vivo*, remains largely unknown. A key question to the cytokine researchers today is not only which cytokines can potentially be involved in the pathophysiology of a disease or immune response, but when do these cytokines function and which of their many activities are relevant *in vivo*. The complexity is derived from the fact that the expression of a given cytokine is invariably influenced by other cytokines, forming networks of 'cytokine cascades'. Such cascades may represent important features of cytokine action in programmed growth, differentiation and function of cells. The intricate ways of their action, often exhibiting pleiotropic or synergistic cooperations, make the study of cytokine biology rather incomplete and often contradictory using conventional systems.

It is not surprising therefore, that various investigators considered transgenic expression of cytokines to be valid tools for the study of their function. This book represents what has been achieved to date.

In vitro approaches of adding single recombinant cytokines or a combination of several cytokines, as well as *in vivo* approaches using recombinant cytokines or monoclonal neutralizing antibodies in animal model systems have clearly the potential of yielding misleading results. In *in vitro* systems, it is impossible to know whether the quantities of the cytokine in the system resemble the quantities that would be present under physiological or pathological conditions. Whether the timing or the availability of other substances at the site of the effect may in fact be responsible for the observed phenomena, cannot be verified. In *in vivo* situations the use of cytokines or neutralizing antibodies may raise similar concerns. Most cytokines have very short half-lives when administered *in vivo* and receptors for most cytokines are believed to be widely distributed. It is reasonable to assume that most of the effect one observes when injecting a cytokine is systemic rather than local. From this point of view, the use of antibody may be more effective. Antibodies

have a much longer half life, thus one can assume that the antibody will block the endogeneous cytokine both systemically and locally. However, the antibody may not be potent enough to neutralize the effects, or may have non-specific effects. Using antibodies to soak up the cytokine in the bloodstream may leave open the question of whether all the activity has truly been removed. For these and additional reasons, the approach of transgenic overexpression of cytokines, especially site specifically, and their specific elimination and production of homozygous knockouts is very promising. This book clearly demonstrates the feasibility of the approach for these biologically important substances. Although analysis of many of these lines is still in the early stages, the initial results are quite fascinating.

A general issue, which seems to arise from the transgenic cytokines is that a cytokine can have so many functions, which becomes evident from its overexpression, and yet appear to have so few when tested by loss of function in homozygous knockout mice (for example LIF, IL-2 and IL-4).

It is becoming clearer, as more and more cytokines succumb to gene targeting techniques, that the control system design is incredibly sophisticated. It may be that many control systems have powerful compensation capabilities by which the loss of one participant is overcome through altered action of another factor with analogous functions.

In the case of LIF, long term administration of the cytokine to adult animals causes rapid weight loss, behavioural disorders, ectopic calcifications and bone abnormalities. With respect to these known targets of LIF actions, the consequences of having no LIF are very little (see chapter 7).

In the case of LIF, many of its actions *in vitro* can be reproduced by one or more of a group of cytokines which includes IL-6, IL-11, oncostatin M and ciliary neurotropic factor.

In the case of IL-2 deficient mice, (see chapter 3) again, most of the effects on the immune system are quite subtle. On the other hand, overexpression of IL-2 in transgenic mice caused major effects such as alopecia, pneumonia and an increase in the number of $Thy1^+$ dendritic epidermal cells, when IL-2 was driven by an MHC promoter (Ishida *et al.*, 1989); motor ataxia due to lymphocytic infiltration into the cerebellum, when IL-2 was driven by a mouse metallothionein promoter (Katsuki *et al.*, 1989).

Overexpression of GM-CSF causes blindness because of accumulation of lymphoid cells in the lens and retinal tissues, and muscle wasting (see chapter 12). The lesions developed in GM-CSF as well as the IL-2 transgenic mice do not closely resemble any particular disease state in

humans. In all these transgenic systems, the mechanisms leading to these phenotypes are unknown. Because of the lack of a conceptual framework that could embrace these phenomena with current understanding of immune regulatory mechanisms, a viable mechanistic explanation cannot be proposed at this time.

This brings me to another point: can transgenic cytokines constitute disease models for human diseases?

At the outset of this very short discussion it is well worth confronting the question of what constitutes an ideal model of human disease. The answer is that the ideal model is a disease in an animal that is identical in every aspect to the human disease. By identical I mean that the animal disease is induced by the same primary factors (has the same cause) and is maintained by the same secondary factors (has the same pathophysiology) as the human disease. With this sort of identity one could be sure that the animal disease has an equivalent spectrum of clinical and pathological manifestations, and a similar response to possible therapeutic agents. This rather uncompromising definition of an ideal model of a disease exposes an important theoretical problem with any of the animal disease models so far proposed. The fact is that there are huge gaps in our knowledge of most complex human diseases, both from the point of view of the exogenous and endogenous etiological factors involved and of the nature of the disease mechanisms present. Taking as an example the big family of autoimmune diseases, it is quite clear that all of these diseases are complex and multifactorial in nature. Hence the view that an autoimmune disease model, even one that is not ideal, can be created by deleting or overexpressing a single gene, is naive at minimum. However, such claims have been made time and time again. One such example is Keffer *et al.* (1991).

This view does not imply that we should not study such animal disease in transgenic animals; on the contrary, understanding of these processes occurring in transgenic animals will definitely lead to important insights that will be highly relevant to the understanding of the human disease. The following two examples illustrate the value of such an approach.

People who inherit the MHC class 1 allele HLA-B27 are extremely susceptible to a variety of diseases collectively referred to as spondyloarthropathies. The most common disorder of this group is ankylosing spondylitis, a chronic multisystem inflammatory disease with arthritis, inflammation of the gastrointestinal tract, genitourinary tract, skin, eyes and heart. Two lines of transgenic rats expressing HLA-B27 develop many of the features of the human disease (Hammer *et al.*, 1990). The black box standing between expression of B27 and gastrointestinal inflammation in ankylosing spondylitis has its equivalent in almost every

autoimmune disease. Even with these transgenic rats, it is still not possible to discern how B27 leads to the disorder, or even just to explain why transgenic rats but not transgenic B27 mice develop the disorder. However, the problem should be more tractable with this remarkable animal system.

The second example related to cytokine deficiency and inflammatory bowel disease (IBD) in humans. IBD consists of two major forms: ulcerative colitis and Crohn's disease. Although immunological mechanisms have been implicated, these entities remain largely purely theoretical. No animal models to these disorders exist. IL-10 deficient mice quite unexpectedly develop mucosal inflammation resembling IBD (Kuhn *et al.*, 1993). The inflammation developed by these mice is a panenteritis and consists of pseudopolyps and villous atrophy. A second cytokine gene-targeted mouse that develops IBD-like disease is that with disrupted IL-2 gene and an inability to produce IL-2 (Sadlack *et al.*, 1993). These mice develop a disease that is more like ulcerative colitis, namely the mice develop ulcerations and bloody diarrhea limited to the large intestine. Clearly none of these mice are identical to patients with IBD since such patients with IBD have T lymphocytes that produce IL-2 and IL-10. As with the other syndromes developed in transgenic cytokine mice (see above), the interpretation of the results is far from being clear. Even if these experiments do successfully identify a component of the cytokine network which may be involved in inflammatory bowel disease, we are still a long way from a precise description of events that cause the disease development. Nevertheless, they are important in elucidating the disease because they show that T lymphocyte abnormalities can preferentially manifest as chronic, non infectious intestinal inflammation. Furthermore, the availability of such mice will open new avenues of research, which will result in enhancing our understanding of the processes involved in these complex biological systems.

Towards the end of these short thoughts, I would like to re-emphasize that, despite the power of the transgenic methodology, it is not without significant limitations. An absolute deficiency of a cytokine from the earliest stages of ontogeny might induce pathways of lymphoid cell development and differentiation which do not occur, or are just 'minor' pathways under normal ontogeny. Therefore, the observation that a process occurs in the absence or excess of a cytokine does not prove that that cytokine is not physiologically involved in that process. In this regard, experiments in which cytokines have been depleted by monoclonal anti-cytokine antibody might reflect the acute situation of the absence of that cytokine more accurately than transgenic experiments, because compensatory mechanisms might have had less time

to develop. The reverse situation is possible as well: alteration in physiological pathways observed in transgenic animals are not necessarily the direct result of the manipulation of that single cytokine. Chronic excess or deficiency of a cytokine can induce changes that are an indirect effect of that cytokine.

Although the gene knockout approach will have a very important role in advancing our knowledge of cytokines biology, I believe it will not replace more traditional approaches.

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Strategies for modulation of interleukin-1 *in vivo*: knockout and transgenics

F.F. CSAIKL,[1,2] U.M. CSAIKL[1] and S.K. DURUM[1]

[1]National Cancer Institute, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Building 560, Room 31–45, Frederick MD 21702–1202, USA
[2]Biological Carcinogenesis and Development Program, Program Resources, Inc./DynCorp, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702–1201, USA

Introduction

Interleukin-1 (IL-1) has an enormous range of reported biological activities from effects on the hematopoietic system to the reproductive system and the central nervous system (Durum and Oppenheim, 1989), an observation which provokes a number of fundamental biological questions. Which, if any, of these activities are essential for survival? Is there really redundancy of cytokines, i.e. are IL-1, tumor necrosis factor (TNF) and IL-6 interchangeable? Why are there two IL-1 genes and proteins and do they have different roles? Would chronic over-expression of IL-1 cause inflammation, or would compensatory systems (such as IL-1 receptor antagonist, or IL-1 receptor desensitization) protect the host? To address these questions, we have used transgenic and knockout mouse strategies. Compared with the other contributions to this volume, our experiments on IL-1 are preliminary and in progress; however, it is hoped that descriptions of these approaches will be of interest to readers.

The development of animal models with altered IL-1 expression allows us to explore novel questions in cytokine research. The altered regulation of an IL-1 gene within a living animal can provide valuable information on its normal biological function and may also reveal

specific pathological syndromes. It allows us to evaluate the impact of IL-1 on a variety of processes ranging from differentiation to immunological and inflammatory responses. Generation of a mouse strain with a disrupted IL-1 gene addresses the overall physiological role of this cytokine. It is also possible to create mouse strains with tissue-specific or cell-lineage-specific expression of IL-1 versus its global expression. Breeding with other established strains will allow analysis of multiple transgenes or defects. With the use of these models developmental and tissue-specific problems can be approached.

Although transgene experiments in both tissue culture and *in vivo* systems utilize similar methodologies, *in vivo* models present unique problems. For the success of both approaches, a regulatory sequence has to be combined with an mRNA coding sequence and has to be transferred to a cell, a cell line, an oocyte or a blastocyst. However, it has been found that constructs that are successful for gene expression in established cell lines do not always express *in vivo*, and/or may express in an unexpected cell type. Several possibilities may account for this problem. The complexity and the dynamics of the mammalian genome are still far from being understood. Known promoter sequences for a particular cell type may well be incomplete or minimal versions of the entire regulatory complex. Nucleosomal positioning may be changed due to integration (both random and homologous) or due to the resulting stoichiometric changes in the DNA–protein complexes, leading to unexpected results at the level of expression (Gross and Garrard, 1988; Svaren and Chalkley, 1990). One way to overcome this problem is to optimize the design of the construct. For example, inclusion of heterologous intron sequences seems to stabilize gene expression (Palmiter *et al.*, 1991). Another potential problem results from disruption of another gene (Woychik *et al.*, 1985; Beier *et al.*, 1989). In addition, so-called 'locus control regions' (Townes and Behringer, 1990) might be out of balance and thus influence the assembly of transcription complexes. Whether selection of expressing or nonexpressing cells occurs during the development of a particular animal is an issue of unknown but intriguing importance.

The gene knockout approach utilizing homologous recombination processes can be very informative, but bears its own set of problems. In contrast to transgenic animals which can give much useful information on the pathological effects of a cytokine, the physiological role of the cytokine may be determined as a result of a complete null mutation of a gene. While in transgenic experiments expression constructs are used, in gene targeting techniques the plasmids are designed to inhibit or

disrupt gene expression. By means of homologous recombination, the transfected cell replaces one copy of its gene with the modified one, thus disturbing the proper transcription of one allele. The addition of a genetic marker in the targeting vector enables the cells that integrated the construct to be selected. The major problem at this step is that homologous recombination is a rare event. The complexity of the mammalian genome and versatility of protective mechanisms, which ensure the proper duplication of DNA, guarantee the consistency of genomic propagation. Thus, the 'creation of new alleles' is generally prohibited. An additional problem arises from the integration at 'unprotected' chromosomal positions, thus high numbers of random integrations may very often conceal the few real knockouts. After the successful creation of knockout mice, the characterization of the resulting phenotype may cause additional problems: pre-existing expectations may cause the wrong questions to be asked. Genes involved in the immune system may provide a successful model, because their function is well characterized, and a functional immune system is not absolutely required for survival in an appropriate environment. The knockout approach may also help to elucidate the evolution of the system by revealing unknown functions of these genes outside of the immune system. Indeed, there is evidence for IL-1 activity in animals lacking an immune system (Beck and Habicht, 1986). This chapter outlines our progress in studies of IL-1 expression in knockout and transgenic mouse models.

The IL-1 genes

IL-1 is a cytokine with pleiotropic properties. It is composed of two distinct peptides with different isoelectric points (IL-1α, pI = 5.0; IL-1β, pI = 7.0). cDNA comparison of IL-1α versus IL-1β shows 40–45% nucleotide and 22–26% deduced amino acid homologies in both man and mouse (Durum and Oppenheim, 1989). Both polypeptides are made as precursors without a hydrophobic signal peptide. While processing of the IL-1β precursor is performed by a specific protease (Black *et al.*, 1989), no such activity was found for IL-1α. While both precursor and mature IL-1α have similar biological activity, the IL-1β precursor is at least 100-fold less active than the processed form (Mosley *et al.*, 1987; Jobling *et al.*, 1988). The results of the genomic analysis revealed common exon/intron organization, and the presence of gene duplication signatures indicate a common evolutionary origin for IL-1α and IL-1β. The two IL-1 genes are tandemly linked and localized on

mouse chromosome 2 within a 70-kb region (Silver *et al.*, 1990). Protein purification and cDNA cloning revealed the existence of a third member of the IL-1 gene family, termed the 'IL-1 receptor antagonist' (IL-1RA) showing 28% of homology to IL-1β (Eisenberg *et al.*, 1991). In contrast to both IL-1α and IL-1β the antagonist possesses a conventional leader sequence.

Both biochemical and kinetic analysis indicated the existence of more than one IL-1 receptor (Matsushima *et al.*, 1986). cDNA cloning confirmed the existence of two independent receptors with considerable differences in structure expression, and properties, termed 'type I' and 'type II' (Dower *et al.*, 1992). Currently available sequence information on the IL-1 family of genes is summarized in Table 1.1.

Experiments

The gene knockout experiment

One approach to modulating IL-1 *in vivo* is targeted disruption of the gene. The basic underlying mechanisms, approaches and problems have been described previously (Csaikl *et al.*, 1993). In order successfully to knock out a gene, cloned and sequenced mouse genomic DNA and a suitable embryonic stem (ES) cell line are necessary. The only gene in the IL-1 family currently available for this purpose is the IL-1β gene. We obtained plasmid subclones of IL-1β genomic DNA from John Telford, Sclavo, and used them to generate our targeting construct. We are using the D3 ES cell line which previously has been successfully used

Table 1.1 The IL-1 family of genes*

	Human	Rat	Bovine	Pig	Rabbit	Sheep	Chicken	Murine	
								M.m.	M.d.
IL-1α	D/R	R	R	R	R	R		R	
IL-1β	D/R	R	R	R	R	R		D/R	
IL-1 receptor (type I)	R	R					R	R	
IL-1 receptor (type II)	R							R	
IL-1RA	D/R	R			R			R	R
IL-1β convertase	R								

*Supercomputer-aided search of IL-1 related sequences: 148 citations (14 synthetic). D, genomic DNA sequences; R, mRNA sequences; M.m., *Mus musculus*; M.d., *Mus domesticus*.