Hepatocellular carcinoma: prospects for NK cell immunotherapy?

Naveenta Kumar[1] and Salim I Khakoo[2]

[1]Section of Hepatology, Division of Medicine, Imperial College London, South Wharf Road, London, W2 1PG, UK

[2] Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton General Hospital, Tremona Road, Southampton S016 6YD, UK

Address Correspondence to:

*Salim I Khakoo
Faculty of Medicine
University of Southampton
Mailpoint 811
Level E South Academic Block
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD
Tel 023 8079 6671/5099
Fax: 023 8051 1761
E-mail: S.I.Khakoo@soton.ac.uk

Abstract

Liver disease is a growing cause of death in the UK and the incidence of hepatocellular carcinoma (HCC) is rising (http://www.cancerresearchuk.org/). The combination of an immunosuppressive environment within the liver and suboptimal host anti-tumour immune responses may account for the poor

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survival outcome of HCC. Understanding how tumours evade immune recognition coupled with new insights into the unique immunological environment within the liver will be critical to developing liver-specific immunotherapies.

Key words

Adoptive transfer

Cancer

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Immunotherapy

Natural Killer cells

The Burden of Hepatocellular Carcinoma

HCC is the one of the most common cancers and the second most common cause for cancer death worldwide (http://globocan.iarc.fr/old/FactSheets/cancers/liver-new.asp). On a worldwide basis it occurs on a background of chronic viral hepatitis B (HBV) and hepatitis C (HCV). Thus, interventions such as early anti-viral therapy for these diseases can be preventative for HCC. This has been demonstrated by the reduction in HCC in Taiwan following the introduction of a vaccination program for hepatitis B[1]. Suppressive therapies for HBV and curative therapies for HCV may help reduce the incidence of HCC in the future. However, a lack of accessibility to these treatments in developing nations, coupled with a rising incidence of non-alcoholic fatty liver disease[2], means that HCC is likely to remain a challenging disease for the foreseeable future.

Curative therapies for HCC are in general surgical and performed in patients, who have been carefully selected according to the Barcelona clinic liver cancer (BCLC) staging criteria[3]. However, as a silent killer, liver disease may present beyond the stage where resection is possible. Furthermore, as liver disease usually occurs on a background of cirrhosis, recurrence rates post-resection of the tumour are high due to remaining underlying viral infection, chronic inflammation and cirrhosis[4]. Transplantation is an option for some patients, but the BCLC criteria are strict. These means that at the time of assessment the disease is often too advanced, or patients subsequently become ineligible for transplantation due to a shortage of suitable donors and lengthy waiting times. Alternatives such a radiofrequency ablation and transarterial chemoembolisation may also be offered, as recommended by the

BCLC guidelines[3]. However these may not be curative and HCC recurrence is common following these treatments.

For those ineligible for the above treatments, due to the presence of multi-focal tumour, there is currently a paucity of alternative therapies on offer. Sorafenib, a multi-tyrosine kinase inhibitor, has been demonstrated to increase the median overall survival by 3 months in patients with Child's Pugh A status and a good performance score (Eastern cooperative oncology group [ECOG] 2 or less)[5]. Erlotinib, an Epidermal Growth Factor Receptor (EGFR) inhibitor, was recently tested in clinical trials, but failed to improve disease survival when used together with sorafenib[6]. For those patients in whom sorafenib fails or is not tolerated, Brivanib has been investigated as an alternative[7]. This drug is a VEGF receptor inhibitor and so has anti-angiogenic properties, but it is also a fibroblast growth factor (FGF) receptor inhibitor. Unfortunately, there was no significant improvement in overall survival with this therapy[7]. Thus conventional chemotherapy is currently an inadequate option for individuals with advanced HCC.

Immunotherapy offers an alternative option. Previously, a number of treatments have been explored[8]. Such therapies include using autologous dendritic cells pulsed with tumour cell lysate with or without an additional IL-12 expressing transgene[9,10]. However, the field of oncology has been transformed by the use of the checkpoint inhibitors, anti-CTLA-4 and anti-PD-1[11,12]. With regards to HCC treatment, a large phase II study of the anti-PD1 antibody nivolumab demonstrated an objective response rate of 20%[13]. However HCC has only moderate mutation rates, well below that of melanomas, which are more responsive to checkpoint inhibitors, indicating

that alternative immunotherapeutic strategies are likely to be required[14]. In this review we consider the immunotherapeutic potential for natural killer cells in this difficult to treat disease.

Liver Immunology, The Tumour Microenvironment and NK cells

The liver possesses a uniquely tolerogenic immune environment. Immune cells residing within the liver are exposed to multiple antigens via the portal circulation and so unfavourable immune responses need to be prevented[15,16]. The presence of cirrhosis alters and dysregulates immune responses[17], and infection with Hepatitis B or C virus can also modulate NK cell function[18]. NK cells and cytotoxic CD8+ T cells are involved in tumour surveillance[19,20]. Conversely, tumour cells that acquire mutations may escape from immune cell surveillance and elimination[19,20]. There is sufficient evidence to suggest that the interplay between tumour cells and non-tumour cells residing within and around the tumour, results in a modulation of the non-malignant cells and the local environment to promote tumour survival and growth[21–23].

Natural Killer (NK) cells account for 30-50% of the immune cell infiltrate within the normal liver[24–26]. NK cells mediate anti-tumour effector responses by direct cytotoxic effects with granule release[27], antibody mediated cytotoxic effects via CD16 engagement[28], secretion of interferon gamma (IFNγ)[29,30] and Tumour Necrosis Factor alpha (TNFα) [30], and, cell-surface expression of Fas ligand (FasL)[31] and tumour necrosis factor related apoptosis-inducing ligand (TRAIL)[32]. NK cell function is dependent upon the balance of the inhibitory and activating cell surface receptor repertoire, the ligands expressed on the

target cells and the cytokine environment[33]. Tumour cells may downregulate major histocompatibility complex class I (MHC-I) expression, theoretically allowing escape from CD8+ T cell cytotoxicity. Downregulation of MHC-I surface expression on tumour cells should trigger a powerful anti-tumour NK cell response, in keeping with missing-self recognition[34]. However, although MHC-I down-regulation has not been rigorously studied in primary liver cancer, the studies published to date suggest that MHC-I down-regulation is not a major feature of HCC[35–37]. Furthermore, NK cells residing within tumour tissue may be modified and may not provide the normal response to MHC-I downregulation on tumour cells. Le Maux Chansac *et al.* examined NK cells residing within lung tumour tissue, and found that although these NK cells were capable of direct cytotoxicity when incubated with the MHC-I negative cell line K562, these same NK cells were unable to lyse autologous MHC-I deficient lung tumour [38]. This was predominantly due to a lack of ligands on lung tumour cells for NK cell activating receptors[38]. In addition, Platonova *et al.* examined the phenotype and function of intratumoral NK cells residing within non-small cell lung cancers and, when comparing them to those from peripheral blood or non-tumour tissue, found the NK cell repertoire was altered with a down-regulation of activating receptor expression, including NKG2D and DNAM-1, rendering these NK cells hypofunctional within the tumour microenvironment[39]. A reduction in NK cell activity has also been demonstrated in the livers and peripheral blood of patient with HCC[40–42].

The interactions resulting in such hypofunctional NK cells are not fully understood. One mechanism modulating NK cells is the crosstalk between immune cells in the tumour. Macrophages and NK cells may have peri- and

intra-tumoural interactions in HCC, resulting in an initial NK cell activation but then a rapid subsequent exhaustion of NK cells[42]. Myeloid derived suppressor cells may inhibit NK cell function in HCC[43,44]. Another potential mechanism for NK cell "tolerance" to tumour is the secretion of soluble ligands for NK receptors by tumour cells, as exemplified by Jinushi et al., who found that soluble MICA (MHC class I relate chain A) levels were elevated in some HCC patients, and corresponding peripheral blood NK cells had lower levels of NKG2D expression and impaired activation[45] (Figure 1).

Current therapies for HCC that modulate NK cells

A number of studies have implicated NK cells in protection against HCC. Firstly, intrahepatic and peripheral NK cells from individuals with HCC have reduced levels of NK cell cytotoxicity[41]. Additionally, in an immunogenetic study, protection against HCC was associated with polymorphisms in the 5' flanking regions of MHC class I polypeptide-related sequence A (MICA), an NKG2D ligand[46]. Following resection for HCC, increased expression of NKG2D ligands by the tumour was associated with reduced recurrence and prolonged survival[47]. With regards to current HCC therapeutics, sorafenib reduces the shedding of the stress-induced ligand MICA from HCC cells[48], thus promoting NKG2D mediated activation of NK cells against HCC targets. Similarly, cisplatin has recently been shown to indirectly upregulate an NKG2D ligand, UL16-binding protein 2 (ULBP2), which may therefore boost NK cell cytotoxicity, thus supporting a new role for cisplatin in HCC therapy[49].

Radiofrequency ablation to treat HCC is also associated with a demonstrable increase in NK cell cytotoxicity and IFNγ secretion[50].

Liver transplantation whilst being a curative treatment for HCC, can often be complicated by recurrence of HCC in the transplanted livers, with a more aggressive course due to the systemic immunosuppression required to prevent transplant rejection. Ohira *et al*. found that following partial hepatectomy in mice, NK cells had a lower expression of TRAIL and the early activation marker CD69, and also that these mice were susceptible to liver metastases in the remaining liver upon injection of the Hepa 1-6 hepatoma cell line[51]. In this model, tumour growth was also suppressed using NK cells extracted from the livers of mice treated with polyI:C [51]. Thus they proposed the use of IL-2 stimulated NK cells derived from donor liver explants as a means to treat HCC which has arisen in patients who have undergone liver transplantation[52].

Direct intra-tumoural gene therapy may provide an alternative method of treatment. Harada et al describe a technique of transferring a murine IL-12 plasmid DNA using electroporation directly into tumours implanted in mice treated with systemic tacrolimus[53]. Not only was the resulting inhibition of tumour growth and limitation of metastases in these mice dependent on NK cells, but IL-12 gene therapy also increased the proportion of tumour infiltrating cytotoxic T lymphocytes whilst avoiding rejection of the implanted allogeneic skin grafts[53]. In a rat model of multi-focal HCC, the combination of IL-12 gene therapy and granulocyte macrophage colony-stimulating factor (GM-CSF) have a synergistic effect mediated by both T cell and NK cells [54] (Figure 2).

Boosting NK cell function with antibody therapy

NK cell function is determined by the balance between activating and inhibitory signals from cell surface receptors, which can be readily targeted by therapeutic antibodies. Activating receptors provide key targets for antibody based therapy aimed at boosting NK cell function. CD16 is a potent activating receptor expressed on NK cells[55]. Tumour cells coated with antibody can trigger antibody-depended cell medicated cytotoxicity (ADCC) via CD16, leading to degranulation, with release of perforin and granzymes, and up-regulation of TRAIL. These lead to an increase in tumour cell death. Furthermore interferon-γ production can stimulate local monocytes and macrophages to augment anti-tumour responses. This mechanism of action has been shown to be effective in several antibody-based therapies for cancers other than HCC[56–59]. In terms of surface marker expression, HCCs are heterogenous tumours, and thus antibody therapies that target HCC have been slow to develop. One candidate antigen is glypican-3 (GPC3), which is expressed on approximated 70% of HCC tumours, and not on normal hepatocytes[60–62]. Anti-GPC3 mouse antibodies and the humanized anti-GPC3 antibody (GC33) have been shown to induce ADCC via CD16 on NK cells[63]. Although the phase I trial for GC33 was promising[64], the phase II trial, where patients with advanced HCC who had failed standard systemic therapy were given GC33, did not demonstrate an overall clinical benefit[65,66]. This may be in part be due to under-dosing. However, the efficacy of such antibody based therapies are also limited by lower levels of NK cell activation resulting from shedding of cell surface CD16[59,67], and by CD16 polymorphisms[68].

Furthermore, repeated stimulation of NK cells via CD16 during anti-CD20 antibody therapy has been shown to induce exhaustion of NK cells[69]. This suggests that antibody therapies inducing NK-ADCC via CD16 may either need to be used in combination with another therapy or alternated with a different NK stimulus to maintain efficacy. Newer, bi-specific and tri-specific antibodies binding CD16 and tumour targets are emerging, which may overcome problems due to polymorphisms in CD16[59,70]. Additionally, following the discovery that the matrix metalloprotease ADAM-17 causes CD16 loss post NK cell activation, combination therapies using metalloprotease inhibitors are now being explored[59,71].

NKG2D, NKp46, NKp30, DNAM-1, and NKp44 induced on activated NK cells, provide potent activating signals when interacting with their ligand, triggering degranulation and release of granzyme and perforin, Thus they provide ideal targets to boost NK cell cytotoxicity. NKG2D binds stress-induced ligands, including MICA, MICB and ULBP1, which are expressed by tumour cells[72]. NKG2D has also been highlighted as a possible important receptor in HCC by Chu *et al.*[73]. These authors found that, in patients who develop HCC soon after hepatitis C eradication, there was a rapid down-regulation of NKG2D on peripheral blood NK cells at the end of anti-viral treatment. Sheppard *et al* further described the complex relationship of NKG2D and HCC, using NKG2D sufficient and deficient mouse models with chemically induced HCC. They demonstrated that NKG2D expression may correlate with tumour progression due to the promotion of a chronic inflammatory state [74]. Similarly seemingly contradictory evidence exists for other known activating NK cell receptors. NKp30 is described as an activating receptor on NK cells[55]. However, direct

cell to cell NK interactions via the NKp30 receptor with the expanded pool of myeloid derived suppressor cells (MDSCs) seen in HCC was shown to be associated with inhibition of NK cell activity[43], further complicating its use as an immunotherapy target. Similarly the activating receptor NKp46 was found to be upregulated on circulating NK cells from patients with HCC with a poorer prognosis[75]. Taken together, the simple model that increased activating receptor expression of NK cells is beneficial is unlikely to be correct. Therefore a better understanding of the roles of activating receptors in the context of HCC is required to determine if targeting these receptors would be a valuable therapeutic strategy.

DNAM-1 has been shown to be an important receptor for NK mediated killing of acute myeloid leukaemic cells[76]. The presence of DNAM-1 on Vγ9Vδ2T cells has been shown to be important for the lysis of HCC cell lines[77]. CD96 and the T-cell Immunoglobulin and ITIM domain (TIGIT) receptor, inhibitory receptors expressed on NK cells, share the same ligand as DNAM-1. Thus, one therapeutic strategy may be to manipulate CD155, the ligand for all these receptors, to allow binding and blockade of TIGIT and CD96, but not DNAM-1[78].

Blocking inhibitory NK cell receptors may provide alternative pathways to boost NK cell function. Killer Immunoglobulin receptors (KIRs) provide the most potent inhibitory signals to NK cells when binding self-HLA-C. Human HLA-haplotype mismatched haematopoietic stem cell transplants conducted to treat leukaemia, resulted in efficient NK-mediated killing of leukaemic cells without graft versus host disease[79]. These promising results led to the development of a humanised anti-KIR antibody for clinical use[80]. However, a

phase II trial in smouldering multiple myeloma using the pan-KIR2D antibody IPH2101 was terminated early due to an overall muted NK cell response after the initiation of therapy[81,82]. This again demonstrates that antibody therapy targeting a single checkpoint may yield unexpected results, and combination therapy may allow a more sustained anti-tumour effect. The CD94-NKG2A heterodimer provides another inhibitory NK stimulus when interacting with the non-classical MHC class I, HLA-E, on target cells[83]. Anti-CD94-NKG2A is currently being tested in combination with PD-1 blockade for the treatment of gynaecological cancers [84].

Limitations to antibody therapies again stem from the fact that the liver is a tolerogenic organ, and tolerogenicity is achieved through a delicate interplay of immune interactions. Current experience with antibody-based immunotherapies have resulted in some patients suffering side effects resulting from an uncontrolled reactive immune response from other immune components and fatal rejection of a liver transplant in two cases[85]. As well as being expressed on T cells, PD-1 is expressed on NK cells, and is up-regulated on peripheral NK cells in HCC[85]. Thus modulating the normally tolerogenic environment of the liver using checkpoint inhibitors may trigger a cascade of pro-inflammatory responses resulting in liver injury.

Therapeutic Prospects: NK cell expansion and adoptive transfer strategies

In-vivo manipulation of NK cells by the use of injected cytokines may provide an alternative strategy to use in combination with other therapies. Direct

administration of high dose IL-2 as an infusion has been approved for use in patients with metastatic renal cancer and metastatic melanoma[86], but it has a narrow therapeutic window with a risk of wide ranging systemic toxic effects[87]. IL-15 infusions have been shown to have promising results in expanding cytotoxic CD56^{bright} NK subsets in vivo[88]. However, these therapies need further exploration as they may have unexpected outcomes due to their lack of specificity for NK cells. For example, low dose IL-2 can preferentially expand T regulatory cells which can in turn inhibit NK cell function[89,90].

A promising area in immunotherapeutics is the option to expand and manipulate NK cells ex-vivo in the presence of cytokines and chemokines and transfer these activated NK cells into the patient[91–94]. Cytokines known to activate and promote proliferation of NK cells, such as IL-2, IL-15, IL-12 and IL-18 are all candidates that could be used in this way[87,95,96]. NK cell adoptive transfer using autologous, allogeneic or haploidentical NK cells have been explored. Autologous NK cell ex-vivo expansion and subsequent infusion result in increased cytotoxicity of circulating NK cells[97]. Autologous mononuclear cells incubated with IL-2 and CD3 to produce cytokine-induced killer (CIK) cells have shown promising results as an adjuvant therapy for HCC, with improved recurrence-free survival[98,99]. Allogeniec and haploidentical NK cell infusions have aimed to capitalise on KIR-HLA mismatch, resulting in a loss of inhibition of infused NK cells coupled with tumour-induced activation due to an increase in surface expression of stress-induced ligands on tumour cells[100–103]. Although no major detrimental side effects have been cited for these infusions, direct anti-tumour efficacy has not yet been proven in solid tumours, and issues remain with determining

longevity of infused NK cell responses. The recent encouraging results of a phase I study using cytokine-activated NK cells for leukaemia, represents an encouraging development for adoptive NK cell therapeutics[104].

Therapeutic prospects: Genetically modified NK cells

NK cell lines may provide another therapeutic option, potentially overcoming problems with maintaining a supply of primary allogeneic NK cells. Furthermore, NK cell lines have the advantage of being easier to manipulate genetically, with transfection efficiency being superior to that of peripheral blood NK cells[105–107]. Irradiated NK-92 cell infusions for renal cell carcinoma, malignant melanoma, and other advanced cancers have passed Phase I trials[108,109]. NK-92 cells are $CD56^{bright}$ cells, lack many inhibitory KIR and CD16, express NKG2D and NKp30, can be expanded easily with IL-2, and demonstrate substantial in-vitro cytotoxicity in response to a variety of tumours[110,111]. NK-92 cells can be modified using non-viral transfection methods to express chimeric antigen receptors (CARs) specific to tumour antigens, thus providing more targeted responses in the host[112].

Future prospects and challenges

Recent work from our group has shown that NK cells can be exquisitely sensitive to changes in the peptide presented by MHC-I[113]. The inhibitory KIR recognise both the heavy chain of MHC-I and the bound peptide. Antagonist peptides in low concentration can reduce inhibition of NK cells substantially[114],

and thus this may be relevant to tumour recognition by NK cells, through the expression of neo-antigens. Recently we have also shown that the activating receptor KIR2DS2 can recognise specific peptide:MHC complexes similar to a "broadly cross-reactive" T cell receptor[115]. To date this has only been shown for viral and model peptides, but it is possible that tumour antigens may form part of this paradigm, leading to the opportunity for peptide-based NK cell immunotherapy.

Furthermore, whilst most data on NK cells in HCC has been acquired from peripheral blood cells, it is now clear that the intrahepatic NK cell subpopulation is quite different to that of peripheral circulating cells. Resident hepatic NK cells have a higher proportion of $CD56^{bright}$ NK cells and express liver resident markers CXCR6[26], CD49a[116] and CCR5[117]. They may be long-lived and are regulated by the transcription factor EOMES, with liver NK cells having higher levels of EOMES compared to peripheral circulating NK cells[118]. Recent work has also suggested that they may be less responsive to cytokines than their peripheral blood counterparts[25]. Thus targeting these intrahepatic NK cells may be challenging, especially in the context of a cirrhotic liver. To ensure that NK cell targeted immunotherapies are effective in the liver, more needs to be deciphered about the resident liver NK cell subpopulations, how they differ in HCC, and importantly how they may be activated to generate an optimal anti-tumour response.

Conclusions

Much is yet to be learnt about the use of NK cells, especially their potential for immunotherapy of solid tumours. The liver poses a number of unique problems to the immunotherapist: it is an extremely tolerogenic environment and HCC arises on the background of chronic liver disease, which may be associated with immunological dysfunction and poor tolerance of therapeutics. Better experimental models, such as humanised mice[107] and the recently described ability to culture liver tumour organoids[119], together with our increasing understanding of intra-hepatic NK cells will contribute to improving the prospects for NK cell immunotherapy for this difficult to treat and common cancer. Nevertheless, given the preponderance of NK cells in the liver and the number of studies implicating NK cells in HCC, NK cell based immunotherapy is an exciting future prospect for the management of HCC.

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Figure Legends

Figure 1. Evasion of Natural Killer cell mediated tumour surveillance in Hepatocellular Carcinoma. Tumour cells present self-antigens on MHC-I and thus suppress NK cell activation via KIR despite an increased expression of stress-induced ligands on the tumour cell surface. MDSCs inhibit NK cell cytotoxicity, IFNγ release and NKG2D expression via membrane bound TGFβ1 and the NKp30 receptor on NK cells. Tumour derived monocytes activate NK cells via a CD48:2B4 interaction, which leads to subsequent exhaustion of NK cells. Similarly soluble MICA released from the tumour cell activates NK cells again leading to exhausted NK cells.

Abbreviations: NK- Natural Killer Cell, HCC- Hepatocellular Carcinoma, MDSC- myeloid derived suppressor cell, aNK- activated NK cell, eNK- exhausted NK cell, M- tumour derived monocyte, MHC-I- Major Histocompatibility Complex Class 1, TGFb1- Transforming growth factor beta 1, IFNγ- Interferon gamma, MICA – MHC-I polypeptide-related sequence A, sMICA- soluble MICA, MICB- MHC-I polypeptide-related sequence B, ULBP1- UL16 binding protein 1, KIR- Killer cell Immunoglobulin-like receptor.

Figure 2. Natural Killer cell anti-tumour responses and therapeutic targets. Expansion of autologous or allogeneic/hapoloidentical NK cells or genetically modified NK-92 cells may be used for NK cell therapy. Loss of NK cell inhibition from lack of recognition of self-antigens presented on MHC-I molecules on tumour cells, coupled with activating signals due to an increased expression of stress-induced ligands (MICA) on tumour cell

surfaces results in activation of the NK cell with degranulation releasing perforin and granzyme. Bi-specific and tri-specific antibodies designed to bind tumour antigens and CD16 will induce ADCC and tumour cell lysis. An upregulation in FasL and TRAIL expression on NK cells can induce tumour cell apoptosis. NK-92 cells may be genetically engineered to express CARs, thus allowing specific targeting of tumour cells.

Abbreviations: NK- Natural Killer Cell, CAR- Chimeric antigen receptors, ADCC- antibody dependent cell mediated cytotoxicity, TNFα- tumour necrosis factor alpha, IFNγ- interferon gamma, FasL- Fas ligand, TRAIL- tumour necrosis factor related apoptosis-inducing ligand, TRAILr- TRAIL receptor, KIR- Killer cell Immunoglobulin-like receptor, MHC-I- Major Histocompatibility Complex Class 1, MICA – MHC-I polypeptide-related sequence A.

FIGURE 1

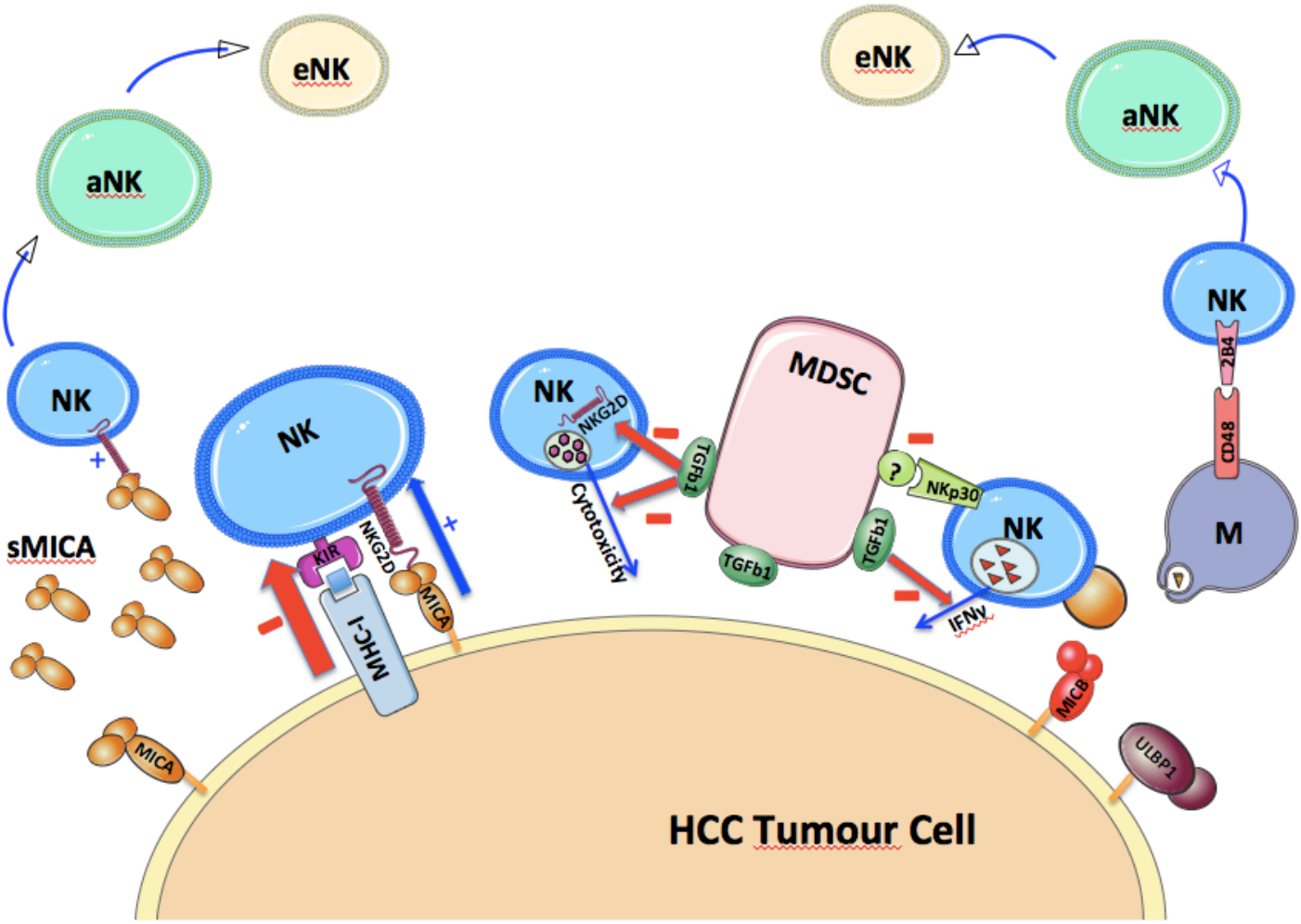

FIGURE 2