CHAPTER 5

Harnessing Natural Killer Cells' Killing Function in Cancer

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INTRODUCTION

Natural killer (NK) cells constitute the subset of lymphocytes that does not need any prior sensitization and that is naturally selectively cytotoxic. NK cells were discovered by Kiessling et al. (1975), while these researchers studied cell-mediated cytotoxicity against tumour cells. The distribution of immune cells (i.e., the ratio of T, B and NK cells) and the number as well as activity of NK cells vary from person to person. These differences provide the basis for the diversity of responsiveness to disease that is observed in a population. In addition, the immune system is influenced by the environment. Notably, the number of immune cells decreases with stress, and the number and activity of immune cells decreases as they get older. The balance of NK cell activity in the body seems to be the most important for maintaining health.

NK cells belong to a group of large granular lymphocytes. They play an important role in the immune system against malignant and infected cells. Unlike T cells, NK cells act as innate immune cells, showing rapid and selective `natural' killing of cellular targets with neither any need for major histocompatibility complex class I expression nor any sensitization step. Despite the innate property of NK cells to kill infected or malignant cells when rapid immune response is required being a great advantage, the ready-to-kill status of NK cells could have detrimental effects as well. Therefore, NK cell activation needs to be tightly modulated by the activation of inhibitory receptors, and this signalling balance orders NK cells whether to kill target cells or to stay in an inactive state.

Despite sufficient evidence that NK cells fight tumour cells, pharmaceutical treatment approaches that target NK cells remain few in number. Nevertheless, the possibility of NK cells to be used as therapeutic agent is increasingly supported by recently approved cancer cell-targeting therapies, which proved that it is possible to appropriately regulate NK cell activity.

The immune system can be considered as a health barometer in that it is activated when a disease occurs; NK cells are at the heart of this immunomodulation.

The Oriental and Western worlds have different philosophies of medical approach. In Western medicine, understanding of the structure and problems of the body based on

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anatomy has been proposed as the rationale approach, and from this Cartesian approach the solution to the disease is subsequently derived. In conventional drug development, this translates in first gaining a clear understanding of the disease target and of its underlying biology and subsequently drugging that target. On the other hand, Oriental medicine emphasises the importance of achieving a harmonization of the whole body with its environment; this concept translates in seeking a natural healing of the disease through the improvement of the homoeostasis and the body as a whole. This approach sometimes leads to the a misunderstanding that Oriental treatments may not always be related to the disease. Remarkably, today's immunotherapeutic methods derived from the Western medicine approach resonates very well with the traditional methods of Oriental medicine in many respects.

Controlling the balance of immunity is in concept similar to that of Oriental medicine of harmonizing our bodies with nature. Oriental medicine uses exercise and herbal medicines with the goal to balance and "harmonize" our bodies. In Western medicine, the practitioners try to control the activity of targets such as receptors by using a variety of pharmaceutical modalities including antibodies and small molecules, with the main objectives of that work on the immune system being to establish or reestablish immune system balance. Considering that NK cells are cells that have immune regulatory functions in the body, this fundamental function resonates echoes many of the bases of Oriental medicine. NK cell–based treatment is being explored in several countries with different regulatory settings. While many reviews have already been written on the subject, in this Chapter, I sought to review the general background of NK cell–based immunotherapy and the activity of clinical research especially focused on Asia.

What Are Natural Killer Cells?

NK cells in human and mouse account for 15% of all circulating lymphocytes. In mice, the NK cells represent 2.5% of the splenic leucocyte composition (Liu et al., 2007). Although NK cells exist in relatively small numbers, NK cells are considered to be the main factor for early innate immune response (Vivier et al., 2011).

NK cells were discovered 40 years ago, but their potential has only recently been highlighted in the field of cell-based and immune-based therapy. When they were first discovered, these cells were regarded as 'impurities that induce cytotoxicity'. Nowadays, on the other hand, these cells are recognised to be very important cells of the immune system as they control microbial infection and tumour progression (Artis and Spits, 2015; Prince and Pickett, 2002; Lee et al., 2007).

What is more, NK cells represent now a new alternative that could replace other traditional cancer treatments including surgery, radiation and chemotherapy (Pietra et al., 2016).

Characteristics of Natural Killer Cells

Importantly, NK cells express neither T cell receptor (TCR) nor B-cell receptor. In human, NK cells express $CD3^{negative}$, $CD56^{positive}$ and $CD16^{positive}$ phenotypes, while mouse NK cells express CD11b, CD49b, NK1.1 and LY49 (Lysakova-Devine and O'Farrelly, 2014).

In human peripheral blood, there are two subtypes of NK cells: $CD56^{bright}CD16^{dim/negative}$ and $CD56^{dim}CD16^{bright}$. $CD56^{bright}CD16^{dim/negative}$ cells produce a high level of cytokines, while inducing low cytotoxicity. In contrast, $CD56^{dim}CD16^{bright}$ cells produce a low level of cytokines, while exhibiting high cytotoxicity. Most NK cells exist in the $CD56^{dim}CD16^{bright}$ type in human peripheral blood; however, a small fraction exists in the $CD56^{bright}$ type (Poli et al., 2009).

NK cells have various activating and inhibitory receptors and secrete several cytotoxic molecules, such as Fas ligand, tumour necrosis factor (TNF) and the proapoptotic protein TRAIL (Zamai et al., 1998).

The Development of Natural Killer Cells

NK cells are derived from the bone marrow. They develop and function independently from the thymus. The development of NK cells requires several steps in humans. The bone marrow–derived CD34+CD45RA+ haematopoietic precursor cells move to the lymph node through blood vessels and subsequently undergo maturation and activation processes by communicating with the dendritic cells (Blanchet et al., 2011). After maturation, NK cells differentiate into two subtypes: $CD56^{bright}$ and $CD56^{dim}$. $CD56^{dim}$ NK cells migrate to the peripheral blood through the efferent lymph, while some $CD56^{bright}$ NK cells remain in the secondary lymphoid tissue and interact with dendritic cells (Guido and Morandi, 2014).

In mice, NK-cell development is initiated from CD122+ precursor NK cells in bone-marrow. The precursor NK cells then sequentially obtained NK cell receptors including NKG2, Ly49, and CD117 (c-kit).

NK cells are characterised by the upregulation of CD49b and CD11b, expression of CD27, and downregulation of CD51, which together lead to the generation of a functionally mature proliferative NK cell population. The maturation of NK cells enables them to be cytotoxic and to produce cytokines (Elpek et al., 2010).

Function of Natural Killer Cells

The main functions of NK cells known to date include the ability to kill tumour cells, virus-infected cells, bacteria and fungi. In this regard, NK cells are expected to play an important role in the antitumour response and the vital immune response against microorganisms.

The cytotoxicity of NK cells is closely regulated by NK activity, and the lytic function of the NK cells is controlled by activating inhibitory signals on the membrane receptors. By using those immune receptors, NK cells could recognise infected cells and tumour cells and naturally eliminate stressed cells without prior sensitization.

The mechanism of regulating NK cell activity is known as the missing self-hypothesis. Abnormal cells decrease or lack the self-identifying molecules such as major histocompatibility complex (MHC) class I, which binds to the inhibitory receptors in NK cells. In addition, the expression of the activating receptors in NK cells is upregulated, where

they can overcome the inhibitory signals in the abnormal cells. With those abnormal cells, NK cells initiate effector functions including cytotoxicity, cytokine production and proliferation. Despite the effective antitumour activity of the NK cells, the contribution of the NK cells in controlling the progression of solid tumours in cancer treatment remains unclear (Mandal and Viswanathan, 2015).

Future Development Approach

Currently, many clinical studies to treat cancer using NK cells are in progress, and some promising results have already been reported. However, NK cell–based treatments remain focused on very limited options as the mechanism of action of NK cells is yet to be elucidated.

Most of the clinical studies on NK cells have been conducted in ways that increase the number of autologous or allogeneic NK cells, which control the route or the frequency of administration (Geller and Miller, 2011). These studies have proven that adoptive NK cell therapy is generally well tolerated and does not induce graft versus host disease (GvHD), unlike T cells (Shah et al., 2015). However, it is difficult to this date to confirm the efficacy of NK cell–based treatment.

Recently, combinational therapies of antibodies or immunotherapy and gene-modified cell therapy using chimeric antigen receptors (CAR) have shown significant improvement in cancer treatment (Mirzaei et al., 2017). Therefore, NK cell therapy is a promising therapeutic area to explore to develop with other immune-oncology approaches including immune checkpoint inhibitors, cytokines or antibodies.

NATURAL KILLER CELLS AND CANCER

To understand the mechanism of action (MoA) of killing tumour cells by NK cells, we need to know what consists of NK cells and how they are activated.

Natural Killer Cell Receptors and Their Ligands

NK cell receptors function as a detection system that determines the cellular response. NK cells regulate their activity through the balance of positive and negative signals from NK cell receptors. There are two types of functionally distinct NK cell receptors; namely inhibitory receptors and activating receptors of NK cells (Srivastava et al., 2003).

The Inhibitory Receptors of Natural Killer Cells

The inhibitory receptors in NK cells recognise self-molecules expressed in the target cells and make NK cells inactive. There are three major types of inhibitory receptors: killer immunoglobulin receptors (KIRs), CD94/NKG2A and Ly49 (Fernandez et al., 2005).

Most inhibitory receptors have immunoreceptor tyrosine–based inhibition motifs (ITIMs) in the cytoplasmic tails of NK cells (Long, 2008). Most of the KIRs have

inhibitory characteristics because the cytotoxic activity of NK cells is inhibited by recognition of the MHC molecules expressed in the target cells.

KIRs (15 genes) are encoded in the leucocyte receptor complex of human chromosome 19q13.4 where other Ig-like receptors are encoded. Their nomenclatures are based on whether those cells have two or three Ig-like external domains (KIR2D or KIR3D) with cytoplasmic domains, which have short receptors (S; without ITIM) or long receptors (L; with one or two ITIM sequences) (Colonna and Samaridis, 1995). The S form is an activating receptor associated with DAP12 (ITAM, positive adaptor molecule), wheeas the L form is an inhibitory receptor containing ITIM (Lanier et al., 1988, 1998).

The characteristics of different KIRs vary depending on their HLA types. Both KIR2DL1 (CD158a) and KIR3DL2 (CD158b) are all specific to HLA-C. On the other hand, KIR3DL1 and KIR3DL2 are specific to HLA-Bw4 and HLA-A, respectively (Chrul et al., 2006). CD94/NKG2A belongs to a family of C-type lectin receptor mainly expressed on the surface of NK cells and a subset of CD8+ T cells. There are seven members in the CD94/NKG2 family: NKG2A, B, C, D, E, F and H. The genes coding those receptors are clustered in natural killer complex (NKC) at human chromosome 12, and Clr (C-lectin–related) genes at mouse chromosome 6 (Browna and Scalzo, 2008). CD94/NKG2A can be inhibited or activated, depending on the members of the complex. The NKG2 receptor is a type II transmembrane dimerizing with CD94 to form a heterodimer. CD94 contains a short cytoplasmic domain and is responsible for signal transduction. CD94/NKG2 receptors bind to nonclassical MHC class I glycoproteins (HLA-E in human and Qa-1 molecules in mouse) (Lee et al., 1998).

Ly49 is a more distinct NK cell receptor in mouse than in human. The Ly49 gene family is encoded as NKC in mouse chromosome 6. The Ly49a receptor was originally identified in the mouse T tumour cells (Nagasawa et al., 1987). Ly49b recognises MHC class I molecules H-2Dd, H-2Dk and H-2Dp; it also binds to H-2Kb (Deng and Mariuzza, 2006).

The Ig-like lectins (CD33rSiglecs), which bind to genes of the LILR family (also called LIR, ILT, and CD85) and CD33-related sialic acid, are included in the nonclassical inhibitory receptors family (Falco et al., 1999; Nicoll et al., 1999). Among the LILR genes, only LILB1 (ILT2/LIR1) encodes the inhibitory receptor of the NK cell (Kuroki et al., 2005). Expression of LILB1 can change depending on the surrounding NK cells ranging from negligible to 75% (Yokoyama and Plougastel, 2003; Yokoyama, 1995; Long, 1999; Parham, 2005; Cosman et al., 1997). Those receptors (regardless of MHC restriction) have inhibitory motifs (ITIMs) in the cytoplasmic domain, where the activation signal is desensitised.

The Activating Receptors of Natural Killer Cells

NK cells express numerous activating receptors, which could be classified into various categories. The main activating receptor groups of NK cells are CD16, NKR-P1 (NK1.1, CD161), NKG2D (KLRK1, CD314), NCR (NKp30, NKp44, NKp46, NKp80) and activating isoforms of human KIRs (Costello et al., 2013). These molecules have no ITIM but instead have ITAM-positive adaptor molecules (DAP12), so they can act as

activating receptors (Hamerman et al., 2011). The most well-characterised activating receptor in NK cells is CD16, which has a low affinity Fc receptor to IgG (FcγRIII) (Perussia et al., 1983). NK cells can mediate antibody-dependent cellular cytotoxicity (ADCC) by FcγRIII (Anegón et al., 1988). Although there are several Fc receptors to IgG, NK cells only express FcγRIII. In addition, apart from the ADCC capacity of NK cells, CD3-CD56+ CD16-NK cells can mediate natural killing (Lanier et al., 1988).

NKRP1 (Kirb1) belongs to the family of lectin-like molecules with type II membrane protein in mice (NK1.1) (Ryan et al., 1992; Giorda and Trucco, 1991). NKR-P1A and CD161 both have an external C terminus; they are also classified as type II membrane proteins (Bartel et al., 2013). NKR-P1A, a receptor coded by the *klrb1* gene, recognises lectin-like transcript-1, a functional ligand. NKG2D binds to ligands (e.g., human ligands MICA, MICB and mouse ligands RAE-1*alpha* and RAE-1*beta*), which are structurally homologous to MHC class I (Radaev and Sun, 2003).

NKG2D is expressed as a disulfide-linked homodimer in both human and mouse NK cells (Lanier et al., 1998a,b). NKG2D is different from other NKG2 molecules, in that it shares very little homology with the other NKG2 molecules (28% instead of 70%) and is not heterodimerised with CD94 (Ho et al., 1998). Remarkably, the expression of NKG2D is not limited to NK cells. It is notably found in γδ TCR+ T cells and CD8+ T cells in human and in most NKT cells and activated CD8+ T cells in mouse (Bauer et al., 1999). In addition, NKG2D does not have any cytoplasmic motif and preferentially binds to the signal chain DAP10 by a YxxM motif for the recruitment of PI3K (Wu et al. 1999). DAP10 acts as costimulatory molecule of NKG2D.

Unlike TCR and immunoglobulins, NCR is a type I transmembrane receptor, which does not go through recombination to be functionally active (Hudspeth et al., 2013a,b). NCR contains ITAM, which activates NK cells. NKp44 has both ITAM and ITIM. NCR was originally known as a receptor that mediates killing tumour-transformed cells and is involved in the control or elimination of many pathogens (Magri et al., 2011). Moreover, NCRs play an important role in immune homoeostasis by regulating the expression of various types of immune cells. The ligands of these receptors include not only self-derived molecules but also pathogenic components (Hudspeth et al., 2013a,b).

Mechanisms of Natural Killer Cell Activation

NK cells can lyse tumour cells and virus-infected cells without prior sensitization. This lysis or cytolytic function is modulated by the balance between inhibitory NK receptors, which specifically bind to MHC (HLA) molecules in healthy cells, and activating NK receptors, which detect stressed cells (Paul and Lal, 2017). When MHC class I molecules are downregulated or eliminated by tumour cells or viral infection, the loss of inhibitory signal from inhibitory receptors leads to the activation of NK cells. This is called 'missing self', and it triggers activation of the NK cells (Hammer et al., 2018).

The activating receptors (e.g., NKG2D) in NK cells could detect self-molecules expressed at higher levels in damaged cells. This is called 'stress-induced self-recognition'. Cell surface

receptors regulate the function of activation, inactivation, proliferation and effectors (Spits et al., 1998; Colucci et al., 2003). Once activated by NK cell receptors, NK cells could exert their cytotoxic effects in many ways. These include cytolytic granule–mediated cell apoptosis and ADCC. NK cells secrete TNF-α, which induces interferon (IFN) gamma and phagocytosis, when it is activated by cytokines or IFNs (Pallmer and Oxenius, 2016).

The Function of Natural Killer Cells

Mature NK cells are morphologically characterised as large granular lymphocytes. These granules contain granzymes (a family of proteolytic enzymes) and perforin (a membrane-disrupting protein) that are responsible for NK cell–mediated killing (Voskoboinik et al., 2015). The interaction between NK cells and its target cells results in the formation of immunological synapses and secretes these granules that lead to specific lysis (exocytosis) of target cells (Krzewski and Coligan, 2012). Furthermore, NK cells can kill tumour cells by using molecules of the TNF family. NK cells notably express both soluble and membrane-bound TNFs. The activation of NK cells induces FAS ligand (FASLG; also known as TNFSF6) and TNF-related apoptosis-inducing ligand (TRAIL; encoded by TNFSF10) on the NK cell surface (Wallin et al., 2003). When bound to those ligands, the death receptors of target cells can activate the caspase enzymatic cascade, inducing apoptosis as a result (Smyth et al., 2005).

An easy experiment to assess the potency of NK cells is to transplant NK cell–dependent tumours in *Tnfsf10*$^{-/-}$ mice (Cretney et al., 2002). NK cells secrete cytokines and chemokines involved in the innate and adaptive immune responses when the NK cells are activated by the interaction with target cell or by cytokines in the tumour microenvironment (Vivier et al., 2011). Although NK cells produce IFN-γ particularly, NK cells could also secrete various interleukins (for example, IL-10), TNF, growth factor (for example, GM-CSF) and chemokines (for example, chemokine (C–C motif) ligand 3 (CCL3), CCL4 and CCL5) (Cooper et al., 2001; Maghazachi, 2010). The secretion of those cytokines recruits other immune cells to the inflammation site and further induces the activation and proliferation of immune cells.

NK cells are an initial and potent producer of IFN-γ. IFN-γ induces the expression of MHC class II molecules on antigen-presenting cells and heavily affects immune reaction, such as the activity of myeloid cells, induction of T helper 1 (TH1) cells and effect on angiogenesis. The activation of macrophage by NK cell–derived IFN-γ was essential in tolerance to chemical carcinogenesis in the primary tumourigenesis mouse model (O'Sullivan et al., 2012).

Natural Killer Cells in the Tumour Microenvironment

The tumour microenvironment is a complex population of cells composed of tumour cells, immune cells and stromal cells. It is a complex network of extracellular matrix, which is responsible for the proliferation, migration and dissemination of tumour cells. Subsets of immune cells include CD8+ T cells, CD4+ T cells, NK cells, and myeloid cells (DC, M2 macrophage, MDSC) (Hasmim et al., 2015). The role of NK cells in the

elimination of circulating tumour cells is well established (leukaemia cells and metastatic cells) (Pende et al., 2009; Wiltrout et al., 1985; Yang et al., 2003). The antitumoural function of NK cells in solid tumour is frequently mentioned in terms of its high infiltration capability, inverse correlation of natural cytotoxicity activity and cancer incidence, rapid tumour growth in NK cell–depleted mouse model (Rusakiewicz et al., 2013; Imai et al., 2000; Dithmar et al., 1999; Kataoka et al., 1999; Mailloux et al., 2010).

CXCR3 is a main chemokine receptor, which plays a role in NK cell migration to tumours, depending on the concentration gradient of tumour-derived chemokine ligands including CXCL9, 10 and 11 (Wennerberg et al., 2014; Bingle et al., 2002). The high expression of CXCL10 increases the infiltration of CXCR3-positive NK cells to tumour cells in melanoma (Wennerberg et al., 2014). However, the infiltrated NK cells often show inhibited phenotype in solid tumours. This is known to result from the fact that not only microenvironmental factors have an inhibitory effect on NK cells in the tumour microenvironment but also the tumour cells themselves have suppressive properties, including affecting and suppressing NK cell activation and function (Vitale et al., 2014).

NK cells communicate with DC and M2 macrophages. In the regulation of NK cell cytotoxicity, the inhibitory effect by transforming growth factor beta (TGF-β) is reported when NK cells are cultivated with DCs. The STAT3 phosphorylation in DC is associated with an increase in TGF-β secretion, which suppresses NK cell activity. The inhibition of TGF-β is reported to result in the recovery of the NK cell function (Sarhan et al., 2015). TGF-β production in DC can be induced when stimulated by the coculture of immature DC and lung carcinoma cells (Dumitriu et al., 2009) or LPS (Zhang et al., 2005). IL-6 and IL-10 secreted by DC also suppress NK cell activity (Pérez-Martinez et al., 2012). Nevertheless, some studies showed that DC could activate NK cell function. Particularly, IL-15–stimulated DC increase the expression of the activating receptors including NKp30 and NKp46 on the surface of NK cells (Anguille et al., 2015). This activation requires cell contact and membrane-bound IL-15 in DC (Ferlazzo et al., 2002).

Macrophages also play an important role in the regulation of NK cell activation in the tumour microenvironment. Macrophages and other myeloid cells are the main population of immune infiltration cells inside the tumour tissue (Bingle et al., 2002; Gabrilovich et al., 2012). They are differentiated into tumour-associated macrophages (TAM), after expression of TAM markers like CD206 (Mantovai et al., 2002). The exposure of TAM to tumour-derived cytokines, such as IL-4, IL-10, IL-13, and M-CSF, can transform TAM into polarised type II or M2 macrophages with immunosuppressive property and induce tumour progression (Mantovai et al., 2002). M2-polarised macrophages contribute to immunosuppression through the production of immune suppressive factors such as IL-10 and TGF-β (Quatromoni et al., 2012).

Recently, the role of myeloid cells including macrophages in the immunosuppression of NK cells can be better understood in the involvement of the A2AR receptor (Cekic et al., 2014). The removal of A2AR in myeloid significantly activates macrophages via M1

polarization, reduces lung metastasis, increases the expression of CD44 in tumour-associated NK cells and T cells and increases the number and activity of NK cells and antigen-specific CD8+ T cells in lung infiltrate (Cekic et al., 2014). In the xenografted lung carcinoma model, the increase in surfactant protein-A has been reported to reduce tumour growth and increase NK cell recruitment and its activity at tumour sites (Mitsuhashi et al., 2013).

Effect of Suppressors in the Tumour Microenvironment

The activity and number of NK cells can be affected by various factors in the tumour microenvironment. These factors that regulate NK cells are discussed below.

Regulatory T Cell

The immunosuppressive function of regulatory T cells (Tregs) suppresses the activation of NK cells and other immune cells (Zou, 2006). Treg directly inhibits the cytolytic function of NK cells and the expression of CD69 (Trzonkowski et al., 2004; Xu et al., 2014). These results indicate that NK cell inhibition by TGF-β produced by Treg is at least one of the mechanisms where NK cell inhibition is mediated by Treg. In vivo, Treg deficiency has been shown to induce the expression of IL-15Rα in DC and to increase the proliferation of NK cells (Terme et al., 2008). In a murine model, this deficiency seems to be similar to the elimination of NKG2D-mediated tumour (Smyth et al., 2005).

Suppression of Natural Killer Cell Activity by Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSC) are included in the myeloid subsets associated with tumour-induced immunosuppression (Gabrilovich and Nagaraj, 2009). MDSCs include immature macrophage, granulocyte and DC. The expansion and immune suppressive function of these cells have been demonstrated in tumour-bearing mouse and cancer patients (Marvel and Gabrilovich, 2015). Therefore, the activation of NK cells is inversely correlated to the expansion of MDSCs (Liu et al., 2007; Li et al., 2009). In addition, the inhibition of NK cells by MDSC has been shown to be dependent on cell contact via membrane-bound TGF-β of MDSC (Li et al., 2009) or inhibition of perforin expression in NK cells and STAT5 activity (Liu et al., 2007). The MDSCs of liver cancer patient exhibit an inhibitory effect on NK cell activity when they are cocultured with NK cells. This inhibition was also found to be dependent on cell contact and was shown to inhibit the expression of NKp30, an activating receptor in NK cells (Hoechst et al., 2009).

Regulation of Natural Killer Cell Activity by Tumour Cells

Tumour cells in many cancers try to escape CD8-dependent T cell killing by lowering the expression of MHC-1 molecule on the surface, making them more vulnerable to NK cell–dependent killing. Cancer cells also upregulate NKG2D ligands after activating NFκB or Sp transcription factors (Raulet, 2003). However, tumour cells have many strategies to suppress NK cell–mediated cytotoxicity. Indeed, NK cells of multiple myeloma

patients constitutively express the inhibitory receptor programmed cell death protein 1 (PD-1), compared with healthy donor NK cells (Benson et al., 2010). Inhibition of NK cell activity by PD-1/PD-L1 can be recovered by using lenalidomide or blocking antibody. Also, tumour cells secrete various immunosuppressive cytokines such as TGF-β. In this regard, neuroblastoma cell–derived TGF-β was reported to downregulate activating receptor NKp30 (Castriconi et al., 2013). Melanoma cells reduce NK cell–mediated cytotoxicity by inhibiting the expression of activating NK cell receptors including NKp30, NKp44 and NKG2D (Pietra et al., 2012). These inhibitory effects are mediated by the production of IDO and PGE2 by melanoma cells. Tumour cells can release soluble NKG2D ligands by proteolytic cleavage, resulting in the downregulation of NKG2D and the impairment of NK cell lytic function (Groh et al., 2002; Song et al., 2006).

However, the secretion of soluble NK cell receptor ligands does not only influence the inhibitory effect on NK cells. In fact, the vesicles containing HLA-B–associated transcript 3 (BAT3), an NKp30 ligand, were reported to activate NKp30 (Pogge von Strandmann et al., 2007); furthermore, shedding of MULT1, NKG2D ligand, was known to increase NK cell activity and expression of NKG2D on the surface (Deng, 2015). Recently, the induction of *c-myc* was reported to increase NKG2D ligands in leukaemia cells resistant to cytarabine (Nanbakhsh et al., 2014). The modification of *c-myc* is associated with various cancers in human (Miller et al., 2012). Therefore, the question arises whether any relationship exists between the expression of *c-myc* transformed in solid tumours and the activating ligand of NK cells.

The Role of Natural Killer cells in Cancer Immunosurveillance and Immunoediting

The role of NK cells in tumour immunosurveillance was first mentioned when the cancer incidence rate was reported to be high in individuals deficient in NK cell function, due to genetic disorders such as Chédiak–Higashi syndrome and X-linked lymphoproliferative syndrome in the 1980s (Roder et al., 1980). In addition, tumour growth and metastasis were explained in mutant mouse with damaged NK cell activity (Talmadge et al., 1980) and in mouse treated by antibody that kills NK cells (Gorelik et al., 1982).

Several studies reported the reduction of NK cell function in cancer patients and their families (Nakajima et al., 1987; Pross and Lotzová, 1993; Schantz et al., 1986; Strayer et al., 1984; Hersey et al., 1979). Moreover in a long-term epidemiological study, it was reported that people with low NK cell function have higher risk of developing various cancers (Imai et al., 2000).

NK cell deficiency is characterised by a lack of NK cells or NK cell function, which results from genetic mutation of genes such as GATA2 and MCM4. NK cell deficiency increases the prevalence rate of malignant tumours (Gineau et al., 2012). The main immunodeficiency effect on NK cells indicates how important NK cells are in tumour immunosurveillance. However, this immunodeficiency not only affects NK cells but also other immune cells. Consequently, it lowers NK cell activity and increases the risk of

cancer. NK cells have been shown to control the growth and metastasis of transplanted tumours in many mouse models (Smyth et al., 2002). However, these studies are too limited to confirm the selective killing of primary tumours by NK cells without any specific target molecules that can be genetically derived or antibody-mediated.

In recent comprehensive transcriptional profiling studies on mouse leucocyte population, all genes expressed by NK cells were also observed to be expressed by other immune cells, such as subsets of T cells or ILCs (Bezman et al., 2012). Mouse NK cells express NK1.1 (also named as KLRB1) in many models (Kirkham and Carlyle, 2014). Notably, NK1.1 is also expressed in invariant NKT cells, some activated CD8+ T cells and ILCs. The restricted marker of NK cells may be NKp46, but this receptor is also expressed in γδ T cells and subpopulations of other ILC groups (Hudspeth et al., 2013a,b). RAG-deficient (e.g., $Rag1^{-/-}$ or $Rag2^{-/-}$) mouse can be used for excluding the effect of B and T cells in study on antitumour effect, but the effect of other molecules expressed by NK cells and other ILC subsets should be considered (Robinette et al., 2015). Although NK cells are reported to infiltrate primary tumours in mouse and human, the question of their role for killing tumours arises because their population seems to be too small (Albertsson et al., 2003). Considering the lack of NK cells in solid tumours that is commonly observed and the relative portion among the immune cells in the circulatory system, the main role of NK cells is expected to prevent cancer metastasis (Gorelik et al., 1982). Although NK cells are not typically observed in solid tumours, their anticancer role at tumour site cannot be excluded as they significantly contribute to anticancer immune reactions by cytolytic function and the release of various chemokines and cytokines, which could induce or activate tumour-infiltrating T cells or myeloid cells.

New reagents and imaging technologies that could analyse the population of leucocytes infiltrated to tumours in a more sensitive and dynamic way to evaluate the recruitment and function of NK cells are under development. The ability of NK cells to attack and eliminate their potential target cells is regulated by the balance between activating and inhibitory signals (Pallmer and Oxenius, 2016). Although most tumours maintain the expression of MHC class I, their expression is downregulated in response to selective pressure by CD8+ T cell. These downregulated tumours could be removed by NK cells because the signals inhibiting NK cells are reduced.

Alternatively, when the ligands corresponding to NK cell activating receptor are upregulated and exceed the inhibitory signal, tumour cells, including those that even maintain the full expression of MHC class I, can be eliminated. The most distinguished ligand for NK cell–activating receptors expressed in transformed cells is the NKG2D ligand (Raulet et al., 2013). The NK cell activation induced by the expression of NKG2D ligand in tumour cells is sufficient to overcome the inhibitory signal transferred from MHC class I receptors, enabling NK cells to remove tumours exhibiting normal levels of MHC class I (Cerwenka et al., 2001; Diefenbach et al., 2001). In addition, mice deficient in the NKG2D gene ($klrk1^{-/-}$) or mice treated with NKG2D-neutralizing antibody are more vulnerable to primary tumourigenesis, a phenomenon that confirms the

role of NKG2D in tumour immunosurveillance (Ullrich et al., 2013). Whether NKG2D-mediated protection is conferred by NK cell, T cell or both remains to be elucidated.

One of the escape mechanism of the tumours to avoid this surveillance is to release NKG2DLs by ligand cleavage mediated by metalloproteinases to reduce the number of ligands at the surface of tumour cells (Groh et al., 2002; Kaiser et al., 2007; Salih et al., 2002; Wu et al., 2004; Fernández-Messina et al., 2010; Salih et al., 2006). Soluble NKG2DLs were found in the serum of patients with many types of cancer (Salih et al., 2008). As a result, this molecule can be used as diagnostic biomarker (Holdenrieder et al., 2006). Another escape mechanism is that tumour cells secrete soluble factors (lactate dehydrogenase, etc.) and induce the production of NKG2DLs in healthy host myeloid cells (Crane et al., 2014).

Regardless of tumour cells or healthy host cells, the chronic stimulation of NKG2D by ligand-bearing cells induces the downregulation of NKG2D and desensitization of the NKG2D pathway and damages NKG2D-dependent tumour immunosurveillance (Champsaur et al., 2010; Oppenheim et al., 2005). The therapeutic strategy that could maintain or induce the expression of NKG2DLs on tumour cell surface and prevent the desensitization of NKG2D receptors in NK cells and T cells should provide an attractive target for immunotherapy. Recent mouse model studies confirmed that soluble high-affinity NKG2DLs and MULT1 could stabilise the expression of NKG2D on the NK cell surface and increase NKG2D-dependent antitumour activity (Deng et al., 2015).

Several other mechanisms that allow tumours to escape NK-mediated surveillance have been reported. For example, platelet-activating tumours can escape from NK cell recognition by inhibiting the expression of activating ligands and expressing highly expressed ligands of NK cell inhibitory receptors, leading to direct escape from NK cell recognition, or indirectly release TGF-β and other immunoregulatory factors (Kopp et al., 2009; Placke et al., 2012). In addition, tumours themselves secrete immunoregulatory factors that impair the activation of immune cells, especially NK cells. Factors that inhibit NK cell effector function include TGF-β (Wilson et al., 2011), prostaglandin E2 (Holt et al., 2012; Pietra et al., 2012), indoleamine 2,3-dioxygenase (IDO) and adenosine (Hoskin et al., 2008). Remarkably, these factors cannot only suppress NK cell function but also block the maturation process of NK cells by downregulating the expression of IL-15 receptors (Richards et al., 2006).

Tumours may produce IL-10, which is known for its antiinflammatory and immunosuppressive properties. However, IL-10 was reported to activate some tumour cells or NK cells (Mocellin et al., 2005; Park et al., 2011a,b). Thus, the function of IL-10 for modulating NK cells remains unclear.

NATURAL KILLER CELL–BASED IMMUNOTHERAPY

NK cells are innate lymphoid cells characterised by cytotoxic activity. When they recognise cancer cells or infected malignant cells, they immediately initiate the elimination process without any specific activation mechanism.

Overview of Natural Killer Cell–Based Immunotherapy

NK cells are the main immune cells that stay at the forefront of immune function and antitumour activity in the body. Not depending on antigen specific receptors such as T and B cells, NK cells express various innate immune receptors on their surfaces, which could distinguish normal cells from infected and cancer cells (Long et al., 2013).

In other words, the activity of the NK cell is maintained and regulated by comprehensive signal transduction that is induced by recognition of the targets by various activating and inhibitory receptors on the surface (Long et al., 2013).

These NK cells proved to directly inhibit the development and metastasis of cancer cells among various immune cells present in the body and even effectively remove cancer stem cells. Thus, from the viewpoint of anticancer immunotherapy, NK cell adoptive therapy has several advantages over T and B cells. As those advantages are gaining attention, many clinical studies of NK cell–based anticancer immunotherapy have been attempted and promising results on many cancers have already been reported (Rezvani and Rouce, 2015; Chouaib et al., 2014). The relationship between NK cell activation and inhibition in intracellular signal transduction molecules in many cancer patients and progression of the disease has been elucidated (Sutlu and Alici, 2009). In addition to the direct treatment of disease (cancer) using NK cells, their use for detecting cancer or predicting therapeutic effect by checking the activity of the NK cells are under progress in many ways. The development of anticancer immunotherapy using NK cells should be based on the understanding of the many receptors of the NK cells as described above.

For example, though 2B4 receptors induce activation of NK cells associated with NKG2D or DNAM-1 receptors, when cytokines such as IL-2 and IL-15 are engaged, the activation of NK cells is significantly facilitated. As induction of activation is reported to be possible by one activating receptor (Watzl, 2014), studies on the mechanism of activation and inactivation of NK cells by a combination of various receptors should be given priority.

Expansion and Manipulation of Natural Killer Cells for Clinical Practice

Manufacturing is a first key point that needs to be well developed so as to secure the proliferation ability and activation level of NK cells and to obtain sufficient functional NK cells considering their purity and condition (Imai et al., 2005). NK cells can be isolated and obtained from many sources including cord blood, bone marrow, peripheral blood and embryonic stem (ES) cells (Sarvaria et al., 2017).

The most commonly used method for isolating NK cells is to acquire peripheral blood mononuclear cells (PBMCs) by blood drawing or leukapheresis. However, as the number of NK cells in PBMCs are low, many scientists focused on the development of an effective method for ex vivo expansion of the NK cells. Numerous R&D projects on process development under Good Manufacturing Practice (GMP) condition for manufacturing clinical-grade NK cells have been conducted (Berg et al., 2009; Siegler et al., 2010).

For the expansion of NK cells, various methods were developed using feeder cells including Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line, genetically modified K562 cells or irradiated autologous cells (Cho and Campana, 2009). Representatively, Campana et al. developed a master bank of GMP-grade genetically modified K562 cells (IL-15, 4-1BB ligands are expressed on the membrane) and proved that large-scale expansion and activation of NK cells was feasible (Fujisaki et al., 2009).

Furthermore, K562 cells designed to simultaneously express MICA, 4-1BB ligand and membrane-bounded (mb) IL-15 or IL-21 were demonstrated to be useful for the utilization of ex vivo expansion of NK cells (Gong et al., 2010). Especially the mb IL-21–modified K562 cells were found to enhance tumour killing of the NK cells by proliferation of NK cells, elongation of telomere and decrease of senescence (Denman et al., 2012) compared with mb IL-15-modified K562.

During the process of large-scale ex vivo expansion using irradiated EBV-transformed lymphoblastoid cell line, activating receptors and death receptor ligands of NK cells are increased, and the pattern of cytokine secretion is modified, leading to an increase in the cytotoxicity against tumour cells. The efficacy of these expanded NK cells was confirmed in phase I clinical trials in advanced metastatic tumour and haematological malignancies, which used autologous NK cells (Berg et al., 2009).

In recent development, NK cells could be expanded with high activation level using various factors including cytokines without any feeder cells. The method for acquiring and culturing NK cells under feeder-free protocol progressed from small scale using flask into automated large-scale using bioreactor. It should be noted that the expression level of NKp44 was higher in NK cells expanded by large-scale bioreactor, than in NK cells expanded by flask (Sutlu et al., 2010).

NK cells can be acquired and isolated from umbilical cord blood (UCB) where haematopoietic stem cells (HSCs) reside. For acquiring HSCs from UCB, CD34+ cells should be selectively isolated. Studies on using many effective cytokines for securing NK cells from these CD34+ cells and expansion of NK cells have been reported. As well as basic methods using antibody sorting, an automation process using specific equipment, was established and optimised (Kao et al., 2007; Spanholtz et al., 2010, 2011).

UCB-derived NK cells have CD56+ characteristics and the expression levels of NKG2D and natural cytotoxicity receptors are known to be consistently high. In particular, UCB-derived CD56+ NK cell were reported to be very effective at targeting myeloid leukaemia and melanoma cell line, and lysed primary leukaemia cell, even with low effector cells versus tumour cells (E:T) ratio (Spanholtz et al., 2010). In addition, CD34+ cells expanded in the serum-free culture process had significantly higher NK cell differentiation potential than initially isolated CD34+ cells. Methods for achieving greater levels of IFN-γ secretion and the production of NK cell with high cytotoxicity using various feeder cells were devised as well (Kao et al., 2007).

Human NK cells can be differentiated and cultured from bone marrow derived CD34+ haematopoietic progenitor cells. In addition, many attempts to produce NK cells

using CD34+ cell from hESCs and induced pluripotent stem cells (iPSCs) have been made. Recently, several researchers reported a method acquiring NK cells using a two-step culture process starting from human embryonic stem cells (hESCs) (Miller et al., 1994; Mrozek et al., 1996). These hESCs-derived NK cells exhibited CD94+ CD117 low characteristics and showed much more effective antitumour response in in vivo xenograft mouse model than UCB-derived NK cells (Woll et al., 2009). Also, hESC- and iPSC-derived NK cells are known to have advantages over peripheral blood-derived NK cells in terms of genetic modification and improvement in survival under in vivo condition (Knorr and Kaufman, 2010). Because of its potential, various studies on NK cells derived from various source of CD34+ stem cells have recently been performed (Table 5.1).

Autologous Natural Killer Cells

Early research on NK cell immunotherapy focused on the improvement in antitumour activity of endogenous NK cells. Autologous NK cells can be activated and enforced by systemic administration of several cytokines including IL-2, IL-12, IL-18, IL-21 and type I IFNs (Cheng et al., 2013).

In these early studies, patient's blood was collected by leukapheresis and administered back to the patient after sorting NK cells by CD56 beads. It was normal to coadminister IL-2 for stimulation and expansion of the injected NK cells (Burns et al., 2003; Rosenberg et al., 1985). However, the method showed only limited efficacy. The observed limitation resulted from several factors, and the stimulation by cytokines in vitro was observed to increase NK cell activity and cytotoxicity to the target, but the activity in the body after in vivo injection is limited.

Factors that cause the differences in response shown between in vitro and in vivo were identified as follow: (1) life-threatening side effects, such as vascular leak syndrome by IL-2 injection; (2) suppression of NK cell function by expansion of Tregs with IL-2; and (3) lack of antitumour effect by self-HLA–mediated autologous NK cell inhibition (Cheng et al., 2013; Stern et al., 2013; Yoon et al., 2010). The ways to overcome these phenomena is a necessary step to further progress here. One of the approaches includes blocking the interaction between NK cells surface inhibitory receptors and HLA class I ligands using anti-KIR antibody such as lirilumab (Foley et al., 2014).

However, those early studies based on a simple isolation and injection approach had two further limitations: the number of NK cells that could be isolated by leukapheresis and by typical aphaeresis were low and the injected NK cells did not sufficiently expand in the body (Berg et al., 2009). In addition, as an alternative to IL-2 therapy that induces expansion of Tregs as well as NK cells, studies on the expansion of only NK cells were actively performed. As a result of these efforts, a method using IL-15 was developed. Notably, IL-15 proved superior to IL-2 in tumour response by increasing expansion and survival of NK cells in the body and decreasing the induction of Tregs in many preliminary studies (Rosario et al., 2016). Thus, studies on other cytokines that could induce the expansion and proliferation of endogenous NK cells as well as adoptive NK cells

Table 5.1 Expansion of Natural Killer Cells In Vitro for Clinical Practice (Cheng et al., 2013).

	Starting Material	Initial Cell Number	Medium	Stimulators[a] Feeder Cells	Culture Instrument
Cord blood–derived NK cells	CD34+ cell from cord blood (ClinicMACS)	(0.89–6.34) $\times 10^6$	Glycostem Basal Growth Medium + 10% HS	SCF, IL-7, IL-15, IL-2, Flt3L, TPO, G-CSF, IL-6, LMWH	VuelifeTM tags, WAVE Bioreactor System 2/10, BIOSTAT CultiBag RM system
	CD34+ cell from cord blood (ClinicMACS)	(0.84–2.50) $\times 10^6$	Glycostem Basal Growth Medium	SCF, IPO, IL-7, Flt3L, IL-15, IL-2, G-CSF, GM-CSF, IL-6, LIF, MIP-1α	24-well tissue culture system
Stem cell/ iPSC-derived NK cells	CD34+ CD45+ cells (H9 hESC cells)	–	RPMI 1640 + 15% defined foetal bovine serum, DMEM/Ham F12 + 20% heat-inactivated human serum AB	IL-3, IL-15, IL-7, SCF and Flt3L; feeder cells; M210-B4; AFT024	–
	BM CD34+	–	Dulbecco's medium supplemented with 12.5% foetal calf serum, 12.5% horse serum	IL-2, feeder cells; stromal cells from irradiated BMMNC	–
PBMNCs	CD3-CD56+ cells from PBMNc (ClinicMACS)	(0.40 ± 0.16) $\times 10^6$	CellGro SCGM serum-free medium, 5% AB human serum	IL-2, IL-15, anti-CD3 monoclonal antibody OKT3	Baxter LifeCell culture bags
	CD3-CD56+ cells from PBMNc	3.0×10^6	SCGM medium and 10% foetal bovine serum	IL-2; feeder cells; K562-mb15-41BBL	VueLife bag system
	CD56+ cells from PBMCs	(9.5–85.8) $\times 10^6$	Alpha-MEM, 20% foetal bovine serum	IL-15, HC	–
	CD56+ cells from PBMCs (ClinicMACS)	2.0×10^8	X-VIVO 20 10% heat-inactivated human AB serum	IL-2; feeder cells; EBV-TM-LCL cells	Flasks and bags
	PBMCs	2×10^6 NK cells	SCGM medium and 10% foetal bovine serum	IL-2; feeder cells; K562-mb15-41BBL	G-Rex100 flasks

Culture Time/ Acquired Cell Number	Fold Proliferation	Purity	Cytotoxicity	Phenotype. Cytokine Production	References**
6 weeks; $(1.6–3.7) \times 10^9$	1435–2657	>90%	K562(>40%, 10:1)	$CD56^+$, $CD3^-$, $NKG2D^+$, $NCRs^+$, $CD161^+$, $CD314^+$, $CD244^+$	[155]
14–35 days; $(1.9–7.8) \times 10^9$	$\sim 10^4$ (freshly UCB), $\sim 10^3$ (frozen UCB)	>95%	K562, Lama, Kasumi, BLM and FM3 (>75%); KG1a (~30%) (18h 1:1)	$CD56^+$, $CD3^-$, $NKG2D^+$, $NCRs^+$, $CD107^+$, $2B4^+$, $CD161^+$, IFN-γ	[154]
30–35 days	~100	>37.5%	K562, MCF7, PC3, (55%–80%), NTERA2, and UB7 (20%–30%)	$CD56^+$, $CD45^+$, $CD16^+$, $CD94^+$, $NKG2D^+$, $NKp46^+$, $CD158a^+$, $CD158b^+$, IFNγ	[159, 162, 163]
–	~690	>75%	K562 (80%, 6.6:1)	$CD3^-$, CD56+, CD2+, CD7+, CD8+, CD16+	[157]
19 days $(85.5 \pm 17.2) \times 10^8$	268.3 ± 66.8	100%	K562 (>60%, 10:1)	$CD3^-$, CD56+, NKG2D+, NCRs+, DNAM-1	[166]
7 days	09.5 (33–141)	83.1% (72.9%–85.9%)	K562, HL-60, KG1 and U937 (>40%, 4:1)	$CD3^-$, CD56+, NKG2D+, NCRs+	[165]
20–23 days	23 (3.2–131.3)	97.9% (82.7%–99.6%)	K562, (23.2%, 7.0%–54.7%, 1:1)	$CD3^-$, CD56+, NKG2D+, NCRs+	[196]
21 days; 3×10^{10}	490 ± 260	$84.3\% \pm 7.8\%$	RCC $(27.6 \pm 9.3\%, 1:1)$	$CD3^-$, CD56+, CD244+, CD48+, NKG2D+, sFasL, IFN-γ, GM-CSF, TNF-α, MIP-1α, MIP-1β	[164]
8–10 days	209 (38–338)	61% (54%–70%)	K562, U266 and Raji (>40%, 5:1)	$CD3^-$, CD56+	[197]

Continued

Table 5.1 Expansion of Natural Killer Cells In Vitro for Clinical Practice (Cheng et al., 2013).—cont'd

	Starting Material	Initial Cell Number	Medium	Stimulators[a] Feeder Cells	Culture Instrument
	PBMCs	–	Serum-free medium and 10% heat-inactivated human plasma	rhIL-2; OK432; anti-CD16	Cell culture bags
	PBMC	1.5×10^6	cRPMI	IL-2; feeder cells; K562-mbIL15-41BBL cells	T25 or T75 culture flasks
	PBMCs	1.5×10^6	CellGro SCGM serum-free medium, 5% human serum	IL-2	Wave Bioreactor System 210
NK cell lines	CD3-depleted PBMCs	107 CD3-depleted cells	AIMV media, 10% hu AB serum	IL-2; feeder cells: OKT3-loaded autologus PBMC	Cell culture bags
	NK92	$(2.5 \times 10^5$/mL $\times$ 25 mL	X-VIVO serum-free media amino acids and 2.5% human AB plasma	IL-2 (500 IU/mL)	1 L Vuelife culture/bag
		1×10^7/bioreactor	Optimised clinical-grade media	IL-2 (100–500 IU/mL)	Controlled stirred bioreactor
	NKG	$(1 \times 10^5$/mL) $\times$ 200 mL/bag	α-MEM medium, 10% foetal bovine serum +10% horse serum	IL-2 (100 IU/mL)	WAVE Bioreactor

GM-CSF, granulocyte monocyte colony-stimulating factor, *hESCs*, human embryonic stem cells; *IFN*, interferon; *iPSCs*, induced pluripotent stem cells; *NCR*, natural cytotoxicity receptor; *NK*, natural killer; *PBMCs*, peripheral blood mononuclear cells; *SCF*, stem cell factor; *UCB*, umbilical cord blood.

[a]Ex vivo expansion of NK cells for clinical immunotherapy under Good Manufacturing Practice conditions.

Culture Time/ Acquired Cell Number	Fold Proliferation	Purity	Cytotoxicity	Phenotype. Cytokine Production	References**
21 days	637–5712	78.9% ± 11.6%	K562, Raji and Daudi (>20%, 3:1)	$CD3^-$, CD56+, CD158a+, CD158b1/b2+, CD159a+, CD69+, NKp30+, NKp44+, NKp46+, IFN-γ, TNF-α	[198]
14 days	165 (4–567)	45.6% (7.4%–76.4%)	K562, MCF-7, LNCaP, DU145, PC-3	$CD3^-$, CD56+, NKG2D, NCRs+	[199]
21 days; 9.8×10^9	Mean 77-fold	Mean 37.5%	K562 (>25%, 10:1)	$CD3^-$, CD56+, CD244+, CD11a, CD69+, NKG2D, NCR+	[200]
21 days; $(4.70 \pm 2.10) \times 10^{10}$	-	≥93%	898 (82 ± 12%, 10:1)	$CD3^-$, CD56+, CD16+, NKG2D+	[176]
15–17 days; 1×10^9/bag	>200	≥80% (viability)	K562 (72%), Raji (58%) (10:1)	$CD3^-$, CD56+, IL-6, IL-8, IL-10	[170, 171]
11–16 days; 10^{10}/bioreactor	>1000	>95% (viability)	Highly lytic to leukaemia, lymphoma, malignant melanoma, prostate cancer, squamous cell carcinoma, breast cancer	Positive: CD56, CD7, CD11a, CD28, CD45, CD54 Negative: CD1, CD3, CD4, CD8, CD14, CD16, CD20, CD23, CD34, HLA-DR	[131]
12–14 days; 1010/bag	>1000	>90% (viability)	K562 (>50%), Ho-8910 (>60%), Daudi (>70%), LoVo (>35%) (10:1)	CD56+, CD16+, $CD27^-$, $CD3^-$, $\alpha\beta TCR^-$, $CD4^-$, $\gamma\delta TCR^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD161^-$, CD45+, CXCR4+, CCR7+, $CXCR1^-$, $CX3CR1^-$, IFN-γ, TNF-α, IL-6, IL-10	[127]

more effectively are actively underway. Remarkably, compared with traditional methods of simple isolation followed by postinjection for stimulation, newly developed methods enabled the use of many NK cells that are highly activated.

Early expansion was conducted in a medium containing a single cytokine, such as IL-2 or IL-15, for generally 14 days leading to a 10- to 20-fold expansion (Mao et al., 2016). Later developed protocols that implement cytokine-containing media with feeder cells such as allogeneic PBMC, T cells, EBV-transformed lymphoblastoid cell line or genetically modified K562 cells could induce the proliferation of NK cells much more effectively and enabled 80- to 10,000-fold expansion in 14–21 days of culture (Cho and Campana, 2009).

Several clinical trials using ex vivo expanded autologous NK cells are underway, and many studies for more effective culture protocols are being conducted. Frei et al. recently reported the method for NK cell expansion that starts from CD3-aphaeresis products using α-MEM–containing vitamin B3 derivative nicotinamide, a strong inhibitor of NAD+-dependent enzymes that regulate the redox response. Research has been conducted not only on the combination of cytokines to increase NK cell expansion efficiency but also on genetic engineering to increase the therapeutic efficacy of the NK cells themselves (Rezvani and Rouce, 2015). However, unlike T cells, the transduction efficiency rate using common viral vectors is low, and thus transduction results in NK cells apoptosis. However, mRNA transduction by electroporation has recently been identified as an effective method to manipulate NK cells; what is more, this method has the advantage of enabling safer clinical protocols as no viral vector is used. Recently, many in vitro/in vivo studies of genetically modified NK cells by transducing CD19-CAR coding mRNA by electroporation in B-cell malignancies clearly showed an increase in cytotoxicity (Ghorashian et al., 2015).

Allogeneic Natural Killer Cell

Albeit the development of ex vivo expansion methods for autologous NK cell described above widened the potential therapeutic uses of NK cells in cancer immunotherapy, several limitations were revealed. The number, activity and characteristic expansion rates of NK cells greatly vary, depending on patient's health condition, thus limiting a consistent and effective use of these cells. As a result, to overcome the limitation of autologous approaches, attempts have been undertaken to enable instead allogeneic NK cell immunotherapy by expanding NK cells sourced from healthy donors.

This allogeneic approach may result from the discovery that the mechanism of recognizing and attacking nonself or cancer cells is different between T cells, B cells and NK cells, among immune cells. Over the past decade, the application of allogeneic NK cells by their ex vivo activation or expansion has emerged as a promising cancer immunotherapy treatment. Particularly, the administration of haploidentical NK cells considering KIR/HLA alloreactivity showed safety and impressive clinical activity in AML patients (Mehta and Rezvani, 2018). Moreover, anti–PD-1 and anti–PD-L1 monoclonal antibody (mAb)

and the immunomodulatory drug lenalidomide have recently been shown to increase the trafficking of NK cells to tumours, to increase sensitivity to antibody-dependent cell-mediated cytotoxicity (ADCC) of NK cells and secretion of cytokines and to suppress the function of Tregs (Giuliani et al., 2017). Moreover, anti–CTLA-4 mAb increases ADCC of NK cells and inactivates Tregs as well as increases TNF secretion in melanoma cell by binding of FcγRIII (CD16) to tumour (Romano et al., 2015). This result indicates that the combined administration of mAb can be effective in terms of increasing NK cell–mediated antitumour effect in cancer patients. Moreover, the adoptive cell transfer of allogeneic NK cells showed cytotoxicity on malignant cells, according to the KIR mismatch principle beneficial to the patient (Martín-Antonio et al., 2017).

For allogeneic applications, ex vivo expansion should be performed using various cytokines (IL-2, IL-15, IL-21, etc.) in lymphocytes of healthy donors, the resulting NK cells typically show activated alloreactivity (Granzin et al., 2017). Furthermore, NK cell function can be stimulated by blocking inhibitory KIRs using mAb in the pretreatment procedure (Dahlberg et al., 2015).

Natural Killer Cell Line

The induction of NK cell function results from a deficiency in KIR ligands; consequently, this phenomenon therefore suggests the possibility of using an NK cell line as a source for allogeneic approaches.

There are seven typical NK cell lines, including NK-92, YT, NKL, HANK-1, KHYG-1, NK-YS and NKG (Cheng et al., 2012). Among these, the antitumour activities of NK-92, KHYG-1, NKL and NKG have already been well documented (Zhang et al., 2017). Particularly, YT, NK-YS and HANK-1 are used in biological research of EBV-associated lymphoma and leukaemia (Klingemann et al., 2016).

These cell lines were recognised as ideal candidates for establishing an ex vivo expansion system in GMP condition, and related studies were initiated. Among those cell lines, only the NK-92 cell line to date was proven to be well tolerated and potentially effective in a clinical trial with progressive malignant melanoma and renal cell carcinoma (RCC) (Tonn et al., 2001, 2013). This cell line is currently developed by NantKwest (Torrey Pines, CA, USA) (previously ConKwest). Also, KHYG-1 cell line derived from the patient with abnormal p53 gene expression has been demonstrated to be applicable for NK-cell–based immunotherapy (Sun et al., 2015). Notably, KHYG-1 was observed to have higher cytotoxicity than NK-92 (Suck et al., 2006).

NKG cell line was first isolated and characterised in China: its potential for cancer immunotherapy was confirmed in a xenograft mouse model (Cheng et al., 2011). NKG cells exhibit natural killing ability, ADCC response and a proliferation tendency similar to that of $CD162^{+}$ $CD56^{dim}$ NK cells. Remarkably, this cell line could maintain the characteristics of original NK cells. One of the great advantages of using NK cell line as they do not undergo senescence and their in vitro expansion can be carried out under GMP condition (Table 5.2).

Table 5.2 Natural Killer Cells (Autologous, Allogenic, Genetic Modified) in Tumour Immunotherapy (Cheng et al., 2013).

	Administration	Stimulation	Effector	Clinical trail	Limitation	References
Autologus NK cells	Stimulation with cytokines in vivo; adoptive transfer after activation/ expansion ex vivo	Cytokine: IL-2, IL-12, IL-15, IL-18, IL-21, type IFN Antibody: KIR Ab	Upregulated adhesion molecules NKp44, perforin, granzyme, FasL and TRAIL; enhanced proliferation ability and cytokine production	Limited activity; metastatic RCC, malignant glioma and breast cancer	Toxicity of systemic cytokine administration; cytokine-activated NK cell apoptosis; suppressed by recognition of self-MHC molecules	[80–100]
Allogenic NK cells	Adoptive transfer after ex vivo activation/ expansion; infusion of unstimulated donor NK cells	IL-15/hydrocortisone, soluble factors, immobilised molecules, cellular activators	Greater tumour killing activity	Safe with minimal toxicity; successful for cancer immunotherapy, including metastatic melanoma, renal cell carcinoma, Hodgkin's disease and poor-prognosis AML; advanced nonsmall cell lung cancer	Rejection by a patient's immune system	[101–105]
NK cells via antibody-dependent cell-mediated cytotoxicity	Systemic administration	Tumour-specific monoclonal antibodies; altered antibody including class switching, humanization, point mutation; coadministering cytokines (IL-12, IL-2, and IL-21), TLR agonist (CpG) or agonist antibodies (anti-4 1BB); antibodies with linked cytokines (immunocytokines)	Higher cytotoxicity to Ab-coated target cells	CD20-specific mAb(rituximab) in non-Hodgkin's lymphoma patients; HER-2–specific mAb(Trastuzumab/ herceptin) in patients with metastatic breast and gastric carcinomas; humanised anti-GD2 mAb in melanoma, osteosarcoma and soft-tissue sarcoma patients		[106–124]

NK cell lines	Adoptive transfer after ex vivo expansion	Expanded in vitro as necessary	High cytotoxicity to tumour cells; cytokine production	Safe and successful antitumour effects NK92; advanced malignant melanoma and renal cell carcinoma	Rejection by a patient's immune system	[125–139]
Genetic modification of NK cells	Adoptive transfer after genetic modification	Cytokine transgene; overexpression of activating receptors by genetic modification; siliencing of inhibitory receptor expression by RNA interference; retargeting NK cells by using chimeric receptor	Increased tumour cell–killing efficiency; stronger intracellular signals for activating NK cell cytotoxicity	Successful antitumour effects; IL-2-NK-92; IL-15-NK-92; IL-15-NKL; SCF-NK-92; anti-HER-2/neu-CD3 δ, anti-CEA-CD3δ, anti-CD33-D3δ, anti-CD19-CD3δ, anti-CD20-CD3δ	Limited specificity of NK cells via cytokine transgene	[140–150]

AML, acute myeloid leukaemia; *FasL*, Fas ligand; *IFN*, interferon; *KIR*, killer cell immunoglobulin–like receptors; *MHC*, major histocompatibility complex; *NK*, natural killer; *RCC*, renal cell carcinoma; *SCF*, stem cell factor; *TLR*, toll-like receptor; *TRAIL*, tumour necrosis factor–related apoptosis-inducing ligand.

COMBINATION THERAPY WITH NATURAL KILLER CELL–BASED IMMUNE THERAPY

In cancer immunotherapy, combination treatments are increasingly being investigated. This trend also permeates in adoptive NK cell therapies with the search of synergistic combinations with conventional pharmaceutical agents to synergise the efficacy of tumour killing.

Cytokine Combination

The ability of NK cells to control tumours and virus infection is largely dependent on their responses to the local environment they encounter, their synthesis of cytokines including IFN-γ and TNF-α and the engagements of different receptors that induce the activation of the cytolytic pathway. As IL-2, IL-12, IL-15, IL-18, IL-21 and type I IFNs are recognised as strong activators of NK cell function, these cytokines regulate NK cell activity (Colucci et al., 2003; Becknell and Caligiuri, 2005; Skak et al., 2008). Among these cytokines, IL-21 has been described as an important regulation factor of NK cell function, which could increase ADCC, cytotoxic activity and proliferation (Roda-Navarro et al., 2006). Synergistic interactions between IL-21 and IL-15 are shown both in IFNγ production in vitro and in the cytotoxicity of the in vivo murine model (Strengell et al., 2003; Kishida et al., 2003; Nakano et al., 2006). Furthermore, the combination of IL-21 with IL-15 or IL-2 has a synergistic effect on NK cell proliferation, IFN-γ secretion, and cytotoxicity to K562 cell line (de Rham et al., 2007). Because of this characteristic, IL-21 was assumed to have an antitumour activity, and its potential effect as an antitumour agent was verified in an in vivo animal model (Søndergaard et al., 2007; Brady et al., 2004; Di Carlo et al., 2004; Moroz et al., 2004).

The IL-21 receptor is mainly expressed in T, B and NK cells; it binds to IL-21, which is produced by activated T cells (Ozaki et al., 2000). IL-21R$^{-/-}$ mice have normal number of NK cells. When IL-21 was used to challenge NK cells from this murine strain, their proliferation and survival was decreased in the presence of IL-2 or IL-15, but peripheral differentiation and increased NK cell cytotoxicity to tumour cell lines was observed in parallel (Brady et al., 2004). IL-21 is an attractive cytokine for antitumour immunotherapy, as it stimulates both NK cells and CD8+ T cells.

For ex vivo NK cell expansion, membrane-bound IL-21 expressed in K562 feeder cells could be effective (Denman et al., 2012).

Furthermore, in an immunocomplex study, rhIL-21 increased soluble CD25 in both CD25+ T cell and NK cells and also increased the expression of IFN-γ, perforin and granzyme B (Davis et al., 2009). According to in vitro studies, IL-21 does not only directly act on chronic lymphocytic leukaemia (CLL) cells but also increases ADCC of NK cells to CLL cells (Gowda et al., 2008). Therefore, rhIL-21 seems to have a modest clinical effect on solid tumour, favourable toxicity profile and evidence of patient's biological regulation in vivo (Todd et al., 2014).

IL-2 is one of the first cytokines that was clinically used in an attempt to induce antitumour immunity (Jiang et al., 2016). As a single agent, a high concentration of IL-2 induced

remission of the disease in a few patients with RCC and metastatic melanoma, despite the mechanism of action involved remains unclear (Davar et al., 2017). Nonetheless, it has been suggested that the ligation of IL-2/15$\gamma\beta_c$ in immune cells could contribute to clinical activity but significant toxicity is associated with rhIL-2 concentration (Gollob et al., 2003).

As a result, the potential therapeutic effect of lower doses of IL-2 was subsequently explored with the objective to maintain biological activity, while decreasing the toxicity of this molecule (Wang and Smith, 1987). At the time of these studies, most of the CD56+ CD3-NK cells were expanded from $CD56^{bright}$ $CD16^{+}$ NK cell subsets (Bernstein et al., 1995; Caligiuri et al., 1993). While these NK cells act against NK cell–sensitive targets (K562) and mediate ADCC, they clearly need additional stimulation of high concentration of IL-2 to mediate LAK activity against NK cell resistant tumours.

With the minimal impact on peripheral proliferation, low concentrations of IL-2 enable in vivo survival and differentiation from progenitors into $CD56^{bright}$ NK cells (Fehniger et al., 2000). As a result, low concentration IL-2 therapy expands and activates NK cell in vivo.

IL-15 is another cytokine, the biology of which is similar to that of IL-2, and its ability and function to stimulate NK cell development and homoeostasis are also worth noting (Marcais et al., 2013).

Based on the efficacies that were observed with T and NK cells, clinical research on rhIL-15 (in the absence of IL-15Rα) was thus conducted in solid tumours (melanoma, RCC: NCT01021059, NCT01369888; advanced cancers: NCT01572493, NCT01727076); and research on whether it supports NK cells after adoptive transfer in leukaemia patient (NCT01385423) was also conducted.

IL-15 has thus been gaining increasing attention as an NK cell modulator for immunotherapy even more so that it does not induce a Treg as compared with IL-2 that shows a clear toxicity. Furthermore, it is possible to increase the functions of both NK cells and T cells, which may result in increased cross talk between these cell types, hence ultimately increasing their antitumour immunity (Malhotra and Shanker, 2011).

After it was found that IL-15 requires IL-15Rα for effective ligation of IL-2/15$\beta\gamma_c$ in vivo, the coadministration of IL-15/IL-15Rα complexes in NK cells was evaluated in several studies (Rubinstein et al., 2006; Stoklasek et al., 2006), which demonstrated in vivo the immunotherapeutic potential of IL-15 therapy.

IL-18 is a member of the proinflammatory IL-1 family that is secreted by activated phagocytes along with IL-12 (Dinarello et al., 2013). Notably, cloned proinflammatory monokine IL-18 has been recently proved to be a strong stimulant for the production of IFN-γ in resting human NK cell (Okamura et al., 1995a,b). Moreover, IL-18 in NK cells has been described as being a costimulatory cytokine that has synergistic effect with IL-12 and IL-15, as IL-12 signalling upregulates IL-18R expression in T cell (Yoshimoto et al., 1998). In the presence of IL-12, IL-18 induces significant and dose-dependent increase in IFN-γ protein level measured in supernatant of CD56+ NK cell culture (Kannanet al., 2011). The combinations of IL-18 and IL-12 are found to induce a very distinct subset of NK cells, typically these cells $CD56^{bright}$, which produce a relatively large amount of IFN-γ

as compared to a larger number of $CD56^{dim}$ subsets (Poli et al., 2009). Individually, IL-18 and IL-12 fail to induce the synthesis of IFN-γ by human NK cells; a finding that is consistent with the previous findings that optimizing IFN-γ production by NK cells requires the costimulation of more than one monokine (Carson et al., 1994). While IL-18Rα is constitutively expressed in unstimulated NK cells and can only induce NK cell proliferation, the addition of IL-15 highly increases the proliferation of these cells (French et al., 2006).

IL-12 is a proinflammatory and immunomodulatory cytokine produced by macrophages that are activated by infection. IL-12 plays an important role in inducing not only NK cell cytotoxicity but also type 1 cytokine responses (Trinchieri et al., 1995; Trinchieri, 1997). As other monokines such as IL-15 and also TNF-α enhance the production of IFN-γ on combination with IL-12, these data provide additional evidence that IL-12–derived signal could be important in monokine stimulation to achieve sufficient levels of IFN-γ production (Carson et al., 1994, 1995).

Radiotherapy

Radiation therapy kills tumour cells by inducing necrosis and mitotic catastrophe mainly through apoptosis and often through DNA damage from the tumour microenvironment (Golden and Apetoh, 2015). In some cases, radiation induces autophagy and senescence in tumour cells. Cells of the immune system can divide rapidly and thus are damaged by radiation exposure. Radiation exposure induces apoptosis not only in T and B lymphocytes but also in mature NK cells and induces lethal damage to bone marrow stem cell precursors (Barao and Murphy, 2003). However, radiation treatment under certain conditions can also boost the immune response. Particularly, radiation exposure can provide appropriate antigens for cross-presentation by host antigen-presenting cells that can induce antigen-specific immune response (Park et al., 2014).

Although NK cells are more radioresistant than T and B lymphocytes in rats (Zarybnicka et al., 2013), they are still sensitive to a high dose of radiation, a phenomenon that clearly interferes with the cytotoxicity of NK cells against tumour cells.

Despite complete binding to tumour cells after irradiation, NK cells are unable to degranulate tumours as they cannot be activated after forming a conjugate. Interestingly, NK cells cultured with IL-2 do not seem to lose their antitumour cytotoxic function under irradiation (Zarcone et al., 1989).

On the contrary, low dose radiation alone was observed to increase the natural cytotoxicity of NK cells (Robert J. Canter et al., 2017). Similar to X-ray irradiation, NK cell–resistant T24 bladder transitional carcinoma cells show an increased sensitivity to killing by blood lymphocytes (Uchida et al., 1989). This increase in NK cytotoxicity can be maintained for long periods of time by immediate coculturing with IFN-α after irradiation (Uchida et al., 1989). Increased NK cell cytotoxicity is induced in NK cells without any phenotypic changes such as expression of NK1.1, NKG2D, CD69 and 2B4 or without any change in the rate of the early or late apoptosis (Shin et al., 2010; Sonn et al., 2012).

In vitro results similarly confirmed the results attained in in vivo mouse models where mice exposed to low dose radiation showed stimulation of innate immunity, while inhibiting proinflammatory response (Sonn et al., 2012). Moreover, in those experiments low-dose irradiation was observed to boost the cytotoxic effect of NK cells on tumour cells in vivo when NK cells were injected to mice with tumour cells as a mixture after irradiation.

Collectively, these results suggest that low-dose radiation could regulate NK cell sensitivity to tumour cells, and this leads to increased tumour killing.

Cancer Cell–Targeting Drugs

Many targets of current cancer therapies are expressed not only in cancer cells but also in immune cells. As a result, numerous cancer therapies not only influence cancer cell survival and proliferation but also affect the immune system. However, as many cancer-targeting drugs are generally tested for their safety and efficacy in xenograft model lacking a functional immune system, this particular phenomenon is often unclear (Richmond and Su, 2008).

Current studies reported that radiotherapies or chemotherapies such as Ara-C, cisplatin, and 5-Fu can induce an increase in the expression of NK-cell–activating ligands, thereby increasing NK cell recognition and killing (Bracci et al., 2014). More recently, several drugs with clear efficacy have been demonstrated to increase NK cell–mediated tumour killing (Romagne and Vivier, 2011; Childs and Carlsten, 2015). For example, the proteasome inhibitor, bortezomib, which was successfully used for multiple myeloma, can induce the expression of the ligands of NK cell–activating receptor (Niu et al., 2017). Another example is the immunomodulatory (IMid) drug, lenalidomide, which was approved for multiple myeloma and myelodysplastic syndrome. Lenalidomide not only impacts cancer cells and angiogenesis but also regulates immune responses by increasing the number of NK cells in the cancer periphery (Carotta, 2016). However, the precise role of lenalidomide in NK cells remains unclear. Lenalidomide may indirectly increase NK cell activation by upregulating ligands in tumour cells and by inducing expression of NK cell stimulatory cytokine such as T cell–derived IL-2 or directly increase NK cell activation by lowering the threshold for NK cell activation (Lagrue et al., 2015; Fionda et al., 2015). A better understanding of the mechanism of lenalidomide in NK cells would be crucial in designing combination therapy rationally.

However, it is reported that cancer-targeting drugs do not always increase the activity of immune cells, but in some cases, they could have deleterious effects on the immune system. For example, ibrutinib is a new irreversible inhibitor of Bruton's tyrosin kinase, which shows promising effect in the treatment of mantle cell lymphoma and CLL. The combination of chemotherapy and rituximab is currently the best treatment option in CD20+ B-cell malignancies, and therefore the potential of ibrutinib and rituximab combination represents an attractive therapeutic option. Recent studies reported that ibrutinib antagonises ADCC of rituximab by irreversible binding to IL-2 inducible tyrosine kinase, which is required for FcR-stimulated NK cell function in CD20+ B-cell lymphoma (Kohrt et al., 2014; Da Roit et al., 2015).

Another example is ruxolitinib, a small molecule inhibitor of JAK 1/2/3 signalling pathway. Ruxolitinib was recently approved for the treatment of myelofibrosis (MPN). As several cytokines regulate NK cell development and function through the JAK/STAT signalling pathway, the number of circulating NK cells in patients treated with ruxolitinib decreases dramatically. Furthermore, in vitro studies proved that ruxolitinib strongly inhibits the cytokine-induced cytolytic activity of NK cells (Schönberg et al., 2015a). Despite these observations, NK cell reduction by ruxolitinib treatment could be reversed, as NK cell level was recovered to normal in patients who withdrew ruxolitinib treatment (Schönberg et al., 2015b).

Thus, when combined with NK cell–based immunotherapies, appropriate scheduling of therapeutic drugs is crucial for a safe and efficacious treatment.

Antibody-Dependent Cellular Cytotoxicity

NK cells play an important role in cancer immunotherapy by a variety of mechanisms that include the targeting of tumour antigen by mAbs. While activating receptors recognise stress-induced ligands, inhibitory receptors prevent normal cells from attacking.

In the case of ligand binding followed by phosphorylation, these activating receptors can activate downstream kinases, leading to the induction of the degranulation of NK cells and cytokine secretion (Lanier, 2008). On the other hand, once the inhibitory receptors are phosphorylated, they recruit phosphatase and deactivate signalling kinase, which results in the inhibition of NK cells (Lanier, 2008). The activity of NK cells is regulated by the balance between these activating and inhibitory signals induced by these receptors.

NK cells can be 'specifically activated' by certain Fc receptor expressed on the cell surface. NK cells express FcγRIIIA/CD16a and/or FcγRIIC/CD32c, which can bind to the Fc portion of immunoglobulin, which transmit an activation signal into the NK cells (Morel, 1999). FcγRIIIA is often associated with FcεRI-γ chains or CD3-ζ chains within the cell membrane or with heterodimer of the two chains. Both FcεRI-γ and CD3-ζ chains have immune tyrosine–based activating motifs (ITAM) on their cytoplasmic tail.

Unlike most activating receptors in NK cells, FcγRIIC has ITAM on its cytoplasmic tail. In FcγR binding, these ITAMs are phosphorylated, resulted in degranulation, cytokine secretion and finally tumour cell lysis of the NK cells through the signal transduction mechanism (binding to tyrosine kinases ZAP-70 and Syk and activation of PI3K, NF-κb and ERK pathways) (Smyth et al., 2005).

Antibody-dependent NK-mediated tumour killing occurs through several pathways, which include (1) exocytosis of cytotoxic granules; (2) TNF family death receptors signalling and (3) proinflammatory cytokine release, such as IFNγ (Smyth et al., 2005). Uptake of perforin and granzyme by target cells and TNF family death receptor signalling induce target cell apoptosis (Smyth et al., 2005). In the meantime, IFNγ secreted by NK cells activate surrounding immune cells to promote antigen presentation and adaptive immune response (Srivastava et al., 2013).

IFNγ production and cytotoxicity were considered as two distinct features of other NK subsets. However, increasing evidence suggests that $CD56^{dim}CD16^{+}$ NK cells, which are a main cytotoxic NK subset, are responsible for mAb-mediated tumour killing and can also produce IFNγ after activation (Poli et al., 2009; Kinder et al., 2015; Juelke et al., 2010; De Maria et al., 2011).

In humans, there is polymorphism in both FcγRIIIA and FcγRIIC, which affects the FcR function. Thus, FcγRIIIA and FcγRIIC genotypes may influence the interaction between immunoglobulin and those receptors, which leads to the difference in efficacy of mAb therapy on individual genotypes; the impact of this genotypic variation alone would suggest the need to implement a parallel biomarker strategy. Some factors (including the FcγR genotype and the antibody Fc backbone of patients) create an opportunity to consider treatment options in a precision oncology approach in an effort to attain superior responses in patients when performing mAb therapy.

Here, mAbs work by ADCC with cells expressing FcRs. Tumour-specific mAbs that recognise tumour-selective antigen on the surface of tumours are used as cancer therapy. These therapeutic mAbs target and attack tumour cells. They include direct toxic molecules to target cells, the inhibition of proliferation of target cells, the blocking of inhibitory signals for immune cells and the direct target killing by immune cells through ADCC (Scott et al., 2012).

Trastuzumab is an anti-human epidermal growth factor receptor 2 (HER2) mAb used for the treatment of HER2-positive breast cancer, as well as many other cancer types that overexpress HER2. HER2 is a member of the human epidermal growth factor receptor (EGFR) family, which induces the activation of signalling pathways that promote cell proliferation and survival by dimerization with other EGFR family members. Although the expression of HER2 is limited in normal cells, it is overexpressed in many tumours, which makes HER2 an ideal target for the treatment of HER2-positive cancer. Trastuzumab was first approved by the FDA in 1998 for the treatment of HER2-positive metastatic breast cancer (Mayer, 2009).

Trastuzumab not only prevents the dimerization of HER2 but also mediates ADCC against HER2-positive tumour cell in vitro. In this regard, the main effector cells are NK cells expressing FcγRIIIA. Based on the observation, a mutant trastuzumab that lost the ability to bind to FcγR as well as antitumour activity in the Xenograft breast tumour model suggests that ADCC is crucial in the antitumour effect of anti-HER2 mAb therapy in vivo. When activating receptor FcγRIIIA is deficient in the same animal model, antitumour response to trastuzumab is notably reduced. However, when the inhibitory receptor FcγRIIB is lacking, there is an increase in the antitumour response in mice (Clynes et al., 2000).

Cetuximab is a mAb that was approved by the FDA for the treatment of EGFR-expressing metastatic colorectal cancer, metastatic nonsmall cell lung cancer and head and neck cancer (Messersmith and Ahnen, 2008). Cetuximab responds to human EGFR and can interrupt tumour growth by receptor blockade. In vitro studies suggest that part of the antitumour activity by cetuximab is mediated by ADCC, and cetuximab-mediated in vitro ADCC is associated with NK cell FcγR polymorphism of effector donors (Kurai et al., 2007; Monteverde et al., 2015).

Rituximab is a chimeric IgG1 mAb targeting CD20, which is a B-cell differentiation antigen. There are several mechanisms that could explain the antitumour effects of rituximab: complement-dependent cytotoxicity, direct target cell apoptosis, antibody-dependent phagocytosis and ADCC (Lievre et al., 2006). In a B-cell lymphoma xenograft mouse model, antitumour effect of rituximab was observed to be significantly reduced in FcγR$^{-/-}$ nude mice or in FcγR-blocked mice (Clynes et al., 2000; Moga et al., 2008).

As ADCC has been shown to be an important contributor to the antitumour activity of many mAb therapies, the enhanced immune activation of effector cells might be an ideal adjunct therapy to increase the ADCC activation of mAbs. In addition to the ADCC capability of NK cells, NK cells can stimulate the activity of other immune processes by the secretion of other cytokines such as IFNγ. Thus, it can provide a link that could initiate additional immune response that could attack target tumour.

A strategic approach to increase NK cell function and ADCC is to administer ex vivo the expanded and activated NK cells. During the ex vivo expansion of NK cells, these activated effectors become the basis for more effective tumour killing and could be administered into cancer patients.

There are several methods for expanding and activating NK cells ex vivo. In the presence of irradiated feeder cells such as EBV-LCL cells or genetically modified K562 cells expressing membrane-bound IL-15 and 4-1BBL, NK cells preferentially proliferate 200-to 400-fold within a 21-day culture (Berg et al., 2009; Fujisaki et al., 2009). NK cells expanded ex vivo using modified K562 cells show better antitumour activity in in vitro and in vivo mouse model (Fujisaki et al., 2009). Moreover, ex vivo–expanded NK cells could mediate ADCC by combination with tumour antigen–specific antibodies and exhibit much better antitumour efficacy.

FcγRs act as a receptor for the Fc portion of IgG immunoglobulins and a linker of the innate immune system in the humoural system. In humans, there are three classes of FcγR: FcγR Ⅰ, FcγR Ⅱ and FcγR Ⅲ. These receptor classes are expressed characteristically in various immune cells. NK cells express FcγR Ⅱ C and FcγR Ⅲ A with low or moderate affinity to IgG. All people with a normal immune system express FcγR Ⅲ A in most of their NK cells. In contrast, half of people express FcγR Ⅱ C in their NK cells. Genetic variation exists in both FcγR Ⅱ C and FcγR Ⅲ A, and this variability could change the avidity and expression of FcγR for IgG molecules.

Consequently, genotypic profiles of NK cells could prove to be a very valuable diagnostics tool to understand the scale of ADCC response of NK cells in each patient. KIRs and FcR genotypes are very helpful in predicting clinical results of ADCC, which include mAb therapy (Koehn et al., 2012).

Targeting Immune Suppressive Signalling

The secretion of TGF-β by tumour cells or the microenvironment has a vast impact on tumour progression and immune system. During cancer progression, TGF-β plays an important role in tumour immune escape.

TGF-β level is often elevated in the serum of cancer patients; this increased level is often associated with systemic inhibition of the immune system and poor prognosis (Dancea et al., 2009).

Like CD8+ T cells, NK cells in patients with increased TGF-β level show reduced cytotoxicity, whereas the expression of NKG2D and NKp46 is decreased, and the expression of NKG2A is increased (Archana Gopal Kulkarni et al., 2014). On the other hand, NK cells derived from patients treated with anti–TGF-β mAbs could restore activating receptor expression, proliferation and cytokine secretion when ex vivo treated (Dahlberg et al., 2015).

To decipher the direct effect of TGF-β with NK cell receptor expression and NK cell function, human NK cells was incubated in the presence of TGF-β. It was observed that in these cells, NKp30 and NKG2D are downregulated, whereas IL-15, which induces NK cell proliferation and IFN-γ secretion, was inhibited (Chester et al., 2015). As a result, targeting TGF-β signalling in NK cells is an attractive immunotherapy in cancer patient with high TGF-β level. However, there is a need for the development of moderate but potent inhibitors with low toxicity because of the many functions of TGF-β in normal tissues, cancer cells, tumour environment and immune cells (Herbertz et al., 2015).

Recent approaches to inhibit TGF-β signalling including ligand traps, antisense oligonucleotides, receptor kinase inhibitors and peptide aptamers (Carotta et al., 2016) have shown promising results with signals of efficacy in preclinical and clinical studies and limited toxicity (Connolly et al., 2012).

Checkpoint Blockers

The most promising approach to activate therapeutic antitumour immunity is the blockade of immune checkpoints (Pardoll, 2012). Under normal physiological conditions, immune checkpoints play an important role in maintaining self-tolerance (prevention of autoimmunity) and in protecting tissues from damage when the immune system responds to pathogenic infection.

To maintain self-tolerance and modulate the inflammation resulting from tissue damage, immune checkpoints regulated the inhibitory pathway embedded in the hardware within the immune system, which is critical for regulating the amplitude of the physiological immune response in the peripheral cellular tissues. Tumours modify the normal immune checkpoint pathway as a main mechanism of immune tolerance, especially to antigen specific T cells. As many immune checkpoints are initiated from ligand–receptor interactions, they can be easily blocked by antibodies or be regulated by a recombinant form of ligands or receptors (Pardoll, 2012).

In the case of T cells, fundamental amplification and quality of the response is initiated from the antigen recognition by TCR and is regulated by the balance between the costimulatory and inhibitory signals. Both agonists of costimulatory receptors and antagonists of inhibitory signals, which result in the amplification of antigen-specific T cell responses in T cells, are used in recent clinical trials (Peggs et al., 2009).

The antibody of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) is the first immune checkpoint inhibitor approved by the FDA. Ipilimumab (anti–CTLA-4 mAbs) blocking CTLA-4–mediated signalling in T cells was approved in 2011 for the treatment of inoperable metastatic melanoma. When ligated, CTLA-4 is an inhibitory receptor that limits T cell activation by sending negative regulatory signal to the TCR (Chester et al., 2015). CTLA-4 controls T cell activity at low level. Antibody blockade of CTLA-4 in mouse model induces antitumour immunity. Clinical studies using antagonistic CTLA-4 antibodies proved their efficacy in melanoma. Despite the high frequency of immune-related toxicity, CTLA-4 inhibitors increased survival in two-randomised phase III trials (Pardoll, 2012).

The discovery that PD-1 limits T cell effector functions in tissues opened the possibility of achieving sustained clinical responses by strengthening antitumour immunity. When PD-L1, the ligand for PD-1 is upregulated, tumour cells block the antitumour immune response in the tumour microenvironment. At the early stage of cancers, PD-1 pathway blockade induces sustained tumour inhibition in various tumour types. The FDA approved pembrolizumab, nivolumab and other immune checkpoint inhibitors targeting PD-1 or its ligand PD-L1. This represents a very important milestone in the rapidly expanding field of cancer immunotherapy (Chester et al., 2015).

What is more, NK cells in patients with multiple myeloma and renal carcinoma that express PD-1 on their surfaces and engage with PD-1 signalling exhibit a reduced cytolytic potential. However, it was observed that a combination treatment consisting of the anti–PD-1 antibody pidilizumab (CT-011) with patient-derived PD-1+ NK cells increases NK cell–mediated killing of autologous cancer cells in vitro (Benson et al., 2010). Despite these advances, the therapeutic benefit of PD-1+ NK cells in cancer patients is not yet fully understood, despite the main therapeutic effect clearly resulting from the reactivation of exhausted T cells. However, the contribution of NK cells in the observed therapeutic benefit cannot be excluded, especially in haematological malignancies (Brad Jones et al., 2008). Another category of immune inhibitory molecules includes IDO expressed in both tumour cell and invasive myeloid cells and some metabolic enzymes such as arginase that are produced by MDSCs. These metabolic enzymes suppress the immune system by locally reducing essential amino acids necessary for synthesis and metabolism in lymphocytes or synthesizing specific natural ligand for cytosolic receptor, which could modify the function of lymphocyte (Pardoll, 2012).

TIM-3, also known as HAVCR2, is another immune checkpoint that is being tested in preclinical models to check the potential of therapeutic intervention to restore the depleted T cells in cancer patients. Resting T cells express low levels of TIM-3, the expression of which is strongly upregulated in activated and depleted T cells (Brad Jones et al., 2008). Antibody-mediated blockade of TIM-3 signalling can recover depleted phenotypes of CD4+ and CD8+ T cells in melanoma patients who exhibit inhibitory functions of TIM-3 in T cells. Similar to PD-1, the expression of TIM-3 is not limited to T cells, and it can also be detected in murine and human NK cells (Ndhlovu et al., 2012).

TIM-3 is in fact expressed by all human NK cells and upregulated in cytokine-activated NK cells. Currently, the functional role of TIM-3 in NK cells is highly controversial. Ndhlovu et al. (2012) recently reported that cross-linking by anti–TIM-3 antibodies in human NK cell line NKL or human PBMC–derived NK cells significantly reduces their cytolytic activity. In contrast of these findings, Gleason et al. (2012) reported that the activation of TIM-3 by ligand cal-9 actually increases IFN-γ production in NK cells.

A more recent report tested the effect of TIM-3 blockade in NK cells derived from advanced melanoma patients. Notably, da Silva et al. identified that TIM-3 surface expression increases with cancer progression, and a phenotype of depleted TIM-3+NK cells usually results in a poor prognosis (Liu et al., 2017). More importantly, while TIM-3 activation is normal when TIM-3+NK cells derived from melanoma patient are incubated with anti-TIM-f3–coated beads, the secretion and degranulation of IFN-γ are significantly reduced (Sun et al., 2016). The function of TIM-3 in NK cells is thus still controversial and further studies on the role of TIM-3 in NK cells derived from cancer patient are required.

The heterodimer CD94/NKG2A is another checkpoint inhibitor complex that is expressed in both T and NK cells. The NKG2A chain of CD94/NKG2A receptor contains two immunoreceptor Tyr-based inhibitory motif (ITIMs) in the cytosolic tail, and the HLA-E/NKG2A interaction results in dominant inhibitory signalling, which induces a strong reduction in NK cell effector function. Some solid cancers and haematological malignancies upregulate HLA-E expression as an immune escape mechanism to avoid killing by NK and T cells. Monalizumab (previously IPH2201) is an anti-NKG2A checkpoint inhibitor that is currently under clinical trial, where its efficacy in head and neck cancer and ovarian cancer is evaluated (Carotta, 2016).

ONGOING CLINICAL TRIALS

While allogeneic T cell treatments, such as donated lymphocyte infusion, are associated with a significant risk of GvHD, NK cells could mediate an antitumour effect without inducing GvHD. As a result, immunocellular therapy using high-purity NK cells constitutes a very attractive alternative adoptive cell therapy that is already being investigated in various clinical trials around the world.

Natural Killer Cell Adoptive Transfer

As highlighted earlier, there are two main approaches to NK cell transfer: autologous or allogeneic, that is, respectively, NK cells can be obtained from the patient or from healthy donors.

Autologous NK cell transplantation

Autologous NK cells are collected from the patient and expanded and activated in vitro in the presence of a cytokine or a cytokine cocktail (Pittari et al., 2015). Although IL-2

was mainly used for this purpose, more functional NK cells could be produced with a combination of IL-12, IL-15 and IL-18. Alternatively, irradiated genetically engineered K562 cells express cytokines such as IL-15 and IL-21 as well as costimulatory molecules and as such could be used as feeder cells (Rushworth et al., 2014). The expanded and activated NK cells are administered back to the patient, sometime with cytokine (IL-2 in most cases) to maintain the function and expansion of the infused NK cells. Although autologous NK cells can recognise active signals, such as stress molecules against cancer cells, their anticancer effect can be limited by the inhibitory signals from self-HLA molecules.

Allogeneic NK-cell transplantation

NK cells can be collected from HLA-matched or haploidentical (partially matched) donors. Although NK cells are expanded in a similar process as that used to expand cells for autologous transplantation, ideally T cells should be removed to avoid GVHD. In this setting, if the haploidentical donor does not express KIRs that recognise the HLA molecules of the patient, donor NK cells can perform a most ideal response as the cells would not receive any inhibitory signal from the patient's cancer cells (Geller and Miller, 2011).

CARs can be engineered and expressed by autologous NK cells, allogeneic NK cells, or an NK cell line such as NK-92. The canonical CAR architecture comprises a fusion of the hinge region, a transmembrane domain, one or more stimulatory molecule and an antigen-binding domain (specific scFv of mAb) (Zhang et al., 2017). CARs may include a CD3ζ chain as a main signal transfer region. In addition, one or two costimulatory domains from CD28 or CD137 can be added. Such constructs lead to sustainability and improvement in function (Quintarelli et al., 2018). While the first generation CAR does not harbor a stimulatory domain, second and third generation CARs have one costimulatory and two costimulatory domains, respectively. CAR engineering conveys antigen specificity in NK cells. The binding of the CAR domain on the tumour antigens eliminates cancer by delivering a strong active signal that induces cytotoxicity of the NK cells (Mehta and Rezvani, 2018).

The ideal protocol for manufacturing NK cells for clinical trials should reproducibly produce a sufficient number of NK cells with high purity and efficacy to produce multiple doses. The infused NK cells should be expandable in the body and should confer antitumour activity with the potential for tumour clearance.

Doses containing a large number of NK cells ranging from 5×10^6 to 5×10^7 CD3-CD56+ cells/kg are typically required in clinical trials. Recently, doses as high as 1×10^8 CD3-CD56+ NK cells/kg were tested (Guillerey et al., 2016) (Tables 5.3 and 5.4).

Development Status of Immune Cell Therapy in Asia

Regulatory changes implemented in various Asian nations, perhaps best exemplified by the new regulatory pathway enacted by the Japanese PMDA, have promoted the translation of adoptive cell-based therapies in the region.

Table 5.3 Clinical Development Status of Natural Killer (NK) Cell Therapies as a Cancer Immunotherapy (Koehl et al., 2016).

NK cell product	NK Source of Cells	Culture Characteristics	Patient Characteristics (Disease)	Result	Country	Phase	References
NK cell line, NK92	Allogeneic	–	Progressive renal cancer, malignant melanoma	Inoculation toxicity of NK-92 is generally modest. One patient with grade 3 fever and grade 1 fever. Grade 4 hypoglycemia. All toxicities were temporary and resolved. No need to withdraw.	United States	I	Arai et al., 2008
NK cell line NK92	Allogeneic	–	Solid cancer	Inoculation-related or long-term toxicities were not observed. Three-quarters of lung cancer patients had antitumour response.	Germany	I	Tonn et al., 2013
PBMNC (peripheral blood mononuclear cells)	Autologous	Stimulated by IL-2 and galactosylceramide	Progressive and recurrent NLCLC	Fever, temporary arrhythmia and headache occurred in inoculation, but main toxicity (more than grade 2) or severe side effects were not observed. There was no antitumour response.	Japan	I	Motohashi et al., 2006
PBMNC	Autologous	Stimulated by IL-2 and Hsp70 protein	Colon or lung cancer	Excluding insomnia in one patient and pruritus in two patients; negative side effects were not observed. There was no antitumour response.	Germany	I	Krause et al., 2004
PBMNC	Autologous	Used 10^8 radiated (3000 rad) autologous PBMC as feeder cell in 10^7 CD3-depleted cell Stimulated by IL-2 and OKT3	Stage IV malignant melanoma or renal cancer	One patient had temporary dyspnea, which needed supplemental oxygen after cell infusion. There were no cell infusion-related toxicities. There was no antitumour response.	United States	–	Parkhurst et al., 2011a,b

Continued

Table 5.3 Clinical Development Status of Natural Killer (NK) Cell Therapies as a Cancer Immunotherapy (Koehl et al., 2016).—cont'd

NK cell product	NK Source of Cells	Culture Characteristics	Patient Characteristics (Disease)	Result	Country	Phase	References
PBMNC	Autologous	Cultured in CD3+ depleted, CD56+ sufficient and radiated EBV-TM-LCL feeder cell Stimulated by IL-2	CLL and solid cancer	Most common treatment related side effects included grade 1–2 fever, renal failure, oedema and hypotension. Two patients had acute thyroiditis, which could lead to hypothyroidism, which might require thyroid alternative therapy.	Sweden	I	Lundqvist and Childs, 2011
CD34+ haematopoietic progenitor cells	Allogeneic	CD34+ progenitor cell was differentiated to NK cell by using IL-15, IL-21 and hydrocortisone	High-level MDS (MyeloDysplatic syndrome), ALL, AML	There was no complexity related to inoculation. There was no efficacy in two active leukaemia patients.	Korea	Pilot study	Yoon et al., 2010
Donor-derived PBMNC	Allogeneic	NK/NK-like T cell (CD56+) was cultured using IL-2 and anti-CD3 antibody(OKT3)	Colon cancer, liver cancer, renal cell carcinoma, CLL	Low-grade fever due to inoculation up to 1 week. Complex symptoms of cough, haemoptysis and bloody stool. Liver cancer patient had stable disease, and there was no antitumour response.	Sweden	I	Barkholt et al., 2009
Haploidentical allogeneic NK cells	Allogeneic	PBMNC derived, CD3+ depleted, CD56+ sufficient, IL-2 stimulation	Two paediatric patients with high-level ALL and one patient with ALL	All three patients had seen efficacy after several weeks/ months. Two patients died in next relapse because of infection.	Germany	I	Koehl et al., 2005
Haploidentical allogeneic NK cells	Allogeneic	PBMNC derived, CD3+ depleted, CD56+ sufficient, IL-2 stimulation	Neuroblastoma, AML, ALL	There was inoculation-related fever. Vomiting and change in blood pressure were seen in two patients. 44% of high-risk patients are alive.	Germany	I/II	Brehm et al., 2011

Haploidentical allogeneic NK cells	Allogeneic	PBMNC derived, CD3+ depleted, IL-2 stimulation	Relapsed ovarian cancer or breast cancer	In inoculation, difficulty in breathing, hypoxia, fever, hypertension, hypotension, fatigue, oedema, pneumonia, rash, nausea and muscle pain were observed. Expected low grade 1, 2 toxic reactions occurred in most patients, with 11 patients progressed to grade 3. But did not show grade 4 toxicity. Two patients progressed to passenger lymphocyte syndrome.	United States	II	Geller et al., 2011
Haploidentical and autologous NK cells	Allogeneic and autologous	PBMNC derived, expanded, or activated by K562 genetically modified in way that 41BB-ligand and IL-15 binding surface are expressed	High-risk myeloma	There was no side effect in seven patients. In one patient, 7 days after cell inoculation, 90% of circulating WBC were derived from donor NK cell.	Unites States	–	Szmania et al., 2015
Donor CD56+ NK cells	Allogeneic	Cultured with IL-15 and hydrocortisone for 20–23 days	Adenocarcinoma or squamous cell carcinoma	No severe side effects. Two patients with partial response and six patients with stable disease were recorded.	Greece	I	Iliopoulos et al., 2010
Donor-derived IL-15/4–1BBL-activated NK	Allogeneic	Donor-derived IL-15/4–1BBL–activated NK using HLA match, T cell–depleted autologous haematopoietic stem cell transplant	Nine paediatric patients and high risk solid cancer adolescents	Five out of nine patients experienced acute GVHD grade III/IV.	United States	I	Shah et al., 2015

Table 5.4 Clinical Development Status of Natural Killer Cell Therapies as a Cancer Immunotherapy (Yang et al., 2015).

NCT Number	Age Group	Patient	Phase	Title	Condition	Status	Country
NCT01944982	Child/ adult	10	I/II	Salvage Therapy With Chemotherapy and Natural Killer Cells in Relapsed/ Refractory Paediatric T Cell Lymphoblastic Leukaemia and Lymphoma	Relapsed/ refractory paediatric T cell lymphoblastic leukaemia and lymphoma	Terminated	Spain
NCT01795378	Child/ adult/ senior	85	I/II	Safety and Efficacy Study of Donor Natural Killer Cells Given After Haploidentical Haematopoietic Cell Transplantation (DNKI-II)	Acute myelogenous leukaemia, acute lymphoblastic leukaemia	Completed	Korea
NCT02130869	Child/ adult	35	I	A Pilot Study of Immunotherapy Including Haploidentical NK Cell Infusion Following CD133+ Positively-Selected Autologous Haematopoietic Stem Cells in Children With High Risk Solid Tumours or Lymphomas	Neuroblastoma, lymphoma, high-risk tumour	Recruiting	US
NCT02280525	Adult/ senior	44	I	Cord Blood Natural Killer (NK) Cells in Leukemia/Lymphoma	Leukaemia	Recruiting	United States
NCT01787474	Adult	30	I/II	IL-21-Expanded NK Cells for Induction of Acute Myeloid Leukemia (AML)	Leukaemia	Recruiting	United States
NCT01823198	Child/ adult	72	I/II	Natural Killer (NK) Cells with HLA Compatible Hematopoietic Transplantation for High Risk Myeloid Malignancies	Leukaemia	Recruiting	United States
NCT02271711	Child/ adult	24	I	Fourth Ventricle Infusions of Autologous Ex Vivo Expanded NK Cells in Children with Recurrent Posterior Fossa Tumors	Brain cancer	Recruiting	United States
NCT02074657	Child/ adult	10	II	Injection of NK cell after activated and expanded after chemotherapy	Recurrent/ malignant paediatric acute leukaemia	Completed	Spain

NCT01884688	Adult/ senior	20	II	A Study of Autologous Expanded Natural Killer Cell Therapy for Asymptomatic Multiple Myeloma	Asymptomatic multiple myeloma	Completed	United States
NCT02316964	Adult/ senior	10		Decitabine, Donor Natural Killer Cells, and Aldesleukin in Treating Patients with Relapsed or Refractory Acute Myeloid Leukemia	Adult acute myeloid leukaemia, recurrent adult acute myeloid leukaemia, secondary acute myeloid leukaemia	Recruiting	United States
NCT02100891	Child/ adult/ senior	20	II	Phase 2 STIR Trial: Haploidentical Transplant and Donor Natural Killer Cells for Solid Tumors	Ewing sarcoma, neuroblastoma, rhabdomyosarcoma, osteosarcoma, CNS tumours	Recruiting	United States
NCT01904136	Child/ adult	45	I/II	NK Cells to Prevent Disease Relapse for Patients High Risk Myeloid Malignancies	Leukaemia, myeloproliferative diseases	Recruiting	United States
NCT01974479	Child/ adult/ senior	20	II	Pilot Study of Redirected Haploidentical Natural Killer Cell Infusions for B-Lineage Acute Lymphoblastic Leukemia	B-cell acute lymphoblastic leukaemia	Recruiting	Singapore
NCT02118285	Adult/ senior	20	I	Intraperitoneal Natural Killer Cells and INCB024360 for Recurrent Ovarian, Fallopian Tube, and Primary Peritoneal Cancer	Ovarian cancer, fallopian tube carcinoma, primary peritoneal carcinoma	Suspended	United States
NCT02259348	Child/ adult	18	II	Repeat Transplantation for Relapsed or Refractory Hematologic Malignancies Following Prior Transplantation	ALL, AML, CML, JMML, MDS, NHL,	Terminated	United States
NCT02118415	Adult/ senior	90	II	Targeted Natural Killer (NK) Cell Based Adoptive Immunotherapy for the Treatment of Patients With Non-Small Cell Lung Cancer (NSCLC) After Radiochemotherapy (RCT)	NSCLC stage IIIA/B	Recruiting	Germany

Continued

Table 5.4 Clinical Development Status of Natural Killer Cell Therapies as a Cancer Immunotherapy (Yang et al., 2015).—cont'd

NCT Number	Age Group	Patient	Phase	Title	Condition	Status	Country
NCT02185781	Adult/ senior	6	I	Phase I Study of Adoptive Immunotherapy With Enriched and Expanded Autologous Natural Killer (NK) Cells for Patients With Ph+ Acute Lymphoblastic Leukemia (ALL)	ALL, complete haematologic remission (CHR), persistent/recurrent minimal residual disease (MRD)	Recruiting	Italy
NCT02409576	Child/ adult/ senior	20	I/II	Pilot Study of expanded, Activated Haploidentical Natural Killer Cell Infusions for Sarcomas	Ewing Sarcoma, osteosarcoma, rhabdomyosarcoma	Recruiting	Singapore
NCT01857934	Child/ adult	42	II	Therapy for Children with Advanced Stage Neuroblastoma	Neuroblastoma	Recruiting	United States
NCT02229266	Adult/ senior	56	II	Randomised Controlled Phase-2 Trial to Determine the Efficacy of Adoptive Immunotherapy With NK Cells in High-risk AML	Acute myeloid leukaemia	Recruiting	Germany
NCT02477787	Adult/ senior	96	II	Randomised Study of Haploidentical Hct and Subsequent Donor nk Cell Infusion in High-risk AML and MDS	Acute myelogenous leukaemia	Recruiting	Korea
NCT02123836	Child/ adult/ senior	20	I	Natural Killer Cells in Acute Leukaemia and Myelodysplastic Syndrome	MDS, acute leukaemia	Recruiting	Singapore
NCT01807611	Child/ adult	110	II	Haploidentical Donor Hematopoietic Progenitor Cell and NK Cell Transplantation for Hematologic Malignancy	Leukaemia, lymphoma	Recruiting	United States
NCT02395822	Adult/ senior	24	II	MT2014-25: Haplo NK With SQ IL-15 in Adult Relapsed or Refractory AML Patients	Acute myelogenous leukaemia	Terminated	United States
NCT02399735	Adult	18	I	Safety Study of NK Cells From Sibship to Treat the Recurrence of HCC After Liver Transplantation	HCC	Recruiting	China
NCT01947322	Adult	11	I/II	Haploidentical NK-cell Infusion in Acute Myeloid Leukemia	Acute myeloid leukaemia	Active, not recruiting	France

NCT02465957	Child/ senior	24	II	QUILT-3.009: Study of aNK Infusions in Combination With ALT-803 in Patients With Stage III (IIIB) or Stage (IV) Merkel Cell Carcinoma (MCC)	Stage IIIB merkel cell carcinoma, stage IV merkel cell carcinoma	Recruiting	United States
NCT02370017	Adult/ senior	68	II	Combined Effect of Natural Killer Cell and Doublet Chemotherapy in Advanced NSCLC as the 1st Line Treatment	Non-small cell lung cancer	Recruiting	Korea
NCT01875601	Child/ adult	51	I	NK White Blood Cells and Interleukin in Children and Young Adults with Advanced Solid Tumors	Solid tumours, brain tumours, sarcoma, paediatric cancers, neuroblastoma	Completed	
NCT02030561	Adult/ senior	29	I/II	NK Cell Infusions With Trastuzumab for Patients With HER2+ Breast and Gastric Cancer	Breast cancer, gastric cancer	Recruiting	Singapore
NCT01898793	Child/ senior	24	I	Cytokine-induced Memory-like NK Cells in Patients With Acute Myeloid Leukemia (AML) or Myelodysplastic Syndrome (MDS)	Leukaemia, myeloid, acute	Recruiting	United States
NCT01807468	Child/ adult	12	II	Haploidentical Stem Cell Transplantation and NK Cell Therapy in Patients with High-risk Solid Tumours	Neuroblastoma	Active, not recruiting	Korea
NCT02481934	Adult/ senior	5	I	Clinical Trial of Expanded and Activated Autologous NK Cells to Treat Multiple Myeloma	Multiple myeloma	Completed	Spain
NCT01853358	Adult/ senior	22	I	Phase I of Infusion of Selected Donor NK Cells After Allogeneic Stem Cell Transplantation	Blood cancer	Active, but not recruiting	France

Japan

Among Asian countries, Japan has a long history of R&D in immune cell therapy. In Japan, immune cell therapy was considered as medical practice before 2014 when the new act on regenerative medicine was proposed. Therefore, hospitals that had the appropriate equipment and facility, could collect a patient's blood under patient's consent and medical doctor's order, culture mixed immune cells including cytotoxic T cells, γδ T cells and NK cells and infuse them back to the patient as medical treatment. However, the specific types type of immune cells were not specified, and the treatments were not controlled either. Although robust clinical experience and know-how regarding culture protocols in immunocellular therapy were thus generated, the resulting products were rarely developed.

So as to facilitate the development of cell therapy products, Japan enacted a new law, the Act on the Safety of Regenerative Medicine (RM Act) on 25 September, 2014, which led to a significant revision of the regulatory process of regenerative medicine products by the Japanese Pharmaceuticals and Medical Devices Agency (PMDA).

Drugs under the RM Act are categorised and separately managed by developmental difficulty and the risk of cell source (Trounson and McDonald, 2015): The higher class is class 1. It includes cell therapy derived from iPSCs and ES cells. Class 2 includes cell therapy derived from allogeneic sources or adult stem cells such adipocyte, bone marrow–derived MSCs, etc. Class 3 corresponds to autologous cell therapies, such as immunotherapy using a patient's own T cells and NK cells. Each class has a specific procedure to verify the safety and efficacy of the new cell therapy under study. The higher the class, the greater the number of approval steps. This RM Act aims at guiding development to ensure safety and promote stem cell research and trials. However, this kind of treatment is not covered by health insurances, thus patients must pay their own treatments by themselves. According to the RM Act in Japan, any manufacturing facility operated by commercial parties should obtain a 'manufacturing license' by PMDA, whereas medical institutions need to obtain a 'practice qualification' and submit an annual report to the health authority. Before this RM Act, Japanese doctors were allowed to perform such treatments if they estimated that these treatments were necessary, hence no plan needed to be submitted and no treatment report needed to be submitted to the authority. All this has changed with the RM Act, thus increasing the credibility of the procedure (Table 5.5).

With the revision of the PMD Act, the pharmaceutical pathway for cell therapy products in Japan became more adaptive and flexible than those of other countries. In the new scheme, if a therapy is proven in the clinic to be well tolerated by patients, the new product can receive a conditional approval and resubmitted for final approval within 7 years (Konomi et al., 2015) (Table 5.6).

China

China is very active in the field of CAR-T cell therapy. Until 2010, most clinical trials for CAR-T cell therapy were conducted in the United States. The first CAR-T cell

Table 5.5 Classifications of Regenerative Medicine (RM) According to Their Risk to Humans. Regenerative Medicines are Divided into the Following Three Classifications According to Their Risk to Humans.

Classification	Example of Cells Used for RM	Example of Procedure
Class 1 (high risk)	iPSCs, ESCs, gene-transduced cell, xenogeneic cells, allogeneic cells	Transplant of autologous iPSCs-derived retinal pigment epithelium cell, ex vivo gene therapy
Class 2 (intermediate risk)	Autologous adult stem cell	Autologous mesenchymal stem cell therapy for cirrhosis
Class 3 (low risk)	Autologous adult cell	Cancer immunotherapy

Table 5.6 Clinical Trial Status for Natural Killer (NK) cell Therapies in Japan.

Number	Phase	Information	Condition	Status	Sponsor
UMIN000013378	I	Study on combination therapy of IgG1 antibody and NK cell	Progressive and relapsed stomach cancer and colon cancer	Open public recruiting	Kyoto Prefectural University of Medicine

therapy in China was listed in the clinical database in 2012. Thereafter, China has made a huge leap in total clinical numbers. Among all the countries, the number of clinical trials for CAR-T cell therapy conducted in China accounted for 9% in 2012, 41% in 2015, and 72% in 2016, showing a dramatic increase (Liu et al., 2017).

Thirty-eight institutions and hospitals in China were identified as sponsors of CAR-T cell therapy, and half of the Chinese clinical trials registered in 2016 were for commercial purposes (http://celltrials.info/2017/02/13/car-cell-china/).

PersonGen Biomedicine Co., Ltd. (Suzhou, China) received approval for conducting clinical trials for repeated dosing (on day 0, 3 and 5) of third generation (scFv bound to signal domains of TCRζ, CD28 and 4-1BB) CAR-transduced NK-92 cells. Children and adults who plan to receive a haematopoietic stem cell transplant may be enrolled for the study, which is targeting intractable CD19+ malignant tumour (NCT02892695), CD33+ bone marrow malignant tumour (NCT02944162) and intractable CD7+ leukaemia/lymphoma (NCT02742727). In addition, a clinical trial for relapsed, and intractable solid cancer targeting MUC1+ is also in progress (NCT02839954) (Rezvani et al., 2017) (Table 5.7).

Korea

Cell therapy products in Korea are mainly involved in the development of cell storage, cell culture and manipulation technology with small- and mid-sised venture companies. Since 2002, more than 15 cell therapy products received a marketing approval from MFDA, the Korean regulatory agency. Most licenced cell therapy products are stem cell therapy products and very few anticancer cell therapy products.

Cell Therapy Products Approved in Korea.

	Company	Product	Classification	Indication	Permission Date
1	SEWON CELLONTECH	Chondron	Autologus bone marrow cell	Knee cartilage defect	2001.01.30
2	TEGO SCIENCE	Holoderm	Autologus Keratinocytes	Burns	2002.12.10
3	TEGO SCIENCE	Kaloderm	Allogenic keratinocytes	Burns Diabetic foot ulcer	2005.03.21 2010.06.24
4	Biosolution	KeraHeal	Autologus Keratinocytes	Burns	2006.05.03
5	Jw CreaGene	CreaVax-RCC inj.	Dendritic cells	Metastatic renal cell carcinoma	2007.05.15
6	GC Cel	Immunecell-LC	Activated T lymphocytes	Hepatocellular Carcinoma	2007.08.06
7	SEWON CELLONTECH	RMS Ossron	Autologus bone marrow stem cells	Ossification	2009.08.26
8	ANTEROGEN	Queencell	Autologus Mesenchymal Stem cells	Regeneration of subcutaneous adipose tissue	2010.03.26
9	S.BIOMEDICS	Cure Skin	Fibroblast	Acne scar	2010.05.11
10	PHARMICELL	Hearticellgram_AMI	Autologus bone marrow stem cells	acute myocardial infarction	2011.07.1
11	MEDIPOST	CARTISTEM	Allogenic Umbilical Cord Blood-derived Mesenchymal Stem Cell	Osteoarthritis	2012.01.18
12	ANTEROGEN	Cupistem	Autologus Adiposse-derived Mesenchymal Stem cells	Crohn's fistula	2012.01.08
13	CORESTEM	NEURONATA-R	Autologus bone marrow stem cells	Amyotrophic lateral sclerosis	2014.07.30
14	Biosolution	KeraHeal-Allo	Allogenic keratinocyte	Re-epithelialization of deep 2nd degree burns	2015.10.16
15	TEGO SCIENCE	Rosmir	Cultured Autologus Fibroblasts	Nasojugal groove	2017.12.27

Table 5.7 Clinical Trial Status for Natural Killer (NK) Cell Therapies in China.

Number	Phase	Condition	Sponsor	Target
NK Study				
NCT02399735	I	HepatoCellular carcinoma	Choogsan University Tertiary Hospital	
NCT02734524	II	NSCLC	Southwest Hospital, China	
CAR-NK Study				
NCT02944162	I I	AML ANLL	PersonGen BioTherapeutics	Anti-CD33 CAR-NK cells
NCT02742727	I II	AML Precursor T cell lymphocytic leukaemia – lymphoma T cell prostate cell leukaemia T cell large granular lymphocytic leukaemia Peripheral T cell lymphoma, NOS Angiogenesis T cell lymphoma External incision NK/T cell lymphoma, nasal cavity Enteropathy type intestine T cell lymphoma Hepatocyte forming T cell lymphoma	PersonGen BioTherapeutics	Anti-CD7 CAR-pNK cells
NCT02839954	II	HCC NSCLC Pancreatic cancer triple negative invasive breast cancer Malignant neuroblastoma Colon cancer Stomach cancer	PersonGen BioTherapeutics	Anti-MUC1 CAR-pNK cells
NCT02892695	I II	ALL CLL	PersonGen BioTherapeutics	Anti-CD19 CAR-pNK cells

In Korea, dendritic cell therapy product (CreaVax-RCC inj.) developed by JW CreaGene Co. Ltd. and an autologous T-lymphocyte therapy product (Immuncell-LC) developed by GC Cell Co. Ltd. (Seoul, Korea) for liver cancer are marketed as a first generation immune cell therapy. Subsequently, driven by the global development of cancer immunotherapy, the field of immune cell therapy research has become extremely buyant in this country where the number of clinical studies is rapidly increasing.

In terms of the number of approved cell therapy products, South Korea has allowed the commercialization of a relatively high number of products compared with other countries. However, most of them are simple expansion of cells attained in the presence of different combinations of cytokines. Recently, an engineered cell therapy expressing TGF-β was approved for the treatment of osteoarthritis; this is the first gene therapy product that was approved in South Korea (Evans et al., 2018).

CAR-T and CAR-NK therapeutics are currently being developed by several firms in South Korea using gene editing technologies; these companies include GC Lab Cell, Chabiotech, ToolGene and KRIBB. While the United States is focussing on T cell, like CAR-T, and Japan is relatively focussing on NKT, Korean companies including GC Lab Cell, Chabiotech and VaxCell Bio tend to focus on NK cell therapy (Table 5.8).

CLINICAL DEVELOPMENT STATUS OF CHIMERIC ANTIGEN RECEPTOR NATURAL KILLER CELLS

Ex vivo–expanded primary human NK cells produce cocktails of cytokines of a different spectrum than that of T cells, as they notably release GM-CSF, IL-3 and IFN-γ. As a result, CAR-NK cells could represent a complementary treatment option for CAR-T cells, either in combination or sequentially. To this date, promising preclinical data on CAR-NK-92 targeting broad cancer targets such as CD19, CD20, CD244 and HER2, as well as CAR-NK, have already been reported (Glienke et al., 2015).

Many scientific questions and regulatory hurdles should be dealt with, before CAR-NK treatments could be envisaged to treat a larger group of patients. Although powerful methods for isolation, expansion, and transduction of NK cells have been elucidated, the preparation of NK cell therapeutic doses still remains burdensome. Notably regarding engineering techniques, compared to lentiviral vectors, retroviral vectors exhibit the advantage of a significantly higher transduction rate; however, these vectors have a higher risk of insertional mutagenesis (Baum and Fehse, 2003; Wu and Dunbar, 2011).

Although some studies reported up to 80%–90% of transfection in NK-92 cells by clinical-grade electroporators (Shimasaki et al., 2012), the transfection rates reported in many studies that used primary NK cells expanded ex vivo was about 10%, which was very disappointing (Boissel et al., 2009). Furthermore, the expression of CAR molecule is short-lived, perhaps less than 7 days (Zhao et al., 2010). Despite these remaining technical hurdles regarding transduction efficiency, phenotype and survival of the NK cells, the existing data already show that these hurdles can be overcome (Boissel et al., 2003) (Table 5.9).

Table 5.8 Clinical Trial Status for NK Cell Therapies in Korea.

NO.	Sponsor	Product Name	Title	Phase	Patient Condition	NK Source of Cells
1	Vaxcell Bio	Vax-NK/HCC	Study on safety of NK cell in liver cancer patient who had Intra-arterial chemotherapy	I	Liver cancer	Autologous
2	Seoul Asan hospital	Donor NK-cell	Injection of donor derived NK cell after haploid HSCT Phase 1-2a clinical study	Investigator-initiated trial	Refractory acute leukaemia	Allogeneic
3	Seoul Asan hospital	Allogeneic donor NK	Randomized comparative clinical studies to evaluate the efficacy and safety of donor natural killer cell donors with homologous hematopoietic cell transplant alone, or after transplantation	Investigator-initiated trial	High-risk AML and MDS	Allogeneic
4	Daejeon Catholic Hospital (Park Seok Young)	EBIH	Phase II clinical trials for the efficacy evaluation of standard 2 chemotherapy and self-amplified natural killer cells and lymphocyte (ANKL) therapy as 1st line treatment	Investigator-initiated trial	Progressive NSCLC	Autologous
5	MTS Bio	SMT-NK injection	Safety evaluation of natural killer cells from healthy blood	I	Progressive femoral cancer	Allogeneic
6	Geesam Hospital	NKTM	Single-center, single arm, open-label, phase I/II study for evaluation of safety and efficacy of self-derived activated lymphocyte	Investigator-initiated trial	Stage 4 solid cancer	Autologous
7	Medicell	Medi-NK	Phase 1 clinical trial of haploidentical natural killer (NK) cells used in pemetrexed combination therapy	I	Stage 4 NSCLC	Allogeneic
8	Green Cross Lab Cell	MG4101 NK-cell	Evaluation of efficacy and safety after administration of MG4101 (allogeneic NK cell treatment) in patients with liver cancer treated with TACE	IIa	Liver cancer	Allogeneic

Continued

Table 5.8 Clinical Trial Status for NK Cell Therapies in Korea.—cont'd

NO.	Sponsor	Product Name	Title	Phase	Patient Condition	NK Source of Cells
9	Catholic Univ. Seoul St. Mary Hospital	NKM self-activated lymphocyte	Phase II clinical trial for the efficacy of NKM self-activating lymphocyte combination therapy in breast cancer patients receiving docetaxel/ Trastuzumab chemotherapy	Investigator-initiated trial	HER-2 overexpressing progressive metastatic breast cancer	Autologous
10	Binex	TK-cell injection (Autologous-derived activated lymphocyte)	Safety and efficacy of FOLFOX-4 standard chemotherapy alone, and combination of FOLFOX-4 standard chemotherapy and TK-cell	II	Relapsed and unresectable progressive colon cancer	Autologous
11	Binex	TK-cell injection (Autologous-derived activated lymphocyte)	Safety and efficacy of FOLFOX-4 standard chemotherapy alone, and combination of FOLFOX-4 standard chemotherapy and TK-cell	II	Relapsed and unresectable progressive gastric cancer	Autologous
12	Binex	TK-cell injection (Autologous-derived activated lymphocyte	Evaluation of efficacy and safety of RFA and TK-cell for combination treatment	II	Progressive gastrointestinal cancer	Autologous
13	Chabiotech	The Autologous Killer Cell	Safety and efficacy of combination of chemotherapy and immune cell therapy	Investigator-initiated trial	Recurrent epithelial ovarian cancer	Autologous
14	Chabiotech	The Autologous Killer Cell	Investigator-initiated I/II trial to observe efficacy and safety after administration of immune cell therapy The Autologous Killer Cell	Investigator-initiated trial	Recurrent glioblastoma	Autologous
15	Green Cross Lab Cell	MG4101	Studies on the injection of natural killer cells after haploid HSCT	Investigator-initiated trial	Recurrent high-risk solid tumour patient after autologous HSCT	Allogeneic

Table 5.9 Preclinical Studies of Chimeric Antigen Receptor (CAR)–Human Natural Killer (NK) Cell and CAR-Expressing NK-92 Cell.

Article		Target
Preclinical Studies on CAR NK-92 Cell		
Boissel et al. (2009)	Chronic lymphocytic leukaemia	CD19
Boissel et al. (2003)	Lymphoblastic leukaemia	CD19; CD20
Chang et al. (2013)	Improvement in NK cytotoxicity	NKG2D
Chu et al. (2014a)	Multiple myeloma	CS1
Cherukuri et al. (2012)	Neuroblastoma	GD2
Jiang et al. (2014)	Multiple myeloma	CD138
Feuchtenberger et al. (2008)	Leukaemia and lymphoma	CD20
Sahm et al. (2012)	Breast cancer	EpCAM
Schonfeld et al. (2014)	Epithelial originated tumour (lung metastasis of breast and renal cancer)	HER-2
Tassev et al. (2012)	EBV-positive T cells	EBNA3C
Uherek et al. (2002)	Epithelial originated tumour (breast cancer, ovarian cancer, and epithelial cancer)	HER-2 (ErbB2)
Zhang et al. (2013)	Melanoma	GPA7
Preclinical Studies on CAR-Human NK Cell		
Alsamah and Romia (2014)	HER-2–expressing cell lines	HER-2
Altvater et al. (2009)	Neuroblastoma	CD244
Chu et al. (2014b)	Burkitt lymphoma	CD20
Imai et al. (2005)	Leukaemia	CD19
Kruschinski et al. (2008)	Ovarian cancer	HER-2
Li et al. (2010)	OP-132 cell line–derived CD19+ B-ALL	CD19
Shimasaki et al. (2012)	Leukaemia	CD19
Clinical Studies		
NCT00995137	Genetically modified haploidentical natural killer cell infusions for B-lineage acute lymphoblastic leukaemia	St. Jude Children's Research Hospital
NCT01974479	Pilot study of redirected haploidentical natural killer cell infusions for B-lineage acute lymphoblastic leukaemia	National University Health System, Singapore, Dario Campana
NCT03056339	Umbilical and cord blood–derived CAR-engineered NK Cells for B lymphoid malignancies	MD Anderson Cancer Centre

PERSPECTIVES

It is no exaggeration to say that we are currently living in an era of anticancer immunotherapy. Many combinations of immune-controlled drugs including immune checkpoint inhibitors are being investigated, and the expectation would be that good combination might come out from these trials. Especially after the approval of the first CAR-T products, the gene therapy field is switching to a higher gear with the exploration of many more genetically engineered cell therapies. An important line of research is to address in parallel the multiple cause of cancer as well as the typical heterogeneity of solid tumours.

A critical consideration to keep in mind is that the immune system has evolved to achieve homeostasis and to keep infectious agents in check while maintaining tissue integrity, rather than to tip the balance towards activation or inactivation. This concept is important as cancer could be viewed as akin to a chronic infection/inflammation, a phenomenon hinted by the exhaustion of T cells typically observed in solid tumour patients. NK cells play a critical role for controlling the immune system and as such a deeper understanding of the biology of NK cells might hold the key to dramatically enhance immuno-therapy efficacy and tolerability. In the near future, many combination trials between immunocellular therapy products and other immune-controlled intervention such as antibodies and small molecules will be performed to overcome cancer the breakthroughs achieved to this date are most likely only the beginning of a revolution in solid cancer treatments that will bring hope to today's no-hope patients.

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