Cytokine Knockouts

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Foreword

My personal history in the field of cytokines had an initial period of several years during which my student and then colleague, Werner Müller, tried in vain to attract me to them. My interest always vanished when I was confronted with complex data pointing to functional redundancy of cytokines in cell culture systems. When gene targeting in the mouse germline became possible, this frustration came to an end. We and others immediately embarked on analyzing the in vivo function of cytokines and the problem of functional redundancy with this powerful new approach. The early cytokine gene knockouts performed by colleagues in Würzburg (IL-2) and by ourselves (IL-4 and IL-10) seemed to give clear answers and at the same time led to surprises: Each of these cytokines apparently had its own special and irreplaceable function, and this function could be quite distinct from what had been anticipated from functional experiments in vitro. Although the latter finding is of course a wonderful incentive for further research, the former is pleasing in a general sense since it highlights the value of each of those one hundred thousand genes or so in our genome, cherished by evolution to become respectable members of the community. Even in the present era of "genomics" there will be no way around the careful functional analysis of each gene by itself.

At this stage, through the efforts of many groups worldwide, a large number of cytokine and cytokine receptor genes have been inactivated in the mouse germline, and the corresponding mutant mice have provided a wealth of novel information, assigning to almost all of these genes a unique function in vivo. Much of this information is assembled in *Cytokine Knockouts,* which thus marks a turning point in the history of cytokine research. Only now, because of the gene targeting approach, are we beginning to grasp the fundamental and,

to my understanding, amazingly general importance of cytokines in the control of the immune system's development from early on in ontogeny and its function in immunological defense.

Immunological research always relates to medicine. It appears natural in retrospect that cytokine deficiencies in mice often gave rise to states of disease. Because of our earlier ignorance about the true in vivo function of cytokines, these diseases were often unexpected and turned out to represent counterparts of human diseases whose origins are not yet understood. The inflammatory bowel diseases in the early IL-2 and IL-10 knockouts (resembling ulcerative colitis and Morbus Crohn, respectively) are examples of this kind. The impact of such mouse mutants on medical research is obvious.

All these new insights and developments in the field of cytokine research are amply documented in this timely book, although the reader should not expect a complete account of what has been achieved in this huge area. The book should be most useful for everybody who wants to learn about those thrilling, novel leads into the biology of the immune system and the pathogenesis of human diseases.

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Preface

The technique of gene targeting or "knockout" has swept through biomedical research of the 1990s as if it were the Occam's razor of biology. The technique provides an acid test of the function of a gene (for recent reviews of the knockout technique, *see* refs. *1* and *2*). It involves creating deletions in one designated gene in an embryonic stem cell line, and then producing mice with just one damaged gene from that cell line. The mice are then tested for physiological abnormalities. The logic is "You don't know what you got til it's gone" (Joni Mitchell). Hundreds of genes have now been knocked out and the results have changed many paradigms.

This book is a collection of reviews on the major cytokine knockouts studied to date. A cytokine is a peptide that one cell uses to signal another cell. The cytokine binds to high affinity receptors on the target cell, eliciting intracellular responses. The target cell can be in intimate contact, as in the case of a T-lymphocyte signaling a B-lymphocyte (via CD40 ligand-CD40). The target cell can also be at a considerable distance, for example, IL-1 produced in a local inflammatory site travels via the blood and triggers a reaction in the hypothalamus leading to fever. There are hundreds of such cytokines mediating inflammation, growth, differentiation, apoptosis, adhesion, and chemotaxis.

Surprises

Cytokine research has been revolutionized by knockouts. There had accumulated an enormous body of literature on the effects of cytokines, much of which fell under sharp scrutiny in the wake of research using knockouts. This began with the first cytokine knockout, IL-2, published in 1991 *(3)*. IL-2 had been discovered as a T-cell

growth factor and studied as such for over a decade: The knockout mice (Chapter 1) caused much chagrin everywhere by producing T-cells quite readily, instead of succumbing to a mysterious inflammatory process. IL-2 is indeed critical, but not for what was thought, since it appears to play a role, not in growth, but in programming CD4 cells for death.

GM-CSF was regarded as an important inducer of production of granulocytes and monocytes. The knockout results (Chapter 21) showed that this is not its vital activity, which appears instead to be activating macrophages to dispose of lung surfactant.

Lymphotoxin was studied as a cytotoxic factor, whose most likely roles would seem to be killing cells that were harboring intracellular pathogens or disposing of otherwise undesirable cells. It came as a complete surprise that lymphotoxin knockouts (Chapters 7 and 8) exhibited a lack of lymph nodes and Peyer's patches, implicating an organogenic role.

Several knockouts were lethal, demonstrating a far broader function than anticipated from their discoveries in inflammatory processes. GP130 was initially discovered as an element in the signal transducing chain for receptors of a number of inflammatory cytokines. The knockout (Chapter 16) is lethal to embryos, producing failure in heart development and severe anemia, with the mice also showing deficiencies in primordial germ cells. LIF receptor, also discovered based on its inflammatory role, is embryonically lethal (Chapter 18). $TGF\beta_3$ knockout is perinatally lethal and causes cleft palate (Chapter 19). In all these cases of knockouts leading to early death, it has been difficult to test inflammatory processes to determine cytokine roles.

Inflammatory Effects

$TGF\beta_1$ knockout (Chapters 19 and 20) revealed its vital function as a kind of "speedtrap on the inflammation superhighway." T-cells, unchecked by $TGF\beta_1$, trigger deadly cellular infiltration of vital organs. It remains perplexing just what critical process is controlled by $TGF\beta_1$—Are these self-recognizing T-cells whose self-tolerance is maintained by $TGF\beta_1$? Alternatively, do these T-cells recognize foreign antigens, then proliferate unchecked, or infiltrate

the wrong organs unchecked, because of increased integrin-mediated adhesion? Other surprising findings about $TGF\beta_1$ were its role in maintaining genome stability and the phenomenon of maternal transfer of this cytokine.

IL-2 knockout (Chapter 1), contrary to expectation, resulted in overproliferation of T-cells and lethal bowel inflammation. It is suggested that IL-2 prepares activated T-cells for apoptotic death.

IL-6 knockout (Chapters 14 and 15) substantiated its role as a key mediator of inflammation. Several features of the global inflammatory response depend on IL-6 triggered by gram-positive bacteria or turpentine injection; these include the acute phase response of the liver and the fever response of the brain. Local inflammation itself, mimicked by turpentine injection, is also IL-6 dependent.

Shock

It was expected that septic shock, induced by high doses of endotoxin, would be dependent on cytokines, based on the efficacy of blocking the cytokines with antibodies, soluble receptors, and receptor antagonists. This had prompted development of anti-IL-1 and TNF reagents to protect septic patients. However, the knockouts of IL-1b (Chapter 9), IL-1RI (Chapter 10), and TNFRp55 (Chapters 5 and 6) did not protect mice from high doses of endotoxin. The simplest explanation is that endotoxin needs no intermediary cytokine to induce endothelial cells to produce nitric oxide, but instead directly relaxes smooth muscle and thus reduces blood pressure below a critical level. Similarly, the acute phase response, which requires IL-6 when the mouse is challenged with gram-positive bacteria, is triggered by endotoxin directly acting on hepatocytes with no cytokine intermediary (Chapter 14). On the other hand, cytokines could still be essential mediators of shock if there were several different lethal combinations produced, such that removing IL-1 alone or TNF alone would not be sufficient. Support for this comes from the knockout of IFNg receptor, which reduced endotoxin shock (Chapter 13), perhaps because IFNg acts synergistically in several different lethal pathways.

ICE knockout (Chapter 11) did have a protective effect on endotoxin shock. But the ICE knockout could not be simply blocking

IL-1b processing, the property for which ICE was discovered, since IL-1b knockout does not protect from shock. ICE does affect the production of several different cytokines, perhaps giving hope to the possibility of therapeutically blocking combinations of cytokines in shock. Or ICE may affect some (as yet unknown) noncytokine function in shock; for example, it has been shown to mediate apoptotic signals, but we do not yet know how that property would be involved in shock.

A different experimental system has been used to model shock in some studies: galactosamine priming, followed by the introduction of endotoxin. This is lethal to mice because of hepatotoxicity, rather than the vascular collapse that results from high doses of endotoxin given alone. TNFRp55 knockout (Chapters 5 and 6) did protect mice from this type of shock.

Immunity

Lymphocytes are far more oblivious to cytokine knockout than anticipated from their vigorous responses to them in vitro and in vivo. A large body of observations before the knockouts had shown potent effects of cytokines on lymphocytes, but most are apparently not critical.

The interactions of T-cells with antigen-presenting cells, T-cells, and B-cells, and of T-cell precursors with thymic stroma must now be viewed as primarily cognate. The major stimuli mediating these cellular interactions do not diffuse away from the producing cells, as was once widely believed; for example, soluble cytokines were thought to be critical products of antigen-presenting cells. CD40 ligand (Chapter 4) is a cognate signaling molecule, anchored to the T-cell membrane, and required for signaling B-cells and macrophages during cell contact. Whether CD40 ligand should be termed a "cytokine" or not does not seem important, but it is interesting that its gene family contains both anchored and soluble species. IL-6 knockout did reduce IgG and mucosal IgA production (Chapters 14 and 15), and it is proposed to act as a growth factor for plasmablasts; however, it is not clear whether the relevant IL-6 derives from T-cells since activated macrophages are also a good source. Other cytokines shown to augment Ig production are LTα (IgA) (Chapter 8), IFNγ (IgG2) (Chap-

ter 13), TNFR (IgG) (Chapters 5 and 6), and IL-4 (IgE) (Chapter 1). T-cell-dependent immunity against leishmania was impaired in CD40L (Chapter 4) and IFNγ (Chapter 13) knockouts, reflecting a deficiency in TH1 type immunity.

IL-7 and its α and γ receptor components (Chapters 2 and 3) are very important for lymphoid development and would appear to be an exception to the lymphocyte's preference for immobilized signals, since IL-7 is secreted. However, IL-7 has a glycosaminoglycan binding site and most of it is probably anchored to extracellular matrix rather than being freely diffusible (Kitazawa et al., unpublished observations).

Nonlymphoid arms of the immune system, on the other hand, clearly require soluble cytokines. Defense against many types of virus requires the interferons (Chapter 13). Defense against listeria requires the activation of macrophages to kill the bacteria, and is defective in IFNγ (Chapter 13), TNF (Chapters 5 and 6), and IL-6 (Chapters 14 and 15) knockout mice. NF-IL6 (Chapter 17) knockout mice are also sensitive to listeria; their macrophages fail to lyse intracellular bacteria for unknown reasons, perhaps because a cytokine-induced gene is required. Antiviral immunity requires the interferons (Chapter 13) and IL-6 (Chapters 14 and 15). Neutrophil entry into an inflammatory site requires IL-8R (Chapter 12).

Hematopoiesis

gp130 is required to expand pluripotential stem cells in the embryo (Chapter 16); just part of this requirement is as a receptor for IL-6, which at least in the adult mouse, optimizes hematopoiesis (Chapters 14 and 15). G-CSF is required for normal levels of neutrophils (Chapter 23). A low level of neutrophils is produced in the G-CSF knockouts, suggesting that this cytokine could be required for expansion of the granulocytic precursors, but not for their differentiation. GM-CSF was surprisingly not required for hematopoiesis (Chapter 21). c-mpl is required for platelet production (Chapter 24). Eosinophil production after nematode infection required β_c (Chapter 22).

Are the numbers of blood cells actually regulated, or is production stochastic? There are examples of regulation. For example, irra-

diated mice, depleted of progenitors, increase production of all lineages. Lineage specific regulation occurs for example via hypoxia, which induces erythropoietin and hence erythropoiesis. Neutrophil production appears to increase in mice transferred from germ-free to conventional environments (Chapter 23), suggesting that immune responses or activated phagocytes produce a cytokine with this activity. Lymphoid production, if it could be selectively stimulated, would be an obvious target for AIDS therapy. Lymphopoiesis depends on IL-7 (Chapters 2 and 3). Is IL-7 production regulated, or is the number of lymphoid cells controlled at much later stages?

G-CSF heterozygous mice (with one knockout and one normal allele), show an intermediate number of hematopoietic stem cells, (Chapter 23). In other words, the magnitude of G-CSF signaling controls the vigor of the proliferative response. However, a heterozygous effect was not observed for gp130 (Chapter 6) or c-mpl (Chapter 24) knockouts, even though they are required hematopoietic factors. What does this imply about hematopoietic regulation? There seem to be two possibilities. One, that the factor is made in excess, so half the production is still more than enough. Two, that the amount of factor is limiting, but its production is regulated by demand, as in the example of erythropoietin production and hypoxia. The second possibility appears to be supported by the effects of giving exogenous factor, which increases production of the cell type, but there could be other explanations for this effect, such as recruitment of progenitors that would normally not encounter the endogenous product.

Effects of Germ-Free vs Conventional Housing

Several of the knockouts, IL-2 (Chapter 1) and TGFβ_1 (Chapters 19 and 20) show much more severe phenotypes in conventional environments than in clean ones. In these mice, normal flora or pathogens are not just replicating uncontrollably, as in immunodeficiency, but are probably inducing immune and inflammatory responses that are not controlled properly. Such uncontrolled immune responses are reminiscent of the murine strains with natural mutation in *fas*L and *fas* (reviewed in ref. *4*), and knockout of CTLA-4 *(5)*—mutations that affect the life-span of lymphoid cells.

IL-8R knockout (Chapter 12) showed increased granulopoiesis in a conventional environment, but not in a germ-free one. This suggests that a site of inflammation calls for neutrophils, and when neutrophils do not arrive, calls for more production.

CD40L knockout (Chapter 4) showed neutropenia in a conventional environment, but not in a germ-free one. This suggests that granulopoiesis can be increased by immune responses controlled by the CD40–CD40L interaction.

Background Gene Effects

The severity of phenotype is influenced by background genes in several cases. Balb/c mice die early from IL-2 knockout, C57BL/6 mice die late (Chapter 1). TGFβ_1 knockout induces colonic inflammation in 129xC57BL/6 mice versus stomach inflammation in 129xCF-1 mice (Chapters 19 and 20). This indicates that other genes can ameliorate or exacerbate these immune and inflammatory effects and has implications for the many human diseases that cannot be reduced to one simple gene.

LIFR knockout mice die at birth on a C57BL/6 background, but on a 129 background, they die at various times *in utero* (Chapter 18). gp130 knockout mice show variations in hematopoiesis among individuals, possibly because of the mixed genetic background (Chapter 16). It is indeed remarkable that biological processes as fundamental as hematopoietic development could differ between strains of mice.

Lack of Redundancy

Because cytokines have so many similar activities, it has become widely assumed that they are redundant, the idea being that T-cell proliferation, for example, is so important that several cytokines are endowed with mitogenic activity. Hence knockout of just one cytokine, perhaps IL-2 or IL-4, would not eliminate T-cell proliferation. But the double knockout of IL-2 and IL-4 also did not eliminate T-cell proliferation. Instead it now appears that T-cells do not need cytokines to proliferate. However, this is not an example of redundancy.

If cytokines were redundant, then combining knockouts of redundant cytokines would produce more than their additive effects. There is a biological precedent for redundancy among the *src*-family kinases: knockout of *src, fyn,* or *yes* alone produce subtlely varied phenotypes, but combined knockouts of *src* + *fyn* or *src* + *yes* are perinatally lethal and *fyn* + *yes* shows glomerulosclerosis *(6)*. So far, cytokine knockouts have not shown such synergistic effects. TNF and lymphotoxin (Chapters 5–8) might have been expected to be redundant, since they can use the same receptor; but their combined knockout produces a phenotype that is the sum of the two individual knockouts. Likewise for the potential redundancy of IL-1α and β, knockout of IL-1β (Chapters 9 and 10), resulted in a more subtle phenotype than many expected, so it could be presumed that IL-1α would cover for the β deficiency; but knockout of IL-1RI, which serves both IL-1α and β, produced nearly the same phenotype as the IL-1β knockout. Moreover, IL-1β knockout did not elicit a compensatory rise in IL-1α production.

Of course this does not mean that redundancies will not be observed with combined knockouts in the future. But the lesson to date is that, despite their extensive overlap in activities, each cytokine has its own unique importance.

Human Disease Models

A number of cytokine knockouts mimic human diseases. Human hyper-IgM syndrome was known to be based CD40 deficiency, which was verified by knockout of CD40L (Chapter 4). Congenital neutropenia can result from mutations in GCSF receptor, as reflected in the GCSF knockout (Chapter 23). Human X-linked severe combined immunodeficiency results from mutations in the common γ chain of cytokine receptors and a similar phenotype is observed in γ chain knockout mice (Chapter 1).

Other knockouts produce a phenotype like that for the human disease, although they are probably not the target gene in humans. NF-IL6 knockouts exhibit Castleman's disease, perhaps because of constitutively high levels of IL-6 (Chapter 17). GM-CSF knockouts exhibit alveolar proteinosis (Chapter 21). IL-2 knockouts exhibit colitis (Chapter 1).

Knockout of IL-6 implicates this cytokine in several pathogenic states (Chapters 14 and 15). The mice do not develop oil-induced plasmacytomas and are resistant to osteoporosis following ovariectomy. These findings hold great promise for the use of cytokine knockouts in general to identify their roles in pathological processes, and hence to indicate targets for therapeutic intervention.

Open Ends

Overall, the knockouts have had great impact on cytokine research because they not only answer important questions, but they raise many more. How does IL-2 program T-cells for death and does this relate to its growth effect? How can colitis appear in IL-2 deficiency, but not receptor deficiency? What are T-cells reacting to in the absences of $TGF\beta_1$—self or environment? Why is GM-CSF such a good stimulus of hematopoiesis—Is it really not used? How does LT control lymph node, Peyer's patch, and spleen follicle formation; what cells make the LT that triggers these formations and what cells respond? How does IL-6 within the brain mediate the fever response? The IL-1 system is so elaborate, containing two cytokines, α and β, IL-1β-converting enzyme, two inhibitors, IL-1RA and IL-1RII, and is a target of poxvirus, yet the knockouts have subtle phenotypes in local inflammatory responses—is this the survival value of this complex system, one that has been preserved for one hundred million years of evolution? What does ICE control that mediates shock? How do CD40L and IL-8R regulate granulopoiesis? How do $\alpha\beta$ intraepithelial lymphocytes develop normally in IL-7 knockout mice whereas all the other lymphocyte populations are blocked? What are the ligands for gp130 and LIFR that are essential for embryonic development, regulate glycogen storage, and the proliferation of hematopoietic stem cells and primordial germ cells? What genes are controlled by NF-IL6 that are required for killing listeria in phagosomes or killing tumor cells? How does IRF-1 control the numbers of CD8 T-cells? It is our hope that answers to these and many more questions regarding the physiological roles of the cytokines will be prompted by these studies.

In closing, we felt a need for a single volume that consolidated much of the cytokine knockout data. The chapters here treat some of the most important experiments conducted in cytokine research, and much is to be learned from reading, comparing, and reflecting on their results. Finally, many thanks to Paul Dolgert of Humana Press for facilitating the efforts that have led to this volume.

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