REPORT

Is NF- κ B a useful therapeutic target in rheumatoid arthritis?

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There is increasing evidence that NF- κ B is a major, if not the major transcription factor regulating inflammation and immunity. While this implies that blocking NF- κ B might be therapeutically beneficial, it raises clear questions regarding the balance between efficacy and safety. In this brief review we discuss the effects of NF- κ B blockade in rheumatoid arthritis, inflammation and immunity, and consider possible therapeutic targets within the NF- κ B family.

WHAT IS A GOOD THERAPEUTIC TARGET?

OPERATIONAL:	Efficacious AND safe
PREDICTABLE?	Rate limiting step Big difference between health and disease
REALITY:	Needs confirmation by clinical trial

Figure 2 Requirements for a good therapeutic target.

NF- κ B is a family of transcription factors central to immunity and inflammation.¹ NF- κ B molecules exist as homo- or heterodimeric complexes formed by combinations of five distinct DNA binding subunits, p50/RelA, RelB, c-Rel, p50, and p52, and are, under most circumstances, found in the cytosol bound to I κ B proteins. However, in response to various stimuli that include physical and chemical stress, viral and microbial products (for example, lipopolysaccharide (LPS)), and inflammatory cytokines (for example, interleukin (IL)1 and tumour necrosis factor (TNF α)), I κ B proteins are rapidly phosphorylated, ubiquitinated, and degraded, freeing NF- κ B to translocate rapidly into the nucleus to regulate gene expression (fig 1).² I κ B kinases are central to that process as they regulate I κ B phosphorylation.

In rheumatoid arthritis (RA) synovium the activation of NF- κ B has been detected using immunohistology, for example, with antibodies that detect NF- κ B translocation in the nucleus, where it needs to be for functional effects.³ Furthermore, antibodies which bind to the sites on NF- κ B subunits

hidden by I κ B have also been used and also help functionally to demonstrate its activation in synovium in vivo.⁴ However, these studies are subject to possible artefacts. The biopsy procedure is undoubtedly stressful, and in itself might induce NF- κ B. As this translocates to the nucleus within two minutes, it is difficult to guard against artefact. Notwithstanding this possible cause of artefact, the presence of NF- κ B need not imply that NF- κ B is a rate limiting step. That requires further functional analysis.

In this paper, we will review the evidence suggesting that NF- κ B might be an interesting target. This will focus on mechanisms which predict efficacy; the available data preclude any detailed discussion of potential safety issues (fig 2).

MATERIALS AND METHODS

Adenoviral vectors

Recombinant, replication deficient, adenoviral vectors encoding *E coli* β -galactosidase (Adv β Gal) or having no insert (Adv0) were provided by Drs A Byrnes and M Wood (Oxford, UK). An adenovirus encoding porcine I κ B α with a cytomegalovirus promoter and a nuclear localisation sequence (AdvI κ B α) was provided by Dr R de Martin (Vienna, Austria). Viruses were propagated and titred as previously described.⁵

Infection of cells from synovium

Synovium from rheumatoid patients undergoing joint replacement surgery was dissociated by digestion with collagenase and DNase. The total mixture of cells, with T cells, macrophages, and fibroblasts as the most abundant cells, was resuspended in serum-free RPMI at 1×10^6 cells/ml and immediately infected by recombinant adenoviruses at a multiplicity

Figure 1 Schematic diagram of NF- κ B activation.

Abbreviations: APC, antigen presenting cell; DC, dendritic cells; ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte macrophage colony stimulating factor; IKK2, I κ B kinase 2; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; m.o.i., multiplicity of infection; PBS, phosphate buffered saline; PSI, proteasome inhibitor; RA, rheumatoid arthritis; [3 H]TdR, [3 H]thymidine; TIMP, tissue inhibitor of matrix metalloproteinase; TNF α , tumour necrosis factor α .

Figure 3. TNF α production in rheumatoid synovial cell cultures is NF- κ B dependent. (A) More than 90% of rheumatoid synovial cells can be infected with replication deficient adenoviruses at an m.o.i. of 40 as shown by measuring β -galactosidase activity by FACS. Rheumatoid T cells, macrophage-like and fibroblast-like cells are all efficiently infected. (B) Rheumatoid synovial cells were infected with an adenovirus overexpressing I κ B α (AdI κ B α) or a control adenovirus without insert (AdV0) at an m.o.i. of 40. Supernatants were collected after 48–72 hours and examined for TNF α production by ELISA.

of infection (m.o.i.) of 40. After two hours, the adenovirus-containing RPMI was removed and fresh RPMI containing 5% fetal calf serum and 1% penicillin/streptomycin was added. After 48–72 hours supernatants were collected and assayed by enzyme linked immunosorbent assay (ELISA) for the presence of cytokines and matrix metalloproteinases.

Dendritic cell maturation

Mononuclear cells were isolated from single donor platelet-phoresis residues as described. Immature dendritic cells were generated from purified monocytes cultured at 10⁶ cells/ml with granulocyte macrophage colony stimulating factor (GM-CSF) and IL4 in 100 mm Petri dishes. On day 6, non-adherent cells were collected and analysed, or transferred to new Petri dishes. The cultures were supplemented with monocyte conditioned medium and TNF α at final concentrations of 20% vol/vol and 10 ng/ml, respectively. Fresh GM-CSF and IL4 were present throughout the culture period. In some experiments, cells were washed out of supplemented cytokine or monocyte conditioned medium at day 6 or day 9 before use in phenotypic or functional assays.

Analysis of dendritic cell infectability

All samples were analysed on a FACScan flow cytometer using the CellQuest software (Becton Dickinson, San Jose, CA). Analysis was carried out on a population of cells gated by forward and side scatter to exclude dead cells and debris.

Dendritic cell surface markers were studied using monoclonal antibodies to HLA-DR, CD86, and CD25 (phycoerythrin conjugated; Pharmingen), HLA-DQ (FITC conjugated; Pharmingen), CD80 (phycoerythrin conjugated; Pharmingen).

RESULTS

Adenovirus encoding I κ B α enables definition of many NF- κ B dependent activities in rheumatoid synovial tissue

We noted that adenoviruses can infect the great majority (>90%) of rheumatoid synovial cells, even the T lymphocytes, which are usually hard to infect (fig 3).⁶ This makes it possible to ask directly what mediators depend on NF- κ B by the adenoviral technique. The merits and uses of this technique were discussed in detail in a previous supplement of this series.⁷ The proinflammatory cytokines, in general, are inhibited by NF- κ B blockade. This is perhaps not surprising. What is of interest is that the degree of inhibition is variable, ranging from about 90% for IL6, to 30–40% for IL1, to very small or no effects for CXC chemokines (fig 4).^{6,8,9} Although these results are open to many interpretations, clinically they suggest that NF- κ B blockade will have a broad anti-inflammatory effect. At the more speculative end of the scale, these data imply that there are other key transcription factors. Even more hypothetical is the insight this may yield as to why there is a need for multiple proinflammatory mediators, which, when generated, have very similar biological

Figure 4 IkB α overexpression selectively inhibits cytokine expression in RA joint cultures. It blocks proinflammatory but not anti-inflammatory cytokines and mediators (IL10, IL1RA, sTNFR, IL11).

effects—for example, TNF α , IL1 α , IL1 β , GM-CSF. In our opinion, this is because their production is regulated differently.

A concept we have elaborated is that it is not only the level of proinflammatory cytokines that needs consideration, it is also the ratio of proinflammatory/anti-inflammatory mediators. These anti-inflammatory mediators include IL10, IL1RA, sTNFR, IL11, IL4, IL13. So a relevant question is the effects of AdvIkB α on anti-inflammatory mediators (fig 4). There is a modest effect on sTNFR, minimal on IL1RA, and not on IL10 or IL11.¹⁰ IL4 and IL13 were not assessed as they are rarely or variably expressed in RA synovium. Thus, NF- κ B blockade will have a major impact in the pro/anti-inflammatory balance, as the proinflammatory but not the anti-inflammatory mediators are reduced (fig 4).

The effect of NF- κ B blockade on matrix metalloproteinases (MMPs) was also assessed by the same approach. This can be done on various cells of the joint. Figure 5 shows the results on synovial tissue. Again the effect would be beneficial, as there is reduction of MMP-1 and MMP-3, but not their inhibitor, TIMP-1.¹⁰ Effects on angiogenic mediators are under analysis.

Effects of NF- κ B inhibition on the immune response

NF- κ B was first described as its name suggests, in B lymphocytes.¹¹ Thus its importance on the immune system is not unexpected. We have used the adenoviral system to probe the function of human dendritic cells, the cells that initiate immune responses, and then confirmed the results using the drug PSI (proteasome inhibitor), which covalently binds to and hence blocks proteasome function and subsequently blocks dendritic cell function. We have reported elsewhere that DC are infected effectively by adenoviruses, with up to 100% expressing marker genes such as β -gal (fig 6).¹²

Adenoviruses encoding IkB α interfere with antigen presentation in the mixed lymphocyte response

The mixed lymphocyte response is accepted as an in vitro model of a strong primary response, as seen in allotransplantation. It is much more convenient to study using human cells than other T cell responses. Thus it was chosen to evaluate DC antigen presentation in view of the fact that DC are the most powerful cells to induce a mixed lymphocyte response. As few as 30 DC can induce a measurable response from 10⁵ T cells (fig 7).¹²

Figure 7 shows the results of a comparison of AdvIkB α and Adv0, with normal uninfected DC. This demonstrates the dramatic effect of down regulating DC function by AdvIkB α . This

may be surprising if one believes that adenoviruses are highly proinflammatory and, if so, it might be suspected that at least Adv0 should have activated the DC. The dramatic diminution of DC function by AdvIkB α suggests that the staining results, which indicate that >90% of cells are infected, are probably correct.¹² Furthermore, they show that the NF- κ B family of transcription factors controls the antigen presenting function of DC. Which of these subunits is critical to DC function cannot be determined as a large excess of IkB α would inhibit them all. As 100 normal or Adv0 infected DC were highly active in the mixed lymphocyte response, the fact that 10⁴ DC infected with AdvIkB α were not stimulatory was interesting, demonstrating a profound immune inhibition.

Figure 5 IkB α down regulates the production of MMP-1 and MMP-3 but not TIMP-1. Rheumatoid synovial cells were infected with an adenovirus overexpressing IkB α (AdvIkB α) or a control adenovirus without insert (Adv0) at an m.o.i. of 40. Supernatants were collected after 48–72 hours and examined by ELISA for the presence of MMPs and TIMP-1.

Figure 6 Dendritic cells take up adenovirus effectively. (A) Mature dendritic cells were generated by five days' culture in 50 ng/ml GM-CSF and 10 ng/ml IL4 and a further three days in monocyte conditioned medium. Then they were plated on a 96 well, flat bottom plate at a density of 2×10^5 cells/well and left either uninfected with an m.o.i. ranging from 40 to 500 of an adenovirus without insert (Adv0) or an adenovirus overexpressing β -Gal expression. Fluorescein-di-(β -D)-galactopyranoside (Sigma) was used as a substrate of β -galactosidase and cell fluorescence was analysed by FACS analysis. (B) and (C) 10×10^6 cells for each condition were left either uninfected or infected with Adv0 or AdvIkBα at an m.o.i. of 300. Two days after infection, cells were left unstimulated or stimulated with LPS (50 ng/ml) for 60 minutes. Cytosolic and nuclear extracts were then prepared and examined for IkBα or p42/44MAPK (loading control) expression by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (B), or for NF- κ B DNA binding activity by electrophoretic mobility shift assay (C).

Mechanisms of AdvIkBα inhibition of DC function

The marked efficiency of inhibition by AdvIkBα made it imperative to understand the mechanisms by which it was mediated. As several sets of molecular mechanisms play a part in the antigen presenting function, these were all examined.

Peptides bound to the major histocompatibility complex (MHC) are the target of recognition by the T cell receptor for

Figure 7 AdvIkBα inhibits the mixed lymphocyte reaction. Mature DC were left uninfected, infected with Adv0, or infected with AdvIkBα at an m.o.i. of 300. DC were then plated in graded doses for 10^5 purified, allogeneic T cells in triplicate in a 96 cell, round bottom microtitre plate on day 1 after adenovirus infection. Proliferation was determined on day six by a $[^3\text{H}]\text{thymidine}$ ($[^3\text{H}]\text{TdR}$) uptake assay. Each point represents the mean (SEM) of three separate experiments.

antigen. Hence, the degree of MHC expression has long been a major factor in antigen presenting cell (APC) function. By flow cytometry, it was shown that the HLA class II antigens, HLA-DR and HLA-DQ, were each down regulated about five-fold (fig 8).¹²

The costimulatory ligands for the CD28 surface receptors on T cells, CD80 and CD86, are important in lowering the threshold for T cell activation. Therefore, their degree of expression is also critical to the stimulatory ability of DC and their expression is up regulated during DC maturation. AdvIkBα reduced CD80 and CD86 expression 5–10-fold, a degree which would be expected to reduce T cell response significantly (fig 8).¹² Cytokines are important in T cell activation, and one—namely, IL12, is particularly important for activating T helper 1 T cells, the T cells that make most of the IL2, and, therefore, contribute in a major way to T cell proliferation. IL12 production was, essentially, abolished by AdvIkBα (fig 8).

There are reports that blocking NF- κ B predisposes certain cells to apoptosis. One possibility was that AdvIkBα infected cells are dying, and, as a result, non-stimulatory. This has been excluded by both direct and indirect assays. Firstly, no apoptosis was demonstrable in these cells over a 48 hour period, in which most of the APC function would be manifest. Secondly, certain functions are up regulated in AdvIkBα infected cells, such as the expression of adhesion molecules ICAM-1 and LFA-1.¹²

Taken together the multiple partial effects on the molecular mechanisms of APC function probably account for the marked loss of APC function. However, other mechanisms are also likely to contribute, as discussed below.

We have extended these studies using the drug PSI. This confirmed that NF- κ B blockade by whatever means profoundly inhibited DC function (fig 9). Obviously, protease inhibition may influence other functions as well as inhibit NF- κ B.

In the light of the profound inhibition of the mixed lymphocyte reaction, we were interested in evaluating whether the blockade of NF- κ B might also be due to immunological tolerance. T cells exposed to DC inhibited by NF- κ B progressed directly into immunological tolerance. This was established by using PSI, a much less cumbersome tool for this purpose than adenovirus.

The initial experiment used purified T cells from one donor, and DC from a different donor, in a mixed lymphocyte

Figure 8 AdvikB α inhibits three critical components of antigen presentation. The effect of AdvikB α infection on dendritic cell antigen presenting and costimulatory molecules as well as the costimulatory cytokine IL12 was studied. IkB α was overexpressed in mature DC for two days and then cells were collected and stained by FACS for HLA (A) or CD80/86 (B), or stimulated by soluble CD40L for 24 hours, and then IL12 production was assayed by ELISA (C).

reaction. The DC were either treated with PSI or with phosphate buffered saline (PBS) for four hours, washed and then co-cultured for 48 hours with T cells. The DC were removed by panning using anti-CD83 and anti-CD86, and the T cells were challenged with DC either pretreated with PSI or PBS for a four day challenge. Tolerant T cells would be those that cannot respond to challenge with fresh DC. This was indeed the case, two days' treatment of DC with PSI prevented subsequent response to fresh DC of the same donor (fig 9).¹³

Figure 9 PSI pretreatment inhibits DC antigen presentation. DC were pretreated with vehicle or PSI (0.1 μ M, 0.5 μ M, 1 μ M) for four hours. After washing, graded numbers from these four batches of DC were plated with 105 allo-lymphocytes on a 96 well plate. Proliferation was determined on day 6 using the [³H]Tdr uptake assay. Each point represents the mean (SEM) of five separate experiments.

Immunological tolerance has a number of different subtypes. An essential difference is whether T cells are still alive or dead. The status of T cells was examined in these cultures. They were not dead, and hence this is a form of tolerance known as clonal anergy.

DISCUSSION

In our view, NF- κ B is a good therapeutic target, but the details of how to block it most effectively remain to be ascertained. The data we have generated in human systems support the work performed in rat models of rheumatoid arthritis where adenoviruses blocking NF- κ B have had beneficial effects.¹⁴⁻¹⁵

The discovery of the IkB kinase 2 (IKK2) by Goeddel, Karin, Mercurio and their groups a few years ago has had a major impact on the way in which pharmaceutical companies are approaching NF- κ B inhibition, chiefly by inhibiting IKK2.¹⁶⁻¹⁹ We have started to look at this in the adenoviral system by making viruses encoding mutants that block IKK2 activity.²⁰⁻²¹ However, there are many routes, some independent of IKK2, that lead to NF- κ B activation, so which of these are essential in RA remains to be ascertained.

The effect of NF- κ B inhibition on the immune response also supports the possibility that NF- κ B is a good target for treatment of RA. There is evidence that T cells play a part in the initiation and perpetuation of rheumatoid synovitis. There is also much belief that DC are a crucial part of the rheumatoid inflammatory pathway, as described in the work of Thomas and Lipsky.²²⁻²³ Hence it is of interest to unravel what controls DC activation. Perhaps not surprisingly, in view of the ancestral role of NF- κ B in haematopoietic activation, it was found that NF- κ B is of major importance. Blocking NF- κ B abrogates APC function, if performed by adenovirus over expressing IkB α or by PSI. What is interesting is the mechanism of this

POTENTIAL PITFALLS OF NF- κ B INHIBITION

1. **Abrogation of useful NF- κ B**
 - apoptosis of liver, possibly CNS
 - immunosuppression and infection: important in T, B, DC, macrophages
2. **Is target chosen appropriate?**
 - IKK2 not involved in all steps
3. **Specificity** – if block proteasome, not specific

Figure 10 Potential pitfalls of NF- κ B inhibition.

immune inhibition. At least four different aspects of APC function were found to be down regulated (figs 8 and 9).

Thus, there was inhibition of the target of T cell receptor recognition of MHC class I and class II, inhibition of expression of costimulatory molecules (CD80 and 86, CD40), inhibition of cytokine (for example, IL12) and chemokine production.

What was not expected was that immunological tolerance would result from the interaction of T cells with NF- κ B inhibited DC.

Overall, this provides a powerful rationale for NF- κ B blockade, in the synovium, as a therapeutic target. The problem is how to deliver it locally. Systemic NF- κ B blockade has potential problems (fig 10). Gene therapy is one approach, but it is arguable that it is a viable one at the present time.

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