NKp30 – a prospective target for new cancer immunotherapy strategies

Pedro Pinheiro[1], Goncalo Justino[1], and M. Matilde Marques[1]

[1]Universidade de Lisboa Instituto Superior Técnico

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Abstract

NK cells are an important arm of the innate immune system, and they constitutively express the NKp30 receptor. NKp30-mediated responses are triggered by the binding of specific ligands, such as tumour cell-derived B7-H6, and involve the secretion of cytotoxic mediators TNF-α, IFN-γ, perforins and granzymes. The latter two constitute a target cell-directed response that is critical in the process of immunosurveillance. The structure of NKp30 is presented, focusing on the ligand-binding site, on the ligand-induced structural changes, and on the experimental data available correlating structure and binding affinity. The translation of NKp30 structural changes to disease progression is also reviewed. NKp30 role in immunotherapy has been explored in chimeric antigen receptor T-cell (CAR-T) therapy. However, antibodies or small ligands targeting NKp30 have not yet been developed. The data reviewed unveils the key structural aspects that must be considered for drug design in order to develop novel immunotherapy approaches.

Introduction

Cancer is one of the major health problems worldwide (Siegel, Miller & Jemal, 2019). Each year the number of cancer cases and associated deaths increases worldwide. Nonetheless, despite the increase in cancer incidence over the past 30 years, the number of deaths has fallen short of the estimated values. Several milestones in cancer therapy are at the origin of the steadily increasing survival rates, and of particular interest are the interleukin 2 (IL-2)-based immunotherapy regimens (DeVita & Rosenberg, 2012).

Immunotherapy was recently added as a major cancer treatment approach, along with surgery, radiotherapy, and chemotherapy. In the early 1960s, it became evident that the immune system was able to prevent the onset of several cancers, as pointed out by some ground-breaking experiments (Brondz, 1964; Granger & Weiser, 1964; Hellstrom, Hellstrom, Pierce & Bill, 1968). The discovery of the T cell growth factor in 1976, later renamed IL-2, further stimulated the studies of the immune response of lymphocytes to cancer cells. The seminal work of Rosenberg *et al* . (Rosenberg et al., 1985) showed that immune modulation could be used to cause the regression of invasive metastases in patients suffering from various types of cancers, including malignant melanoma, colorectal cancer, sarcoma, renal cell cancer adenocarcinoma and oesophageal cancer. This led to the approval of IL-2 for the treatment of renal carcinoma and metastatic melanoma in 1992 and 1998, respectively (Jiang, Zhou & Ren, 2016).

The notion that the immune system could play an important role in cancer treatment led to the development of new antibodies for use as immunomodulatory agents. Patients treated with ipilimumab or rituximab, two monoclonal antibodies designed to promote the immune system response against melanoma and lymphoma, respectively, showed an improvement in overall survival(Hodi et al., 2010; Molina, 2008). These rates increase significantly when these immunomodulators are used in combination with conventional chemotherapeutic

regimens (Castro, Sandoval-Sus, Bole, Rassenti & Kipps, 2008) and other immunomodulators (Shanafelt et al., 2019).

The first application of immunomodulators in cancer therapy was the use of IL-2 and interferon-α (IFN-α). IL-2, as mentioned above, was first used in the treatment of metastatic melanoma in 1984. In this pioneering work, one patient was treated with a high dose of *Escherichia coli* -derived recombinant human IL-2 (rhIL-2). Within two months, all tumours showed extensive necrosis, and were completely eliminated a few months later (Rosenberg, 2014). Following this success, 409 patients with metastatic melanoma and metastatic renal cancer were subject to high dose rhIL-2 regimens. Complete regression was observed in 7% of the melanoma and 9% of the renal carcinoma cases, granting this therapy its approval by the FDA (McDermott & Atkins, 2006). However, it became clear that IL-2 alone was not enough to improve patient survival(Jiang, Zhou & Ren, 2016). Nevertheless, rhIL-2 is currently used, either alone or in combination with other therapeutic agents, in the treatment of a variety of malignancies that include melanoma and renal carcinoma, leukaemia, neuroblastoma and non-small cell lung cancer (Mortara, Balza, Bruno, Poggi, Orecchia & Carnemolla, 2018).

IFN-α, first approved for clinical use in the US in 1986, is another cytokine widely used in cancer treatment. Recombinant human IFN-α type 2b (rhIFN-α2b) is used as a monotherapy or in combination with antineoplastic drugs (Asmana Ningrum, 2014). Like other cytokines, interferons display pleomorphic activities. In the case of IFN-α2b, the anti-cancer activity can be divided into direct and indirect effects. Cancer cell growth is inhibited directly by the induction of cell cycle arrest and apoptosis(Gutterman, 1994). On the other hand, type I interferons, such as IFN-α, have multiple effects on the overall state of the immune system, promoting its activity against cancer cells(Gutterman et al., 1980; Tompkins, 1999).

These discoveries led to the development of new treatments based on the immune system potential to fight cancer. The field of immuno-oncology grew significantly in the last 30 years and today it can be divided into two major strategies: passive and active immunotherapy. Passive immunotherapy comprises the use of antibodies or immunomodulatory molecules, as well as the adoptive transfer of activated immune cells. This last approach includes the isolation and expansion of patient lymphocyte-activated killer (LAK) cells that are primed *ex vivo*with IL-2 to increase their anti-tumour activities (Nagasawa et al., 2012). This *ex vivo* priming of lymphocytes results in a population of highly active T cells that can be expanded and then reinfused back into the patient, with significant results in tumour regression (Nagasawa et al., 2012; Tsurushima et al., 1999), and have also been used in a few trials with somewhat encouraging results (Quattrocchi et al., 1999). The concept of isolating tumour-infiltrating lymphocytes (TIL) or circulating T cells for *in vitro*cultivation, activation and expansion, and subsequent reinfusion, has demonstrated promising results in the treatment of several malignancies, supporting the therapeutic potential of tumour-specific T cells (Sharpe & Mount, 2015). However, TIL isolation requires surgical techniques that may not be possible in patients with visceral tumours in later stages (Fan, Shang, Han, Song, Chen & Yang, 2018), and TAA recognition is MHC-restricted. This means that the anti-tumour activity is only developed against cells presenting foreign antigens bound to MHC molecules. As tumours use immune escape mechanisms based on MHC expression alteration and TAA processing, a fair part of these adoptive cell transfer (ACT) therapies fail to produce significant results. To overcome these problems, genetically engineered T cells have emerged as an alternative (Fousek & Ahmed, 2015).

The branch of active immunotherapy comprises the methodologies that aim to induce a response by stimulating the immune system. This differs from the passive immunotherapy as the molecules/cells used in this case are not produced by the host. In active immunotherapy the immune system is boosted, with either cytokines, immunoadjuvants, or vaccines, to elicit generic or specific responses (Baxter, 2014).

All these techniques have been proven effective in cancer treatment. However, none of these is free from pitfalls. All the immunotherapy regimens currently approved or under study perform extremely well in some cancers and patients but fail completely in others. This was observed early in the immuno-oncology era with IL-2-based treatments that have a response of only *ca* . 7% (Rosenberg et al., 1985). One drawback is the fact that some patients present little or no immune response to their tumours, with the tumour microenvironment being devoid of infiltrating lymphocytes, rendering cytokine-, antibody-, CTL- and TIL-

based therapies useless. This is nowadays recognised as the next big challenge in cancer immunotherapy (Gajewski, 2015).

It is important to mention the economic impact of the available treatments per patient. The cost of standard treatment with one checkpoint inhibitor antibody is around \euro100 000 per year (Fellner, 2012), while a single CAR-T treatment can reach \euro430 000 (Hay & Cheung, 2019). For these reasons, new cost-effective immuno-oncology treatments are necessary to increase the number of patients receiving such regimens and their efficacy. Many authors have focused recently on a subset of lymphocytes – natural killer (NK) cells – that possess the innate ability to detect transformed cells, proposing them as the next "major target in cancer immunotherapy" (Lorenzo-Herrero, Lopez-Soto, Sordo-Bahamonde, Gonzalez-Rodriguez, Vitale & Gonzalez, 2018; Souza-Fonseca-Guimarães, Cursons & Huntington, 2019).

Natural killer cells

Natural killer (NK) cells are a part of the innate immune system. This subset of lymphocytes was first identified in the 1970s as a set of cells that were neither B or T and that could kill target cells without prior sensitization (Greenberg, 1994). The term *natural killer*was devised by Kiessling and co-workers in 1975 (Kiessling, Klein & Wigzell, 1975) to describe these naturally occurring lymphocytes with lytic activity against syngeneic or allogenic cells.

NK cells descend from the same lineage as T cells, thus sharing some of the characteristics of these lymphocytes, such as cytolytic activity and lymphokine (lymphocyte-produced cytokines) production. However, NK cells do not undergo T cell receptor gene rearrangement to create diverse receptors as part of the adaptive immune system (Caligiuri, 2008; Moretta, Ciccone, Mingari, Biassoni & Moretta, 1994). Therefore, the mechanisms through which NK cells lyse tumour and virus-infected cells are regarded as non-specific (Trinchieri, 1989). Recent studies have related altered levels of circulating NK cells and/or activity with cancer development, acute viral infections and autoimmune diseases, as summarized in **Table 1** .

Table 1

NK cells are large granular lymphocytes with a diameter ranging from 7 to 12 μm, depending on the activation state. Upon staining, these cells are easily recognised among other circulating lymphocytes by their reniform nucleus and by the presence of azurophilic granules in the cytoplasm (Whiteside & Herberman, 1994). These characteristics are not exclusive of NK cells, as activated cytotoxic T cells may also present such phenotype. Therefore, for the correct identification of the NK phenotype, CD3 must be absent, and CD56 (neural cell adhesion molecule, NCAM) and CD16 (FcγRIII) must be present. Moreover, NK cells also lack surface immunoglobulins (Ig) and constitutively express IL-2 receptors (Nagler, Lanier & Phillips, 1990; Whiteside & Herberman, 1994). Many other surface markers are present in NK cells, as well in other lymphocytes. However, the unique combination of the $CD3^-$ $CD56^+$ $CD16^+$phenotype defines human NK cells (Abakushina, 2015). Additionally, two subsets of NK cells may be defined, depending on the relative expression of CD16 and CD56. These are called $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^+$, where bright and dim are associated with high and low levels of expression, respectively. The first subset is outnumbered by the second in circulation but constitutes the majority of NK cells in lymphoid tissues. These subsets present different expression levels of other markers and receptors and different cytotoxic activities, with $CD56^{bright}$ being significantly less cytotoxic than $CD56^{dim}$ cells (Poli, Michel, Theresine, Andres, Hentges & Zimmer, 2009). It has been hypothesized that $CD56^{bright}$ cells are an immature form of NK cells that differentiate into $CD56^{dim}$ NK cells, which participate in natural and antibody-mediated cell cytotoxicity (Chan et al., 2007). On the other hand, $CD56^{bright}$ NK cells express higher levels of cytokine receptors, such as the IL-2 receptors αβγ and βγ (IL-2Rαβγ and IL-2Rβγ, respectively), higher levels of some activating and inhibiting receptors, and chemokine receptors. These cells produce higher levels of cytokines upon stimulation, namely of IL-10, an immunosuppressive cytokine, and therefore are thought to have immunoregulatory properties (Cooper et al., 2001).

NK cells also display a broad range of cell adhesion molecules (CAMs) essential for their cytotoxic activities, as these participate in interactions between NK cells, target cells, and accessory cells to generate effective immune responses. Some of these CAMs are upregulated upon activation, including lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18), LFA-3 (CD58) and intracellular adhesion molecule-1 (CD54). Interestingly, upon *in vitro* stimulation with IL-2, the cell surface levels of all these CAMs increase, as do the levels of CD56. Even more interesting is the fact that, upon this increase in CAM expression, the cytotoxic activity of NK cells towards NK-sensitive target cells also increases, and cells that were previously non-susceptible to NK-killing become targets (Robertson, Caligiuri, Manley, Levine & Ritz, 1990).

The cytotoxic response of NK cells

The cytotoxic response of NK cells upon activation is divided into 4 steps – formation of the immunological synapse, microtubule re-organization, lysosome docking to the membrane, and lysosome fusion with the membrane (Paul & Lal, 2017).

In the first, an interface between the NK cell and the target cell is formed, deemed immunological synapse. This occurs when the NK cell approaches the target, either accidentally or "intentionally" due to chemotactic signalling.

Upon contact, CD2 molecules present on the surface of NK cells recognise the presence of stage-specific embryogenic antigen 1 (SSEA-1, also known as CD15 or Sialyl-Lewis^{X}). At this point, if the target cell presents markers for NK cell inhibition, the formation of the immunological synapse is halted. Conversely, if activating receptors are present, tight adhesion between the two cells is promoted by receptor-ligand interactions of high affinity. LFA-1 and MHC-1 molecules present on NK cells bind ligands present on the target cell surface. The formation of these receptor-ligand complexes is enough to activate the cytolytic response, to some extent. However, these processes are considered more relevant in maintaining the immunological synapse than in triggering NK cell cytotoxicity. Full activation of the cytotoxic response depends on the engagement of specific receptors, such as the natural cytotoxicity receptors, present on the surface of NK cells. The immunological synapse is shaped in a way that both cells form a ring-shaped interface. Within this ring, cytotoxic granules and other cytotoxicity mediators are released directly and in a controlled fashion towards the target-cell surface (Orange, 2008; Stinchcombe & Griffiths, 2007). The secretory lysosome exocytosis requires reorganization of the cell cytoskeleton. In this step, the microtubule organizing centre (MTOC) becomes polarized and the secretory lysosomes are transported along the microtubules towards the synapse. Upon reaching the cell membrane, the granules dock and fuse, releasing the contents towards the target. This formally constitutes degranulation (Paul & Lal, 2017; Topham & Hewitt, 2009).

The molecular mechanisms of target-cell killing

As referred before, contrarily to T cells, NK cells do not rely on the somatic rearrangement of receptor genes to accommodate the expression of a variety of receptors. Instead, NK cell receptors are germ-line-encoded and consistently expressed (Biassoni, 2008; Whiteside & Herberman, 1994). The homeostasis of NK cell activity is ensured by a specific set of receptors with activating and inhibitory activities, some of which overlap. Inhibitory receptors contribute to self-tolerance of NK cells, preventing the lysis of normal healthy cells. On the other hand, activating receptors trigger the lytic activity of these cells, prompting the destruction of cells that present activating ligands.

Upon activation, NK cells release a series of lytic enzymes through the degranulation process already described, namely perforins and granzymes. Granzymes induce target-cell apoptosis, but their actions depend on being appropriately delivered by perforins (Boivin, Cooper, Hiebert & Granville, 2009). Together with these enzymes, a wide variety of cytokines is also released, including interferon γ (IFN-γ), tumour-necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, IL-5 and IL-13, chemokine macrophage inflammatory proteins 1α and 1β (MIP-1α, MIP-1β), IL-8, and chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES) (Paul & Lal, 2017).

Besides the direct effect of granzymes (and perforins), cytokines such as IFN-γ also play an important role in inducing target-cell death. IFN-γ, as seen before, is an important activator of macrophages, as well of APCs. Stimulation of the latter, as shown before, upregulates the expression of cytokine IL-12, as well as IL-18 and co-stimulatory molecules CD86, that enhance T_h1 differentiation and CTL function. IFN-γ can also exert antiproliferative effects on tumour cells by enhancing the expression of the cell cycle inhibitor proteins p27Kip, p16 or p2 (Ni & Lu, 2018). TNF-α is also implicated in the collapse of tumour vasculature, but can additionally induce tumour regression by triggering apoptosis, T cell activation (by Tregs' blockage), and neutrophil and monocyte chemoattraction to the tumour region, and by downregulating the immunosuppressive phenotypes of tumours (Josephs et al., 2018).

GM-CSF is a part of the inflammatory cascade, recruiting monocytes and inducing the differentiation, proliferation, and migration of granulocytes (neutrophils, eosinophils and basophils) to the inflammation site. It is also essential for the differentiation of dendritic cells that, as seen before, are responsible for processing and presenting of tumour antigens and for activating cytotoxic T lymphocytes (Yan, Shen, Tien, Chen & Liu, 2017).

IL-5 and IL-13 are associated with B cell growth, eosinophil activation, and regulation of inflammatory and immune responses (Minty et al., 1993). IL-10, on the other hand, seems to have a pleiotropic effect on the immune response. It downregulates T_h1 responses, cytokine secretion, such as TNF-α, IFN-γ and IL-12, and $CD4^+$ T cell activation (de Waal Malefyt, Abrams, Bennett, Figdor & de Vries, 1991; de Waal Malefyt et al., 1991). However, IL-10 can also induce the secretion of granzymes and perforins by $CD8^+$ T cells and potentiate TCR-dependent IFN-γ secretion (Emmerich et al., 2012). Lastly, MIP-1α/1β, IL-8 and CCL-5 have chemotactic activities towards granulocytes, neutrophils and T cells, eosinophils and basophils (Kohidai & Csaba, 1998; Wolpe et al., 1988).

Through the secretion of these cytokines and chemokines, NK cells are able to recruit other immune cells, namely dendritic cells that can infiltrate the tumour tissue and trigger robust and sustained immune responses (Bottcher et al., 2018).

Balance between activation and inactivation of NK cells

NK cells express a wide variety of receptors with activating and inhibiting functions that allow for the fine discrimination between healthy and ailing cells in a matter of seconds; the activating and inhibitory signalling pathways involved are depicted in **Figure 1** . Most of the inhibitory receptors detect the absence of MHC I molecules, while activation receptors probe for the presence of specific ligands that flag the target cells as harmful (Leung, 2014). Because NK cells are extremely cytotoxic when active and can very quickly deliver their lytic response without prior sensitization, contrarily to other lymphocytes, a complex activation/inactivation system tightly regulates their responses. Activation depends on tipping the balance between stimulatory and inhibitory signals, an equilibrium that is dictated by the engagement of different receptors. After cell-to-cell contact, NK cells integrate, within seconds, the signals from their activating and inhibiting surface receptors. Normal cells, expressing normal MHC I molecules, will engage the inhibitory receptors, rendering NK cells unresponsive, without compromising their functionality (*licensing*). Cells lacking MHC I and presenting surface activating ligands will trigger the cytolytic response. A dynamic equilibrium is reached when the target cells present both MHC I molecules and activating ligands. In this case, no response is triggered as the positive and negative feedback loops cancel each other. If more activating ligands are engaged, the activation signal dominates and target-cell lysis occurs (**Figure 2**) (Vivier, Ugolini, Blaise, Chabannon & Brossay, 2012).

The engagement of different receptors is translated into activating and inactivating signals through ITIM and ITAM motifs, present in either the receptors themselves or in adaptor proteins. The ITAM and ITIM signalling cascades are combined intracellularly to determine the response of NK cells. Upon binding of activating ligands, the ITAM motifs are phosphorylated, triggering the recruitment of Syk-family kinases and the subsequent activation of cytolytic responses. However, engagement of inhibitory receptors also leads

to the phosphorylation of ITIM motifs on these receptors, a process that is promoted by the same Src kinase responsible for ITAM phosphorylation. These now phosphorylated ITIMs recruit the tyrosine phosphatases SHP1 or 2, which can in turn terminate intracellular signals emanating from ITAM signalling receptors via their phosphatase activity, rendering NK cells inactive (**Figure 2**)(Linnartz-Gerlach, Kopatz & Neumann, 2014).

Figure 1

Figure 2

As mentioned, most of the inhibitory signals are triggered by ITIM motifs. Activation signals are transduced by ITAM motifs located in the cytoplasmic portion of the receptor or of associated molecules, that include DAP12. The activating receptor NKG2D, constitutively expressed by all NK cells, bears no ITAM motif. Therefore, in order to transduce stimulatory signals, NKG2D is associated with an adaptor subunit designated DAP10 (Farag, Fehniger, Ruggeri, Velardi & Caligiuri, 2002). DAP10 does not contain ITAM motifs either, but is able to recruit PI3K activity upon tyrosine phosphorylation of its YINM motif (Lanier, 2009).

NK cells also present receptors that can either activate or inhibit the response, namely the natural cytotoxicity receptors (NCRs) 2B4 and NKR-P1. The 2B4 receptor has been classified as a multi-functional receptor with an activity (activation or inhibition) that depends on the stage of NK cell maturation. Also, two isoforms of this receptor have been identified, with different intracellular domains (an ITIM and an immunoreceptor tyrosine-based switching motif, ITSM), of which only one transduces stimulatory signals. Similarly, the NKR-P1 receptor family contains several members, of which two contain ITIM motifs and one is associated with a high-affinity IgE receptor (FcεRI) that bears ITAM motifs, thus transducing an activation signal.

Tumour cells are generally under a constant state of cellular stress due to hypoxia, chronic proliferative signals, and genome instability. Not surprisingly, cells under these conditions upregulate KAR ligands, becoming susceptible to NK cell killing. Some mutations, characteristic of carcinogenesis, also downregulate the presentation of MHC I molecules, rendering tumour cells more susceptible to attachment by NK cells. To survive, cancer cells develop mechanisms to evade NK cell killing. One of the proposed evasion mechanisms hypothesizes that tumour cells secrete soluble forms of the activating ligands. Certain human tumours can release soluble forms of MICA and MICB (MHC class I polypeptide-related sequence A and B, respectively), the natural activating ligands of NKG2D. As NKG2D-dependent signalling requires dimerization of the ligands, by saturating the tumour environment with monomeric ligands, tumour cells are able to downmodulate NK cell responses (Groh, Wu, Yee & Spies, 2002). On the other hand, it has been suggested that highly specific NKG2D soluble ligands can reverse completely the desensitization of NK cells and increase tumour regression (Deng et al., 2015).

Other direct evasion mechanisms rely on the secretion of immunosuppressive factors such as IL-10 or TGF-β1 (transforming growth factor β1), or upregulation of MHC I molecules to counteract the stimulating signals of activating ligands. Indirect evasion mechanisms require the activation or inhibition of Tregs, killing of dendritic cells, and even the use of monocytes and macrophages as NK cell desensitizers. Monocytes and macrophages can be reprogrammed by tumour-derived signals and start to express inhibitory molecules, such as TGF-β1, inhibiting the activity of the adjacent NK cells (Peng et al., 2017; Sabry & Lowdell, 2013).

Natural Cytotoxicity Triggering Receptors

Among the known activating receptors, NKG2D is by far the best characterized. It has been argued that, because NKG2D has a signalling pathway that does not overlap with the inhibitory signalling pathway of KIRs, its triggering is less susceptible to inhibitory ligands. At the same time, several studies have highlighted the importance of NCRs as part of the main mechanisms by which NK cells kill tumour targets (Pegram, Andrews, Smyth, Darcy & Kershaw, 2011). In fact, one study demonstrated that lymphoma cells are able

to grow *in vivo* in the absence of NCR^+ NK cells in mutant mice, but in wild-type mice, tumours were completely rejected over time (Halfteck, Elboim, Gur, Achdout, Ghadially & Mandelboim, 2009).

NCRs are expressed exclusively by NK cells and are extremely important in their activation. Of the four NCRs currently known, three are constitutively expressed (NKp30, NKp46 and NKp80) and one is expressed only in activated NK cells (NKp44) (Barrow, Martin & Colonna, 2019; Moretta et al., 2001). Engagement of these receptors by their ligands transduces a strong activation signal to the cell. Signal transduction by NCRs happens through ITAM motifs of associated proteins DAP12, in NKp44, FcεRIγ and CD3ζ in NKp30 and NKp46 (Kruse, Matta, Ugolini & Vivier, 2014; Moretta et al., 2001). NKp80 has been described only recently and its signalling is transduced through a hemITAM motif (Bartel, Bauer & Steinle, 2013). Interestingly, NKp44 presents three alternative mRNA splice variants, one of which bears in its intracellular portion an ITIM motif. The different levels of expression of these variants define the signalling arising from NKp44 engagement (Parodi et al., 2019).

The surface density of NCRs varies amongst individuals and is directly correlated with the ability to actively eliminate tumour cells. Moreover, lysis of certain NK-sensitive tumours can be averted by simultaneously blocking the activity of NKp30, 44 and 46, showcasing the relevance of these molecules (Biassoni et al., 2001; Moretta et al., 2001). Contrarily to what happens to NKG2D, NCRs NKp30 and NKp46 are not upregulated upon cytokine treatment. This demonstrates that the NCR density at the NK cell surface is remarkably stable and its underexpression (termed NCR^{dull}) has been correlated with poor prognosis in cases of leukaemia (Fauriat et al., 2007) and non-small cell lung cancer (Charrier et al., 2019).

NCRs have been shown to recognise a multitude of ligands, including bacterial-, viral- and parasite-derived proteins, as well as stress-induced proteins (Kruse, Matta, Ugolini & Vivier, 2014). NKp30 interacts with a broad range of ligands without an obvious structural similarity, including viral, parasitic and tumoral proteins(Kruse, Matta, Ugolini & Vivier, 2014). NKp30, for instance, has been shown to interact with B7-H6 and BAT3, two proteins that share no homology (Pende et al., 1999). Binding of heparin and heparan sulphate sequences is also a puzzling observation. These sequences are widely expressed in all cells, but NCRs seem to be able to distinguish between the ones presented by tumoral and normal cells (Kruse, Matta, Ugolini & Vivier, 2014).

NKp30 has been regarded as a possible target for future immunotherapies due to its importance in mediating the anti-tumour effects of NK cells in both solid tumours and haematological malignancies (Correia, Fogli, Hudspeth, da Silva, Mavilio & Silva-Santos, 2011; Delahaye et al., 2011; Zhang, Wu & Sentman, 2012).

NKp30 as a mediator of NK activity

The structure of NKp30

The work of Pende and co-workers, in which NKp30 was first identified, demonstrated the importance of this receptor in the lysis of several tumour cell lines (Pende et al., 1999). More recently, its structure has been obtained by X-ray crystallography, revealing features distinct from the other known NCRs (Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011).

The ectodomain of NKp30 contains an immunoglobulin (Ig)-like fold, connected to a transmembrane α-helix via a short stalk domain. The Ig-like domain is comprised of eight β-strands, forming two antiparallel β-sheets, linked by a disulphide bond between Cys39 and Cys108 (**Figure 3**)(Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011; Li, Wang & Mariuzza, 2011; Pende et al., 1999).

Figure 3

Although initially overlooked, this stalk domain has been shown to play an important role in the receptor activity, as it increases ligand binding affinity (Hartmann et al., 2012). Initially considered to include 5 to 6 residues (Pende et al., 1999), the stalk domain considered here is a longer 15-residue domain

(^{129}KEHPQLGAGTVLLLR^{143}) (Memmer et al., 2016). This stalk domain is very sensitive to point mutations, that lead to impaired binding and activity. The last residue of this domain is a cationic arginine. Arg143 is located on the membrane extracellular interface, but upon ligand binding it is translocated into the membrane, strengthening the interaction with the CD3ζ and FCεRIγ Asp residues (Li, Wang & Mariuzza, 2011; Memmer et al., 2016).

The initial structural elucidation of NKp30 identifies it to be monomeric in the crystal phase, in agreement with its behaviour in size exclusion chromatography and on the cell surface, but later work identifies it as a dimer (Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011; Pende et al., 1999). However, the authors also point out that many similarities between NKp30 and the homodimeric NKG2D receptor suggest that NKp30 can exist as a homodimer in which the binding site is formed by the two identical subunits (Li, Wang & Mariuzza, 2011).

NKp30's closest human homologues are CTLA-4 (Kaifu, Escaliere, Gastinel, Vivier & Baratin, 2011), a homodimeric member of the CD28 family, and PD-1, an immune checkpoint protein that guards against autoimmunity(Francisco, Sage & Sharpe, 2010; Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011); a simple homology modelling approach based on the known structure of CTLA-4 originated a homodimeric NKp30 structure, in which the subunit interaction is dominated by a network of salt bridges and hydrogen bonds between Glu8, Arg10, Glu13, Arg106 and Glu110 of both monomers(Kaifu, Escaliere, Gastinel, Vivier & Baratin, 2011). However, this dimer is formed by a top-top approach of the subunits and is unlikely to be relevant in vivo. It has recently been shown that the formation of NKp30 ectodomain dimers leads to a higher affinity towards the B7-H6 ligand. This ectodomain dimerization appears to be driven by the association of the stalk domains of both polypeptide chains (Herrmann, Berberich, Hartmann, Beyer, Davies & Koch, 2014).

Superposition of the NKp30 and the NKp44 structures revealed common features as well as distinct conformations. The core regions of NKp30 and NKp44 are quite similar, but significant differences are found at the interacting region between the two β-sheets, which in the case of NKp30 presents six more H-bonds. An additional α-helix is found between strands in NKp30 that is absent in NKp44. Furthermore, NKp30 lacks the extensive positively charged region that is found in NKp44. Sequence homology is about 30% between NKp30 and NKp44 whilst there is no significant overlapping with NKp46 (Foster, Colonna & Sun, 2003; Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011; Li, Wang & Mariuzza, 2011).

As previously mentiones, signal transduction occurs through the ITAM-bearing adaptor proteins CD3ζ and FCεRIγ. Like the other NCRs, association with the adaptor proteins is promoted by opposing charge residues. NKp30 and NKp46 contain positively-charged Arg residues that interact with Asp residues in CD3ζ and FCεRIγ. On the other hand, NKp44 contains positively-charged Lys residues that interact with Asp residues in DAP12 (Koch, Steinle, Watzl & Mandelboim, 2013). Interestingly, NKp30 has been shown to be expressed in several variants (NKp30a , b , c ,d , e , f) resultant from alternative splicing. Cells transfected with the variants NKp30a and NKp30b were found to produce high amounts of IFN-γ upon NKp30 engagement. Conversely, cells transfected with the NKp30c variant produced small amounts of IFN-γ but high levels of IL-10, the immunosuppressor interleukin. This variant seems to interact poorly with the CD3ζ adaptor protein, in contrast with the a and b variants, which could justify the dual immunomodulatory effect of NKp30 (Delahaye et al., 2011; Siewiera et al., 2015). Moreover, one work has demonstrated that expression of higher levels of the c variant is directly correlated with poor prognosis in gastrointestinal tumour patients (Delahaye et al., 2011).

The role of the positively charged Arg143 residue in the end of the stalk domain in the association of the receptor with CD3ζ, together with the observation that CD3ζ increases cell membrane expression of NKp30, has led to the proposal that this receptor exists in two different assemblies – a signalling-incompetent one and a ligand-induced signalling-competent complex (Memmer et al., 2016), which is consistent with the *licensing* hypothesis for NK cell response (Yokoyama & Kim, 2006).

It is also important to mention that NKp30 is present in the extracellular surface of the cell as a glycoprotein

and that different glycosylation patterns affect the affinity towards its ligands. For instance, glycosylation of Asn42 increases the binding affinity of BAT-3 but severely hampers the interaction with B7-H6 (Hartmann et al., 2012). These structural features, which may vary between individuals, may be the cause of some pathologies and the reason why some patients develop aggressive malignancies even when their NK cell population is normal.

The NKp30 receptor is the product of the *NCR3* gene expression, which can be transcribed as four exons that undergo alternative splicing(Delahaye et al., 2011; Shemesh, Brusilovsky, Kundu, Ottolenghi, Campbell & Porgador, 2018). Up to this date, only isoforms NKp30 a to c, resulting from alternative splicing of exon 4 and corresponding to different intracellular terminal domains, have been studied. NKp30a and NKp30b present a stronger association to the intracellular adaptor CD3ζ (and also to FcεRI-γ), while NKp30c exhibits a lower association to either (Delahaye et al., 2011; Kaifu, Escaliere, Gastinel, Vivier & Baratin, 2011).

NKp30a and NKp30b are typically associated with an immunostimulatory activity of NK cells, and are associated with overall improved survival rate and with better prognosis in gastrointestinal stromal tumours (Delahaye et al., 2011; Rusakiewicz et al., 2017) and in hepatocellular carcinoma (Mantovani et al., 2019), in contrast to a higher morbidity observed in patients that predominantly express isoform NKp30c. A similar trend is observed in the case of some viral infections, namely in chronic hepatitis virus C infection (Mantovani, Mele, Oliviero, Barbarini, Varchetta & Mondelli, 2015), while no clear association between NKp30 expression status and outcome could be found in HIV-1 patients (Prada et al., 2013).

This isoform-prognostic correlation is particularly evident in paediatric neuroblastoma patients, where the pattern of isoform expression is highly correlated to a 10-year event-free survival in multiple cohorts (Semeraro et al., 2015; Semeraro, Rusakiewicz, Zitvogel & Kroemer, 2015). However, this requires further study, as a later clinical study addressing paediatric neuroblastoma response to imatinib treatment failed to identify any correlation between NKp30 isoforms and clinical response (Morandi et al., 2018).

While isoforms NKp30a, NKp30b and NKp30c present a V-type Ig-like extracellular domain, splice variants NKp30d, NKp30e and NKp30f encode a different C-type Ig extracellular domain, coming from an exon 2 alternative splicing, with aminoacid residues 66 to 90 being absent (Hollyoake, Campbell & Aguado, 2005; Kaifu, Escaliere, Gastinel, Vivier & Baratin, 2011; Neville & Campbell, 1999; Shemesh, Brusilovsky, Kundu, Ottolenghi, Campbell & Porgador, 2018). The intracellular domains, resulting from exon 4 expression, are shared between isoforms a and e, between isoforms b and d, and between isoforms c and f (Shemesh, Brusilovsky, Kundu, Ottolenghi, Campbell & Porgador, 2018). A seventh isoform, computer-generated, that matches experimental evidence at protein level, but corresponds only to the extracellular Ig domain of NKp30, has been proposed (Uniprot accession number A0A0G2JKT7) but has not been confirmed yet.

NKp30 polymorphisms in disease

The outcome variability associated with immunotherapy regimens is most likely of multifactorial origin, and a contributing factor is the variability of the immune cell receptors involved in recognition and signalling. A large number of single nucleotide polymorphisms (SNP) of the NKp30-coding *NCR3* gene have been identified, but only a very small number of them have been studied, particularly regarding population resistance to parasite infection (Delahaye, Barbier, Fumoux & Rihet, 2007; Hermann et al., 2006).

A susceptibility locus for mild malaria, a major cause of morbidity and mortality in many developing countries, is located in the MHC region in chromosome 6p21, where *TNF* polymorphisms have been associated with mild malaria. The *NCR3* gene is located just 15 kb distal to *TNF* , and early studies have shown that the *NCR3* -412G>C SNP is associated with increased risk and frequency of mild malaria (Delahaye, Barbier, Fumoux & Rihet, 2007), when compared to the standard *NCR3* -412G allele. A later study confirmed this, but also showed that this association only occurs for mild malaria cases, and not for severe malaria cases (Thiam et al., 2018). Noticeably, STAT4, a transcription factor essential for IL-12 mediated cytotoxicity and IFN-γ production in mouse and human NK cells, binds this promoter region with higher affinity for the G allele (Baaklini et al., 2017). In agreement with this, purified NK cells were found to lyse *Plasmodium*

falciparum -parasitized erythrocytes, through a direct interaction of NKp30 with *P. falciparum*erythrocyte membrane protein–1 (*Pf* EMP-1) (Mavoungou, Held, Mewono & Kremsner, 2007). A more recent study with healthy human volunteers found that parasitemia levels and NK cell activation follow the same trend during the first 18 days post-challenge by the bites of five *P. falciparum* 3D7 strain-infected *Anopheles*mosquitoes, in what is the first study identifying the role of the NKp30 protein against *P. falciparum* (Walk & Sauerwein, 2019).

When considering the role of NKp30 on *Trypanosoma cruzi*infections, Hermann *et al.* found that cord blood NK cells from newborns congenitally infected with *T. cruzi* had a reduced expression of NKp30, which could be due to earlier NK cell activation or to NK cell activity down-regulation by *T. cruzi* itself (Hermann et al., 2006). Later work has shown that NK cells were activated*in utero* , during the first exposure to the parasite, and that the fetuses response to *T. cruzi* is an adult-like one, based on IFN-γ production by CD8+ T cells and through an IL-12 dependent monocyte pathway (Guilmot, Bosse, Carlier & Truyens, 2013; Hermann et al., 2006; Hermann et al., 2010; Sathler-Avelar et al., 2003).

Two other NKp30 SNPs in the promoter region have been studied in healthy Japanese, the -201G>A and -163G>C, but no transcription binding factors are known at these sites; a second set of SNPs were found in the same population, c.111G>A and c.156C>T, but these are synonymous substitutions, and so the phenotype remains unchanged(Sato et al., 2001).

Ligands for NKp30

The NKp30 receptor may be engaged by several ligand types. Interestingly, and even though NKp30 is considered an activating receptor, some ligands have been associated with the opposite response. This is the case of Poxvirus haemagglutinin (HA) that was found to bind NKp30, acting as antagonist. On the other hand, these same antigens are able to trigger the NKp46-mediated response (Jarahian et al., 2011). Human cytomegalovirus tegument protein pp65 (HCMV pp65) is also able to block the triggering signal of NKp30 engaging. This protein interacts directly and specifically with NKp30 without blocking the interaction of this receptor with its activating ligands. Despite being able to form NKp30-activating ligand complexes, the killing action of NK cells is suppressed by pp65, which suggests a downstream effect of this protein on the stimulatory signal transduction. It was demonstrated by Arnon and co-workers that pp65 disrupts the interaction of NKp30 with the adaptor protein CD3ζ, directly reducing the activation state of NK cells (Arnon et al., 2005). The other known ligands for NKp30 have activation effects and their engaging results in cytokine production and perforin and granzyme release. For instance, it was found that molecules expressed on the surface of *Plasmodium falciparum* -infected erythrocytes can be recognised by NKp30 and NKp46. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) was identified as a strong activating ligand of NKp30 and NKp46 that, upon engagement, results in the lysis of the infected cells (Mavoungou, Held, Mewono & Kremsner, 2007).

Despite the importance of these virus- and parasite-infected cell destruction activities, researchers have focused primarily on NK cell tumour-targeting. One crucial finding stimulating this research field was the discovery of heparin and heparan sulphates as activating ligands for NCRs. An initial research paper described, in 2004, that membrane-associated heparan sulphate proteoglycans were involved in the recognition of cellular targets by NK cells. In fact, the NCR NKp44, as referred before, bears an extensive positively charged surface that may constitute a binding site for sulphated carbohydrate structures (Bloushtain et al., 2004). Heparin and heparan sulphate sequences have formal negative charges (in their sulphate groups) that would account for strong interactions. Binding specificities of NKp30, 44 and 46 to these carbohydrates were further investigated, revealing that different receptors bind different monomer sequences (Hecht et al., 2009). However, since NKp30 does not possess a positively charged region, contrary to NKp44, and, as stated before, there is no structure homology between the receptors, the existence of other binding sites in the receptors or the cooperation of ligands to induce the activation response, seems plausible. In fact, some evidences indicate that heparan sulphates are able to bind NCRs but unable to induce cytolysis on their own. On the other hand, the diversity of heparan sulphate molecules with different sequences and chemical

moieties may account for the inconsistent results that have been presented, as these sequences might also interact with inhibitory receptors (Hershkovitz et al., 2008). Besides the wide diversity of heparan sulphate sequences, it is also worth to mention that different NCR glycosylation states may play an important role in the binding of different molecules, especially large molecules that assume secondary and tertiary conformations, like polysaccharides (Brusilovsky, Radinsky, Yossef, Campbell & Porgador, 2014; Hershkovitz et al., 2008).

Other molecules that are also expressed by tumour cells and act as activating ligands of NKp30 are the proteins BAT3 and B7-H6, as seen before. BAT3 (HLA-B associated transcript 3, also identified as BAG-6) is overexpressed by tumour cells in response to stress signals. Under these conditions, the protein is translocated to the cell membrane and released from the cells, engaging the receptor NKp30. Interestingly, cytokine release is only experimentally attained using tumour-released BAT3 and no activation of NK cells is observed when a recombinant protein is used. This suggests that BAT3 only induces the cell response when it is part of vesicles, such as exosomes (Pogge von Strandmann et al., 2007). On the other hand, the other known tumour-derived ligand of NKp30, B7-H6, is able to induce cytokine release in its soluble or membrane-bound form (Gutierrez-Franco et al., 2018; Phillips, Romeo, Bitsaktsis & Sabatino, 2016). Membrane expression of B7-H6 has been widely implicated in the sensitivity of cancer cells to NK cell-mediated cytolysis (Cao, Wang, Zheng, Wei, Tian & Sun, 2015). Conversely, NK cells chronically stimulated with soluble forms of B7-H6 have been shown to downregulate the expression of NKp30, contributing to tumour immune escape (Pesce et al., 2015). It seems that, besides the normal regulation mechanisms presented by NK cells, there is yet another layer of regulation dictating the activity of NK cells. Nevertheless, among all the known ligands for NKp30, B7-H6 has been regarded as a possible target for novel cancer therapies. The basis of the B7-H6-NKp30 recognition has been widely studied in several works, in an effort to highlight the molecular mechanisms behind the strong NK cell response elicited by tumours presenting B7-H6. Protein glycosylation also plays a role, as it has been shown that single, double and triple mutations, where residues Asn42, Asn68 or Asn21 were mutated to Gln, lead to increased K_D values for B7-H6 binding, with the exception of the Asn121Gln, which practically does not affect K_D values (Hartmann et al., 2012).

NKp30's B7-H6 binding site

To understand the basis of tumour recognition by NKp30, several authors have tried to accurately describe the interactions between NKp30 and its natural ligands. As there is no significant sequence homology between the known NKp30 ligands, or similarity in their predicted structure folding, it is not possible to determine a plausible binding site by identifying common features in ligand structures. Site-directed mutagenesis was used to alter the surface charge and/or hydrophobicity of specific areas of NKp30, allowing the identification of a relatively small region where at least one of the known ligands – B7-H6 – interacts (Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011). The B7-H6 binding region of NKp30 is comprised mostly of non-polar amino acid residues, that account for the hydrophobic interactions between the two proteins. Two hydrogen bonds are also involved, as well as a salt bridge. B7-H6 affinity is extremely sensitive to changes in the protein structure, as the interactions of the two proteins are restricted to a small region of NKp30 (residues Ile50-Val53, Leu80, Ser82, Phe85, Leu86, Glu111, Leu113 and Gly114), as seen in **Figure 4** . In fact, selective mutation in a few residues results in marked changes in the receptor affinity towards the ligand – Ser52Arg and Ser52Ala mutants display a 150 and 14 fold affinitiy reduction, respectively, and Ile50Ala mutants display a 14 fold affinitiy reduction; also, the Phe85Ala and Leu86Ala mutants display a 53 fold reduction in the binding affinitiy (Li, Wang & Mariuzza, 2011).

Figure 4

The substitution of Ser52 by an Arg residue has a tremendous impact on the binding affinity of B7-H6. In this mutation, a hydrophobic amino acid residue is replaced by a positively charged one, which completely changes the polarity of the protein surface, dramatically increasing the dissociation constant (K_D). Replacement of Ser52 with another hydrophobic amino acid (Ala in this case) has a much more reduced impact on the binding of B7-H6. Replacement of Phe 85 with a non-aromatic amino acid residue causes an accentuated

decrease in the pocket hydrophobicity. The B7-H6 region that interacts with this portion of NKp30 comprises the residues Thr59 and Pro128, each making sixteen van der Waals contacts with NKp30, that account for 44% of all contacts between the two proteins (Li, Wang & Mariuzza, 2011). This region of B7-H6 forms a projection that seems to bind at the groove formed between Ser52 and Ser82 of NKp30 (see **Figure 4)** , which may be a key feature involved in triggering signal transduction by NKp30.

Further work yielded the X-ray structure of NKp30 bound to B7-H6, providing important information regarding the interaction of these two molecules. Interestingly, B7-H6 interacts with just eleven NKp30 residues, mainly through hydrophobic interactions. The three potentially N -glycosylated residues identified by other authors (Asn residues 42, 68 and 121) are located outside the interface and therefore should not interfere with binding (**Figure 4**) (Li, Wang & Mariuzza, 2011). However, it was argued that different glycosylation patterns may affect the overall 3D structure of the protein, increasing or decreasing binding affinity, as already discussed (Hartmann et al., 2012).

Marked NKp30 conformational changes occur upon B7-H6 binding, as observed by comparison of the two available structures (bound and unbound). A key change is the reduction of the distance between residues 52 and 82, that define the ridge where B7-H6 docks. Moreover, the loop composed by Arg67 and Asn68, located above the ridge, seems to rotate, pushing Asn68 backwards and pulling the Arg67 residue down towards the centre of the ridge. Although the available structures do not allow the identification of an H-bond, the NH moiety of the side chain of Arg67 approaches the C=O of Val53 at about 4Å.

Interestingly, Asn68 is one of the 3 identified glycosylation acceptor sites. Although all these sites are located outside the binding site, the glycosylation of Asn42 and Asn68 is essential for B7-H6 binding; their glycosylation is likely to induce subtle NKp30 conformational changes, helping shape the ligand-binding pocket (Hartmann et al., 2012). On the other hand, BAT-3 binding depends essentially on Asn68 glycoslyation, whereas Asn42 and Asn121 have a lower impact(Hartmann et al., 2012). This indicates that Asn68 is critical for correct ligand binding and subsequent signal transduction.

Although no experimental information exists regarding these conformational changes, it is clear that some degree of modification is required for NKp30-based triggering of NK cells. One particular work demonstrated that, upon binding of B7-H6, conformational changes in NKp30 enable the translocation of Arg143 to a position deeper in the phospholipid bilayer. With this, the positively charged arginine residue aligns with the negatively charged aspartate in CD3ζ or FCεRIγ adaptor proteins, that trigger the NK cell response (Memmer et al., 2016).

Targeting NKp30

B7-H6 is expressed by approximately 20% of available human tumour cell lines and by some primary human tumours (Brandt et al., 2009). It has been widely demonstrated that B7-H6 expression is directly correlated with susceptibility to NK-killing, as engaging of NKp30 by this protein triggers a strong cytolytic response. It has also been suggested that some cancer therapies may increase the stress levels on tumour cells and upregulate the expression of B7-H6, increasing the susceptibility to NK cells (Cao, Wang, Zheng, Wei, Tian & Sun, 2015). Moreover, it has been demonstrated that artificially coating cells with B7-H6 promoted NK cell cytotoxicity, providing a proof of concept that NKp30 engagement may represent a new strategy in cancer therapy (Kellner et al., 2012).

In 2013, a patent was filed describing a method of endowing T cells with receptors that recognise B7-H6-expressing cells, as a novel cancer immunotherapy (Zhang & Sentman, 2013). In that work a bi-specific T cell engager (BiTE) antibody was produced by fusing an anti-B7-H6 antibody fragment with an anti-murine CD3 scFv (single chain variable fragment of antibodies). BiTE was successful in triggering the response of host T cells against B7-H6-expressing cells (Wu, Zhang, Gacerez, Coupet, DeMars & Sentman, 2015). CAR-T cells bearing B7-H6-recognising domains have also been described, demonstrating specific activity against B7H6-expressing tumour cells (Hua, Gacerez, Sentman & Ackerman, 2017). These approaches, although efficient, require the expression of B7-H6 on target cells, something that is tumour type-dependent,

as already referred. Strategies aiming at increasing the expression of this protein in tumour cells have not been explored, probably because a number of reports suggest a positive correlation of the protein levels with tumour metastasis and progression (Xu et al., 2016; Zhang et al., 2018; Zhou, Xu, Chen, Xu, Wu & Jiang, 2015). Therefore, direct targeting of NKp30 has been considered in the development of NK cell-based therapies.

One work focused on triggering the response of NK cells with B7-H6-derived small peptides. In this approach, the peptide sequence present in the projection of B7-H6 that binds to the ridge of NKp30 was reproduced as a soluble fragment. Treatment of NK cells with this molecule induced the release of TNF-α, proving that NKp30 may be targeted using small peptides. This work, however, failed in achieving a complete response of NK cells, as no release of IFN-γ was triggered by the designed peptides and no data regarding the cytolytic activity of NK cells was reported (Phillips, Romeo, Bitsaktsis & Sabatino, 2016).

Despite the advances in NK cell characterization and in the identification of the triggering mechanisms, few therapies using NK cells are available for cancer treatment. In some ongoing clinical trials, autologous NK cells are expanded *ex vivo* and reinfused after stimulation with cytokines, namely IL-2. In another approach, allogenic NK cells are directly administered to the patients, and an immortalized NK cell line (NK-92) is used in some instances. However, most of the ongoing trials rely on the use of specific antibodies aimed at triggering the cytolytic responses of NK cells against cancer cells, by targeting NK cell receptors and cancer cell markers. Some of the therapies were found to be effective and to significantly increase the life quality of the patients, while others presented serious side effects (Dianat-Moghadam, Rokni, Marofi, Panahi & Yousefi, 2018).

Only a few works have focused on NKp30 as a target for the activation of NK cells and as a possible route towards the development of new immuno-oncology therapies. With all the benefits of immunotherapies and as part of the personalized medicine approaches, it is expected that, in the future, new drugs and treatments focusing on harvesting the power of the immune system through NK cells will be developed.

Conclusion

The immune activity of NK cells is mediated by a large number of cell surface receptors that recognize different ligands and mediate different signalling pathways. Natural killer cells are one of the arms of the innate immune system, and are particularly interesting because they are able to differentiate between self and non-self cells, not by the absence of MHC I-presented molecules, but rather by their loss. This finely regulated activity comes from a balance between activating and inhibiting receptors that constantly sense the environment around the cell and generate a response that considers all the stimuli present.

NKp30 is a natural cytotoxicity trigger and its role on NK cell response to several stimuli is well explored, in particular in tumour settings. The usage of efficient NKp30 agonists would constitute a tremendous tool in immuno-oncology because it would simultaneously make use of both the specificity and the potency conferred by this receptor.

NKp30 is the target of several antibodies that have been used in immunotherapy regimens. Due to the inherent characteristics of the process, antibody production and usage is far from being a straightforward option when compared with the use of smaller and more affordable drugs. The development of specific and potent drugs, targeting the binding site of NKp30 and generating the same response, is of utmost importance.

The structure of NKp30 has been resolved both in its free and ligand-bound forms, and the ligand-binding region is well characterized. However, important aspects of NKp30-mediated NK cell activation arise not only from direct ligand binding but also from other affected residues. All structural data available in the literature, reviewed here, indicate that the design of specific and effective drugs requires not only binding to the adequate site, but also the ability to replicate ligand-induced conformational changes, in particular Arg67 displacement.

Targeting NKp30 with small molecules, such as peptides or low molecular weight ligands, should be considered as a new strategy to trigger immune responses against cancer prompted by NK cells. NKp30-targeted therapies have been proven efficient, as demonstrated by CAR-T cell expressing chimeric NKp30 receptors, that were found able to destroy B7-H6+ cells (Zhang, Wu & Sentman, 2012). This strategy, however, presents major drawbacks regarding the production and cost of CAR-T cells. The work of Phillips *et al.* (Phillips, Romeo, Bitsaktsis & Sabatino, 2016) provided important insights regarding the activation of NK cell response with small peptides, but more affordable and comprehensive strategies should be developed in the future as part of a new branch of cancer immunotherapy.

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Tables

Table 1 - Examples of abnormalities in NK cell number or activity associated with clinical symptoms or increased risk in humans.

NK abnormality	Cause	Consequences
— number	Congenital or acquired immunodeficiency (AIDS included) Fanconi's anaemia (bone marrow impairment)	Higher incidence Multiple infection

NK abnormality	Cause	Consequences
	Autosomal recessive SCID	Multiple infection
— activity	Chediak-Higashi syndrome (abnormal lytic granule biogenesis)	Increased risk for
	Griscelli syndrome type 2 (lytic granules do not detach from microtubules)	Susceptibility to
	Wiskott-Aldrich syndrome (defective actin organization at immune synapse)	Multiple infection
	Hermansky-Pudlak syndrome (abnormal lytic granule biogenesis)	Susceptibility to
— number or — activity	unknown	Recurrent sponta
	Acute large granular lymphocyte proliferation	Aggressive leukae
	Chronic NK cell lymphocytosis	Vasculitis, arthrit

Figure legends

Figure 1 –Schematic representation of the NK cell signalling pathway. A) Upon binding of the killing activation receptor (KAR), the ITAM motifs of the intracellular portion are phosphorylated by a member of the Src family. Syk or ZAP70 are activated and further phosphorylate the PI3K complex and activate VAV-1/-2/-3, PLCγ1 and PLCγ2. This will further trigger a cytoskeleton reorganization and affect the calcium flux into the cell, leading to an increase in Ca^{2+}levels. This cascade results in the cytotoxic/cytolytic response of NK cells, with the release of the cytotoxic granules (degranulation) and production of cytokines/chemokines. B) Killing-inhibiting receptors (KIRs), present on the surface of NK cells, may also be engaged by the corresponding inhibiting ligands. These ITIM-bearing receptors are subsequently phosphorylated by Src kinases. This leads to the recruitment of SHP1/2 kinases that terminate the activation signalling emanating from ITAMs, rendering the NK cells inactive. The ultimate activation/inactivation result of these pathways is directly correlated with the number of receptors engaged. Target cells expressing more activation than inactivation ligands will engage more activation receptors resulting in the transduction of more activation signals, triggering the response of NK cells (Koch, Steinle, Watzl & Mandelboim, 2013; Linnartz-Gerlach, Kopatz & Neumann, 2014; Linnartz, Wang & Neumann, 2010; Paul & Lal, 2017; Watzl & Long, 2010).

Figure 2 – NK cell activation/inhibition equilibrium. The relative number of engaged receptor-types dictates the response. A) Normal cells, expressing normal MHC I molecules (inhibitory ligands) do not trigger the cytolytic response of NK cells. B) Stressed cells, such as tumour cells, engaging the same relative number of activating and inhibiting receptors of NK cells, induce a dynamic equilibrium that results in no cytolytic response of NK cells. C) If that equilibrium is tipped over by engaging more activating receptors on NK cells, a cytolytic response will be triggered against the cells presenting the corresponding ligands.

Figure 3 – Structure representation of NKp30. The two antiparallel β-sheets (in yellow) and the two α-helixes (in red) are shown. The two β-sheets are bridged by a disulphide bond between residues Cys39 and Cys108. The stalk domain and the transmembrane α-helix are not represented. Image generated from the published X-ray structure of unbound NKp30 (PDB 3NOI) *(Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011)* .

Figure 4 – NKp30 molecular surface with A) B7-H6-contacting residues (red) and N-glycosylation sites (blue) highlighted. Only one possibly N-glycosylated residue (Asp68) is located near the identified binding site, which comprises residues Ile50, Gly51, Ser52, Val53, Leu80, Ser82, Phe85, Leu86, Glu111, Leu113 and Gly114, marked in red; and B) B7-H6-contacting residues differentiated by type of amino acid residue: polar residues (yellow) and hydrophobic residues (cyan). Image generated with PyMOL*(Schrodinger, 2015)* from the published X-ray structure of unbound NKp30 (PDB ID: 3NOI) *(Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011)* . NKp30 structure representation in its unbound (C, E) and B7-H6-bound forms (D, F) are also shown. Isoleucine 50 is labelled as a positioning reference. Conformation differences between the two forms are observed in the distance between residues 52 and 82, labelled in yellow, that differ by about 1 Å, and in the relative positions of arginine 67 and asparagine 68. The loop composed by arginine 67 (blue) and asparagine 68 (orange) seems to bend upon binding. Asparagine 68 moves away from proline 79 (in red) and

arginine 67 bends down towards the pocket, approaching valine 53 (not highlighted). Image generated from the published X-ray structures of unbound NKp30 (PDB ID: 3NOI) *(Joyce, Tran, Zhuravleva, Jaw, Colonna & Sun, 2011)* and B7-H6 bound NKp30 (PDB ID: 3PV6) *(Li, Wang & Mariuzza, 2011)* .