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Review

NF-κB and the regulation of hematopoiesis

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Abstract

This review will focus on the role of nuclear factor κB (NF-κB) signaling in hematopoietic differentiation. We will also discuss several hematopoietic pathologies associated with deregulation of NF-κB and their potential therapies.
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Abbreviations: BAFF, B cells activation factor; BAFF-R, B cells activation factor receptor; BCR, B Cell Receptor; bm-PMN, bone marrow polymorphonuclear neutrophilic granulocytes; CFU-GEMM, granulocyte/erythroid/monocyte/macrophage colony-forming units; DC, dendritic cell; DN, dominant negative; FTOC, fetal thymic organ cultures; GM-CSF, granulocyte–macrophage colony-stimulating factor; GVHD, graft-*versus*-host disease; HED-ID, hypohidrotic ectodermal dysplasia with immune deficiency; IFN, interferon; Ig, immunoglobuline; IKK, IκB kinase; IL, interleukin; IκB, inhibitor of κB; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; MY, metamyelocyte; MHC, major histocompatibility; NBD, Nemo binding domain; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; NIK, NF-κB inducing kinase; NK cells, natural killer cells; NKT, natural killer-like T cells; NOD, non-obese diabetic; PAI-2, plasminogen activator inhibitor 2; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol myristate acetate; PM, promyelocyte; RA, rheumatoid arthritis; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TAK1, transforming-growth-factor β-activated kinase 1; TCR, T-cell receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant acid phosphatase

Rel/NF-κB transcription factors exert their function as homo- and heterodimers. The nuclear factor κB (NF-κB) family consists of five members (c-Rel, p65/RelA, RelB, NF-κB1/p105, which is processed into p50, and NF-κB2/p100, which is processed into p52). Whereas p65 and p50 are ubiquitous proteins, other members of the family are expressed in specific cell types. c-Rel expression is confined to lymphocytes, monocytes, granulocytes and erythroid cells, whereas RelB is predominantly expressed in dendritic cells (DCs) and lymphocytes. p52 is expressed in the stomach epithelium, DCs, macrophages and lymphocytes. The fact that specific NF-κB proteins are predominantly expressed in hematopoietic cell lineages emphasizes their pivotal role during immune functions.

In resting cells, NF-κB dimers (the p65/p50 heterodimer is the archetypical heterodimer activated by the classical pathway) are retained in the cytoplasm by interaction with inhibitory molecules of the IκB family (IκBα is the predominant inhibitor of the p65/p50 heterodimer). Upon induction of the pathway, the IκB proteins are phosphorylated by the inhibitor of κB (IκB) kinase-complex (IKK-complex). The IKK-complex is a central multiprotein regulator that controls the activation of the classical NF-κB pathway. The best-characterized components of this complex are the two kinases IKK1 and IKK2 as well as the regulatory components NEMO (NF-κB essential modulator) and ELKS.[1,2] Phosphorylation of IκB proteins induces their polyubiquitination, leading to their degradation by the proteasome. NF-κB dimers, released from their inhibitor, can then translocate to the nucleus, where they bind their DNA responsive elements, leading to the activation of a plethora of target genes. Several tumor necrosis factor (TNF) receptor family members (LTβR, BAFF-R, CD40, RANK, p75TNFR, HVEM, CD27, CD30, 4–1BB, GITR, BCMA, OX40, TACI) have been shown to be able to activate the alternative NF-κB signaling pathway leading to the formation of p52/RelB dimers. The specific expression of these receptors on lymphocytes suggests an important role for the alternative pathway in B- and T-cell development or activation. The alternative pathway activates IKK1 homodimers via the NF-κB-inducing kinase (NIK), followed by phosphorylation of p100, which is subsequently polyubiquitinated and processed into p52. This review will focus on the role of NF-κB signaling in hematopoietic differentiation (Figure 1). We will also discuss several hematopoietic pathologies associated with deregulation of NF-κB and their potential therapies.

NF-κB Signaling within the Myeloid Lineage

Mice deficient for IκBα show an increase of granulocyte/erythroid/monocyte/macrophage colony-forming units (CFU-GEMM), suggesting that the classical NF-κB pathway contributes to the differentiation of these lineages.[3] Moreover, deletion of both cRel and p65 affects common myeloid early progenitors.[3] These mice present several hematopoietic defects including a reduction of spleen colony-forming unit progenitors. Granulocyte–macrophage colony-stimulating factor (GM-CSF) interacts with its receptor to induce the differentiation of a myeloid progenitor into granulocytes and macrophages. Interestingly, it has been shown that IKK2

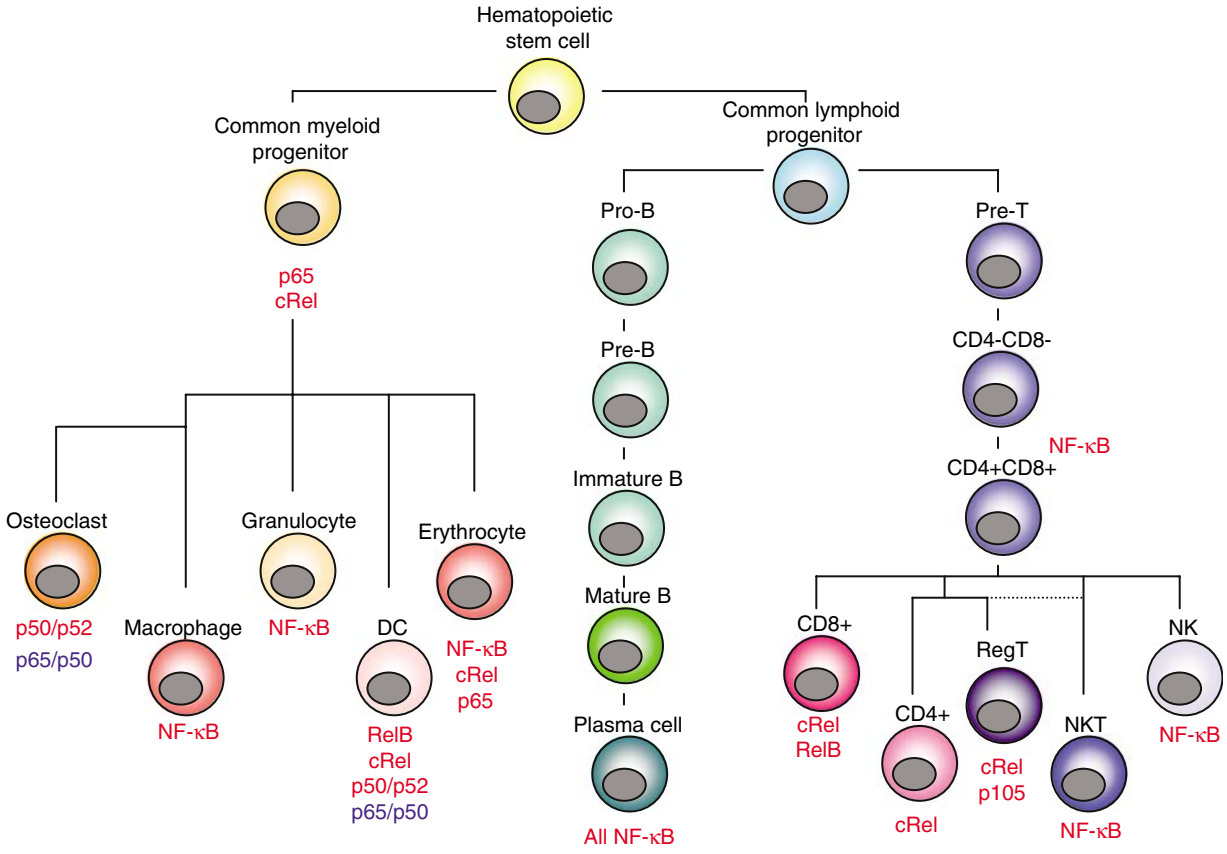

Figure 1 NF-κB in hematopoietic differentiation. The tissue specificity of the expression of NF-κB member underlines the important of this transcription factor in hematopoiesis. Whereas p65 and p50 are ubiquitous proteins, other members of the family are expressed in specific cell types. c-Rel expression is confined to lymphocytes, monocytes, granulocytes and erythroid cells, RelB is predominantly expressed in dendritic cells (DCs) and lymphocytes, and p52 is expressed in the stomach epithelium and also in DCs, macrophages and lymphocytes. Several mouse models allowed the characterization of which NF-κB members were predominantly involved in the different steps. In the figure, NF-κB indicates that the specific member is not yet identified. For granulocytes, osteoclasts, B and T cell lineages, more precise explanations are present in the following figures

interacts with the α-chain of the GM-CSF receptor during this process.[4] NF-κB has been shown to be involved in the differentiation and activation of macrophages, granulocytes, osteoclasts, DCs and erythrocytes.

NF-κB and macrophage activity

Macrophages are particularly important in establishing innate immunity. Upon microbial infection, these cells phagocytose cell debris and produce hydrolytic and proteolytic enzymes as well as cytokines and growth factors. Engagement of microbial molecules with Toll-like receptors (TLRs) leads to the activation of NF-κB to induce expression of proinflammatory cytokines, chemokines and antiapoptotic proteins. Macrophages also present antigen to activated T cells and thus contribute to part of adaptive immunity.

Whereas no NF-κB activity can be detected in monocyte precursors, their differentiation into macrophages induced by phorbol myristate acetate (PMA) is correlated with IKK activation and subsequent NF-κB activity.[5] During this process, NF-κB protects the cells from apoptosis. TNF receptor-associated factor 6 (TRAF6)-deficient macrophages showed a decreased NF-κB activity concomitant with an increased apoptotic response. The authors proposed that p21 (Waf1/Cip1) is an important NF-κB target gene involved in survival. In accord with this finding, macrophages deficient in IKK2 were deficient in apoptosis.[6] Another antiapoptotic gene involved in NF-κB-induced survival in macrophages is the bcl-2 family protein Bfl1/A1. More recently, it has been shown that transcription of the serpin plasminogen activator inhibitor 2 (PAI-2) is induced by the cooperation of both CREB and NF-κB.[7]

A new function of IKK1 has been identified in macrophages. Two different groups showed that whereas IKK2 is involved in NF-κB activation in macrophages, IKK1 actually inhibits NF-κB activation in these cells. Lack of IKK1 activity (deficiency or expression of a dominant negative (DN) protein) enhanced NF-κB activation and was accompanied by elevated antigen-presenting activity towards T cells and increased inflammation and bacterial clearance.[8,9] Different mechanisms were suggested to underlie this phenomenon. Lawrence *et al*. have recently shown that the enhancement of NF-κB activity in macrophages from mice that express kinase-deficient IKK1 (AA) instead of wild-type IKK1 is because of the modulation of the phosphorylation of p65 at serine 536 leading to an accelerated turn over of the transcription factor. However,

Li *et al*. were unable to detect the lipopolysaccharide (LPS)-induced p65 degradation in fetal liver derived macrophage from IKK1-deficient embryos. On the other hand, these authors detected an enhanced IκB-α degradation leading to increased NF-κB activity. They hypothesized that IKK1 limits IκB-α kinase activity of IKK1/2 heterodimers. The reason for the discrepancy between the two studies is not clear at this point but could be explained by the fact the two groups were using different models (IKK1 kinase dead *versus* absence of IKK1).

NF-κB, granulocyte differentiation and apoptosis

It has been reported that NF-κB plays a role in granulocyte differentiation (Figure 2a). The late stage of granulopoiesis is marked by cellular morphological changes. This late stage takes place in the bone marrow, where the cells undergo differentiation from a promyelocyte stage (PM) to a metamyelocyte (MY) stage and finally into bone marrow polymorphonuclear neutrophilic granulocytes (bm-PMNs) that migrate to the blood to become peripheral blood PMNs. Microarray analysis of the different stages of differentiation show an upregulation of chemokines and cytokines, as well as their respective receptors, at the metamyelocyte (MY) to bm-PMN transition (e.g. interleukin (IL)-1/IL-1R or TNF/TNFR).[10] The authors hypothesize that an autocrine loop is responsible for constitutive NF-κB activity. Further experiments are however needed to confirm the importance of NF-κB signaling during granulopoiesis.

From a clinical point of view, the inhibition of granulocyte differentiation could be very interesting. An important step in the resolution of inflammation is the deletion of proinflammatory granulocytes by apoptosis. Whereas the lifespan of the cells is short in the circulation, granulocytes life is prolonged at the inflammatory site. The first evidence that granulocyte cell death is prevented by NF-κB has been provided *in vitro* by blocking NF-κB-activity with several NF-κB inhibitors (SN50, curcumin, pyrrolidine dithiocarbamate, MG-132), which show increased apoptosis of human neutrophils.[11] More recently, inhibition of IKK/Nemo interaction with Nemo-binding domain (NBD) peptide, a potent inhibitor of the classical NF-κB pathway, was shown to increase constitutive cell death which was also observed following induction with LPS.[12]

NF-κB and osteoclast differentiation

Bone volume and calcium homeostasis is maintained through a constant bone formation by osteoblasts, while bone

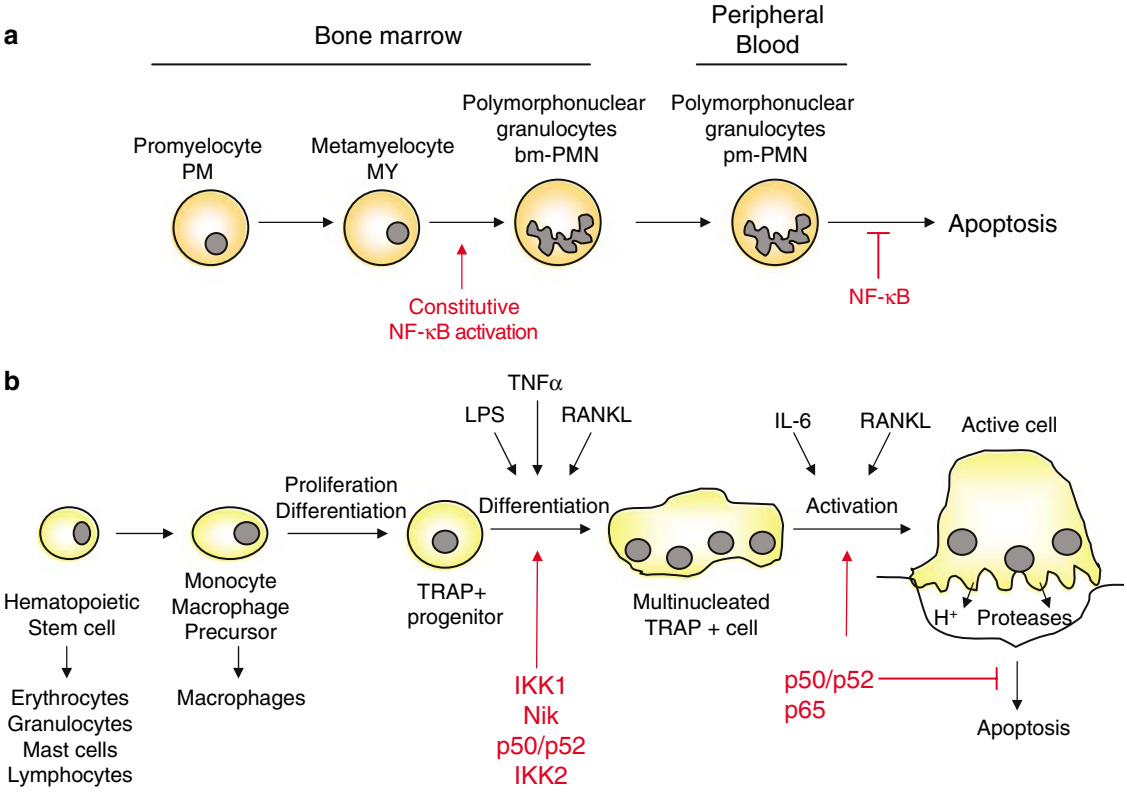

Figure 2 NF-κB in granulocyte and osteoclast differentiation. (**a**) In the bone marrow, granulocytes undergo differentiation from a promyelocyte stage (PM) to a metamyelocyte (MY) stage and finally into bone marrow polymorphonuclear neutrophilic granulocytes (bm-PMNs). These cells migrate to the blood to become peripheral blood PMNs. Whereas NF-κB constitutive activity has been demonstrated in the bone morrow, more experiment are needed to fully understand its role. In addition, numerous studies underline the importance of NF-κB as the transcription factor involved in the survival of granulocytes, thanks to its antiapoptotic properties. (**b**) Osteoclasts differentiate from hematopoietic cells of the monocyte/macrophage lineage. First, monocyte/macrophage precursors divide and differentiate into tartrate-resistant acid phosphatase (TRAP)-positive cells. In the second step, these TRAP-positive cells fuse into multinucleated cells. The final step is the maturation into active cells that are attached to the mineralized bone matrix. Osteoclast bone resorption is mediated by secretion of protons and proteases into a tight compartment created between the osteoclast and the bone matrix. NF-κB has been implicated in this differentiation process as well as in the survival of the cells

resorption is carried out by osteoclasts. Whereas osteoblasts are derived from undifferentiated mesenchymal cells, osteoclasts differentiate from hematopoietic cells of the monocyte/macrophage lineage. Osteoclast development can be summarized as a three-step process (Figure 2b). First, monocyte/macrophage precursors divide and differentiate into tartrate-resistant acid phosphatase (TRAP)-positive cells. In the second step, these TRAP-positive cells fuse into multinucleated cells. The final step is the maturation into active osteoclasts that are attached to the mineralized bone matrix. Osteoclast bone resorption is mediated by secretion of protons and proteases into a tight compartment created between the osteoclast and the bone matrix. Bone loss is often observed at sites of inflammation. LPS, expressed by gram-negative bacteria, induces the activation of NF-κB via toll-like receptor 4 (TLR4) and TRAF 6. It has been shown that LPS not only protects osteoclast progenitors but can also induce their fusion. Finally, cytokines such as TNFα and IL-1 are produced at inflammation site. These cytokines induce NF-κB and stimulate osteoclast progenitor survival and differentiation.

None of the single knockouts of NF-κB family members in mice show defects in osteoclast differentiation; however, mice deficient for both p50 and p52 develop an osteopetrosis phenotype characterized by an increase of bone mass due to a defect in osteoclast differentiation.[3] The discovery of the RANK (receptor activator of NF-κB)/RANKL (receptor activator of NF-κB ligand) pathway has led to a better understanding of the regulation of osteoclasts by osteoblasts. RANKL-expressing osteoblasts interact with RANK receptor expressed on osteoclasts, leading to osteoclast activation. Knockout mice, deficient for this receptor or its ligand, display a prominent osteopetrotic phenotype which confirms the importance of these proteins in osteoclast differentiation.[13] As its name implies, RANK stimulation induces a signaling cascade leading to NF-κB activation. Whereas it has been shown that TRAF 1, 2, 3, 5 and 6 are able to interact with RANK *in vitro*, *in vivo* studies have demonstrated an essential role only for TRAF6 in osteoclast differentiation.[13] Indeed, TRAF6 (−/−) mice develop an osteopetrosis phenotype with defects in bone resorption and tooth eruption.

Upon TRAF recruitment, an intermediate complex, composed of transforming-growth-factor β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2, has been proposed to transduce the signal from TRAF6 to the IKK complex.[14] Mizukami *et al*.[15] showed the formation of a RANK–TRAF6–TAB2–TAK1 complex in RAW264.7 cells. However, to date, there are no *in vivo* studies confirming the role of TAB2 and TAK1 in the formation of osteoclasts.

Another kinase involved in RANK signaling is NIK, which induces the processing of p100 into p52 after RANK stimulation of various cell types. NIK-deficient bone marrow cells are unable to differentiate into mature osteoclasts upon RANK stimulation.[16] However, no osteopetrotic phenotype has been observed in NIK-deficient or mutant (aly/aly mice expressing a mutant NIK protein) mouse models under basal conditions.[16] However, after RANKL delivery, the number of osteoclasts is reduced in NIK-deficient mice compared to wild-type animals.[16] The importance of NIK in osteoclast development has been confirmed in a serum transferred arthritis (STA) model. While inflammation was shown to induce RANKL secretion and osteoclast differentiation in wild-type animals, it was demonstrated that NIK-deficient mice show a reduction of bone erosion.[17] This phenotype was specific to osteoclasts as no differences were detected in the inflammatory response.

The role of the IKK complex in osteoclast differentiation has been the subject of several investigations. *In vitro* experiments using IKK1-deficient fetal liver cells demonstrated the involvement of IKK1 in RANKL-mediated osteoclastogenesis.[18] Chaisson *et al*. described a normal number of TRAP$^+$ osteoclasts, but also showed a decrease in the number of multinucleated cells. Processing of p100 into p52 was also impaired after RANKL treatment. These results, together with the result of the NIK studies, indicate that the NIK/IKK1 pathway is not involved in basal osteoclastogenesis, but is important for stimulated differentiation such as during inflammation or cancer.[18] The importance of IKK1 in *in vitro* differentiation of osteoclasts and in p100 processing to p52 upon RANKL stimulation has been confirmed using bone marrow cells expressing a kinase-dead IKK1.[19] However, this study did not confirm the *in vivo* role of IKK1. On the contrary, this study showed that IKK2 is required for osteoclast differentiation both *in vitro* and *in vivo*. Indeed, mice with an inducible deletion of IKK2 (ikkβΔ) develop an osteopetrotic phenotype resulting from a highly reduced number of osteoclasts in the bone.[19] IKK2 was shown to be involved in two different steps of osteoclast differentiation. Firstly, it has been shown that IKK2 protects osteoclast progenitors from TNFα-induced apoptosis. In the absence of both TNFR1 and IKK2, osteoclast progenitors are protected from TNFα-induced death; however, they remain unable to fully differentiate into multinucleated giant cells, indicating a role for IKK2 in the final step of differentiation. Results from another study were in accordance with the result described above: the bone-resorbing activity of osteoclasts was reduced after inhibition of the NF-κB pathway by a kinase dead IKK2, while this activity was enhanced by a superactive form of IKK2.[20] RANK stimulation is therefore able to activate both the classical and the alternative NF-κB pathway.

NF-κB and DCs differentiation and maturation

DCs are pivotal players in establishing adaptive immunity by presenting antigens and stimulating T cells. Microbial stimuli, proinflammatory cytokines or CD40L-expressing T cells induce the maturation of DCs.

As previously mentioned, RelB and p52 are specifically expressed in DCs. RelB knockout mice lack DC as expected in the thymus and also in the spleen.[3] RelB deficiency does not affect all types of DCs. It has been shown that RelB knockout mice display normal levels of immature langerhans cells and RelB deficiency does not interfere with the development of lymphoid CD8a$+$ DC development.[3] However, more recently, it has been shown that only few DCs can be obtained after transplantation of RelB-deficient bone marrow in irradiated hosts and that these cells are deficient in the processing/presentation of antigen.[21] RelB translocation to the nucleus is not regulated by IκB proteins but by p100. It has been shown that p100-deficient DCs express

more major histocompatibility (MHC) class II and costimulatory molecules, consistent with an increased capacity to activate T cells.[22] Furthermore, the expression of the inhibitory domain of p100 suppresses DC differentiation by blocking them at a precursor stage.

In addition to deficits found in RelB single knockout mice, DCs derived from cRel-deficient bone marrow fail to efficiently activate T cells, even if these cells undergo normal maturation after LPS treatment.[23] No other single NF-κB family member knockout mouse has shown impaired DC development or function, probably due to redundancy of these members. However, double knockout studies indicate that other NF-κB proteins could be involved in DC differentiation. Mice deficient for both p50 and p52 also display reduced numbers of DCs.[3] Moreover, mice transplanted with fetal liver cells deficient for both p50 and p65 showed virtually no DC in the spleen.[24] These authors did not observe any difference at the myeloid precursor level, as macrophage differentiation was not affected. They suggested that the DC phenotype in the spleen is due to a lower survival of these cells in the absence of p50 and p65.

Inhibition of NF-κB activity by blocking of upstream components of the pathway has also been shown to impair DC development. Indeed, IκBα overexpression inhibits the maturation of DCs, probably by preventing the expression of MHC-II and costimulatory molecules.[25] In another study, it was reported that T-cell-mediated activation of DCs is impaired in the presence of DN IKK2, whereas this mutant has no effect on LPS treatment.[26] Tas *et al*.[27] confirmed the role of the IKK complex in DC maturation by showing that the NBD-peptide blocks DC maturation induced by LPS. As a consequence, T-cell proliferation induced by DCs is reduced, as is T_h-polarization of naïve T cells (both T_h-1/T_h-2). Taken together, these results point to the role of both classical and alternative NF-κB pathways that are pivotal for DC maturation. Blocking LPS-induced NF-κB activation by the application of TPCK blocks the maturation process of these cells.[28] In addition, pretreatment of DCs with PSI, an irreversible proteasome inhibitor, decreases the antigen-presenting function of these cells.[29] As discussed above, it is clear that NF-κB proteins play a crucial role in DC maturation. However, they are also involved in the survival of mature cells.

NF-κB and erythropoiesis

The importance of NF-κB signaling in erythropoiesis is still poorly defined. However, some studies suggest a role for some NF-κB family members. The first study showed that, during early erythroid proliferation, p65, p50 and p52 are highly expressed and display a constitutive DNA-binding activity. Later on, their levels decrease during maturation.[30] When c-Rel−/−, p65−/− fetal liver-derived hematopoietic progenitors were transplanted, they failed to promote the survival of lethally irradiated mice.[31] The mice transplanted with the double knockout cells presented a lower hematocrit level and were prone to anemia. Together, these results suggest an *in vivo* function for NF-κB signaling in erythropoiesis, but more studies will be necessary to understand the exact mechanism.

NF-κB and Lymphopoiesis

In 1986, NF-κB has been discovered as the major transcription factor involved in the regulation of the immunoglobulin light chain κ, implicating a crucial role for NF-κB in lymphocyte development. Since then, much has been learned about the role of NF-κB in lymphopoiesis.

The NF-κB pathway has been shown to be important for the differentiation steps of not only T and B cells but also for their homeostasis. As several reviews in this issue will discuss the role of NF-κB in B- and T-cell development and function in detail, we will only briefly summarize the wealth of information available on this subject (reviews in this issue).

Before discussing the role of NF-κB signaling in B- or T-cell maturation in more detail, it is worth pointing out that certain NF-κB protein deficiencies prevent lymphocyte development in general, indicating a role for NF-κB in survival of hematopoietic stem cells before they differentiate into T-cell or B-cell progenitors. Fetal liver-derived hematopoietic stem cells from p65- or IKK2-deficient mice fail to graft irradiated animals probably because they do not produce Bcl-2 to overcome the high TNF levels in the bone marrow. Also, NEMO deficiency leads to the absence of lymphocytes.[32]

NF-κB and T cells

The thymus is the main organ supporting T-cell development where precursor T cells ($CD44^{low}$) differentiate into CD4–CD8 double negative cells. This step of differentiation can be separated in four stages, I–IV, which can be characterized by expression of specific cell surface markers (CD44 and CD25). CD4–CD8 double negative thymocytes subsequently differentiate into double positive $CD4^+CD8^+$ cells. After positive and negative selection, $CD4^+CD8^+$ cells ultimately differentiate into single positive $CD4^+$ or $CD8^+$ cells. These cells migrate to the periphery and the lymphoid organs where they exert their function (Figure 3).

Transgenic mice expressing a luciferase reporter gene under the control of a NF-κB-dependant promoter were used to demonstrate NF-κB activity in the thymus of embryos as well as in the thymus, bone marrow, spleen and lymph nodes of adult animals.[33] Interestingly, p65 was then described to be present in the cortex of the thymus while RelB and c-Rel were predominantly expressed in the medulla, indicating that different NF-κB family members display different localization within the thymus. The role of NF-κB family members in the different stages of T-cell development was subsequently analyzed in more detail using several knockout and transgenic models.

IκB-ε-deficient mice showed a decreased number of thymocytes in stage III.[3] Transgenic mice expressing a constitutively active IKK2 molecule under the control of a T-cell-specific promoter displayed increased numbers of stage IV cells,[34] indicating that the pre-T-cell receptor (pre-TCR) activates NF-κB which will in turn provide an anti-apoptotic signal to control the number of cells. In accordance with this hypothesis, expression of a transdominant IκBα isoform in fetal thymic organ cultures (FTOC) induced a lower number of double positive $CD4^+CD8^+$ cells, suggesting a

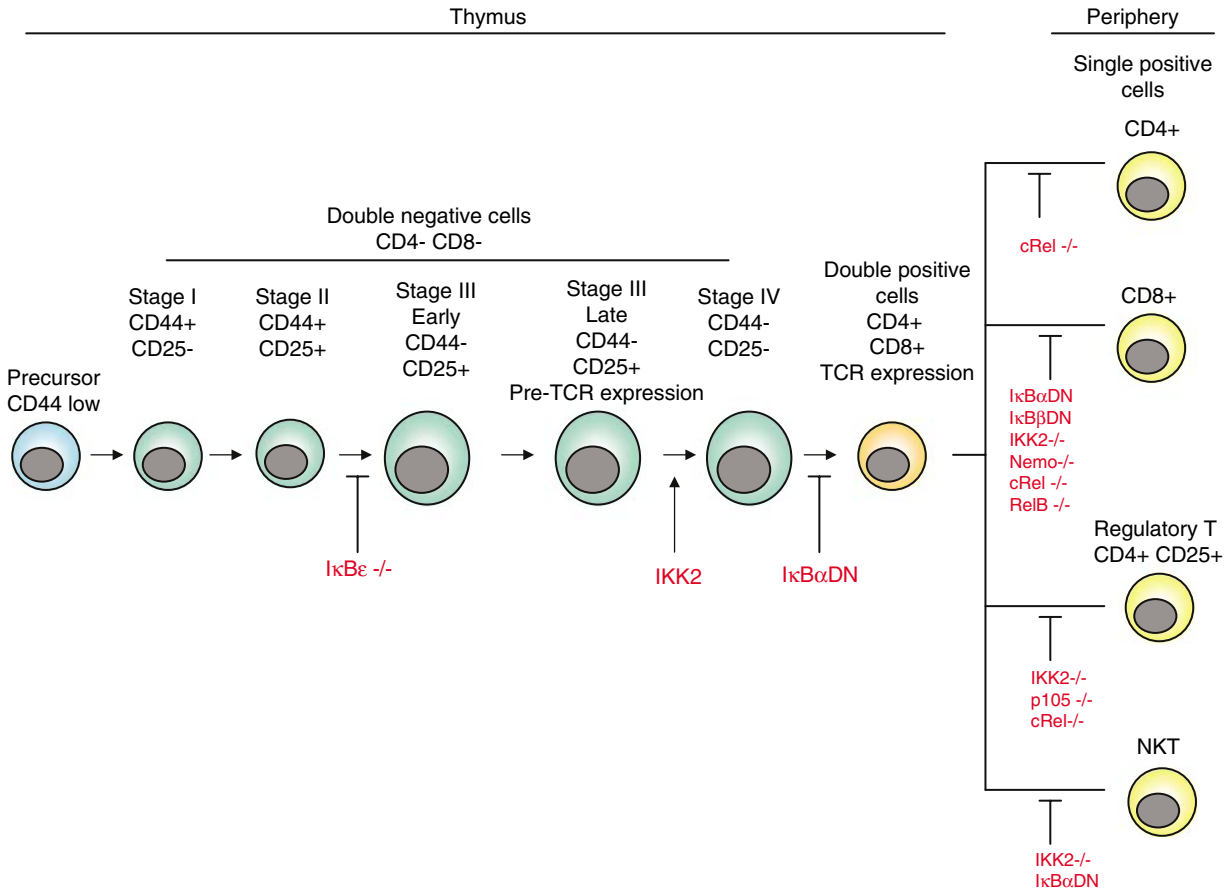

Figure 3 NF-κB in T-cell differentiation. T-cell development is a very organized process taking place in the thymus. Precursor T cells ($CD44^{low}$) differentiate into $CD4^-CD8^-$ double negative cells. Their cell surface markers allow the identification of four stages of double negative cells: in stage I, the cells are $CD44^+CD25^-$; in stage II, CD25 can be detected (cells $CD44^+CD25^+$); in stage III, the expression of CD44 is lost (cells $CD44^-CD25^+$) and in stage IV, the cells do not express these two markers anymore (cells $CD44^-CD25^-$). The thymocytes subsequently differentiate into double positive $CD4^+CD8^+$ cells, which, after positive and negative selection, differentiate into single positive $CD4^+$ or $CD8^+$ cells. These cells migrate to the periphery and the lymphoid organs where they exert their function. Different mouse models allowing the characterization of the role of NF-κB in T-cell differentiation are indicated. IκB DN: IκB dominant negative, super-repressor of NF-κB activity

role for NF-κB during differentiation from stage IV to the double positive stage.[35]

Transgenic mice expressing IκBα or IκBβ under the control of a T-cell-specific promoter have been generated and show some differentiation defects at later stages,[3] leading to impaired T-cell proliferative responses, impaired T-cell-dependant cytokine production as well as a decrease of $CD8^+$ T cells in the periphery.

Surprisingly, NF-κB has been reported to have a proapoptotic function during the negative selection of double positive $CD4^+CD8^+$ thymocytes. Indeed, double positive thymocytes expressing transdominant IκBα were less sentitive to apoptosis.[3] Similar results were obtained after expression of DN IKK2.[36] On the contrary, another study suggested an antiapoptotic function of NF-κB during negative selection. This study was performed with a new IκB family member (IκBNS) which can be induced by TCR stimulation.[37] The authors showed that NF-κB could be blocked by overexpression of IκBNS, and that this increased anti-CD3ε-induced apoptosis of FTOC. Further studies are however necessary to confirm the role of this new IκB protein.

Using transdominant IκBα mice, Hettmann *et al*.[38] demonstrated a role for NF-κB signaling in positive selection of $CD8^+$ single positive cells. All together, these observations suggest that during positive selection, classical NF-κB signaling seems to have an antiapoptotic function, in contrast to the situation described above for negative selection. The antiapoptotic function of IKK2 has been independently confirmed in several studies. Inactivation of IKK2 or Nemo leads to an increase in apoptosis during the last stage of thymocyte differentiation.[39]

A subpopulation of the T cells, the natural killer-like T cells (NKT cells), is also maturating in the thymus and NF-κB has also been implicated in the differentiation of these cells. The number of mature NKT cells is decreased in mice with a RelB deficiency, mice without IKK2, aly/aly mice (mutated NIK) and in mice expressing transdominant IκB-α.[3]

Single positive $CD4^+$ or $CD8^+$ thymocytes migrate from the thymus to the periphery. NF-κB also plays a central role during the later stages of T-cell development. Several groups have put considerable effort into understanding the role of the IKK complex in mature T-cell function. Dominant negative

forms of IKK1 and IKK2 have no effect on the development of peripheral T cells.[36] Similar results have been confirmed in mice with a T-cell-specific IKK2 deletion; the expression of kinase dead IKK2, however, decreased the number of $CD4^+$ and $CD8^+$ cells in the spleen and lymph nodes.[39] The latter group also demonstrated that IKK2 is essential for the development of three subpopulations of T cells, including memory T cells, NKT cells and $CD4^+CD25^+$ regulatory T cells and that mice with T-cell-specific NEMO deletion display a number of $CD4^+$ and $CD8^+$ cells. The clinical importance of NEMO in T-cell development has also been shown in human patients in which NEMO mutation contributes to immunodeficiency.[40]

NF-κB and B cells

The development of mature/activated B cells from B-cell progenitors is a very well characterized process that takes place via defined intermediate developmental stages (Figure 4). Committed B-cell progenitors develop from the stem-cell pool in the bone marrow and develop via the intermediate pro-B-cell- and pre-B-cell stages into immature B –cells, which will leave the bone marrow and travel to the peripheral lymphoid organs to undergo final step of maturation. These cells can develop into activated B cells, memory B cells or plasma B cells upon proper stimulation.

In general, mice with single NF-κB protein knockouts (except the ones mentioned above) predominantly display deficiencies in B-cell activation but much less in B-cell maturation, suggesting a redundancy in NF-κB protein function during B-cell maturation. Mice deficient in p105 display decreased numbers of marginal zone B cells and peritoneal B1 cells.[41,42] cRel-deficient mice show defects in memory B-cell differentiation and germinal center B cells.[3] p100-deficient mice have reduced numbers of follicular B cells.[3] However, p100 knockout mice also display a disrupted spleenic microarchitecture.[3] Similarly, RelB knockout mice display multiorgan inflammation, spleenomegaly, disorganized B- and T-cell areas, lack of germinal centers, lack of spleenic marginal zone structures, reduced expression of homing chemokines, and lack of immune responses.[43] It is difficult to make a statement about the role of p100 and RelB in B-cell development, because the impairment in B-cell maturation could be caused by endogenous NF-κB signaling deficiencies or be a result of defects in spleenic architecture which do not allow B-cell maturation. Altered microarchitecture of secondary lymphoid organs is also observed in bcl-3-deficient mice.[3] These mice failed to produce antigen-specific antibodies.

Marginal zone B cells are exceptions to the rule as these cells are more sensitive to knockout of individual NF-κB family members. These cells are completely lost in the case of p100-, p105- or RelB knockout, while they are partially blocked in mice lacking cRel or p65.[41,44,45]

Knockout of multiple NF-κB proteins leads to more dramatic B-cell phenotypes. Chimeras deficient for both p105 and p65 do not have any $B220^+$ lymphocytes at all,[46] whereas p105 and p100 double deficient cells arrest at the T1 maturation

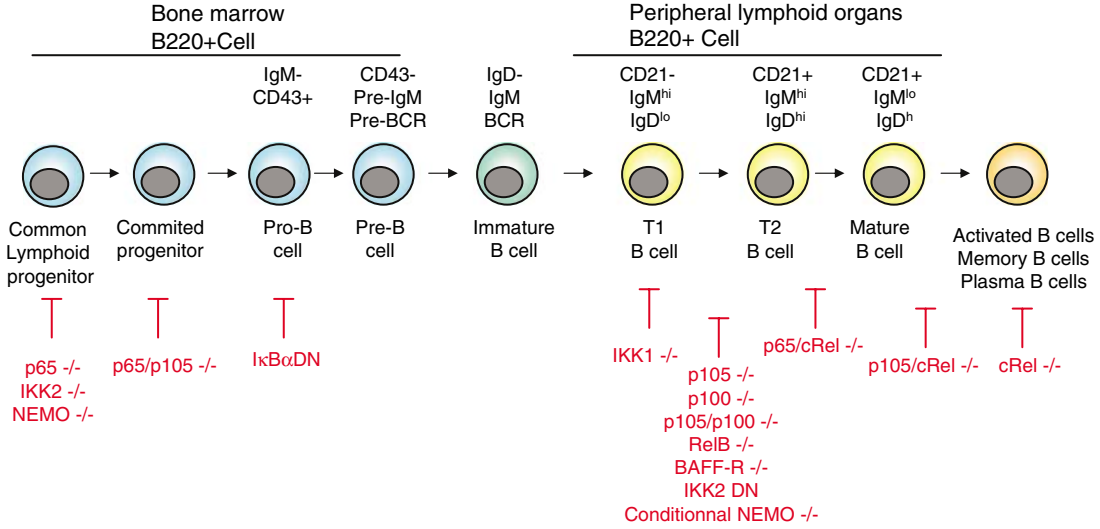

Figure 4 NF-κB in B-cell differentiation. Committed B-cell progenitors develop from the stem-cell pool in the bone marrow and develop via the intermediate pro-B-cell- and pre-B-cell stages into immature B cells. Several important events take place during these developmental steps. VDJ recombination during the pro-B-cell stage gives rise to the generation of the heavy chain of the B-cell receptor (BCR), which initially pairs with surrogate light chains to form the pre-BCR that is characteristic of pre-B cells. Pre-BCR-expressing cells undergo expansion and light-chain gene rearrangements. When the light chains are synthesized, they will pair with the heavy chain to form the BCR (surface IgM). At this developmental stage, the cells are coined immature B cells. The former B-cell maturation stages can be distinguished by the expression pattern of cell surface markers. All B cells, including committed B-cell progenitors, are $B220^+$. Pro-B cells are IgM^-CD43^+, while pre-B cells are IgM^+CD43^-. Once matured into immature B cells, the cells will leave the bone marrow and travel to the peripheral lymphoid organs. Arriving there, they progress from transitional 1 (T1-) B cells into T2-B cells. The latter can further maturate into resident marginal zone B cells or into mature (follicular) B cells (also known as conventional B cells or B2 cells) that can enter circulation. T1, T2 and mature B cells can be distinguished by their Ig-expression status. Mature B cells can develop into activated B cells, memory B cells or plasma B cells upon proper stimulation. Altogether this cascade of events ensures a tight control of B-cell numbers and specificity. NF-κB has been involved in different step of the B-cell differentiation. Numerous knockout studies allowed the dissection of the role of the different members of NF-κB family. DN: dominant negative

stage.[3] In addition, chimeras deficient for both cRel and p65 display reduced T1 and T2 cell numbers, and an absence of mature B cells.[3] Finally, combined deletion of p105 and cRel results in defects in the proliferation and survival of mature B cells without affecting earlier steps.[42] Altogether these results show that the different NF-κB proteins are important at different stages of B-cell maturation.

When the block is placed more upstream in the NF-κB pathway, the phenotypes are more dramatic. Upon reconstitution with IKK2- or NEMO-deficient stem cells, no lymphocytes could be detected in chimeric mice.[47] Blocking of classical NF-κB signaling by overexpression of DN IκBα results in a block after the pro-B-cell stage.[48] IKK1 deletion, involved in the alternative pathway, also leads to severe B-cell maturation defects in radiation chimeras. Senftleben *et al*.[49] and Kaisho *et al*[50] reported that in such mice B-cell maturation, germinal center formation, the formation of secondary lymphoid organs, serum immunoglobuline (Ig) levels, antigen-specific immune responses and the cleavage of p100 are severely impaired. It has been shown that BAFF-R signaling is the critical signal leading to activation of the alternative NF-κB pathway at the transition point from the T1-B-cell stage to the T2-B-cell stage. B cells activation factor receptor (BAFF-R) signaling is also essential for the survival of T2 -cells and mature B cells.[44] Indeed, BAFF-deficient mice have similar B-cell deficiencies as BAFF-R-deficient mice and p105/p100 double knockout mice.[51] However, the classical NF-κB pathway also plays a role in the survival of T1-B cells. Conditional NEMO knockout or knock-in of mutant IKK2 reduces the number of transitional B cells and mature B cells.[47] Together, these results show that both the classical- and the alternative NF-κB pathways (and therefore both IKK2 and IKK1, respectively) are critical for B-cell maturation and B-cell survival.

NF-κB in NK cells

NK cells fight invading pathogens by secreting cytolytic proteins (perforin, granzymes) and cytokines (TNFα and interferon γ (IFNγ)). A role for NF-κB in NK cell differentiation has recently been described.[52] Immature NK cells present constitutive NF-κB activity and this activity is regulated during NK differentiation. In addition, chimeric mice deficient for both IκBα and IκBε possess reduced numbers of NK cells, especially in the spleen. Finally, the authors show that IκBα- and IκBε-deficient NK cells have a defect in the secretion of IFNγ.

NF-κB is also involved in regulating the activity of NK cells. Mutations in Nemo have been identified in several patients suffering from an X-linked syndrome characterized by hypohidrotic ectodermal dysplasia with immune deficiency (HED-ID). In three patients, the number of circulating NK cells was unaffected but the cytotoxic activity of these cells was impaired.[53]

Hematopoietic Diseases and Therapy

Several diseases are the result of hematopoitic deregulation. NF-κB is involved in several of these and notably in lymphoma, arthritis, atherosclerosis and diabetes. Additionally, NF-κB was suggested to play an important role in the allograft rejection. Here, we will briefly discuss the role of NF-κB in these diseases.

NF-κB in cancer

The first indications that NF-κB could play a role during oncogenesis were found in studies with the retroviral oncogene v-Rel, whose cellular homolog is c-Rel. Overexpression of v-Rel induces the development of T-cell lymphoma. Later, constitutive NF-κB activity has been reported for several human cancers including T- and B-cell lymphomas.

Inflammation and NF-κB go hand-in-hand. Therefore, it is not surprising that NF-κB is believed to be involved in the development of inflammatory diseases. Recently, it has been suggested that several chronic inflammatory diseases are strongly associated with cancer. It has been proposed that the reactive oxygen and nitrogen species secreted by proinflammatory cells at the site of inflammation transforms premalignant cells by inducing accumulating DNA damage. Additionally, the growth and progression of tumors can be stimulated by certain cytokines, growth factors, proteases and antiapoptotic signals delivered by inflammatory cells. Gene-modified mouse models have established the link between inflammation, NF-κB and cancer. Three independent groups demonstrated that blocking NF-κB (by deletion of IKK2 or by overexpression of a transdominant negative IκBα) is able to decrease tumorigenicity.[54–56] Anti-inflammatory drugs could therefore have a beneficial effect in cancer therapy.

The presence of skeletal abnormalities is a common observation associated with poor prognosis and complications in patients with cancer. Increased osteoclast activity leads to the development of osteolytic lesions in breast metastases, thyroid, lung and renal cancer as well as in neuroblastoma and multiple myeloma.[57] A 'vicious circle' has been described in which tumor cells directly or indirectly induced factors (such as IL-1, IL-6, TNF-α, MIP-1α and RANKL) that stimulate osteoclastic bone resorption. In turn, factors released from the degraded matrix (TGF-β, fibroblast growth factor, insulin-like growth factors (IGFs)) stimulate the tumor growth. One of the therapeutic strategies developed to block pathological osteoclast differentiation in cancer is therefore to block RANK signaling.[58]

NF-κB in arthritis

NF-κB plays an important role in the development of rheumatoid arthritis (RA), a chronic inflammatory joint disease. It has been well documented that NF-κB, in particular the p65/p50 heterodimer, is constitutively activated in synovial tissue of patients.[59] This activation leads to the induction of numerous genes coding for proinflammatory proteins secreted at the site of inflammation such as cytokines (e.g. IL-1, TNFα, IL-6, IL-8), COX-2 and iNOS.[60] NF-κB is also proposed to be involved in synovial hyperplasia through its antiapoptotic properties.[60] The injection of NF-κB decoy oligonucleotides has been shown to improve the pathology in a rat arthritis model, by facilitating synovial apoptosis. The role of NF-κB in

the initiation of RA pathology has been confirmed in various animal models. In a murine collagen-induced arthritis and in a rat adjuvant-induced arthritis model, it has been shown that the clinical manifestation of the disease is preceded by NF-κB activation.[61] A lot of effort has been invested to understand the upstream component responsible for NF-κB activation in the synovial tissue. IKK2 is the main kinase involved in cytokine-induced NF-κB activation in fibroblast-like synoviocytes.[62] Interestingly, injection of activated IKK2 induces the development of arthritis in rats, whereas intra-articular injection of a DN IKK2 in a rat adjuvant-induced arthritis is able to decrease the pathology.[63] In support of this, a selective IKK2 inhibitor, SC-514, has been shown to partially inhibit NF-κB in synovial fibroblasts.[64]

One of the complications of chronic inflammatory diseases, such as inflammatory arthritis, is the loss of bone resulting from a massive influx and activation of osteoclasts at the site of inflammation. The first proof that blocking NF-κB could potentially be therapeutically efficacious was obtained after the administration of a DN IκB in a mouse model of inflammatory arthritis.[65] In this model, transdominant IκBα was able to block NF-κB activation in arthritic cells, and significantly attenuated osteoclast recruitment and bone erosion. Later on, two different groups successfully inhibited NF-κB activation by blocking IKK activity using a cell-permeable NBD peptide. They showed that NBD peptide disrupts the association of NEMO with both IKKs and blocks TNFα-induced NF-κB activation in several cell lines.[66] In mouse models of arthritis, this NDB peptide inhibited osteoclastogenesis but also prevented the inflammatory bone destruction.[67] Finally, curcumin has been shown to inhibit *in vitro* NF-κB activation and consequently inhibits both RANKL- and TNFα-induced osteoclast differentiations.[68]

NF-κB in atherogenesis

Atherosclerosis is considered a chronic inflammatory disease in which macrophages play a crucial role. One of the early steps of the disease is the recruitment of monocytes to the artery wall, which further differentiate into macrophages. The first role of macrophages is protective by uptake of oxidized low-density lipoproteins (oxLDL). The accumulation of this lipid in the macrophage is thought to cause transformation of macrophages into what is called a foam cell which play a proatherogenic role by inducing an inflammatory response. Interactions between macrophages and T cells are responsible for the chronic inflammatory state of the disease that recruits smooth muscle cells, and induces their proliferation. Smooth muscle cells in turn secrete extracellular matrix proteins and facilitate the formation of a fibrous plaque.

NF-κB, the primary inflammatory transcription factor, plays a crucial role in the development of this disease. The first indication of its involvement is from the detection of constitutive NF-κB activity in atherosclerotic lesions. At these lesions, several factors inducing NF-κB activation have been detected. They include modified LDLs, metabolic factors, microbial products or agents and cytokines. In response, NF-κB activates a great number of proatherogenetic genes such as cytokines and leukocyte adhesion molecules (TNFα, IL-6, MCP-1, V-CAM, etc.). These various studies implicate a proatherogenic function of NF-κB, at least in the early phase of the disease. This has lead to the investigation of the use of IKK inhibitors for therapy of atherosclerosis.

It has recently been demonstrated that NF-κB can exert an antiatherogenetic effect since macrophage specific IKK2 deletion increased atherosclerosis in low-density lipoprotein receptor (LDLR)-deficient mice.[69] In addition, LDLR-deficient mice transplanted with p50-deficient bone marrow developed smaller atherosclerotic lesions, characterized by the absence of foam cells. The authors postulated that this apparent contradiction could be explained by the fact that p50 homodimers decreased NF-κB activity and therefore results in reduced pathology.[70] Finally, mutation of A20, a protein downregulating NF-κB activity, results in reduced atherosclerosis.[71]

These opposing roles of NF-κB (pro- *versus* antiatherogenic) were explained in part by results obtained in another model. It has been proposed that early NF-κB activation has a proinflammatory effect but that later NF-κB activity is essential for inflammation resolution. Clearly, more studies are necessary to fully understand the complexity of NF-κB signaling involved in atherosclerosis.

NF-κB in diabetes

Type 1 diabetes is an autoimmune disease characterized by the destruction of the insulin producing β islet cells of the pancreas. While $CD4^+$ and $CD8^+$ T cells have been shown to destroy pancreatic islets in diabetes, it is the antigen-presenting cells that are responsible for the differentiation defect of T cells that leads to this aberrant behavior. It has been shown that patients as well as non-obese diabetic (NOD) mice present a developmental defect in DC.[72] As previously discussed, NF-κB plays an important role in DC development and maturation. Therefore, a role for NF-κB in diabetes was hypothesized. IKKs hyperactivity has been demonstrated to be responsible for elevated NF-κB activation in NOD mouse DCs and the subsequent increase of IL-12 production in these mice.[73] The same group showed that hyperactivation of NF-κB results in enhanced APC presentation.[74] The importance of NF-κB and DCs in diabetes has recently been confirmed, since *in vivo* administration of DCs in which NF-κB was blocked prevents the development of diabetes in NOD mice.[75]

Although type 2 diabetes is characterized by peripheral insulin resistance, it has been hypothesized that obesity, inflammation and type 2 diabetes could be linked. Firstly, it has been observed that obese patients present an elevated level of inflammatory cytokines that cause hepatic insulin resistance.[76] Additionally, a role for NF-κB has been found in the development of insulin resistance. Salicycate, known to inhibit NF-κB activation, prevents fat-induced insulin resistance in rodents.[77] In addition, targeted disruption of IKK2 decreases obesity- and diet-induced insulin resistance. Cai *et al*.[78] reported that a high fat diet increases IKK2 activity and the subsequent activation of NF-κB explains the higher level of inflammatory cytokines. Furthermore, mice expressing constitutively active IKK2 in hepatocytes develope a type 2 diabetes phenotype, while hepatic knockout of IKK2 restores

liver insulin responsiveness.[79] Arkan *et al*. further demonstrated an important role of myeloid cells in the development of the pathology, as specific deletion of IKK2 in myeloid cells improved insulin sensitivity. This changes the previously perceived etiology of type 2 diabetes as it previously was not known how NF-κB dysfunction in immune cells could contribute to this disease.

Treatment strategies for diabetes targeting NF-κB are under development. It has been shown in a mouse model that supplementation of *N*-acetylcystein, a known inhibitor of NF-κB, is able to reduce hyperglycemia and to attenuate the severity of insulin-dependent diabetes.[80] In addition, patients with diabetes type 2 present a reduced plasma glucose level after treatment with thiazolidinediones. Interestingly, this new class of insulin-sensitizing drugs has been shown to inhibit also NF-κB.[81] Finally, salicylates including high doses of aspirin improve glycemia in patients.[82] In the future, new drugs inhibiting the IKK complex could be tested for their effects on diabetes.

NF-κB in graft rejection

Allograft rejection is mediated by undesired activation of immune cells. Patients under life-long immunosuppressive drugs are predisposed to viral and bacterial infections. Therefore, the design of a strategy that specifically targets alloreactive T cells might prevent the severe secondary effects.

Several studies suggest that inhibiting NF-κB activation has therapeutic potential. Cardiac graft rejection was slower when the transplantation is performed in p50- or p52-deficient mice.[83] More convincing data were obtained with c-Rel-deficient recipients. In the later case, allograft survival could be observed during prolonged periods of time.[84] Additionally, it has been shown that mice expressing a transdominant IκBα do not reject transplanted hearts.[85] The graft tolerance of these mice seems to be because of T-cell deletion as overexpression of Bcl-xL reinstated rejection of the cardiac allograft.[86]

Several strategies to block NF-κB have been under investigation in this disease. NF-κB decoy oligonucleotides have been shown to have a beneficial effect on heart transplantation,[87] whereas pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor) seems to improve liver graft transplantation.[88]

In allogenic stem cell transplantation, another complication is graft *versus* host disease (GVHD). In GVHD donor, T cells kill recipient cells, predominantly by secreting cytokines. Again, blocking NF-κB appears to be a good treatment strategy. PS-341, a proteasome inhibitor known to inhibit NF-κB activation, protected mice from GVHD.[89] Very interestingly, graft *versus* tumor responses were not abolished after systemic PS-341 treatment of mice with advance tumors. More recently, PS-1145, an IKK inhibitor, has been shown to be protective against GVHD.[90] This inhibitor exhibits less toxicity than PS-341, probably because proteasome inhibitors are not very specific. However, both drugs protected the host by decreasing the quantity of cytokines secreted by the graft cells.

Perspectives

NF-κB has been shown to be a pivotal player in hematopoiesis. Especially in lymphopoiesis, the contribution of each member of the NF-κB family has been studied elaboratively. It has been shown that specific NF-κB proteins are involved at specific stages of differentiation, in protection from apoptosis, as well as in cellular homeostasis of B- and T cells. Whereas the role of NF-κB in lymphopoiesis is well established (and will be reviewed in more detail elsewhere in this issue), the importance of NF-κB in the development of other hematopoietic lineages is also recognized. In this review, we tried to give an overview of the data available on the role of NF-κB in the development of the myeloid lineage. These data strongly suggests that NF-κB is a major player in the development of this lineage as well. Additionally, it is now recognized that deregulation of NF-κB contributes significantly to many diseases in which the function of specific hematopoietic cell types is affected. It will be of importance to identify the specific target genes of the various NF-κB family members in these different cell types. Inhibition of NF-κB activity is an attractive treatment strategy for hematopoietic diseases in which NF-κB is chronically activated. However, a hallmark of many hematopoietic diseases is that these diseases manifest themselves systemically, indicating the necessity of systemic treatment, which potentially can lead to toxic side effects when NF-κB is inhibited in cells that are not involved in the disease. It is therefore essential to study the role of NF-κB in the regulation and deregulation of hematopoiesis in more detail to obtain a better understanding of which NF-κB pathway (or family member) needs to be inhibited in any particular disease. The development of inhibitors of specific NF-κB proteins or NF-κB pathways will contribute to the amelioration of the therapies.

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