NK cells and type 1 diabetes

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Abstract
Type 1 diabetes (T1D) is characterized by an immuno-mediated progressive destruction of the pancreatic β cells. Due to the ability of NK cells to kill target cells as well as to interact with antigen-presenting and T cells, it has been suggested that they could be involved in one or multiple steps of the immune-mediated attack that leads to T1D. Abnormalities in the frequency and activity of NK cells have been described both in animal models and patients with T1D. Some of these alterations are linked to its onset while others seem to be a consequence of the disease. Here, we discuss the main characteristics of NK cells and review the studies that investigated the role of NK cells in T1D, both in mouse models and humans.

Keywords: Type 1 diabetes, T cells, NK cells

Introduction
Type 1 diabetes (T1D) is characterized by an immuno-mediated progressive destruction of the pancreatic β cells (Kelly et al. 2003). Although the precise mechanisms involved in its pathogenesis are still unclear, it is known that autoreactive T cells have a central role in the process. Due to the ability of NK cells to kill target cells as well as to interact with antigen-presenting and T cells, it has been suggested that they could be involved in one or multiple steps of the immune-mediated attack that leads to T1D. Here, we discuss the main characteristics of NK cells and review the studies that investigated the role of NK cells in T1D, both in mouse models and humans.

NK cells
NK cells are large granular lymphocytes that do not express B or T cell receptors and participate in the innate immune response. Besides playing important roles in defence against malignancies and infectious diseases, NK cells are implicated in the graft-versus-host disease, the regulation of hematopoiesis and are capable of interfering in the adaptative immune response (Flodstrom et al. 2002). Under normal circumstances, they are primarily located in the peripheral blood, bone marrow, spleen and liver (Moretta et al. 2002; French and Yokoyama 2004; Sinkovics and Horvath 2005). In humans, NK cells are identified by the lack of the surface marker CD3 and the presence of CD56 with or without CD16.

NK cells act through the cytotoxic destruction of their target cells. Unlike cytotoxic T lymphocytes, they exert their effector function without the need for previous in vitro or in vivo activation. This characteristic makes them remarkably suited to mediate the first line of defence against pathogens, as part of the innate immune response (Baxter and Smyth 2002; Colucci et al. 2003). When activated, NK cells induce apoptosis of the target cells mainly through the exocytosis of perforin and granzyme. They also secrete pro-inflammatory cytokines such as interferon gamma (IFNg), tumor necrosis factor (TNF), granulocyte–macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory protein 1α and 1β (Backstrom et al. 2004).
NK cells recognize the target cells through specific molecules on the surface of these cells. These molecules may be either inadequately present or absent due to cell infection or its transformation. In the first situation, this could be due to non-self molecules encoded by pathogens or, alternatively, self-molecules only expressed in a higher frequency in certain adverse conditions. The second scenario implies the absence of molecules normally present on the surface of the autologous cells, like MHC class I. These alterations could render these cells susceptible to NK lysis (Raulet 2004). Generally, this second mechanism alone does not trigger cytotoxicity, unless it is combined to the anomalous expression of other molecules on the target cell surface.

There are receptors on the surface of the NK cells that can trigger cell stimulation or inhibition. Briefly, these receptors are coupled with intra-cellular signaling adapters that contain activation or inhibition sites based on their tyrosine residues. These are called, respectively, immunoreceptor tyrosine-based activation motifs (ITAM) and immune tyrosine based inhibitory motifs (ITIM) (Lanier and Bakker 2000).

Two main categories of NK inhibitory receptors were identified in humans: the heterodimer CD94:NKG2A, specific for HLA E molecules; and the killer cell immunoglobulin-like (KIR) receptors, that recognize HLA A, B and C molecules. KIR receptors, however, are not always inhibitory. They belong to a diverse family of receptors, both stimulatory or inhibitory, codified by genes in the chromosome 19 and expressed mainly by NK cells, but also by some subgroups of T lymphocytes (Vilches and Parham 2002; Middleton et al. 2005). Based on the combination of KIR genes, two major groups of haplotypes can be defined, A and B. While genes encoding inhibitory receptors predominate in the former, two or more activating KIR genes are present in the latter (Slik et al. 2003).

NKG2D and natural cytotoxicity receptors (NCRs) are the main NK activating receptors. NCRs (NKP30, NKP44, NKP46 and NKP80) are expressed exclusively in NK cells and belong to the superfamily of immunoglobulins (Pende et al. 1999; Sivori et al. 1999; Moretta and Moretta 2004). Their ligands are not completely defined, but it is known that at least part of them (NKP44 and NKP46) recognize viral hemmaglutinins. Heparan sulphate proteoglycans have also been shown to act as ligands for NKP46 and NKP30 (Arnon et al. 2001; Mandelboim et al. 2001; Bloushtain et al. 2004). The NKG2D receptor is expressed by the majority of human NK cells, but also in other lymphocytes such as CD8 + . It is codified by the NK receptor gene complex in chromosome 12. Differently from the other NKG2 receptors, which are inhibitory and form dimers with the receptor CD94, the receptor NKG2D is a homodimer that recognizes a variety of ligands induced by stress, such as non-classic HLA class I molecules MICA and MICB, as well as ULBPs (Raulet 2003; Andre et al. 2004).

Although numerous receptors have been implicated in NK cells activation, it is not known if any specific receptor alone is capable of triggering the NK effector function by itself. A synergic interaction between multiple receptors seems to be necessary for triggering the cytotoxicity and cytokines production. Therefore, NK cell function depends on the balance of various signals from stimulatory and inhibitory receptors, as well as the expression of their corresponding ligands (Hoffman 1980).

**NK cells in autoimmune diseases**

Approximately 5% of the population in the Western countries are affected by autoimmune diseases (Flodstrom et al. 2002). Although there is a strong genetic component determining susceptibility to these diseases, some kind of environmental factor is usually also necessary to trigger their appearance. Under normal conditions, self-tolerance mechanisms prevent intra-thymic maturation and activation of autoreactive lymphocytes, what is called central tolerance (Janeway et al. 2005). However, a small proportion of the autoreactive cells escapes from this process, undergoes maturation and reaches the peripheral circulation. To prevent the autologous cells against destruction by these autoreactive cells, there are also peripheral mechanisms that can prevent the action of these autoreactive or even destroy them, what is known as peripheral tolerance. When there is a failure in the central or peripheral self-tolerance mechanisms, immune reaction to self-antigens can occur, leading to autoimmune diseases.

NK cells might have a direct influence in the development of autoimmune diseases, destroying cells in their target organs, or, alternatively, have an indirect role, regulating the adaptative immune response (Kos and Engeman 1996; Shi et al. 2000). This modulation could occur in various steps of the autoimmune response. Due to their potential to interact with antigen-presenting cells (like dendritic cells), NK cells could interfere in the priming of autoimmune responses. They might also influence the downstream response, as it is known that they can affect the proliferation and generation of B and T autoreactive lymphocytes. Although NK cells might prevent the presentation of self-antigens by dendritic cells, avoiding the autoimmune response, they also seem to be necessary for the initiation of auto-reactive B and/or T cell response (Sinkovics and Horvath 2005). On the other hand, NK cells can also secrete cytokines that suppress B and T cell responses or even destroy them (French and Yokoyama 2004).

Given their cytolytic ability, NK cells have been potentially involved in the pathogenesis of diseases...
caused by tissue destruction, as are most autoimmune diseases. It has been shown that NK cells can kill autologous cells (Hansson et al. 1981; Morse et al. 2001). However, under normal conditions, molecules on the surface of the autologous cells, especially MHC class I, engage inhibitory receptors on the surface of NK cells preventing them from delivering a lytic signal and making them self-tolerant. Loss of self-tolerance can occur if an autologous cell loses the expression of MHC class I (Flodstrom et al. 2002). The exact mechanism involved in the development of self-tolerance by the NK cells is not completely understood.

NK cells have been identified in target organs of patients suffering from autoimmune diseases (Flodstrom et al. 2002). Although this phenomenon might be explained solely by the migration of NK cells as part of any inflammatory process, independently of its cause, there is evidence that NK cells are capable of attacking autologous cells (Hansson et al. 1981; MacKay et al. 1986; Nakamura et al. 1990; Morse et al. 2001) which may indicate that they could contribute to the development of autoimmune cell destruction. As infectious disease have been implicated as potential triggers for many autoimmune diseases and one of the main roles of NK cells is the protection against infections, a link between NK cells and autoimmune diseases would seem reasonable. NK cells might either promote or suppress autoimmune diseases triggered by infectious diseases. By rapidly clearing the infection, NK cells could limit the immune-mediated tissue damage (Paya et al. 1989; Fairweather et al. 2001). On the other hand, a vigorous attack towards infected autologous cells could result in significant destruction of the target tissues (Flodstrom et al. 2002), which could lead to the release of self-antigens. As the inflammatory milieu caused by the infection may induce the priming of adaptive immune response, this can cause to the activation of normally quiescent autoreactive lymphocytes reacting to the recently spread self-epitopes.

Curiously, several small studies during the 80s have shown a reduced number and/or function of NK cells in the peripheral blood of patients with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, Sjogren syndrome and T1D (Hoffman 1980; Herold et al. 1984; Negishi et al. 1986; Lorini et al. 1994; French and Yokoyama 2004). In many of these studies, however, NK cell frequency was established based on non-specific markers that did not exclude NKT or other cells from the counts. NKT cells are T regulatory cells involved in the development of peripheral tolerance that share some receptors with NK cells but, differently from NK cells, present CD3 on their surface.

A reduced frequency or function of NK cells in the peripheral blood of patients with autoimmune diseases could represent a primary defect involved in the pathogenesis of the disease or a secondary effect of the disease itself or its treatment. In many cases, the treatment of autoimmune diseases includes corticosteroids. Although it has been shown recently that these drugs may reduce NK killing capacity (Mavoungou et al. 2005), studies in recently diagnosed patients with SLE and dermatomyositis in whom corticosteroid treatment had not been started show that the NK dysfunction cannot be attributed to the treatment (Yabuhara et al. 1996; O’Gorman et al. 2002). Although a deficiency of NK cells could be involved in the appearance of autoimmune diseases in general, interestingly, the main clinical characteristics of patients with complete and selective NK cells deficiency are not autoimmune diseases, but recurrent infections, especially the ones caused by herpesvirus (Biron et al. 1989). On the other hand, patients with NK related lymphocytosis frequently present autoimmune diseases (Tefferi et al. 1994).

Recently, it has been recognized that certain expression patterns of activating and/or inhibitory receptors on NK cells may be linked to the development of autoimmune diseases. A predominance of KIR activating receptors over inhibitory ones was reported in psoriatic arthritis (Martin et al. 2002). An increased expression of MICA in the sinovium of patients with reumathoid arthritis was also shown (Groh et al. 2003).

**NK cells in mouse models of type 1 diabetes**

The two most intensively studied rodent models of autoimmune T1D are the nonobese diabetic mouse (NOD) and the BioBreeding (BB) rat. Both develop autoimmune mediated destruction of the pancreatic β cells after a variable period of insulitis similarly to human T1D. These animal models have been extremely important to help us elucidate the mechanisms involved in the development of T1D. The early stages of the disease process leading to T1D are characterized by insulitis, followed by β cell destruction in the later stages (Kelly et al. 2003). When most insulin-secreting cells are lost, T1D usually becomes clinically evident. The rate of progression from insulitis to T1D is variable and can range from a rapid destruction with a very early onset of the disease to a slow process with a late and quite often insidious clinical presentation. The precise mechanisms involved in the development of insulitis and its progression to necrosis and massive β cell destruction is still yet to be elucidated. Autoreactive T-cells have been shown to have a critical role in this process (Eisenbarth 1986). Although autoantibodies against the major antigens related to T1D are produced, they seem to be a consequence of β cell destruction and a wide spread of antigens present on these cells (Baekkeskov 1982).
A link between the development of diabetes and NK cells was suggested in the 80s. At that time, it was shown that, in diabetes prone BB rat, splenic NK cells were capable of destroying pancreatic islet cells in these animals (MacKay et al. 1986; Koever 1988; Nakamura et al. 1990). More recently, the potential of NK cells to destroy islet cells was also demonstrated in NOD mice (Flodstrom et al. 2002). However, NK cells do not seem to be essential for the development of T1D, at least in animal models. Although the depletion of NK cells prevented the development of T1D in mice treated with streptozotocin (Maruyama et al. 1991a) and cyclophosphamide (Maruyama et al. 1991b), it did not result in similar prevention in mice that spontaneously develop T1D (Ellerman et al. 1993; Sobel et al. 1995) or in BB rats (Edouard et al. 1993; Ellerman et al. 1993). The role of NK cells in the pathogenesis of T1D could be a modulation of the intensity and aggressiveness of the β cell destructive process. Indeed, Poir et al. (2004) have demonstrated a higher frequency of NK cells in the pancreatic infiltrate of BDC 2.5 transgenic mice with a B6.H-2b genetic backgound, known to present a rapid progression from insulitis to T1D, than in BDC 2.5 transgenic mice with a NOD genetic background, which present innocuous insulitis with a rare and slow progression of the disease a higher frequency of NK cells in the pancreatic infiltrate of BDC2.5/B6.H-2b transgenic mice. Flodstrom et al. (2002) suggested that NK cells could have a particularly important role in T1D induced by virus. According to this hypothesis, pancreatic β cells could have an abnormal response to IFN that would render them susceptible to viral infections and subsequent cell death induced by the NK cells. This could trigger T1D not only directly, causing β cell lysis, but also indirectly, as in genetic predisposed individuals the NK mediated damage could contribute to the release of self-antigens that could prime autoreactive T cells and trigger the disease.

Systemic abnormalities of NK cells have also been described in animal models for T1D. It has been shown that diabetes-prone BB/W rats have an increased frequency and activity of NK cells when compared with diabetes resistant rats of the same species (Woda and Biron 1986), although the opposite has been observed when intestinal NK cells were studied (Todd et al. 2004). Numeric and functional abnormalities in NK cells have also been reported in NOD mice and been implicated in the etiology of T1D (Kataoka et al. 1983; Poulton et al. 2001; Johansson et al. 2004). Poulton et al. (2001) have found a decreased numbers of peripheral NK cells in these animals with an increased frequency in the bone marrow, suggesting that a defect in NK export could be involved. Kataoka et al. (1983) described a depression of natural killer activity in 12-week-old NOD mice. A particularly interesting abnormality in NOD mice is a deficient activity of the receptor NKG2D in NK cells. In these animals, upon activation NK cells from NOD mice but not from C57BL/6 mice expressed NKG2D ligands, which resulted in downregulation of the receptor NKG2D. It is not yet known whether this abnormality is involved in the induction of diabetes in NOD mice (Ogasawara et al. 2003). Interestingly, a study performed by the same authors has shown that activation of NKG2D in CD8 + cells is essential for the progression from insulitis to diabetes in NOD mice (Ogasawara et al. 2004).

**NK cells in humans with type 1 diabetes**

A few small studies in the 80s and 90s have shown a reduction of the frequency of NK cells in the peripheral blood in patients with T1D, especially in the ones with recent onset (Chandy et al. 1984; Herold et al. 1984; Pozzilli et al. 1984; Gupta et al. 1986; Wilson et al. 1986; Hussain et al. 1987). A few authors, however, have found a numeric deficiency of NK cells independently of the disease duration and suggested that this abnormality could be persistent and possibly genetically determined (Hussain et al. 1987). On the other hand other studies performed at that time did not find any abnormality in the frequency of NK cells in the peripheral blood (Herold et al. 1984; Scheinin et al. 1990). Two important concerns with those early studies are the small numbers of patients included and the use of non-specific markers such as H25, Leu7 (CD57) and Leu11a (CD16) (Scheinin et al. 1990; Baxter and Smyth 2002) to identify the NK cells. H25 is no longer available, but in any case bound to T cells in addition to NK cells. While Leu 7 (CD57) is not only only expressed in T cells but also absent on some NK cells, Leu11a (CD16) is present on monocytes, macrophages and some granulocytes in addition to NK cells. Recently, we have confirmed with a larger sample and more specific markers at the Joslin Diabetes Center (Boston, MA) that patients with recently diagnosed T1D have a slight reduction in the frequency of NK cell in the peripheral blood when compared to controls or patients with long-standing disease (Rodacki et al. 2006). However, the biological relevance of this finding is questionable, since the difference was quite modest. Nevertheless, this slight reduction could be related to the existence of insulinopenia or, alternatively, to a polarization of NK cells to the pancreas as a contributing factor for the β-cell destruction.

Functional abnormalities have also been reported in NK cells of patients with T1D. A reduced lytic capacity, as determined by cytotoxicity assays, was also reported in those earlier studies, both in recently diagnosed patients and/or the ones with long-standing T1D (Negishi et al. 1986; Lorini et al. 1994). AS for the numeric abnormalities, these results were not
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universally confirmed (Nair et al. 1986; Hussain et al. 1987). In our recent analysis at the Joslin Diabetes Center, there was a reduced surface expression of the activating receptors NKP30 and NKP46 as well as lower mRNA levels of IFNγ and perforin in NK cells of patients with long-standing T1D, but not in those recently diagnosed, when compared to controls without T1D. This indicates that there is a reduction in the activity of NK cells in patients with long-standing T1D but not in individuals with recent onset disease, probably as a consequence of the disease rather than a cause. The discrepancy between these results might be related to the criteria used to define the recent-onset group, varying from within 1 month to up to 1 year after the diagnosis. In our cohort, all recently diagnosed patients were tested within the first month of the disease.

The reduced activity of NK cells in patients with long-standing T1D but not in those recently diagnosed suggests that this could be a consequence of the disease. Either a direct effect in the NK cells or an abnormality that could interfere in their function could be potentially implicated, such as an impaired secretion of cytokines capable of interfering in NK cells function or alteration in ligands to the NK cells activating receptors. It is not yet known if the abnormalities in NK cell function have any influence in the risk of infectious diseases or neoplasias in patients with T1D.

Lanier et al. had demonstrated that activated NK cells in NOD mice displayed a low level of NKG2D. We compared the expression of this receptor in patients with T1D and controls and found a similarly reduced expression of NKG2D in the first group, independently of the duration of the disease (Rodacki et al. 2006). This suggests that there is a deficiency of NKG2D expression not only in NOD mice but also in humans with T1D. It is possible that this abnormality is involved in the induction of T1D.

A few studies have evaluated KIR genes in patients with T1D. While Van der Slik et al. (2003) have shown a predominance of genes encoding activating KIR genes in patients with T1D when compared to controls. Nikitina-Zake et al. (2004) have demonstrated that KIR2DL2, 2DS2 e 2DS3 were more prevalent in patients with T1D than controls without diabetes. These results suggest that certain KIR genes might influence the susceptibility to T1D, which would be reasonable since the KIR repertoire determines self-tolerance in NK cells and also influence the activity of T cells, both autoreactive and regulatory (Vilches and Parham 2002). It is possible that increased frequency of certain KIRs might be associated with autoreactivity in T1D. In our analysis, we confirmed the association between KIR2DS3 and T1D and also found interesting associations between this gene and HLA class II linked to T1D susceptibility. While HLA DR B1*03 was negatively associated with KIR2DS3, for HLA DR B1*04 a positive association was found (Rodacki et al. 2006). Combinations of HLA and KIR genes have been linked with susceptibility of autoimmune diseases (Rajagopalan and Long 2005).

Conclusion

Abnormalities in the frequency and activity of NK cells have been described both in animal models and patients with T1D. Some of these alterations are linked to its onset while others seem to be a consequence of the disease. The elucidation of the exact role of NK cells in the pathogenesis of T1D is important in order to explore whether possible related immune interventions may affect the risk of T1D or delay its age of presentation. It is also important to clarify if NK abnormalities are involved in the risk of infections or neoplasias in patients with T1D and if any intervention can be used to correct this problem.

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References
