

Showcase on Research

Cytokine Signals and Target Validation in Inflammation

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The Centre for Functional Genomics and Human Disease at the Monash Institute of Reproduction and Development is dedicated to the investigation of the role of genes in the causation of, or predisposition to, certain diseases. These diseases include infections, cancer, neurodegeneration and inflammation, all being prominent features of Down syndrome. Thus this syndrome and chromosome 21 provide our primary model of genotype-phenotype correlation (1). The Centre has assigned part of its program to the CRC for Chronic Inflammatory Diseases. This includes some candidate genes whose encoded products are strongly associated with inflammation. The candidates include interferons (IFNs) and their cognate receptors (IFNARs), which have been shown to be important in macrophage development and responses to proinflammatory stimuli. Further candidates include the Ets1 and Ets2 transcription factors which are activated by proinflammatory stimuli and regulate the expression of some proinflammatory genes. Both of these classes of candidates are involved in the development and/or activation of macrophages or related lineages of cells, which is a central theme of the CRC. Furthermore, the expertise of the staff of the Centre in the use of gene targeting to establish murine models of human disease (2,3) will be extensively used in the target validation phase of the CRC program.

Interferons as central mediators of inflammatory signalling

Interferons are a family of cytokines originally discovered by their antiviral properties, and now recognised to have pleiotropic actions in host defence (4). Type I IFNs include the highly homologous 15 subtypes of α , plus β , ω and τ which are produced by many cell types - predominantly lymphocytes, macrophages, dendritic cells and fibroblasts - in response to virus and other pathogenic organisms. However,

there is also considerable evidence that type I IFNs are produced and act locally in the apparently healthy animal (5). Type II IFN γ has no amino acid identity to type I IFNs, and is produced by T cells and NK cells in response to mitogenic stimuli. The type I IFNs bind to cognate receptor components designated IFNAR1 and IFNAR2, which are distinct from the IFN γ receptors IFNGR1 and IFNGR2.

Recent studies have demonstrated that type I IFNs could be key regulators in inflammation (Fig. 1) because they are necessary for the normal development of myeloid lineage cells particularly macrophages, they are essential mediators of responses to proinflammatory stimuli and they regulate a number of genes of known importance in inflammatory responses.

We have previously generated mice with a null mutation in the *Ifnar1* component of the type IFN receptor that are highly susceptible to viral infection, but otherwise are apparently healthy. The only phenotype detected to date in unchallenged *Ifnar1*^{-/-} mice is a deficiency in the development of myeloid lineage cells in the bone marrow (6). Surprisingly, bone marrow macrophages (BMM) derived from *Ifnar1*^{-/-} mice were defective in their responses to M-CSF, LPS and TNF α . This was manifest in reduced proliferation in response to M-CSF and the lack of the antiproliferative

response to LPS and TNF α . Furthermore the activation of cyclinD2 and iNOS by LPS did not occur in *Ifnar1*^{-/-} cells, nor in the presence of IFN-neutralising antibodies (7-9). These (but not all) effects of LPS were due to the secondary production of type I IFNs (superoxide and survival are still induced/activated by LPS in *Ifnar1*^{-/-} BMM). Therefore, these data place type I IFNs in a central part of inflammatory responses and highlights the importance of considering the cytokine milieu and cytokine cross talk in our interpretation of host response to disease stimuli. Our current work will address the mechanism of crosstalk between LPS and possibly other proinflammatory stimuli and type I and II IFN responses, the nature of the difference between *Ifnar1*^{-/-} and *Ifnar2*^{-/-} mice and the susceptibility of *Ifnar*^{-/-} mice to models of inflammation such as rheumatoid arthritis and chronic obstructive pulmonary disease (COPD), established at other nodes of the CRC.

The Ets transcription factors and inflammation

The Ets-transcription factors are winged helix-loop-helix proteins that bind DNA through interaction with both the major groove and the minor groove leading to local bending of the DNA helix. Ets-2, like other Ets transcription factors, binds to promoter elements containing

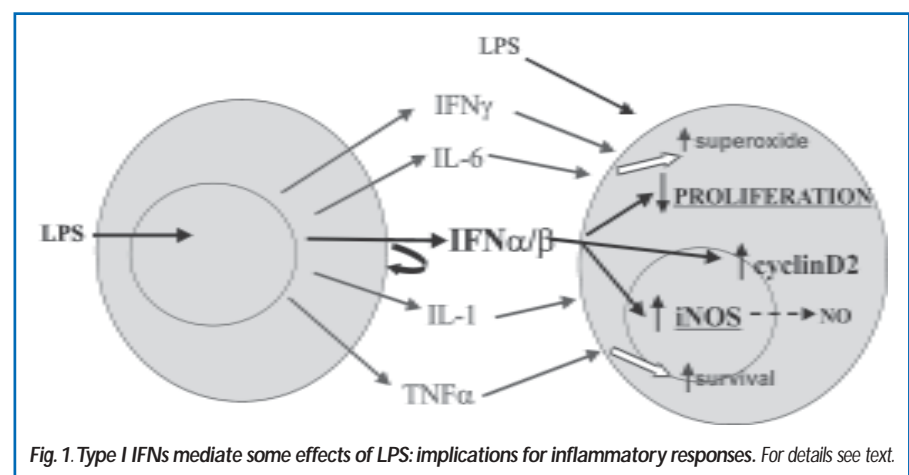


Fig. 1. Type I IFNs mediate some effects of LPS: implications for inflammatory responses. For details see text.

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nucleotide sequences with a GGA(A)/(T) core (Ets Binding Site). Ets-2 is widely and dynamically expressed throughout murine embryonic development being highly expressed in developing limb buds, the distal tail, in developing bone, tooth buds, epithelial layers of the gut, nasal sinus and uterus, developing lung, adult lymphoid tissues, skin and several regions of the developing and adult brain (10). Scientists at the Centre have been working for many years on the contribution of Ets2 to the pathophysiological features of Down syndrome, cancer and apoptosis. Indeed, transgenic mice overexpressing Ets2 provide a partial phenocopy of features of Down syndrome, in particular the abnormalities of bone formation (11).

There is evidence for a role for Ets-2 in the survival and differentiation of macrophages. Colony-stimulating factor 1 (M-CSF) is one of the major cytokines that regulates the survival, proliferation, and differentiation of macrophages and is required to kill neoplastic cells or microorganisms. This activation by CSF-1 is accompanied by increased expression of Bcl-X_L and Ets-2 (12). In response to CSF-1 the protein (MAP) kinases p42 and p44 were activated and found to phosphorylate Ets-2 on thr72, resulting in increased stability of the protein. Conversely, depriving primary bone marrow-derived macrophages of CSF-1 induces programmed cell death by apoptosis and decreased expression of anti-apoptotic Bcl-X_L protein and Ets-2. Thus, persistent activation of the Ras/Raf/MAP kinase pathway by CSF-1 is necessary for post-translational modification of Ets-2 and its stabilisation in macrophage lines. Ets-2 is upregulated in mature macrophages by pro-inflammatory stimuli such as LPS and oxidative stress (13,14). In addition to its upregulation by proinflammatory stimuli, interest in the role of Ets-2 in inflammation also centres on its target genes such as u-PA, stromelysin 1, collagenase 1 and p53 (15). Furthermore, Ets-2 is reportedly increased in human inflammatory diseases including, shear stress in synovocytes and RA patients (16,17).

Attempts to generate Ets-2 null mice to validate the role of this gene in disease have demonstrated that the null

phenotype is fetal lethal (Wilson, T.J. *et al.*, unpublished work). A targeted mutation in Ets-2 was reported to generate fetal lethality due to a defect in trophoblast cell migration into the endometrium (18). However, this mutant Ets-2 allele produces a truncated protein, which could either have a partial function or act as a dominant negative. Therefore the phenotype of this mouse line is difficult to interpret with respect to the *in vivo* function of Ets-2. We have generated conditionally targeted Ets-2 +/- and -/- ES cells, in the process of generating conditionally targeted mice. These will be able to be mated with transgenic mice expressing the Cre enzyme regulated by either inducible, macrophage-specific (e.g. c-fms) or T cell (e.g. CD2)-specific promoters. We will use these model systems to validate the role of Ets-2 in the development and function of macrophage lineage cells and in models of inflammation.

Ets-1 is the Ets family member with the highest amino acid identity to Ets-2. Like all Ets factors, the homology in the DNA binding domain is 97% and overall is 76%. Despite the similarity in structure, Ets-1 appears to have different functions to Ets-2. Ets-1 expression in adults is more restricted to lymphoid tissues; it is highly expressed in during angiogenesis in embryonic development and in wound healing (15). Ets-1 is also expressed by activated mast cells (19). Like Ets-2, Ets-1 can be activated via the MAP kinase pathway in response to UV light or proinflammatory stimuli such as LPS or TNF α (20). Ets-1 also regulates the expression of downstream target genes that are important in macrophage development/action like GM-CSF (21). Interestingly, Ets-1 synergises with another proinflammatory transcription factor, NF κ B in the transactivation of the GM-CSF promoter (21). There appears to be an autocrine loop with respect to proinflammatory factors, whereby Ets-1 is regulated by, and also regulates, the expression of TNF α . Ets-1 also has been shown to regulate the expression of other important macrophage stimulating and proinflammatory proteins including, collagenase, stromelysin and u-PA and VEGF-R (15).

Like many Ets factors, the null mutation in Ets-1 is fetal lethal (22; and

Xu, D., Wilson, T.J., and Hertzog, P.J. *et al.* unpublished work). We have generated Ets-1 null ES cells to study the consequences of this mutation on signalling and to examine the impact on the *in vitro* development of macrophages and their responses. Currently, we are generating conditionally targeted mice in order to investigate the role of Ets-1 in murine models of inflammatory disease.

Inflammation and Down syndrome

Down syndrome (DS) is caused by an extra copy of chromosome 21. Overexpression of some or all of the genes associated with this supernumerary chromosome are thought to contribute to the pathophysiological features of this syndrome. These include skeletal abnormalities, cardiac and neurodegenerative disease, and predisposition to inflammatory disease. DS is associated with an increased incidence of joint hypermobility, a number of immunological abnormalities and an increased incidence of autoimmune phenomena such as arthritis. Individuals with DS have a high incidence of cervical spine instability marked by degenerative changes and 8 – 28% have hip disease caused by severe arthritis (23,24).

The pathophysiological features of DS also occur in the general population only in DS they occur at higher frequency and earlier onset. Therefore understanding the genes of human chromosome 21 (HSA21) [HSA is for *Homo sapiens*] that contribute to the DS features is important not only to understanding the genetic basis of DS but also to establishing principles or providing candidate genes for these diseases in the general population.

The work of the Centre is focused on HSA21 genes that contribute to immunological changes, altered predisposition to cancer and neurodegeneration; there are also candidate genes that are relevant to chronic inflammation. From the discussion above, it is clear that the HSA21 genes, *lfnar1*, *lfnar2* and *Ets-2* are important both in the development of macrophages and in their responses to proinflammatory stimuli. In addition there is evidence that other HSA21 genes

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might contribute to pro-inflammation tendencies in DS. One example is ADAMTS1, an aggrecanase that cleaves versican, a component of skin. Overexpression of this gene might contribute to the distribution of collagen VI that is different in DS fetal skin compared to normal; DS is associated with a number of cutaneous abnormalities, e.g. dermatitis, elastosis, perforans serpiginoza and skin infections (25).

HSA21 also encodes the superoxide dismutase 1 (SOD1) gene that is an important antioxidant gene. SOD1 catalyses the detoxification of superoxide radicals to hydrogen peroxide; H_2O_2 is then detoxified further to water by glutathione peroxidase (GpX) or catalase. Since catalase is expressed at low levels in some organs like brain, the ratio of SOD:GpX is an important determinant of susceptibility to oxidative damage (26). Over expression of SOD1 as occurs in DS predisposes to neurodegeneration and ageing-related damage presumably due to an accumulation of H_2O_2 . Similarly, the GpX $-/-$ mice that we generated are susceptible to systemic oxidative damage as well as brain-specific ischaemic injury (27,28). These redox-sensitive genes such as SOD and GpX are also important in regulating inflammatory responses as signalling through NF κ B is redox-sensitive.

Validation of candidate genes by gene targeting

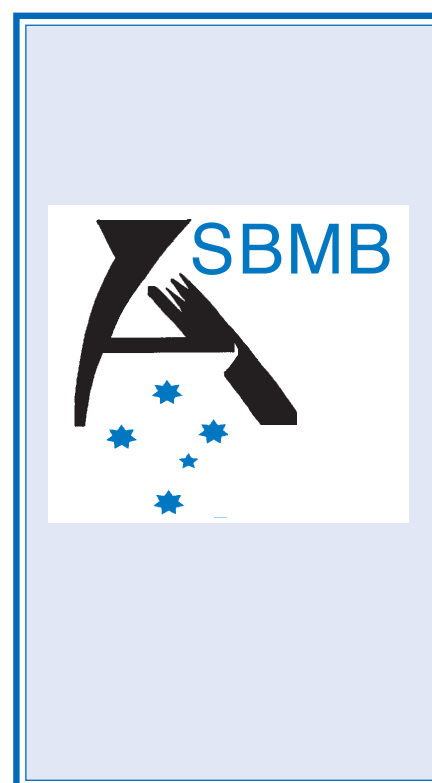
As described elsewhere (see article by David Hume), there is a comprehensive program in the CRC involving work with human disease tissues and animal models of inflammatory disease to identify genes that are candidates for having a central role in disease causation and therefore presenting as targets for therapeutic intervention. An integral part of the gene target validation will be to generate gene targeted mouse models, either mice with null mutations or overexpression transgenic mice. Not only is this a powerful means of establishing *in vivo* function, but it also can be an important aspect of establishing safety (2,3,6,29). For example, if generating a null mutation in every cell in the body results in a

healthy animal, then it is likely that targeting the gene product by other strategies, such as by small molecule inhibitors or inhibitory antibodies, will not have adverse side-effects. This principle was evident in the mice we generated with a null mutation in the Cathepsin K gene, which provided a validation of targeting this enzyme in bone disease (29). The Centre has extensive experience in the generation of gene targeted mice (1-3, 6,11,29) and has recently established a company to provide this service, called IngenKO, which is part owned by Monash University, to incorporate additional expertise and resources in this area.

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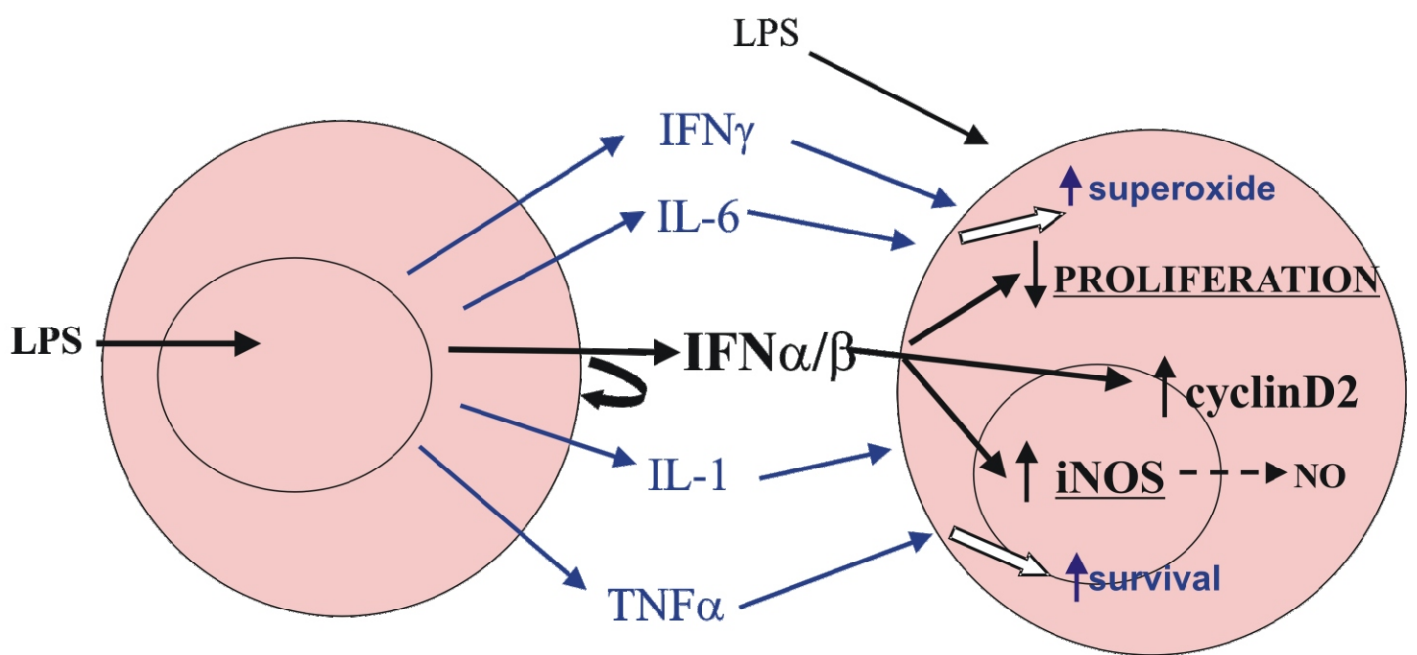


Fig. 1