

PhD
**PROGRAM IN TRASLATIONAL
AND MOLECULAR MEDICINE**



**University of Milano-Bicocca
School of Medicine and Faculty of Science**

**Cytokine-induced killer (CIK) cell cultures for
the adoptive immunotherapy of hematological
malignancies: characterization and new
therapeutic strategies for clinical application**

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Chapter 1

Introduction

The hypothesis that the immune system can recognize and eliminate tumor cells was first postulated in 1909 by Paul Ehrlich. However, at this time the hypothesis could not be tested experimentally, due to the limited knowledge on the cellular and molecular basis of the immune system. In the fifties however, experimental evidence in mice demonstrated that immunization with chemically and virally induced tumor cells could mobilize tumor-specific immune responses that were able to reject the original tumor upon re-challenge. The results of these experiments suggested an antigenic nature of tumors and gave rise to the field of cancer immunotherapy.

1.1 Cancer immunotherapy

Cancer is a major cause of death throughout the world and even though cancer treatments have constantly improved patient survival during the last decades, new therapeutic strategies are needed. Standard cancer therapies, including surgery, radiation and chemotherapy, cure most of cancer patients by efficient removal of

the primary tumor. However, when the cancer metastasize, these conventional therapies are often ineffective. One alternative treatment strategy is cancer immunotherapy, ie, the use of the components of the immune system to fight cancer.

In the 1890s William Coley, a New York surgeon, observed that sarcomas spontaneously regressed upon streptococcal infections. This led him to administer a streptococcus extract, named Coley's toxins, to sarcoma patients, achieving a cure rate of 10%. He is therefore considered the "father of immunotherapy". The field of cancer immunotherapy has since evolved into a complex variety of therapeutic approaches. These are commonly divides into two main categories: passive and active cancer immunotherapy; the last one can be further divided into specific and non-specific immunotherapy depending on its action in the recipient (Figure 1). Active immunotherapy aims mainly to elicit the body's own response to attack the tumor cells, whereas passive immunotherapy relies on therapeutics that can directly mediate the killing of the tumor.

Examples of active immunotherapy include the systemic delivery of cytokines, the introduction of adjuvants into the tumor microenvironment, and cancer vaccines, whereas immunization with monoclonal antibodies and adoptive transfer of tumor-specific T lymphocytes are components of passive immunotherapy.

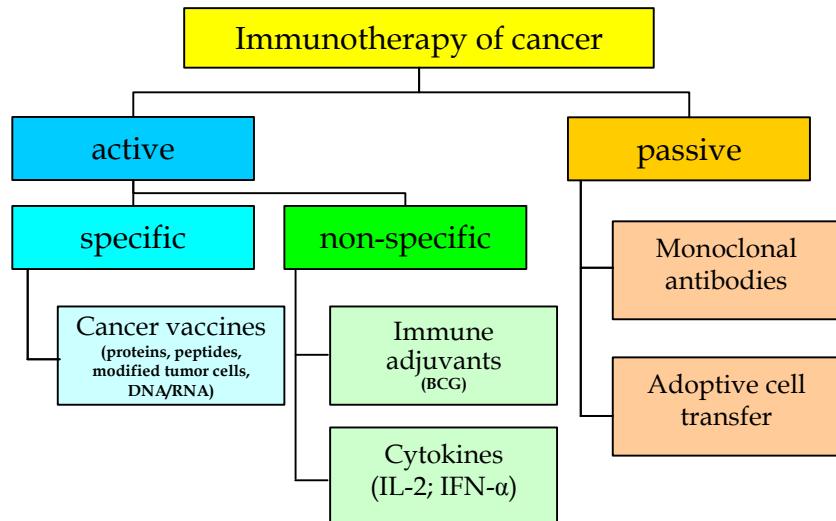


Figure 1. Categories of cancer immunotherapy.

1.1.1 Active cancer immunotherapy

Non-specific active immunotherapy includes the administration of cytokines which can boost immune responses through the recruitment and maturation of a variety of immune effector cells. Treatment with IL-2 and IFN- α , which were the first cytokines to be approved by the American Food and Drug Agency (FDA), has shown clinical responses in patients with malignant melanoma and renal cell carcinoma¹⁻³. Several other cytokines, such as IL-7, IL-12, IL-21 and granulocyte macrophage colony-stimulating factor (GM-CSF) are under clinical investigation for their therapeutic potential as stand-alone agents or as adjuvant⁴.

Another example of non-specific cancer immunotherapy is the administration of immune-activating antigens (adjuvants). Vaccination against tuberculosis using an attenuated mycobacterial

strain called Bacillus Calmette-Guerin (BCG) is successfully used in clinic to treat bladder cancer during the last three decades⁵.

Cancer vaccines are an example of specific active immunotherapy because they do not generally boost the immune system, but rather give it a specific target to identify antigens and attack the tumor. The aim of specific active cancer immunotherapy is to induce a long-lasting antitumor immune response, ie, immunological memory, preferably composed of both the humoral and cellular arm of the immune response. The majority of cancer vaccines have the objective to lead to the generation of tumor-specific CD8⁺ cytotoxic T lymphocytes (CTLs). These CTLs recognize the tumor cells by their cell surface expression of tumor antigens presented as processed peptides on MHC class I molecules. However, CD4⁺ T cells, recognizing peptides presented by MHC class II molecules, are probably equally important in antitumor immunity and have gained more interest in recent years. Vaccines are either prophylactic, namely administered to healthy individuals in order to prevent the occurrence of disease, or therapeutic, ie, administered to the already diseased patients with the intention to treat. The first prophylactic cancer vaccine to be clinically approved was the hepatitis B virus (HBV) vaccine, used to prevent HBV induced hepatocellular carcinoma⁶. Two more recently approved preventive cancer vaccines are Gardasil® and Cervarix®. They protect against infection by specifically two types of HPV, types 16 and 18, which cause approximately 70% of all cases of cervical carcinoma⁷. Most cancer vaccines, for non-virally induced tumors, are therapeutics since they are intended for use in patients with already apparent

tumors. They can accordingly be used to treat minimally residual disease, to prevent recurrence after primary removal of the bulky tumor, and to increase the tumor free survival.

Generally, antigens expressed by the tumor are weakly immunogenic, which can result in inadequate presentation to T cells. So, therapeutic vaccines must bypass immune-regulatory mechanisms that have already led to tumor tolerance. Consequently, several strategies for cancer vaccination and vaccine delivery have evolved and are being investigated in animal models as well as in clinical trials. Cancer vaccines can be based on: 1) *recombinant peptides and proteins*; 2) *whole tumor cells*, either autologous or allogeneic, that are inactivated by irradiation and/or genetically modified to express co-stimulatory factors; 3) *viral vectors* engineered to express tumor antigens; 4) *dendritic cells* loaded with tumor antigens of different forms, such as tumor cell lysates, proteins, peptides or mRNA; 5) *DNA or RNA* encoding the tumor antigen.

1.1.2 Passive cancer immunotherapy

Passive cancer immunotherapy involves administration of “ready-made” effector molecules or cells. Due to the short lived in vivo activity of these effectors, this category of immunotherapy is typically dependent on repetitive administrations. Examples of specific passive cancer immunotherapy include immunization with monoclonal antibodies and adoptive transfer of donor stem cells, ie, allogeneic stem cell transplantation, or in vitro expanded tumor-

specific T lymphocytes, natural killer (NK) cells, $\gamma\delta$ T cells or cytokine-induced killer (CIK) cells.

Monoclonal antibodies

The concept of using antibodies to selectively target tumors was proposed by Paul Ehrlich over a century ago. The advent of hybridoma technology in 1975 enabled the production of monoclonal antibodies (mAbs). Owing to their origins in mice, these monoclonal antibodies were typically immunogenic in humans and had poor abilities to induce human immune effector responses, thereby limiting their clinical applicability. Later advances in antibody engineering provided flexible platforms for the development of chimeric, humanized and fully human monoclonal antibodies which satisfactorily addressed many of these problems. Over the past decade, the effectiveness of antibodies in treating patients with cancer has been increasingly recognized^{8,9}.

Antibodies are grouped into five classes based on the sequence of their heavy chain constant regions: IgM, IgD, IgG, IgE and IgA. Of the five classes, IgG is the most frequently used for cancer immunotherapy. Antibodies can be subdivided into two distinct functional units: the fragment of antigen binding (Fab), and the constant fragment (Fc). The Fab contains the variable region, which consists of three hypervariable complementarity-determining regions (CDRs) that form the antigen binding site of the antibody and confer antigen specificity. Antibodies are linked to immune effector functions by the Fc fragment, which is capable of initiating

complement-dependent cytotoxicity (CDC), binding to Fc receptors for IgG (Fc γ Rs) on effector cells and initiating antibody-dependent cellular cytotoxicity (ADCC), and binding to the neonatal FcR (FcRn) (Figure 2)^{10,11}. FcRn has an important role in the maintenance of serum IgG levels and can contribute to the long half-life seen with this isotype¹².

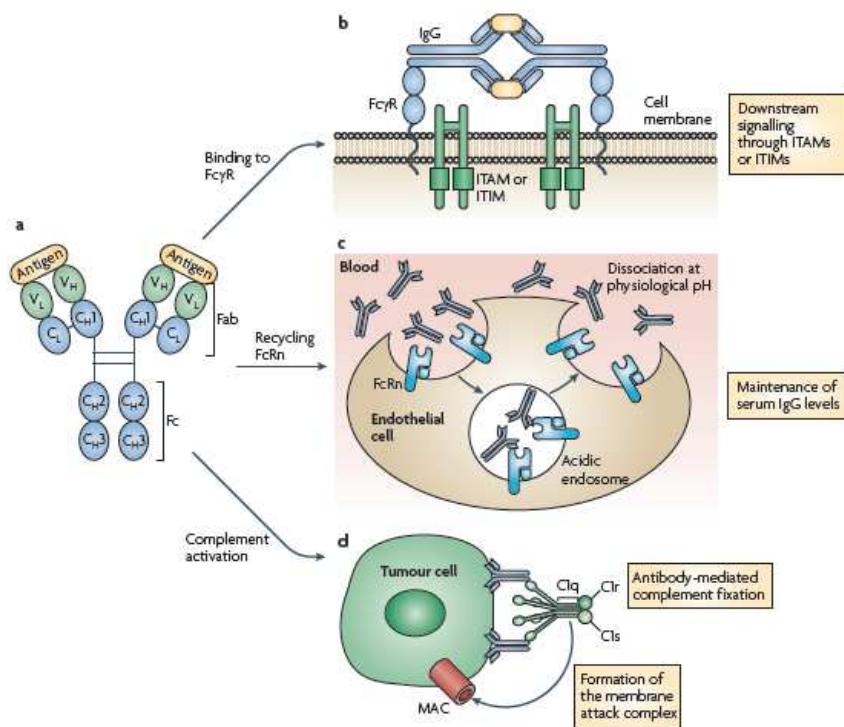


Figure 2. IgG structure and function. (a) IgG molecules are tetramers of ~ 150 kDa, which comprise a pair of identical heavy and light chains linked by disulphide bonds (blue bars). The heavy chains contain a variable (V) domain (the V_H domain; green) and three constant (C) domains, the C_{H1} domain, C_{H2} domain, and C_{H3} domain (blue). By contrast, the light chains contain a V domain (the V_L domain; green) and a single C domain (the C_L domain, blue). Highly selective binding of antigen (orange) is a common

hallmark of antibodies. This is mainly mediated by six loops, which are known as the complementarity-determining regions (CDRs), three of which are present in each of the V_H and V_L domains. **(b)** Antigen coated with IgG can bind Fc receptors (Fc γ R) on immune effector cells and initiate signalling through immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine-based inhibitory motifs (ITIMs). **(c)** IgG can bind neonatal Fc receptor (FcRn) on endothelial cells to maintain serum IgG levels. **(d)** IgG can also bind to tumor cells and recruit complement component 1q (C1q) to initiate the complement cascade, resulting in tumor cell lysis by the membrane attack complex (MAC). (*Adapted from Weiner LM et al, Nat Rev Immunol, 2010 10: 317-327*)

Monoclonal antibodies can be directed towards a number of cancer-associated antigens, including tumor cell surface antigens, vascular growth factors, tumor-associated stroma, and host immune checkpoints. Many of the tumor-expressed antigens targeted by therapeutic antibodies are growth factor receptors, such as, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), that show increased expression during tumorigenesis. By blocking ligand binding and/or signalling through these receptors, monoclonal antibodies may serve to normalize growth rates, induce apoptosis and/or help sensitize tumors to chemotherapeutic agents. Monoclonal antibodies directed against tumor antigens can mediate their effector functions also through modulating immune effects, such as, ADCC and CDC. In addition, antibodies that target crucial events in the tumor microenvironment have shown therapeutic benefit in preclinical and clinical settings. For example, many solid tumors express vascular endothelial growth

factors (VEGFs), which bind to its receptor on the vascular endothelium to stimulate angiogenesis. Targeting vascular growth factors can inhibit angiogenesis and prevent tumor growth. As an alternative to directly targeting tumor cells, numerous antibody-based therapeutic strategies have been developed to target cells of the immune system with the goal of enhancing antitumor immune response. Some example is the targeting of immunoregulatory coreceptors (ie, CD40, CTLA4) and antibody-based strategies aimed at reversing tumor-mediated immunosuppression (anti-TGF- β).

Antibodies could also have the potential to deliver cytotoxic agents to tumor cells. MAbs conjugated to radioactive isotopes or chemotherapeutic drugs have shown therapeutic efficacy mainly in haematological malignancies.

In 2010 there were ten antibody-based therapies for the treatment of cancer licensed by the FDA, for such diverse malignancies as breast cancer, lymphoma, leukemia, and colon cancer (Table 1).

Generic name (trade name; sponsoring companies)	Target	Antibody Format	Cancer Indication
Unconjugated antibodies			
Rituximab (Rituxan/Mabthera; Genentech/Roche/Biogen Idec)	CD20	Chimeric IgG1	Non-Hodgkin lymphoma
Trastuzumab (Herceptin; Genentech/Roche)	HER2	Humanized IgG1	Breast cancer
Alemtuzumab (Campath/MabCampath; Genzyme/Bayer)	CD52	Humanized IgG1	Chronic lymphocytic leukaemia
Cetuximab (Erbitux; ImClone Systems/Bristol-Myers Squibb)	EGFR	Chimeric IgG1	Colorectal cancer
Bevacizumab (Avastin; Genentech)	VEGFA	Humanized IgG1	Colorectal, breast and lung cancer
Panitumumab (Vectibix; Amgen)	EGFR	Human IgG2	Colorectal cancer
Ofatumumab (Arzerra; Genmab/GlaxoSmithKline)	CD20	Human IgG1	Chronic lymphocytic leukaemia
Immunooconjugates			
Gemtuzumab ozogamicin (Mylotarg; Pfizer)	CD33	Humanized IgG4	Acute myelogenous leukaemia
⁹⁰ Y-Ibritumomab tiuxetan (Zevalin; Biogen Idec)	CD20	Mouse	Lymphoma
Tositumomab and ¹³¹ I-tositumomab (Bexxar; GlaxoSmithKline)	CD20	Mouse	Lymphoma

Table 1. Therapeutic monoclonal antibodies approved for use in oncology.

(From Weiner LM et al, *Nat Rev Immunol*, 2010 10: 317-327)

Adoptive cellular therapy

Cell based immunotherapies are proven to be effective for some cancer, in particular hematologic malignancies. The adoptive transfer of leukemia-directed effector cells to patients with hematologic malignancies includes different approaches. Beside the well-established infusion of donor derived T cells, there are attempts to use in vitro generated cytotoxic T lymphocytes that were selected for their tumor specificity. The alternative approach in the adoptive transfer of antitumor effector cells is to infuse NK cells, $\gamma\delta$ T cells, or CIK CD3 $^+$ CD56 $^+$ cells which are not MHC-restricted and therefore present a reduced risk to develop graft-versus-host disease (GVHD).

T cells

High-dose chemoradiotherapy followed by rescue from the resulting ablation of normal bone marrow with a hematopoietic stem cell transplantation (HSCT) has become standard therapy for many hematologic malignancies. One problem with this treatment is GVHD, due to allogeneic donor-derived T cells injuring the foreign normal tissues of host. However, malignant cells that survive chemoradiotherapy are also of host origin and patient who develop GVHD have lower relapse rates from an associated graft-versus-tumor (GVT) effect (Figure 3). T cells mediate this antitumor activity, as confirmed by the complete responses sometimes observed in patients who receive infusions of donor T cells to treat relapse after HSCT and in recipient of non-myeloablative allogeneic HSCT regimen in whom, because of the absence of high dose chemotherapy, all antitumor effects result from GVT activity¹³. Ongoing efforts to define antigenic targets with limited tissue distribution, permitting donor lymphocytes to preferentially target malignant cells and not critical normal tissues, coupled with methods to generate and/or select T cells with such specificity, should provide a much-needed refinement to this approach. An alternative to using allogeneic T cells to mediate antitumor responses has been isolating autologous tumor-reactive T cells, expanding the cells *in vitro*, and then reinfusing the cells back into the patient.

This approach circumvents many of the obstacles to generating an adequate response *in vivo*, as the nature of the APCs and components of the microenvironment can be more precisely

controlled in vitro¹⁴. However, this strategy has required the development of methods to extensively manipulate T cells in vitro with retention of specificity and function, such that after infusion the cells will survive, migrate to and eliminate tumor cells.

On this basis, the spectrum of adoptive T cell therapy strategies can be divided pragmatically into two areas:

1. Non-specific expansion
2. Antigen-specific enrichment

Non-specific expansion includes the use of donor lymphocyte infusions (DLI) in the allogeneic stem cell transplant setting, autologous tumor-infiltrating lymphocytes obtained from tumor sites and expanded ex vivo, and the non-specific activation of effector cells in the peripheral blood using antibodies to the TCR-CD3 complex. Antigen-specific enrichment involves in vitro manipulation that preferentially expands or selects for T cells expressing a T-cell receptor (TCR) of given specificity, or genetic modification that endows a population of lymphocytes with desired target specificity¹⁵.

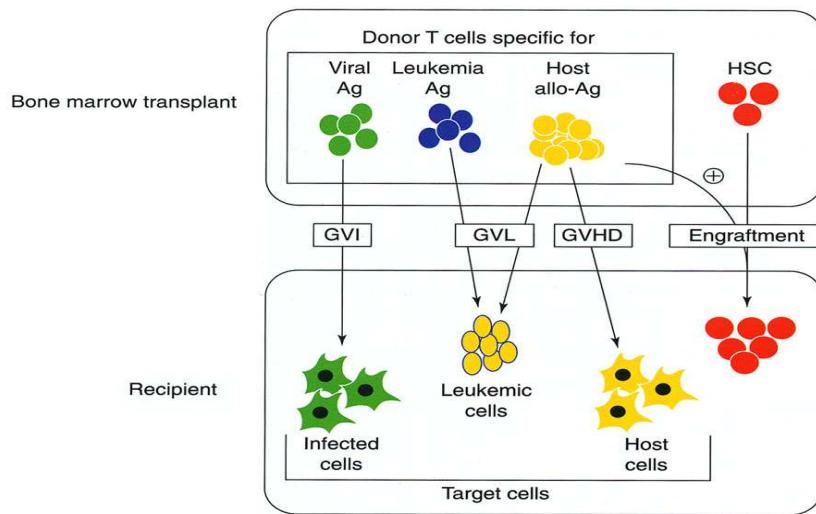


Figure 3. Antigen-specific activity of donor T cells is important in GVHD, GVL and GVI effects and engraftment. Donor T-cell populations in the allograft contain alloreactive T cells, which become activated and proliferate on recognition of specific MHC or minor histocompatibility antigens (mHAGs) on host cells. These alloreactive T cells can have cytolytic activity against host cells, which contributes to the development of graft-versus-host disease (GVHD). Donor T-cell populations in the allograft can also contain leukemia-reactive T cells, which can recognize leukemia-associated antigens and exert cytolytic activity against leukemic cells. Both leukemia-reactive T cells and alloreactive T cells, which recognize alloantigens on the leukemic cells, mediate graft-versus-leukemia (GVL) activity. Donor T-cell population in the allograft can also contain virus-reactive T cells, which can recognize viral antigens and exert cytolytic activity against virus-infected cells.

Donor-derived lymphocyte infusion

The first evidence that T cells play a role in mediating antitumor responses in humans was observed in patients receiving T cell replete or T cell-depleted marrow for allogeneic stem cell

transplantation almost 30 years ago¹⁶. Patients receiving syngeneic (identical twin) and T-cell depleted marrow cells experienced rates of leukemia relapse after transplant that were more than twice as high as patients receiving unmanipulated (T cell replete) marrow from allogeneic donors. Furthermore, patients developing GVHD were even more likely to experience leukemia-free remission than those who did not develop GVHD¹³. Taken together, these results suggest that the allogeneic effect of donor lymphocytes plays an important role in eradicating residual leukemia. The directed use of donor lymphocytes infusion (DLI) for the treatment of leukemic relapse after transplant represents an extension of these findings and has been most successfully implemented in patients who relapse following transplantation for chronic myeloid leukaemia (CML)^{17,18}.

Usually DLI consists of a crude mixture of lymphocytes collected by lymphocytapheresis and is applied in HLA-matched HSCT. T cell DLIs are not recommended in partially mismatched or haploidentical HSCT because of the high risk to develop GVHD. Efforts have also been made to introduce a suicide gene into donor lymphocytes, so that higher T cell doses to eradicate leukemic cells can be used while reserving the ability to later eliminate donor lymphocytes *in vivo* should GVHD be observed¹⁹. For example, lymphocytes transduced with the Herpes Simplex virus-thymidine kinase (HSV-TK) suicide gene, undergo cell death as the HSV-TK preferentially converts low-dose ganciclovir (which can be safely administered to patients), into a toxic intracellular metabolite. While the use of DLI has been most effective for chronic myeloid leukemia relapse (durable complete response 60% to 75%), it is largely

experimental therapy for patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myeloma, and lymphoma, where responses have been significantly weaker (< 30%)¹⁵. In an effort to boost these responses, investigators have turned to the use of donor lymphocytes that have undergone in vitro activation. In one form of this approach, microbeads coated with antibodies to the TCR-CDR3 complex (anti-CD3) and a T cell costimulatory molecule (anti-CD28) were used to activate and expand apheresis peripheral blood mononuclear cells (PBMCs) collected from donors for infusion. In a Phase I study, this strategy proved to be partially effective for patients relapsing post-transplant with ALL, AML and non-Hodgkin's lymphoma (NHL) without excessive GVHD²⁰.

Tumor-infiltrating lymphocytes

Reasoning that more specific lymphocytes may be found in tumor tissue, investigators considered utilizing tumor-infiltrating lymphocytes (TIL) for adoptive immunotherapy. Tumor-infiltrating lymphocytes are mononuclear cells harvested from a tumor excisional biopsy and propagated in vitro, usually with high doses of IL-2 (6000 U/mL). TIL cultures are predominantly CD3⁺ T cells whose responses to tumor cells are MHC-restricted. However, these are difficult to obtain reproducibly from most types of tumor, with the exception of melanoma, where approximately 70% of tumors can yield sufficient TIL for treatment regimens²¹. Antitumor response in patients with metastatic melanoma was observed in 22–35% of cases when TIL were given together with high dose IL-2, which was

required for TIL survival¹⁵. The modest efficacy observed with TIL may be attributed to the limited numbers of tumor-reactive effectors in a largely heterogeneous population and the continued dependence of high doses of IL-2 in vivo. TIL cultures vary in composition and may be constituted predominantly by CD4⁺ or CD8⁺ T cells or contain both of these two subsets²¹. Although it is not clear which type of culture is most effective in therapy, there is some suggestion that CD8⁺ clones alone are less effective than mixed populations. Indeed, CD4⁺ T cells have been demonstrated to play a role in the generation of optimally effective CD8⁺ T cells.

EBV-specific T lymphocytes

Epstein Barr virus (EBV)-specific T cell therapy represents one of the most successful applications of antigen specific adoptive cellular therapy for the treatment of virus-associated cancer.

Patients receiving highly immunosuppressive therapy following stem cell or organ transplantation are at high risk for EBV-associated post-transplant lymphoproliferative disease (PTLD). In immunocompetent individuals, EBV-infected B cells are constrained from developing into a lymphoproliferative disorder by the endogenous EBV-specific CTL population. However, immunosuppressive therapy can lead to unchecked proliferation of EBV-infected B cells. In the post-stem cell transplant setting, donor lymphocyte infusions can lead to reconstitution of EBV-specific CTL response and induce clinical remissions in patients developing PTLD²². However, DLI can often lead to GVHD which could be eliminated through the use of more specific effectors, such as, EBV-specific lymphocytes. Using

autologous EBV-transformed lymphoblastoid cell lines, Rooney and others were able to generate high numbers of EBV reactive CTL ($>10^7$ cells/m²) sufficient for repeated cycles of adoptive transfer and demonstrated that infusions of EBV-CTL could be used to effectively treat and prevent PTLD, when given to patients with rising EBV DNA titers post-transplant^{23,24}.

Tumor-specific T lymphocytes

With respect to cancer not associated with viral infection, early therapies using donor PBMCs reinduced remission in bone marrow transplanted patients with relapsed CML¹⁷. This is a GVL effect but is not transferable for general cancer therapy, although it showed proof of principle that the immune system could combat tumors. However, the majority of tumor-associated antigens are weakly immunogenic self-antigens and natural T cells against these antigens are low in frequency, have low TCR avidity or are anergic, and thus a more robust cellular therapy is needed²⁵. To this end, leukemia-specific CTLs have been generated ex vivo in the presence of dendritic cells presenting the target antigen. Supplemental cytokines are provided during cell culture to support lymphocytes proliferation, survival and differentiation, rendering them possible to expand tumor-reactive T cells to enormous numbers in vitro.

In the autologous setting, CTLs specific for melan-A peptide could be generated from the PBMCs of melanoma patients using dendritic cells pulsed with tumor peptide epitopes as APC, and clinical responses were seen in some patients²⁶. However, this

approach is limited to patients carrying the HLA haplotype required to bind the previously defined immunogenic epitope.

The strategies mentioned above are highly labour intensive and clinical trials of cancer immunotherapy have shown modest and variable response rates, virus-associated malignancies aside, pointing to the existence of additional hurdles. Effective therapies require T cells to persist, expand, traffic, and home to tumors, and mediate effector function. Regarding the possible explanations of the limited clinical response rates, the terminal differentiation of transferred tumor-reactive CD8⁺ T cells appears to be a hindrance. The phenotype of late stage effector CD8⁺ T cells in a progressive differentiation includes a loss of lymph node homing receptor CD62L, a loss of co-stimulatory molecules CD27 and CD28, and a loss of survival cytokine IL-7 receptor CCR7. Although these phenotypes are strongly associated with effective function in vitro, they are evidenced to limit the survival and the effective function of the transferred T cells in vivo^{27,28}. Several strategies, including the enforced expression of costimulatory proteins and telomerase, and the use of short term ex vivo activated T cells, have been used to attempt to extend the lifespan of cultured T cells. IL-15 has also been considered as a possible additive to culture to enhance the survival of expanded tumor-specific T cells²⁹.

Besides a modulation of the status of transferred T cells, to hurdle the suppressive environment in hosts is becoming more and more essential for enhancing the efficacy of passive immunotherapy. T regulatory cells (Tregs) are a subset of T cells which actively suppress T cell activation and prevent autoimmune disease resulting

from pathological self-reactivity. These cells are overexpressed in a wide range of tumors and increased numbers of Tregs correlates with poor prognosis. The marked improvement of adoptive T-cell transfer in lymphopenic host provided indirect evidence of the importance of Treg elimination before adoptive T-cell transfer²⁹.

It is also possible to use integrating vectors to genetically modify T cells before infusion to enhance tumor recognition, cell survival, migration or effector functions; in essence, engineering responses that might not be naturally achievable. For example, T cells recognition of tumors has been imparted by expressing high-affinity chimeric transmembrane receptors with the external recognition structure of an antibody and the signaling domain of a T cell receptor. Survival of transferred cells can also be enhanced by introducing chimeric cytokine receptors¹⁴.

NK cells

Natural killer (NK) cells have been found to substantially contribute to GVT responses, which were previously thought to be largely mediated by T cells alone.

The activation of NK cells is regulated by signals derived from a variety of cell surface activating and inhibitory receptors. Activating receptors include the natural cytotoxicity receptors (NCRs: NKp30, NKp44 and NKp46), NKG2D, 2B4, NKp80 and DNAM-1. The major control of NK cell activation, however, is mediated by their expression of inhibitory receptors that are specific for MHC-class I molecules, which prevent attack of normal (MHC-I expressing) cells in the body³⁰. Some tumor cells and virus-infected cells avoid

recognition by CTL by downregulating their expression of MHC-I, but these escape mutants consequently become targets for NK cells, since they lack the inhibitory receptor ligands. In this way these inhibitory receptors mediate NK cell tolerance toward normal “self” cells, and their lack of engagement provides the mechanistic basis for recognition and attack of tumors that lack these “self” ligands, as shown in Figure 4. Killer cell Ig-like receptors (KIR) constitute the major NK cell inhibitory receptors that recognize cognate MHC-I in humans. KIR have evolved in higher mammals as a polymorphic receptor family encoded by distinct genes, each of which can detect a subset of the wide array of polymorphic human MHC-I molecules (HLA-A, -B, and -C)³¹. Expression from individual KIR genes is independently regulated within each NK cell, and during NK cell development, a repertoire of cells is established, in which distinct NK cells stochastically express different members of the KIR family. In order to achieve functional competence, each NK cell must express at least one KIR family member that recognizes at least one of the expressed “self” MHC-I molecules³². To create further diversity, individuals in the human population inherit different haplotypes of KIR family members, and most individuals express some KIR that do not recognize their own “self” MHC-I molecules³³. Due to such diversity, it is possible that a subset of NK cells from one individual will not be tolerized by the MHC-I expressed by another individual, since appropriate ligands are missing for the available inhibitory KIR. This is known as allogeneic NK cell responsiveness, which can occur under conditions of transplantation.

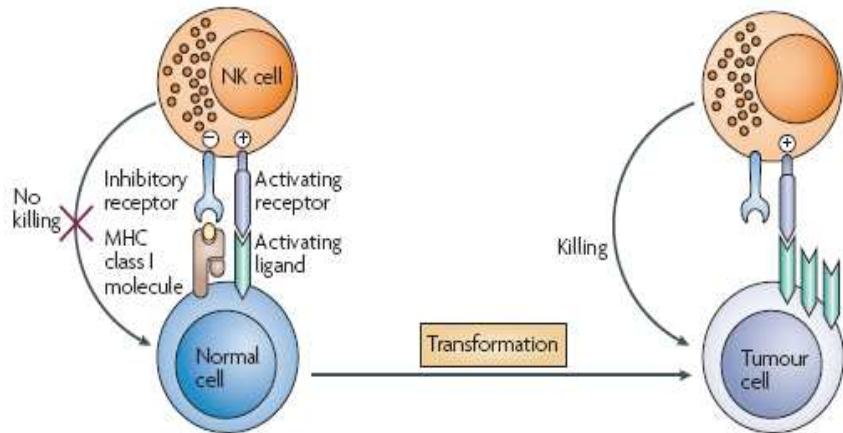


Figure 4. Missing-self recognition of tumor cells. NK cell activation is regulated by a balance between signals mediated through activating and inhibitory receptors. Upon cellular transformation, MHC class I ligands for inhibitory receptors are often reduced or lost. In parallel, cellular stress and DNA damage lead to the upregulation of ligands for activating NK-cell receptors on the tumor cell. Together, these events shift the balance towards NK cell activation and induction of cytolytic effector functions resulting in target-cell killing. (From Ljunggren HG et al, *Nat Rev Immunol*, 2007, 331-339)

Ruggeri et al. first reported that NK cell alloreactivity, due to KIR ligand mismatch, can significantly improve survival advantage when introducing haploidentical HSCT to treat AML³⁴. The benefit was observed when the donor expressed KIRs that lacked MHC-I ligands in the recipient.

In addition to the inhibitory KIR, other members of this receptor family are known to trigger NK cell activation, although their ligands are currently unknown. Further work is clearly necessary to establish the optimal conditions that favour the benefits

of KIR/ligand match/mismatch to promote antitumor NK cell responses after hematopoietic stem cell transplantation.

The direct adoptive transfer of mature haploidentical NK cells (NK cell-based DLI) has been shown to be safe and to improve the outcome of AML in some patients³⁵. Several techniques have also been developed for ex vivo expansion of NK cells. However, some caution must be taken with respect to possible phenotypic changes, lineage deviation and/or selective expansion of specific subsets of NK cells. Another aspect to consider is to what extent in vitro manipulation may alter the ability of NK cells to mediate cell-cell interactions, trafficking and homing. The lessons learned from trials of adoptive T-cell therapy suggest that naïve or short-term ex vivo activated NK cells may have better antitumor potential compared with long-term activated NK cells in the setting of adoptive immunotherapy³⁰.

In patients with metastatic melanoma, human peripheral blood mononuclear cells can be isolated and cultured with IL-2 to generate a class of cells denoted as lymphokine-activated killer (LAK) cells. These cells phenotypically and functionally resemble NK cells. When LAK cells are administered concomitantly with IL-2 into patients with either advanced metastatic melanoma or renal cell carcinoma, complete tumor regression can be achieved in about 10% of cases. Infusion of LAK cells into cancer patients have been performed in several clinical trials but the clinical response rate has only been about 20%³⁶. The difficulty of expanding NK cells in vitro and the likely requirements of high doses of IL-2 coadministration

with is associated toxicity, have so far severely restricted their use in clinic.

Several crucial questions require consideration for the development of successful NK cell-based immunotherapy in the future. These include questions relating to the type of NK-cell preparation to be used (activation, degree of enrichment, and possible selection of specific subpopulations), criteria for donor selection (KIR genotyping and phenotyping, and size of the alloreactive subset), conditioning of patients prior to therapy, clinical context of therapy and, finally, criteria for patient selection and strategies for the identification of susceptible tumors within specific patient groups²⁹.

$\gamma\delta$ T cells and cytokine-induced killer (CIK) cells

Gamma-delta T cells ($\gamma\delta$ T) provide a further option for adoptive transfer with a potential advantage of recognizing phosphoantigens that can be upregulated preferentially on some tumors. $\gamma\delta$ T cells recognize antigens in a non-MHC restricted manner, which may enhance their applicability in a wide variety of cancer patients. These cells can be generated in large numbers from peripheral blood using a phospholigand, therefore showing the feasibility of utilizing this cell subset. Their antitumor effects have been demonstrated in vitro³⁷ and in mouse models³⁸ and their safety demonstrated in Phase I clinical trials^{39,40}, but their widespread application awaits optimization of their effectiveness in humans.

Cytokine-induced killer (CIK) cells are T-cell receptor $\alpha\beta$ CD3⁺CD56⁺ cytotoxic T cells that have demonstrated a MHC-

unrestricted and unspecific cytotoxicity against a variety of malignant cells including fresh tumors and autologous or allogeneic CML progenitors, but have only minor effects on normal hematopoietic progenitor cells. CIK cells are expanded from PBMCs by the timely addition of IFN- γ , IL-2 and the anti-CD3 monoclonal antibody OKT3. The use of this cellular population in adoptive immunotherapy will be discussed below in more details.

1.2 Cytokine-induced killer (CIK) cells

1.2.1 Expansion and phenotype of CIK cells

Cytokine-induced killer (CIK) cells are a heterogeneous population of polyclonal T lymphocytes sharing NK phenotype and functional properties. Such cells were named “cytokine-induced killers”, because they were generated under the influence of cytokines and mediate potent MHC-unrestricted cytotoxicity against a variety of malignant human cell lines. Initially described by Schmidt-Wolf et al. in 1991, CIK cells are efficiently expanded in vitro from PBMCs by the timely addition of IFN- γ , mAb anti-CD3 (OKT3) and IL-2. After 2 – 3 weeks of in vitro culture the expansion of CIK cells is described to range from few to more than 1000-fold⁴¹⁻⁴³. Within the bulk culture of expanded CIK cells two main subpopulations can be distinguished, one coexpressing the CD3 and CD56 molecules (range: 40% to 80%), while the other presenting a CD3⁺CD56⁻ phenotype (range: 20% to 60%). The bulk culture comprises also a small fraction (<10%) of CD3-CD56⁺ NK cells (Figure 5). The antitumor activity of CIK cells has been reported to be associated with the CD3⁺CD56⁺ fraction^{43,44}. CD3⁺CD56⁺ cells were initially described as a minor population of human peripheral blood lymphocytes (1% to 5% of T cells) that mediate MHC-unrestricted cytotoxicity against malignant cell lines⁴⁵.

More extensive phenotypic analysis showed that the majority of expanded CD3+CD56+ cells express the TCR $\alpha\beta$ and are CD8⁺, but CD4⁺ cells can be found as well to a lesser extent within bulk CIK

cultures. Other phenotypic characteristics of CIK cells are the expression of HLA-DR, CD57, CD11b and CD5 molecules, while they are missing the Fc γ receptor CD16, mediator of ADCC mechanisms. CIK cells, unlike classic NKT cells, are independent from CD1 molecules for their expansion and present a polyclonal TCR repertoire.

CIK cells could be generated by addition of INF- γ (1000 IU/mL) on day 0 of culture, anti-CD3 OKT3 (50 ng/mL) and IL-2 (500 IU/mL) on the next day, followed by the subsequent addition of IL-2 during culture⁴³. Antibody anti-CD3 acts as a mitogenic stimulus on T cells that are then expanded in IL-2-containing medium. The addition of IFN- γ on day 0 seems to increase the cytotoxicity and expansion of CIK cells, likely stimulating the monocytes in the culture. Activated monocytes provide both a contact-dependent factor [CD58/ lymphocyte function associated antigen-3 (LFA-3)] and a soluble factor (IL-12) crucial for the expansion and acquisition of a T helper 1 phenotype of CIK cells⁴⁶. Indeed CIK cells secrete IL-2, IL-6 and TNF- α but not IL-4, IL-7 and IL-12. Some works suggest that IL-7 can be used instead of IL-2 to generate CIK cells with high cytotoxic activity. Similarly, IL-12 can be used instead of IL-2 or IL-7 to generate high numbers of CD3 $^{+}$ CD56 $^{+}$ cells with high cytotoxic activity^{47,48}.

Lu & Negrin demonstrated that T lymphocytes, rather than NK cells, are the precursors of CIK cells. T cells and NK cells were isolated from peripheral blood and separately cultivated in CIK conditions. It was observed that only the first could generate CIK cells, while NK cells maintained their initial phenotype throughout

the culture period. The acquisition of CD56 was IL-2 dependent. It seems that CD3⁺CD56⁺ CIK cells are preferentially generated from CD3⁺CD8-CD4- precursors and, to a lesser extent, from CD4⁺CD8⁺ and CD4-CD8⁺ T cells⁴³. Morphologically, CIK cells are large and heavily granulated and can not be distinguished from NK cells.

Besides the classical expansion from circulating PBMCs, it has been demonstrated that a successful expansion of CIK cells can also be obtained from granulocyte colony-stimulating factor (G-CSF)-mobilized bone marrow and from cord blood cells^{49,50}. Recently it has been shown that, after allogeneic HSCT, CIK cells could be efficiently expanded from engrafted patients even during immune-suppression treatment; their expansion degree and functional activity was similar to that observed in their correspondent healthy donors^{44,51}.

Reproducibility and simplicity of the expansion protocol and the availability of clinical grade culture reagents have facilitated the clinical utilization of CIK cultures.

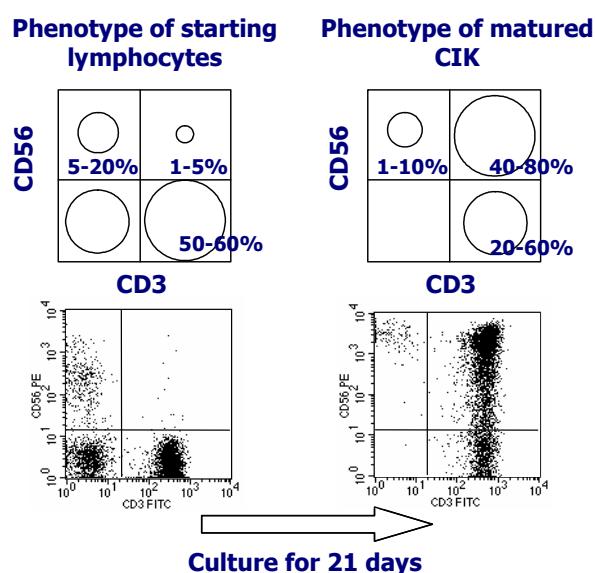


Figure 5. Phenotype at the beginning and at the end of CIK culture.

Culture of bulk peripheral blood mononuclear cells under CIK condition is able to generate an end product comprising of a majority of CD3⁺ cells with a variable CD3⁺CD56⁺ fraction.

1.2.2 Antitumor activity and GVT potential of CIK cells

Mechanism of action and in vitro data

CIK cells are endowed with a potent MHC-unrestricted cytotoxicity against both hematological and solid malignancies and recognize and kill tumor targets without prior exposure or priming. The antitumor activity of CIK cells is mainly restricted to the CD3⁺CD56⁺ fraction. Investigation into the possible explanations for the better cytolytic activities against tumor cells demonstrated for the CD3⁺CD56⁺ cells over its CD3⁺CD56⁻ counterpart revealed that the CD3⁺CD56⁺ cells consist of a higher proportion of CD8⁺ cells compared to the CD3⁺CD56⁻ cell subset, even if no differences in cytotoxicity were observed between the CD8⁺ and CD8⁻ populations of CD3⁺CD56⁺ cells. Furthermore, the CD3⁺CD56⁺ subset is a more terminally differentiated late effector T cell population bearing the CD27⁺CD28⁻ or CD27⁻CD28⁻ phenotypes⁵². In contrast, the CD3⁺CD56⁻ cells are early effector T cells expressing mainly the CD27⁺CD28⁺ and CD62L⁺ phenotypes. The granzyme content is also higher in the CD3⁺CD56⁺ cells, consistent with the report that late effector T cells possess more potent cytotoxicity than early effector T cells.

Over the years, CIK cells have been tested for its antitumor activity against a variety of tumor targets. They were shown to be able to kill t(14;18)-positive lymphoma cell lines but not normal human hemopoietic precursors⁴¹. Doxorubicin- and vinblastine-resistant tumor cell lines expressing high level of P-glycoprotein (Pgp) were also susceptible to CIK-mediated lysis^{53,54}.

CIK cells could be generated from mononuclear cells obtained from patients with newly diagnosed leukemia. The CIK cells generated following culture were free from contamination of the original leukemic cells. It was demonstrated that CIK cells derived from leukemic patients with various chromosomal abnormalities such as t(15;17), t(8;21), Philadelphia chromosome, trisomies, and complex structural abnormalities at diagnosis were totally free of all these chromosomal abnormalities on screening by karyotyping⁵⁵. Hoyle et al. has reported the generation of CIK cells from CML patients that were cytolytic against autologous and allogeneic CML cells. Furthermore, CML colony growth was reported to be suppressed by CIK cells and after 28 days of coincubation, the remaining colonies in culture were exclusively composed of Philadelphia- (Ph-) negative cells^{56,57}. CIK cells could be generated from untreated chronic lymphocytic leukemia (CLL) patients and are cytotoxic against autologous CLL targets^{58,59}. Additionally, CIK cells could also be generated from the marrow or peripheral blood samples from acute leukemia patients collected at diagnosis. These cells were lytic against both autologous and allogeneic AML blasts but not ALL blasts⁵⁵. A comparative gene expression analysis of CIK

cells in response to AML or ALL stimulators revealed a differential regulation of immune-related genes⁶⁰.

Studies have been performed to address the sensitivity of CIK cells to FAS-mediated apoptosis, since the induction of apoptosis in tumor infiltrating lymphocytes has been shown to be a tumor escape mechanism. CIK cells are resistant to FAS-mediated apoptosis, suggesting that they might also be effective against FAS ligand (FASL)-expressing tumors⁶¹.

The mechanisms underlying the cytotoxicity of CIK cells have not been completely clarified, however some key molecules and pathways have recently been identified. Tests with blocking antibodies against CD2, CD3, CD8, CD28, CD56, very late antigen 4 (VLA-4), TCR- $\alpha\beta$, and MHC class I and II molecules failed to inhibit the cytotoxic activity, suggesting an MHC-independent method of target recognition. A significant inhibition was obtained blocking lymphocyte function-associated antigen 1 (LFA-1) and intracellular cell adhesion molecule 1 (ICAM-1), suggesting that cytolysis is dependent on cell-to-cell contact^{53,62}. Treatment of CIK cells with dibutyryl (db)-cAMP, which prevents the conversion of LFA-1 into a high affinity receptor for ICAM-1, inhibited perforin and granzyme release of CIK cells triggered by both mAb anti-CD3 or tumor targets⁶³. The use of immunosuppressive drugs like Cyclosporine and FK506 prevented degranulation of CIK cells induced by CD3-TCR stimulation, but could not block the cytotoxicity triggered by the interaction with tumor targets⁶³.

The molecule that seems to play the most important role in tumor recognition by CIK cells is probably the NKG2D receptor.

Natural-killer group 2 member D (NKG2D) is one member of the c-type lectin-activating receptor family that is evolutionarily conserved and is located within the NK gene complex on human chromosome 12p12-p13⁶⁴. NKG2D is expressed on all NK cells and is a promiscuous receptor that recognizes at least 6 counterligands. These include the MHC-class I-like molecules, MICA and MICB, and members of the ULBP family (ULPB1-4), named for the ability of some members to bind to the UL-16 protein of cytomegalovirus. Other potential NKG2D ligands have been described at a molecular level, but their cell surface expression and NKG2D-binding capacity have not yet been demonstrated. Interestingly, the ligands for NKG2D appear to have a pattern of expression that is relatively restricted to malignant tissue^{65,66}. Given this, NKG2D may be particularly important in the recognition of malignant cells. NKG2D has a significant role in the triggering of IL-2-activated NK cells because ligation induces calcium flux, cytokine release, and cytotoxicity. NKG2D has also been identified on $\gamma\delta$ T cells and CD8⁺ TCR $\alpha\beta$ T cells^{67,68}. In contrast to NK cells, NKG2D crosslinking on antigen-specific CTL clones does not induce cytokine production or calcium flux or trigger cytotoxicity. However, NKG2D signaling does augment cytotoxic and proliferative responses of T cells on antigen encounter, thus qualifying NKG2D as a T-cell costimulatory molecule^{65,68}.

NKG2D expression is upregulated in CIK cells activated and expanded in vitro in the presence of IFN- γ , high-dose IL-2, and TCR-crosslinking with anti-CD3 mAb, and it is not restricted to the CD3⁺CD56⁺ subpopulation. Studies with antibodies blocking the

NKG2D molecules, small interfering RNA experiments, and redirected cytolysis indicated that the majority of the cytotoxicity of CIK cells is exerted through the NKG2D interaction rather than TCR engagement^{69,70}. The action of NKG2D is probably associated with the upregulation of the NKG2D-associated adaptor molecule disulphide adaptor protein-10 (DAP10), involved in the NKG2D activatory signaling, induced by the high dose of IL-2 present in the culture medium of CIK cells⁶⁹. While NKG2D mediates the interaction between CIK cells and tumor targets, the final cytolytic effect is perforin- and granzyme-mediated.

Additional NK cell-activating receptors that may be involved in target recognition by CIK cells need to be further investigated. Immunological manipulations aimed to potentiate the antitumor activity of CIK cells have been explored. Hence, dendritic cells (DC) were cultured and engineered to present tumor antigens to CIK cells, hoping that this might enhance the recognition of tumor cells and its subsequent killing. This was shown to be feasible for multiple myeloma (MM) when the DC were pulsed with target-derived idiotype before coculture with the CIK cells⁷¹. CIK-resistant pancreatic carcinoma target cells also became susceptible to CIK cells that were cocultured with DC loaded with tumor-restricted RNA and CA19-9 peptide⁷². Furthermore, transfection of IL-2 genes into CIK cells to enhance its IL-2 production potentiated their cytotoxicity against a pancreatic cancer cell line following coculture with DC when compared to non-IL-2-transfected CIK cells⁷³. The expression of HLA-class I and II, along with the expression of the co-stimulatory molecules CD40, CD80 and CD86 on DC was up-regulated following

coculturing with CIK cells. Moreover, the proliferation of CIK cells and their expression of the CD3, CD4, CD28 and CD40L molecules were increased following coculturing with DC⁷¹.

Murine studies

Studies have been performed to establish whether CIK might be clinically useful. Animal models with SCID mice were initially used to test the *in vivo* antitumor activity of CIK cells. The first reported data demonstrated the antitumor activity against lymphoma cells. They described how CIK cells were able to effectively purge tumor-contaminated bone marrow, resulting in the increased survival of recipient animals with the majority of them showing no signs of tumor growth for more than 100 days after the injection⁴¹. Following studies demonstrated how the adoptive infusion of CIK cells significantly prolonged the survival of SCID mice that received grafts of human B-cell lymphoma cells (SU-DHL-4), compared with both untreated controls and mice infused with equal amounts of LAK cells. A potent CIK activity was achieved with as few as 1×10^7 cells and found to be independent of exogenous IL-2 administration but seemed to require the active proliferation of CIK cells since their activity was abrogated by irradiation (15 cGy). The beneficial effect of CIK cells was maintained regardless if the infusion was performed intravenously or intraperitoneally⁴³. Also in a syngeneic bone marrow transplantation model, the infusion of CIK cells significantly prolonged the survival of lymphoma-bearing mice. One possible limitation to the value of such studies is that the effector cells were

not MHC matched to the tumor target; thus, alloreactivity could have accounted for the cytotoxicity. To rule out the possible role of allogeneic stimulation, further experiments demonstrated that CIK cells were fully capable of killing autologous CML blasts after engraftment in SCID mice⁵⁶.

CIK cells were demonstrated to be safe in bone marrow transplantation, because there was minimal cytolysis against normal syngeneic bone marrow. Cytotoxicity against allogeneic bone marrow occurred, but was consistently less than the one observed against tumor targets⁷⁴.

Granule exocytosis (granzyme/perforin) and cell surface apoptosis-inducing ligands (FASL, TRAIL and TNF- α) are the two main pathways used by cytotoxic T cells to induce tumor cytolysis⁷⁵. CIK cells generated from mice strains deficient in FASL were fully capable of exerting in vitro cytotoxicity, while CIK cells expanded from perforin-knockout mice had lost their tumor killing ability. Similarly, the adoptive infusion after allogeneic HCT of CIK cells generated from FASL-deficient or wild-type mice protected the animals from a lethal dose of lymphoma cells, while no tumor protection was observed following the infusion of perforin-deficient CIK cells⁷⁴.

With the availability of the bioluminescence imaging (BLI) technology, the in vivo functional activities of CIK cells could be visualized in a real-time fashion in mice inoculated with bioluminescent gene-transfected tumor cells and treated with CIK cells for cellular immunotherapy. To monitor tumor regression by BLI, mice were implanted intraperitoneally with HeLa-*luc* (a

luciferase gene-transfected human cervical carcinoma cell line) and subsequently treated with CIK cells. Mice treated with CIK cells had significant tumor regression or complete eradication compared to saline-treated mice. Similar tumor response was visualized for murine lymphoma cell lines⁷⁶. Using the same strategy, CIK cells were transfected with the *gfp/luc* genes to visualize their trafficking by BLI. Following injection, it was observed that *luc*⁺ CIK cells first reached the lungs within 30 minutes, followed by a general distribution to other sites of the body. By the 7th hour, a population of the labeled CIK cells migrated to the tumor sites and remained detectable at these sites for an additional 9 days with resultant tumor regression⁷⁷.

CIK cells preferentially localized to tumor sites expressing NKG2D ligands and their killing activity was inhibited by the *in vivo* administration of NKG2D blocking antibodies. In the same model it was demonstrated that there was a low expression of NKG2D ligands at GVHD target organ sites, further evidence of the low GVHD risk associated with CIK cells⁷⁸.

Clinical studies

CIK cells can be generated successfully from healthy donors, as well as, from patients treated with chemotherapy for various malignancies and undergoing peripheral blood progenitor cell (PBSC) leukapheresis⁴⁹. Feasibility of large-scale expansion was also reported for cord blood and even from washout of leftover mononuclear cells from cord blood unit bags⁵⁰. The relatively robust

and simple cell culture procedures to expand CIK cells have enabled this approach of adoptive cellular immunotherapy to be increasingly studied. Several phase I trials to test the clinical efficacy of CIK cells on a small number of patients have been reported. The first clinical trial using CIK cells was reported in 1999 by Schmidt-Wolf et al. in Germany using autologous CIK cells electroporated with IL-2 genes for infusion into 10 patients with metastatic renal carcinoma, colorectal cancer, and lymphoma. Circulating CIK cells persisted for up to two weeks after the infusion and an increase in serum levels of IFN- γ , GM-CSF and TGF- β was observed along with an increased cytotoxic activity of total peripheral blood lymphocytes. One patient with follicular lymphoma showed complete response. No major side effect was observed, except for 3 patients who developed fever that spontaneously resolved⁷⁹.

Negrin's group at Stanford reported the treatment with autologous CIK cells of 9 patients with relapsed HD and NHL post autologous transplantation. Besides demonstrating the feasibility of large-scale expansion of CIK cells and the absence of adverse reaction, this trial achieved 2 partial responses and 2 stabilization of disease in the recipients⁸⁰.

An increasing amount of studies dealing with the treatment of patients with solid tumors with CIK cells has been published, with many studies performed in Asia. Unfortunately, these studies are often published only in Chinese.

In one report, patients with hepatocellular carcinoma (HCC) who achieved complete remission with transcatheter arterial chemoembolization and radiofrequency ablation were divided into

two groups, where intrahepatic arterial CIK cell infusion was given to a study group of 45 patients. Relapse at 1.5 years occurred in 31.1% of patients in the CIK group as compared to 85% in the control group, with the difference mainly accounted for by the significantly lower relapses seen at local sites (15.56% vs 65%, respectively)⁸¹. A randomized trial of adjuvant autologous CIK cells after resection of hepatocellular carcinoma showed a significant increase in disease-free survival, while no statistically significant differences were observed in overall survival⁸². Furthermore, an interesting observation was the effect of CIK cells treatment on the HBV viral load in patients with HCC. Shi et al showed a measurable decrease in viral load after one month following CIK treatment⁸³⁻⁸⁵. A recent Phase I study from Italy reported on 12 patients with advanced NHL, metastatic renal cancer or HCC. Along with a very favorable toxicity profile, the adoptive infusion of autologous CIK cells resulted in three complete responses and two stabilizations of disease. Two of the complete responses were observed in metastatic renal cancer and HCC. These patients received the simultaneous subcutaneous injection of low dose IL-2 and IFN- α respectively⁸⁶.

In the allogeneic setting, the first clinical application of CIK cells after allogeneic HSCT in a Phase I trial was reported by our group. Eleven patients with refractory haematological malignancies received the adoptive infusion of donor CIK cells and successfully demonstrated the feasibility and the low toxicity profile of this approach. As often occurs in such settings where other salvage therapies are being used concurrently, it was difficult to assess the efficacy. Nevertheless, 3 patients (1 each with myelodysplastic

syndrome, HD, and AML) achieved measurable response, in terms of improvement in donor chimerism or clearance of disease that could be solely attributable to donor CIK cell infusions. One patient had stable disease, one had hematologic improvement, whereas disease progression and death occurred in six patients. Similarly, GVHD rate was not higher than that of unmanipulated DLI, with grade I and II acute GVHD in 4 patients, 2 of whom progressed to extensive chronic GVHD ⁸⁷. In a similar setting, preliminary data reported by the Stanford University group confirmed the feasibility of adoptive CIK cells infusions and showed evidence of GVT effects with clinical activity. In this report, 10 patients with various haematological malignancies relapsing after allogeneic transplant received cytoreductive treatment followed by donor CIK cell infusions in escalating doses up to 1×10^8 CD3/kg. They reported an event-free and overall survival of 20% and 76% respectively. Grade I acute GVHD occurred in 1 patient and limited chronic GVHD in 2 patients⁸⁸.

The main clinical studies with CIK cells, including both allogeneic and autologous settings, are summarized in Table 2. These early results appear very promising and will hopefully lead to more large and controlled clinical trials in these settings.

Disease	Patients	Type of CIKs	Toxicity	GVHD	Clinical responses
Relapsed AML; HD; CML; ALL; MDS;	11	Allogeneic	None relevant	Acute: 4 (Grade I-II) Chronic: 2	CR (3); SD (1)
Relapsed AML; NHL; MM; HD	10	Allogeneic	*Ventricular arrhythmias: 2 (Grade 3-4)	Acute: 1 (Grade I) Chronic: 2	1 year EFS 20% 1 year OS 76% TTP 90 days (9 - 577)
Colorectal and renal carcinoma; NHL	10	Autologous	Fever: 3	NA	CR (1) SD (3)
Relapsed HD; NHL	9	Autologous	Fever: 1 Mild hypotension: 1	NA	PR (2); SD (2)
NHL; renal carcinoma; HCC	12	Autologous	Fever: 2	NA	CR(3); SD (2)
Advanced NSCLC	59	Autologous (+ Chemotherapy)	None relevant	NA	Increased PFS and OS compared with control group (chemotherapy alone)
Resected HCC (adjuvant setting)	127 (randomized)	Autologous	Fever: 5	NA	Increased DFS compared with control group (no adjuvant treatment)
HCC (adjuvant setting)	85 (randomized)	Autologous	None relevant	NA	Decreased recurrence rate compared with controls (no adjuvant treatment)
Gastric cancer (Stage IV)	57 (randomized)	Autologous (+ chemotherapy)	None relevant	NA	Decreased tumor markers Improved QOL Increased 2 year life-span (compared with chemotherapy alone)
HCC	13	Autologous	Transient fever (most patients)	NA	Reduced tumor volume (3) Improved symptoms Decreased HB-V-DNA load

*One of the two patients experienced also transient elevation of transaminases. One more patient experienced ventricular arrhythmia and transient hypertension during the infusion of CIK cells.
 ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; CML: Chronic myeloid leukemia; CR: Complete response; DFS: Disease-free survival; EFS: Event-free survival; HCC: Hepatocellular carcinoma; HD: Hodgkin's disease; MDS: Myelodysplastic syndrome; MM: Multiple myeloma; NA: Not applicable; NHL: Non-Hodgkin's lymphoma; OS: Overall survival; PFS: Progression-free survival; PR: Partial response; QOL: Quality of life; SD: Stable disease; TTP: Time to progression

Table 2. Clinical studies with the adoptive infusion of CIK cells. (From Sangiolo D et al, Expert Opin Biol Ther, 2009 9: 831-840)

1.2.3 Alloreactivity and GVHD potential of CIK cells

GVHD is the most frequent and severe complication associated with the adoptive infusion of allogeneic lymphocytes after HSCT. Since from early studies, CIK cells appeared to be endowed with a reduced alloreactive potential, compared with conventional T cells, making an appealing and promising alternative to classic DLI. Initial observations came from in vitro studies and were subsequently confirmed by preclinical animal models that helped to highlight crucial mechanisms responsible for the reduced GVHD potential.

The first studies to assess the alloreactivity of CIK cells were based on in vitro mixed lymphocyte reactions (MLR) across major MHC-barriers. Expanded murine CIK cells, in contrast to fresh naïve splenocytes, did not exhibit increased proliferation when cultured with irradiated MHC-mismatched stimulators⁷⁴. In these experiments however, it was difficult to detect the real contribution to proliferation given by the allogeneic stimulation since CIK cells were already actively proliferating under the influence of IL-2. Recently similar experiments have been reported with human CIK cells tested in HLA-mismatched MLR but with low dose of IL-2. It has been shown how CIK cells, when tested as a bulk population, maintained an alloreactive proliferation similar to that observed with fresh lymphocytes. If tested separately, it was clear how the majority of the observed proliferation was due to the CD3⁺CD56⁻ subset while CD3⁺CD56⁺ cells showed only minimal alloreactive capacity⁴⁴.

To assess if the reduced alloreactivity observed in the MLR assays could result in a reduced GVHD risk, the adoptive infusion of

CIK cells was first tested in murine experimental models of HSCT. Bone marrow from C57BL/6 mice was used to reconstitute lethally irradiated BALB/c mice; subsequently increasing numbers of either C57BL/6 derived CIK cells or naïve splenocytes were adoptively infused. Compared with naïve splenocytes, CIK cells infused across MHC-barriers caused minimal GVHD, persisting into the peripheral circulation up to 3 weeks after the infusion and mediating effective GVT⁷⁴. The reduced incidence of GVHD could be explained if the infused CIK cells have a very limited lifespan after the prolonged ex vivo expansion. It is true that CD3⁺CD56⁺ cells present a terminally differentiated late effector phenotype and are endowed with a limited proliferative potential⁵². The CD3⁺CD56⁻ subpopulation, however, represents a considerable percentage of the final bulk population of CIK cells and exhibits an earlier effector phenotype with a greater proliferative capacity^{44,52}. The phenotype of infused cells could thus not be the only explanation for the low GVHD incidence, and a crucial role seemed to be played by certain cytokines produced by CIK cells. Attention has been focused in particular on the abundant production of IFN- γ , spontaneously occurring in expanded CIK cells, and known to be protective against GVHD⁸⁹. Further evidence in support of this hypothesis has been found in an elegant HSCT preclinical experiment, in which CIK cells, generated from IFN- γ knockout mice, rapidly induced lethal GVHD when infused across MHC-barriers, in contrast to the wild-type counterpart that confirmed a minimal GVHD potential⁹⁰.

Recently, HSCT murine models with the infusion of luciferase-expressing CIK cells allowed visualizing the traffic and fate

of these cells in allogeneic HSCT recipients and operating a direct comparison with conventional T cells responsible for GVHD. Infused CIK cells displayed an early homing to spleen and lymph nodes with an expansion peak within the first week. This pattern was similar to that observed with fresh lymphocytes that, however, had a more rapid signal increase and resulted in the death of all recipient mice due to acute GVHD. Allogeneic CIK cells trafficked through the same GVHD target organs but more transiently and with much less infiltration compared with conventional T cells⁷⁸. Furthermore, CIK cells, compared with conventional T cells, demonstrated a significantly lower acquisition of homing molecules, required for the entry of inflamed and GVHD target organs ($\alpha 4\beta 7$, CCR9, E-selectin, CXCR3 and CCR5), and a higher susceptibility to apoptosis. The same experiments confirmed a high and stable production of IFN- γ from CIK cells⁷⁸. Interestingly, with a similar murine model, but in a minor-mismatched setting, it was demonstrated for the first time that proliferation and spreading of CIK cells may be driven also by differences in minor histocompatibility antigens. The observed pattern was similar to that observed in major-mismatched settings but with a lower speed of tissue propagation and a reduced intensity in the peak of photon emission. These findings are particularly interesting because they could be representative of the clinical settings, where the majority of HSCT is performed between HLA-identical siblings that display differences only at the minor antigen level.

Early clinical applications of CIK cells-based adoptive immunotherapy after allogeneic HSCT confirmed the feasibility of

this approach and the reduced propensity of these cells to cause GVHD. Following repeated infusions of donor-derived CIK cells, in patients with hematological malignancies who relapsed after HSCT, our group reported 4 to 11 (36%) cases of acute GVHD (\leq grade II). Three episodes occurred after HSCT from HLA-identical siblings and one after HSCT from a HLA-matched unrelated donor; two cases progressed to extensive chronic GVHD⁸⁷. A similar low incidence of GVHD was described in a preliminary report from the Stanford group (one grade I acute and two limited chronic GVHD events) of ten patients, with relapsed hematological malignancies after allogeneic HSCT, treated with infusions of donor CIK cells⁸⁸.

1.2.4 Novel Development

An exciting development that promised to broaden the application of CIK cell was reported over recent years.

Bispecific antibodies (bsAb) are artificial proteins that carry two different antigen-binding sites. By virtue of their dual specificity, bsAb can trigger effector cells, via a membrane receptor, and at the same time link them to a tumor cell, via tumor-associated antigens. This interaction may lead to increase effector cell targeting and enhance antitumor specificity.

Different works demonstrate that bsAb can be used to increase the modest cytotoxicity of CIK cells against epithelial tumor cell lines expressing different tumor-associated antigens. Primary ovarian carcinoma cells are resistant to CIK-mediated lysis. However, it has been recently demonstrated that the addition of bispecific

antibodies CD3xCA125 or CD3xHer2 (heteroconjugates of anti-CD3 with anti-CA125 or Her2) could efficiently target the CD3+ CIK cells to ovarian carcinoma bearing the specific ovarian tumor antigens and overcome the resistance. This strategy was shown to be effective against autologous primary ovarian tumor cells in vitro and in ovarian tumor-bearing mice. The survival of mice with ovarian carcinoma treated with CIK cells redirected by the bsAb was prolonged compared to control mice treated with CIK cells alone^{91,92}. In an attempt to treat the Ewing's family tumors, the low levels of cell surface Her2/neu expression were employed to redirect ex vivo activated CIK cells to tumor targets, using the bsAb CD3xHer2/neu⁹³. Epithelial cell adhesion molecule (EpCAM) is another tumor-associated antigen highly expressed on many epithelial carcinomas, like gastrointestinal malignancies and non-small-cell lung cancer. Cytotoxicity of CIK cells against a colorectal tumor cell line can be enhanced by the addition of a bsAb directed against EpCAMxCD3⁹⁴. Redirection of CIK cells to tumor target by CD3 leads to an increase in both CIK cells proliferation and apoptosis⁹⁵. BsAb CD5xCD19 increases cytolytic activity of CIK cells against CD19 positive B cell lymphoma in a similar extent to that observed with bsAb CD3xCD19. Therefore, the use of CD5 for CIK cell binding and redirection prevents activation-induced cell death and may thus lead to longer survival of CIK cells in vivo⁹⁶.

Besides using bsAb, other methods of harnessing antigen-antibody affinity to redirect CIK cells to target tumor cells involved the transfection of CIK cells to express tumor specific chimeric receptors (CARs). CARs are, in fact, artificial T cell receptors

constituted by a specific antigen-binding domain (the extracellular domain, consisting of the single-chain variable fragments of a monoclonal antibody), and a signaling region (the intracellular domain), usually taken from the zeta-chain of the TCR/CD3 complex, linked together through a hinge region⁹⁷.

CIK cells are known to be inactive against B ALL targets in vitro, but CIK cells engineered to express the CAR specific for the CD19 antigen could redirect CIK cells to localize into leukemia-infiltrated tissues and become cytotoxic towards CD19-expressing B ALL targets⁹⁸. In particular, anti-CD19 chimeric receptors delivering primary (by CD3 ζ) and costimulatory (by 4-1BB) signals render CIK powerfully cytotoxic against B ALL targets and induce secretion of immunostimulatory cytokines and proliferation⁹⁹. Tumor killing by CIK cells transduced with ζ -CD19 chimeric receptor is predominantly mediated via the perforin/granzyme pathway and is independent of death receptors signaling in primary B ALL¹⁰⁰.

Yoon SH et al modified CIK cells by electroporation with RNA encoding a chimeric immune receptor containing anti-Her2 single-chain variable fragment joined to intracellular portion of CD28 and CD3 ζ in order to redirect CIK to Her2 over-expressing cancer targets specifically. CAR-transduced CIK showed cytotoxicity specific to tumor cell lines expressing Her2 and their adoptive transfer in ovarian cancer xenograft nude mice model led to significant inhibition of tumor growth compared with mock-transduced CIK¹⁰¹. Recently, CIK cells activity against AML has been improved by genetic modification with CAR specific for the CD33 myeloid antigen¹⁰².

Another innovative genetic modification of CIK cells was the transfection of CIK cells with oncolytic viruses, which have the ability to infect only transformed cells. A modified double-deleted vaccinia virus (vvDD) was able to infect CIK cells without affecting its activity. CIK cells were employed as a carrier vehicle bringing the oncolytic virus to tumor cells through the NKG2D receptor which is highly expressed by CIK cells to its ligands MICA and MICB on the tumor cells. VvDD-carrying CIK cells migrated to the tumor sites where the oncolytic viruses were then released to specifically lyse tumor cells. This approach has been successfully shown to work using mouse models for human ovarian tumor and murine breast carcinoma, resulting in reduction of tumor burden and prolongation of survival^{103,104}.

Advances in genetic engineering technologies could further broaden the potential clinical application of CIK cells if the challenge to conform these genetic engineering technologies to Good Manufacturing Practice (GMP) compliance could be overcome.

1.3 Anti-CD20 monoclonal antibodies

Anti-CD20 monoclonal antibodies were the first mAbs to be used for the treatment of cancer. CD20 is a cell-surface marker expressed on mature B cells and most malignant B cells. It was chosen as a target for antibody therapies as it is expressed at high levels and it is not internalized or secreted into circulation after antibody binding. Anti-idiotypic antibodies were first used in the early 1990's with some encouraging results. However, these studies were hampered by the immunogenicity of these murine antibodies. As technology evolved, chimeric antibodies were developed with the emergence of rituximab in animal studies and early phase clinical trials. Most lymphoma patients now receive rituximab at some point in their treatment^{105,106}.

In vitro anti-CD20 mAbs have been shown to act via three distinct mechanisms: complement-dependent cytotoxicity (CDC), antibody dependent cell mediated cytotoxicity (ADCC), growth arrest and apoptosis in certain cell lines (Figure 6)^{107,108}. The last three decades have seen considerable progress in the understanding of the structure and function of the CD20 molecule and in the development of new engineered anti-CD20 mAbs with enhanced functional activities that may lead to superior efficacy¹⁰⁹.

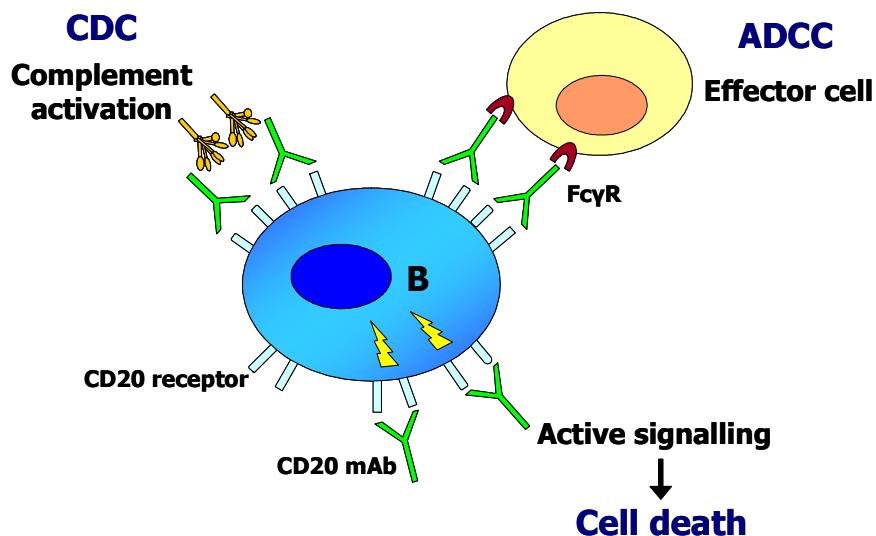


Figure 6. Potential mechanisms of action of CD20 mAb. The three potential mechanisms of action of CD20 mAb are shown. (a) CD20 mAb binding results in clustering of Fc domains; this allows binding of C1q and activation of the classical complement pathway. (b) CD20 mAb bound to B cells can directly recruit effector cells via the Fc γ R receptor family. This results in phagocytosis of CD20 mAb bound cells. (c) CD20 mAb in vitro, in the presence of crosslinking, induces cytosolic Ca $^{2+}$ flux and can induce apoptosis.

1.3.1 The CD20 antigen

CD20 was first identified over 30 years ago and it was one of the first B-cell markers to be isolated. CD20 is first expressed at the pre-B-cell stage during B cell development, maintained throughout B-cell maturation, and lost during final maturation to plasma cells. B-cell activation results in an additional increase in CD20 expression¹⁰⁸. The CD20 gene (*MS4A1*) is located on human chromosome 11q12-13

and is in close proximity to the breakpoint site of t(11:14) translocation seen in some lymphomas¹¹⁰. The *MS4A1* promoter contains potential sites for several transcription factors such as Oct-1, Oct-2, PU.1 and Pip. The mechanisms controlling CD20 expression are poorly understood. CD20 mRNA is transcribed from a single copy gene and is 16 kb long, consisting of eight exons. Two mRNA isoforms, consisting of a more predominant form of 2.6 kb and a less predominant form of 3.3 kb, have been described as products of an alternative splicing mechanism¹¹¹.

The CD20 protein is a non-glycosylated 33 to 37-kDa integral membrane phosphoprotein. It has four transmembrane domains with both the amino and carboxy termini of the protein located within the cytoplasm, and only a single forty-four amino-acid extracellular loop between the 3rd and the 4th transmembrane domain, containing a disulfide bond important for rituximab binding^{111,112}. This configuration, deeply anchored in the membrane, protects from antigenic shedding. Circulating “soluble forms” of CD20 have been detected in the serum of patients with chronic lymphocytic leukemia and high leukemic cell counts¹¹³.

B lymphocytes express three CD20 protein isoforms (33, 35, 37 kDa) resulting from multiple phosphorylation of serine and threonine residues located in the cytoplasmic domain of the CD20 protein. This implies that CD20 is highly regulated by phosphorylation. The 33 kDa protein is the predominant isoform (75% to 80%) while the 35 kDa protein represents only 20% to 25% of total CD20¹¹¹. CD20 is highly phosphorylated in malignant B-cells and nonphosphorylated in nonproliferating B-cells, suggesting that

phosphorylation is associated with proliferation¹¹⁴. In the plasma membrane, the CD20 protein is organized into multimers, usually dimers or tetramers, and has been reported to be closely associated with other proteins, in particular the transmembrane adapter protein p75/80, also named C-terminal Src kinase-binding protein (Cbp), CD40 and MHC class II (Figure 7)^{115,116}.

It has been shown that monoclonal antibody binding to CD20 induces rapid translocation of the molecule to lipid rafts, which are membrane microdomains enriched in cholesterol and sphingolipids. These lipid rafts are platforms for signal transduction, allowing the colocalization of receptors and signaling effectors. The presence of Cbp in association with CD20 in lipid rafts could concentrate a C-terminal Src kinase (Csk) such Lyn, Fyn or Lck, which phosphorylates Cbp, allowing autophosphorylation of Csk and thus inducing kinase activity^{117,118}.

So far, no CD20 ligand has been identified and CD20 does not display the usual structure of a receptor. CD20-deficient mice do not have any obvious defect of B-cell function¹¹⁹. Therefore, the exact function of CD20 remains largely unknown. Some data indicated that CD20 could play an important role in calcium influx across the plasma membranes, sustaining intracellular calcium concentration and allowing activation of B cells.

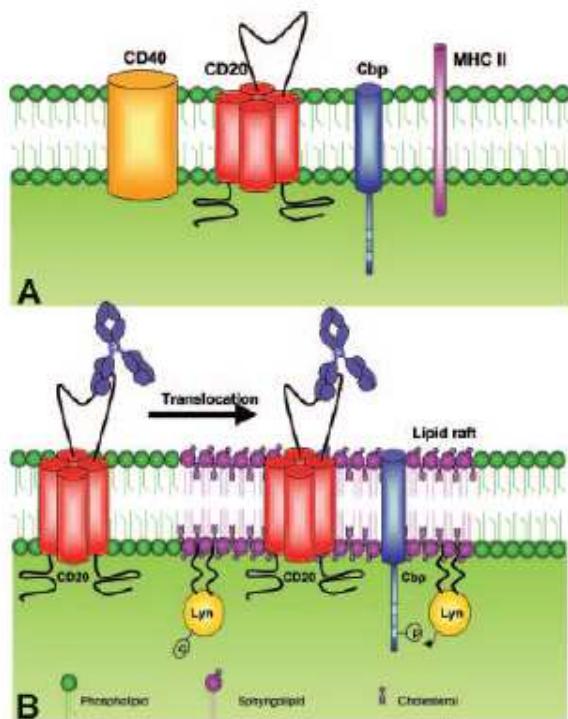


Figure 7. CD20 antigen structure and organization into membrane bilayers. (a) The CD20 protein has 4 cell membrane-spanning domains with both the amino and carboxy termini of the protein located within the cytoplasm. CD20 is closely associated with other proteins, in particular the C-terminal Src kinase-binding protein (Cbp), CD40, and MHC II. (b) Antibody binding to CD20 induces rapid translocation of the molecule to lipid rafts. Cbp can concentrate the C-terminal Src kinases (Csk) such as Lyn, Flyn, or Lck, which phosphorylate Cbp, leading to induction of kinase activity. (From Cartron G. et al.; *Blood*; 2004 104: 2635-2642)

1.3.2 Rituximab

Rituximab (MabThera, Rituxan) is a chimeric mAb, produced by recombinant technology, specifically targeting the CD20 antigen. Rituximab was created by fusing the light- and heavy-chain variable domains of 2B8, a murine monoclonal anti-CD20 antibody, and human κ light-chain and γ1 heavy-chain constant regions¹²⁰. Rituximab is currently indicated in both follicular and aggressive B-cell NHL. It is the first approved targeted treatment in the field of oncology and its impact on the treatment of NHL is evidenced by the short interval between its initial description and its approval by both American and European authorities (1997 and 1998, respectively). Rituximab has revolutionized the management and treatment of B-cell malignancies, increasing the median overall survival of patients with many of these diseases. In combination with conventional chemotherapy such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), it has significantly improved response rates and progression-free and overall survival of patients with diffuse large B-cell lymphoma or follicular lymphoma^{105,106}. In addition to B-cell malignancies, there is a growing interest in the use of rituximab for the treatment of autoimmune conditions, and in particular rheumatoid arthritis (RA) and thrombotic thrombocytopenic purpura (TP). Although the clinical effectiveness of rituximab is no longer in question, its in vivo mechanisms of action have yet to be elucidated. In vitro data suggest that it induces apoptosis, CDC and ACDD, and some evidence suggest that the

immune modulating properties of this antibody are responsible for its efficacy in vivo¹⁰⁷.

Complement dependent cytotoxicity

Rituximab was originally shown to be capable of binding C1q and inducing complement activation leading to cell lysis¹²⁰. Complement dependent cytotoxicity (CDC) is an important effector mechanism in the eradication of foreign agents and neoplastic cells. The complement system consists of classical, lectin and alternative pathways that converge and ultimately generate the same set of effector molecules (Figure 8). The classical pathway requires immunoglobulins, whereas the lectin and alternative pathways are activated primarily by microbial components. The first step in activation of the classical pathway is the binding of the C1q component to Fc portions of target-bound IgG or IgM. This binding triggers a proteolytic cascade resulting in the cleavage of soluble C3 and in the generation of large amounts of C3b fragment, the main effector molecule of the complement system. C3b molecules act as opsonins but also bind to the C3 convertase to form a C5 convertase, leading to the generation of the membrane attack complex (MAC) which forms membrane pores leading to cell lysis through osmotic shock. C3 is abundant in plasma and newly generated C3b is rapidly inactivated unless bound to the C5 convertase complex. C3b can also bind to complement receptors (CRs) expressed on effector cells such as granulocytes, macrophages, or NK cells and induce cell-mediated lysis or phagocytosis, depending on the effector cell complement-

dependent cellular cytotoxicity (CDCC)^{10,11}. CD20 seems to be an excellent target for CDC against numerous cell types in vitro probably, at least in part, because of its high expression and the proximity of the mAb-binding-epitope to the plasma membrane. Furthermore, rituximab's ability to redistribute CD20 into lipid drafts appears to cluster the mAb and greatly enhances its ability to capture C1q and elicit CDC¹¹⁴.

There is conflicting literature on the importance of complement in CD20 mAb activity. Several in vitro studies have demonstrated that rituximab induces CDC in B-lymphoma cell lines¹²⁰ and fresh B-lymphoma cells¹²¹⁻¹²³ and this complement-mediated lysis correlates in part with the level of CD20 antigen expression^{122,124}. The sensitivity to CDC induced by rituximab could be different by subtype of lymphoma¹²⁴. Support for CDC as a key effector mechanism comes from studies in vivo demonstrating that: 1) expression of complement regulatory proteins CD46, CD55 and CD59 is associated with rituximab resistance¹²⁵; 2) complement depletion rapidly occurs in vivo following rituximab infusion and is implicated in infusion related toxicity¹²⁶; 3) replacement of the consumed complement restores the activity of rituximab in CDC assays and might benefit patients¹²⁷. Similarly, in support of this, there are several murine models of lymphoma, in which the role of complement was shown using either C1q knockout animals or C3 depletion in vivo by cobra venom factor^{128,129}. In contrast, other murine models investigating depletion of normal B-cells by CD20 antibodies have indicated no role of the complement¹⁰⁹. Clinical evidence is still lacking in humans to establish a relationship between

complement activation and therapeutic efficacy. A clinical study found that expression levels of complement regulatory proteins failed to predict the clinical outcome of rituximab treatment¹³⁰. Complement regulatory proteins, among which the most important are CD46, CD55 and CD59, are a family of glycosylphosphatidylinositol-anchored proteins that inhibits complement system to prevent host tissue damage. CD55 (decay-accelerating factor, DAF) accelerates the decay of C3 and C5 convertase and CD46 (membrane cofactor protein, MCP) acts as a cofactor for the cleavage of C3b. CD59 prevents pore formation by the MAC¹⁰.

Recent evidence suggests that complement activation can have, on the contrary, a detrimental effect on the antitumor activity of rituximab *in vivo*. First, Beum et al showed that deposition of active complement components facilitated the removal of rituximab-CD20 complexes from the lymphoma cells by FcR-expressing macrophages through the process of shaving, a phenomenon which seems to be exacerbated by the addition of C3b¹³¹. Second, Wang et al showed that complement activation by rituximab resulted in decreased ADCC due to C3 deposition onto the target-bound antibody and reduced binding of opsonized targets to FcγR on NK cells^{132,133}.

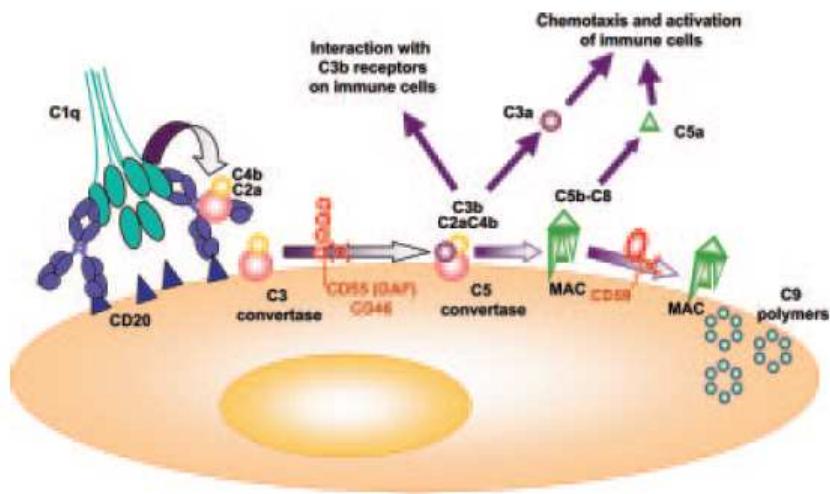


Figure 8. CDC classical pathway (IgG1, IgG3, IgM). The binding of the C1q component to Fc portions of IgG or IgM triggers a proteolytic cascade, resulting in the generation of large amounts of C3b. C3b molecules act as opsonins but also bind to the C3 convertase to form a C5 convertase, leading to the generation of the membrane attack complex (MAC), which kills the target. (From Cartron G. et al.; *Blood*; 2004 104: 2635-2642)

Antibody dependent cellular cytotoxicity

ADCC (antibody-dependent cellular cytotoxicity) is an important effector mechanism in the eradication of intracellular pathogens and tumor cells. The CD20 protein is not shed from the cell surface and does not internalize following antibody binding and therefore appears to be an ideal target for the recruitment of effector cells expressing Fc γ receptors (Fc γ Rs) that bind Fc portion of immunoglobulins. There are 3 classes of Fc γ Rs: Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16). Fc γ Rs can transduce activating signals

through immunoreceptor tyrosine-based activation motifs (ITAMs), or delivery inhibitory signals through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Immune complexes can engage activating and inhibitory Fc γ R on the same cell, providing an opportunity of fine-tune cell activation. The main inhibitory Fc γ R is the single chain Fc γ RIIb, whereas most Fc-dependent stimulatory signals are transduced by Fc γ RI and Fc γ RIIIa (also known as CD16A), both of which require an accessory ITAM-containing γ -chain to initiate signal transduction. Fc γ RI is a high-affinity receptor expressed by macrophages, dendritic cells, neutrophils and eosinophils. Fc γ RIIIa is the primary activating Fc γ R expressed by NK cells, dendritic cells, macrophages and mast cells and is required for NK cell-mediated ADCC. Fc γ RIIIb (also known as CD16B) is a glycophosphatidylinositol (GPI)-anchored protein that, unlike Fc γ RIIIa, does not contain the common γ -chain and is exclusively expressed on human neutrophils (Table 3). Binding of IgG to target cell antigens enables the recognition of these targets by immune effector populations that express Fc γ R receptors, such as NK cells, neutrophils, mononuclear phagocytes and dendritic cells. These cells are then directed toward target cells, inducing either phagocytosis or release of their cytotoxic granules, cytokines, chemokines, proteases and reactive oxygen species, to promote cell killing^{10,11}. Data from both laboratory models and correlative clinical studies suggest that ADCC plays a significant role in the antitumor effects of rituximab¹³⁴. In vitro studies have shown that rituximab induces ADCC in human lymphoma cell lines¹²⁰. Clynes et al showed that the therapeutic effect of rituximab is lost in Fc γ -receptor knockout mice¹³⁵.

Human *FCGR3A* displays a nucleotide polymorphism at nucleotide 559 resulting in an amino-acid substitution at position 158 with either a phenylalanine (F) or a valine (V). This substitution influences the affinity of Fc γ RIIIa for IgG1 with a higher affinity for Fc γ RIIIa-158 VV NK cells¹³⁶. In clinical investigation, different studies have shown that single-agent rituximab is more effective in patients with CD16 polymorphisms associated with higher affinity for human IgG^{137,138}. Patients homozygous for the V158 polymorphism (VV) on CD16 have higher clinical response rates to rituximab than patients who are carriers for F158 (FF or VF), suggesting that ADCC plays a key role in the therapeutic effect of rituximab. Phagocytosis of human rituximab-opsonized lymphoma cells by macrophages has also been demonstrated in vitro¹²⁴.

Cytokines such as IL-2, IL-12, IFN- α , GM-CSF, and G-CSF are known to increase ADCC/phagocytosis by stimulation or expansion of NK cells and macrophages. For example, IL-2 increases ADCC by NK cell expansion and activation¹³⁹, and a correlation between NK cell counts and clinical response was found in a clinical trial combining IL-2 and rituximab in patients with NHL.

	Fc γ RI	Fc γ RIIa	Fc γ RIIb	Fc γ RIIc	Fc γ RIIIa	Fc γ RIIIb
Monocytes/ macrophages	+	+	+	—	+	—
NK cells	—	—	—	+	+	—
Neutrophils	+/-	+	—	—	—	+
B lymphocytes	—	—	+	—	—	—
Dendritic cells	+	+	+	—	+	—
Mast cells	+/-	+	+	—	—	—
Platelets	—	+	—	—	—	—

Table 3. Fc γ receptor expression according to cell type. (From Cartron G. et al.; *Blood*; 2004 104: 2635-2642)

Intracellular signalling and induction of apoptosis

It has also been proposed that mAb binding of CD20 can directly transmit intracellular signals that lead to programmed cell death (PCD)^{114,140}. This was based on early observations of changes in cell growth, including growth arrest with anti-CD20 mAb¹⁴¹. Since then PCD has been demonstrated with a range of lymphoma cell lines, but rarely on primary tumors, and has generally been shown to depend on further anti-CD20 mAb crosslinking¹⁴². Furthermore, not all B-cell lines are sensitive and the cell death pathway evoked is clearly cell-line and stimulus dependent, apparently varying with both the mAb chosen and the degree of hyper-crosslinking delivered. When rituximab is sufficiently cross-linked it is capable of eliciting potent apoptotic responses in sensitive cell-lines via the intrinsic mitochondrial pathway¹⁴³. However, cell death induced by non-hyper-crosslinked anti-CD20 mAb appears to be non-apoptotic and varies considerably depending on the mAb used, rituximab being relatively weak at inducing PCD.

Mechanisms of resistance to rituximab

Despite the expression of antigens such as CD20, patients may not have a response to antibody therapy or resistance to the therapy may develop. Resistance mechanisms have been related to a decrease in effector cells in immune compromised individuals to induce

ADCC, or an increase in complement inhibitors that would alter the efficacy of CDC mediated cell killing. In addition, alterations in apoptotic pathways and inadequate blood levels of rituximab have been suggested. Another interesting mechanism of resistance that has been suggested is loss of CD20 expression after treatment with rituximab¹⁰⁷.

The efficacy of rituximab in lymphoma therapy varies according to tumor burden and subtype. Although follicular lymphoma and diffuse large B-cell lymphoma are relatively sensitive, B-chronic lymphatic leukemia (B-CLL), mantle cell lymphoma (MCL) and small lymphocytic lymphoma are significantly less, such that dose escalation with approximately 6-times more mAbs is required for significant clinical activity. CLL cells have the lowest level of CD20 molecules (8,000 per cell), while other lymphomas have shown over 100,000 per cell, and this may explain why CLL patients have a poor response to rituximab. Beum et al. have showed that CD20 loss following rituximab treatment is mediated by shaving/loss of the rituximab-bound CD20 molecules by monocytes. This shaving reaction was shown in CLL patients and once CD20 levels were below a certain threshold, rituximab was subsequently inefficient at reducing the levels of B-cells¹³¹. However, loss of CD20 cannot fully explain the resistance of rituximab seen in all lymphoma subsets, as both follicular lymphoma and MCL have high levels of CD20 but MCL patients have a significantly poorer response rate to rituximab compared to follicular lymphoma. Probably a combination of resistance factors influence different disease subsets underlying a

growing need to explore these mechanisms in each type of lymphoma in both *in vitro* and *in vivo* models.

1.3.3 Future anti-CD20 antibodies

The success of rituximab has stimulated considerable efforts to develop improved reagents. New waves of CD20 mAbs are in clinical development with the promise of improving outcomes of B-cell malignancies even further¹⁰⁹.

An approach is to generate new unconjugated CD20 antibodies with enhanced functional activities that may lead to superior efficacy. Anti-CD20 antibodies with different functions may be generated either: 1) by selecting antibodies that bind to a different CD20 epitope, which binds in an alternative mode or with changed affinity, resulting in altered intensity or type of functional mechanism; or 2) by engineering the Fc region of the antibody to enhance immune effector functions (Figure 9). The epitope and/or binding mode have been shown to dictate two major types of CD20 antibody effector function profiles, termed type I or type II^{129,140}. Although both types I and II antibodies bind bivalently to CD20, they form distinct complexes with CD20, as inferred from the fact that the B-cell surface can accommodate approximately double the number of type I antibodies compared with type II. Type I antibodies stabilize CD20 on lipid rafts, leading to stronger C1q binding and potent induction of CDC. However, this binding mode triggers only low levels of direct cell death. In contrast, type II antibodies do not stabilize CD20 in lipid rafts and thus exhibit reduced binding to C1q and lower levels of CDC, but they potently induce homotypic

adhesion and direct cell death¹⁴⁰. The majority of CD20 antibodies, including rituximab, veltuzumab, ocrelizumab and ofatumumab are of type I, whereas the prototype type II antibody is the murine antibody B1 (tositumomab) and GA101.

As previously discussed, numerous pre-clinical studies suggest that ADCC is an important mechanism of action for CD20 antibodies. Efforts to modify the Fc domain primary structure, using computational and high-throughput screening have resulted in Fc domains with higher affinity for FcγRIIIa and an enhancement of ADCC^{8,144}. The new CD20-specific antibodies ocrelizumab and AME-133 both contain mutated Fc domains and promote enhanced ADCC compared with rituximab. Modification of Fc domain oligosaccharide content provides another mechanism for enhancing ADCC. Most of the currently used therapeutic antibodies are highly fucosylated owing to the nature of the cell lines used for manufacturing. However, addition of bisecting N-acetylglucosamine or a lack of fucose on IgG1 N-linked oligosaccharide chain can dramatically increase ADCC in vitro and in vivo antitumor activity^{8,144}.

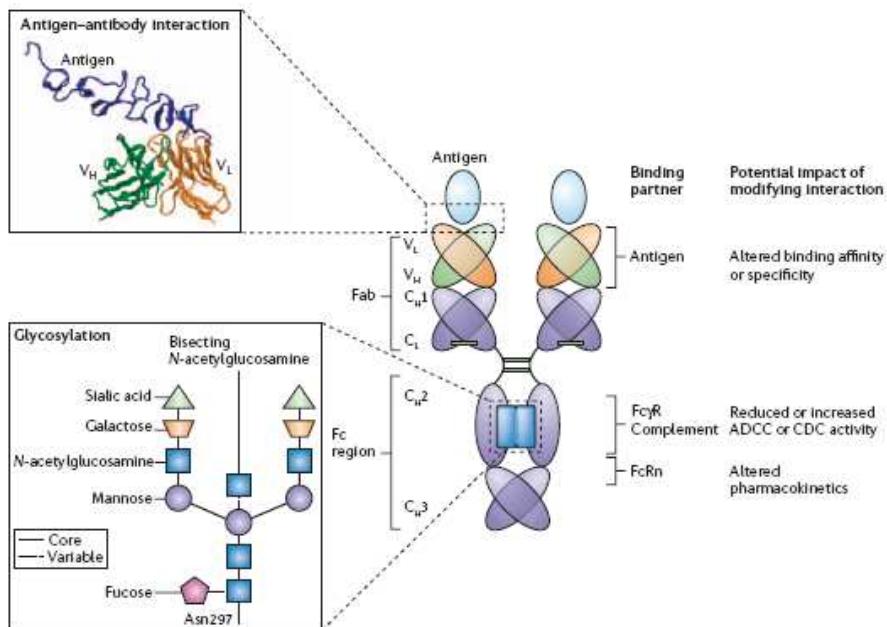


Figure 9. IgG structure and function. Human IgG bound to an antigen on a target cell can subsequently interact through its Fc region with Fc γ Rs expressed by effector cells or with complement component C1q, potentially supporting the destruction of target cells through ADCC or CDC, respectively. Modifying the amino-acid sequence of IgG to tailor the interaction with binding partner is a promising strategy to improve the clinical potential of antibodies. Effector functions (ADCC and CDC) require the presence of the Fc-region glycan (dark-blue) and are crucially influenced by its structure. The N-linked glycan that is attached to the conserved asparagine (Asn) at position 297 comprises a core structure of N-acetylglucosamine and mannose, plus additional carbohydrate residues, which can vary, including fucose, galactose, sialic acid and bisecting N-acetylglucosamine. (From Carter PJ, *Nature Review Immunology*, 2006, 6: 343-357)

There are now at least 7 CD20 mAb in clinical development with many more in preclinical evaluation (Table 4). These new mAb are engineered for potential benefits over the 1st generation rituximab. The modifications include: 2nd generation reagents, where the IgG mAb is humanized or fully human to reduce immunogenicity, but with unmodified Fc region, and 3rd generation mAb which are humanized and have an engineered Fc region designed to improve therapeutic performance by adapting their effector functions.

The former (2nd generation) include ocrelizumab, veltuzumab and ofatumumab, and the latter (3rd generation) includes TRU-015, AME133V, Pro13192 and GA101. Clinically, ofatumomab is the most advanced of these reagents in that it will be the first to seek FDA and EMEA approval for the treatment of CLL. The other two 2nd generation mAb are very similar to rituximab in both their structure and potency, and advantages over rituximab will probably come from their immunogenicity and alternative route of administration. The 3rd generation mAb AME133V, Pro13192 and GA101 are all modified either by amino-acid substitution or by glycoengineering to promote interaction with FcγR, particularly FcγRIIIa¹⁰⁹.

mAb	Clone	Format	Indication	Comments	Phase of development
Rituximab (Mabthera)	C2B8	Chimeric IgG1	NHL/RA	Gold standard	Approved 1997
Ofatumumab (Arzerra)	2F2	Human IgG1	HNL/RA	High CDC and ADCC	Phase III trials
Veltuzumab (Immu-106)	A20	Humanized IgG1	NHL/TP		Phase I/II
Ocrelizumab (PRO70769)	2H7	Humanized IgG1	HNL/RA	High ADCC	Phase III trials
PRO131921	Mut 2H7	Humanized IgG1	CLL/NHL	High ADCC	Phase I/II
GA101 (RO5072759)	Bly1 low fucose + mutation	Humanized IgG1	CLL/NHL	High PCD and ADCC Low CDC	Phase I/II
AME-133	Engineered	Humanized IgG1	Relapsed NHL	High affinity High ADCC	Phase I/II
TRU-015	SMIP*		RA	High ADCC Low CDC	Phase I/II
Y ⁹⁰ -Ibritumomab tiuxetan (Zevalin)	2B8	Murine IgG1 (Y ⁹⁰)	NHL	Low ADCC	Approved 2002
I ¹³¹ -Tositumomab (Bexxar)	B1	Murine IgG2a (I ¹³¹)	NHL	Low CDC	Approved 2003

*Small modular immunopharmaceutical (SMIP) drug composed of human IgG1 Fc and hinge regions (hinge, CH2 and CH3) linked directly to an anti-CD20 scFv.

Table 4. The current development status of anti-CD20 mAb and the key effector mechanisms selected/modified. Adapted from Lim SH et al, *Haematologica* 2010, 95 (1): 135-143.

GA-101

GA-101 is a third-generation humanized and glyco-engineered type II IgG1 anti-CD20 mAb developed by Glycart Biotechnology AG for the potential treatment of B-cell malignancies. It is the first unconjugated humanized type II mAb clinically investigated (B1/tositumomab, the other established type II, is only used as an I¹³¹ radiolabeled format). GA-101 was humanized by grafting complementary-determining region sequences from the

murine CD20 mAb Bly1 onto framework regions with fully human IgG1κ germline sequence and converted from type I to type II during humanization. GA-101 was further engineered to exhibit increased effector functions by alterations to its glycosylation profile and modifications to the elbow hinge region of the molecule, with the aim of increasing both direct and immune-mediated target cell death. The carbohydrates of the Fc region of GA-101 were glyco-engineered using recombinant glyco-engineering antibody production technology (GlycoMAb; Glycart) to produce bisected, afucosylated Fc region-carbohydrates. The host cell was engineered to express $\beta(1,4)$ -N-acetylglucosaminyltransferase III to increase the proportion of the Fc region polypeptides with bisected hybrid oligosaccharides or galactosylated complex oligosaccharides relative to complex oligosaccharides¹⁴⁵.

The glyco-engineered Fc region of GA-101 exhibited a 50-fold greater affinity for the human low-affinity FcγRIIIa than a non-glyco-engineered antibody and the increased binding of GA-101 to FcγRIIIa resulted in an increase in ADCC against CD20-expressing NHL cell lines that ranged from 5- to 100-fold¹⁴⁶. Modification of the elbow hinge sequences within the antibody variable framework regions increased the cell death-inducing activity of GA-101 upon binding to CD20 on target cells, compared with other CD20-directed antibodies¹⁴⁷. GA101 has shown promising activity in preclinical animal models and phase I/II clinical trials in B-NHL and B-CLL^{145,148-150}.

Scope of the thesis

The aims of my PhD thesis project were to characterize the biology and functions of the Cytokine-induced killer (CIK) cells expanded in vitro from peripheral blood mononuclear cells under influence of cytokines and to propose new approaches to optimize their therapeutical efficacy.

The project has developed as follows:

- The first part of the project has the purpose to characterize in detail the predominant subset of the CIK cell cultures ($CD3^+CD56^+$) in relation to the other subsets also present in the bulk culture at the end of the expansion ($CD3^+CD56^-$ and $CD3^-CD56^+$) and to compare them with the circulating $CD3^+CD56^+$ cells. We have considered in particular their cellular origin, phenotype, proliferative capacity and cytotoxic activity.
- The second part of the project wants to evaluate the possibility to improve the antitumor activity of CIK cell cultures by combined use with therapeutic monoclonal antibodies. In particular, we have investigated combining adoptive immunotherapy with CIK cells and anti-CD20 monoclonal antibodies, such as chimeric type I rituximab or the new generation type II GA101, to optimize treatment of B-cell lymphoma.

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Chapter 2

Cytokine-induced killer cells are terminally differentiated activated CD8 cytotoxic T-EMRA lymphocytes

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Abstract

Objective. Cytokine-induced killer cells (CIK) are CD3⁺CD56⁺ T cells with natural killer (NK)-like cytotoxic activity used for the immunotherapy of tumors. We aimed to fully characterize CIK cells and define their ontogeny.

Methods. CIK were generated in vitro by stimulation of peripheral blood mononuclear cells or T-cell subsets with interferon- γ , anti-CD3 and interleukin-2. They were fully characterized in terms of phenotype, cytotoxic activity, and gene expression with respect to circulating CD3⁺CD56⁺ cells, NK cells, and CD56⁻ T cells present in CIK cultures.

Results. We demonstrate that CIK are terminally differentiated CD8 T cells that derive from proliferating CD3⁺CD56⁻CD8⁺ T cells. They express polyclonal T-cell receptor V β chains and have acquired CD56, NKG2D, and large granular lymphocyte morphology, but lack expression of most NK-specific activating (NKp30, NKp44, NKp46) and inhibitory (KIR2DL1, KIR2DL2, KIR3DL1, NKG2A, CD94) receptors and can kill K562 targets. Circulating CD3⁺CD56⁺ cells are also CD8⁺CD16⁻, but are oligoclonal, poorly cytotoxic for K562, and express lower levels of CD56 and NKG2D. Gene profiling of CIK, CD56⁻ T and NK cells present at the end of culture shows that differences are much more limited between CIK and CD56⁻ T compared to CIK and NK cells. Most of the genes upregulated in CIK cells compared to CD56⁻ T cells are part of the tumor necrosis factor gene network.

Conclusions. The CIK phenotype, that is CD45RA⁺, CCR7-, CD62L-weakly positive, CD11a⁺, CD27⁺, CD28-, macrophage inflammatory protein 1 α ⁺, perforin⁺, Fas ligand⁺ coincides almost exactly with that described for the T RA⁺ effector memory CD27 single positive subset of terminally differentiated human memory T cells.

Introduction

Cytokine-induced killer (CIK) cells can be obtained in vitro by sequential exposure of circulating mononuclear cells to interferon- γ (day 0) and monoclonal anti-CD3 antibody OKT3 (day 1) and subsequent expansion in presence of interleukin (IL)-2. CIK are CD3 $^{+}$ CD56 $^{+}$ double-positive T cells that have acquired phenotypic markers of natural killer (NK) cells and show nonrestricted cytotoxicity against tumor cells of several lineages in vitro, in particular hematologic neoplasms ¹⁻⁵. Moreover, they show anti-tumor activity in vivo in mice carrying either murine or human tumors^{1,2,6-8}. More interestingly, CIK cells have shown little or negligible cytotoxicity against normal tissues, including normal bone marrow, thus representing a valid tool for immunotherapy protocols, even in the allogeneic hematopoietic stem cells transplant context^{6,7,9-11}.

Reproducibility and simplicity of the expansion protocol and the availability of clinical grade culture reagents have greatly facilitated the clinical utilization of CIK cultures. Indeed, several clinical phase I studies have already been conducted both in solid tumor¹²⁻¹⁶ and leukemia patients^{17,18}. In most of these trials, autologous cells have been used¹²⁻¹⁷, although in one case, CIK cells of donor origin have shown little toxicity when administered to relapsed leukemia patients following allogeneic hematopoietic stem cells transplant¹⁸.

CIK cells have previously been shown to derive from CD3 $^{+}$ CD8 $^{+}$ T cells¹⁸. Although few CD3 $^{+}$ CD56 $^{+}$ cells have been

observed in peripheral blood, the relationship between in vitro-generated CIK and circulating CD3⁺CD56⁺ cells is not known. Finally CIK culture products are heterogenous in that they also contain CD3⁺CD56⁻ T cells, as well as a small percentage of CD3-CD56⁺ NK cells. We have, therefore, set out to investigate in detail the cellular origin of in vitro-expanded CIK cells, and to fully characterize them phenotypically and functionally.

Methods

CIK cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors or umbilical cord blood by Ficoll-Hypaque density centrifugation (Lympholyte-H, Cedarlane, Hornby, Ontario, Canada). Buffy coats were obtained from the Hospital's Transfusion Center and umbilical cord blood samples from the Obstetrics and Gynecology Unit after informed consent. Forms for consent have been authorized and approved by local ethical committee. To generate CIK cells, PBMCs were resuspended in X-VIVO medium (Cambrex Bioscience, Verviers, Belgium) and stimulated on day 0 with 1000 U/mL recombinant human (rh) interferon- γ (Boehringer Ingelheim, Vienna, Austria), on day 1 with 50 ng/mL anti-CD3 monoclonal antibody OKT-3 (Jassen Cilag, Schaffhausen, Switzerland) and 500 IU/mL rhIL-2 (Chiron Corp., Uxbridge, UK) and expanded in presence of rhIL2 for at least 21 days¹⁹. The K562 cell line was grown in RPMI-1640 complete medium (Cambrex Bio Science).

Immunophenotypic analysis by flow cytometry

The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated CD3 (clone SK7), CD16 (3G8), CD11a (G-25.2), CD45RA (L48), $\alpha\beta$ T-cell receptor (TCR) (VT31), $\gamma\delta$ TCR (11F2) (BD Biosciences, San Jose, CA, USA), phycoerythrin (PE)-conjugated CD3 (SK7), CD56 (NCAM16.2), CD28 (L293), CXCR2 (6C6), CD27 (L128), macrophage inflammatory protein 1 α (MIP1 α)

(11A3), CXCR4 (12G5) (BD Biosciences), NKG2A (Z199), NKp30 (Z25), NKp44 (Z231), NKp46 (BAB281), CD94 (18D3), KIR2DL1/KIR2DS1 (EB6B), KIR2DL2/KIR2DL3/KIR2DS2 (GL183), KIR3DL1/KIR3DS1 (Z27) (Immunotech, Beckman Coulter, Marseille, France), NKG2D (ON72) (R&D Systems, Minneapolis, MN, USA), allophycocyanin-conjugated CD56 (NCAM16.2), CD62L (Dreg56) (BD Biosciences), and CD95L (Caltag, Burlingame, CA, USA), PE-Cy7-conjugated CD4 (SK3) and peridinin chlorophyll-conjugated CD8 (RPA-T8) (BD Biosciences). Analysis was performed either on a FACScan (for double-fluorescence analysis), or a FACS Canto I (for triple and quadruple fluorescence) (Becton Dickinson, San José, CA, USA). Data analyses were performed using the Cell Quest (Becton Dickinson) or FCS Express (De Novo Software, Thornhill, Ontario) softwares, respectively. For analysis of intracellular perforin, cells were fixed and permeabilized by Fix&Perm Reagents and labeled with FITC Conjugated Perforin Antibody Reagent Set (BD Biosciences). For intracellular cytokine staining CD3⁺CD56⁺ and CD3⁺CD56⁻ cells immunoselected from CIK culture at day 21 were first incubated with Staphylococcal enterotoxin B (Sigma-Aldrich, Ayrshire, UK) for 6 hours. Brefeldin A was added in the last 4 hours of culture. Cells were then fixed, permeabilized, and incubated with FastImmune Anti-Hu-TNF α /CD69/CD4/CD3 kit (BD Biosciences). For V β repertoire analysis, cells were stained with CD3- peridinin chlorophyll and CD56- allophycocyanin antibodies and with couples of V β subfamily antibodies conjugated to FITC and PE. The TCR V β test kit was from Beckman Coulter.

CFSE staining and proliferation assays

Cells were labeled with 1 μ M carboxy fluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA), washed and returned to culture. CFSE staining was analyzed on the FACSscan by standard green fluorescence analysis.

Cell selection

Wherever stated, cell isolation by fluorescence-activated cell sorting (FACS) was performed with a Cell Sorter FACSaria Instrument (BD Biosciences) after labeling with the appropriate monoclonal antibodies. For cytotoxicity assays, CD56⁺ and CD56⁻ populations were separated using CD56 immunobeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Circulating CD3⁺CD56⁺ cells were immunoselected from PBMCs by a two-step procedure using the CD3⁺CD56⁺ human NKT cells Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Cytotoxicity assays

Cell-mediated cytotoxicity was measured with a standard 4-hour assay using the fluorescent dye Calcein-AM (Sigma-Aldrich Company) as described¹⁹.

Microarray gene expression analysis

After 21 days of CIK culture, the CD3⁺CD56⁺ (CIK), CD3⁺CD56⁻ (T), and CD3-CD56⁺ (NK) cells obtained starting from one normal donor were sorted on a FACSaria cell sorter. The purity of the fractions analyzed was superior to 97%. RNA extraction, quality control, and gene expression analysis were performed by Miltenyi

Biotech using a validated protocol for microarray analysis of a panel of 1076 immune function-related genes (PIQOR Immunology Microarray). For all the genes, four replicates of each subset from the same culture were analyzed. In order to identify relevant results, very low signal intensities (less than twofold above average signal intensities of the background in both Cy3 and Cy5 channels) were excluded from the analysis. To determine if a pair of comparative data was significantly increased or decreased, we employed the default cut-off values of the software (≤ 0.58 for downregulation or ≥ 1.7 for upregulation). If gene expressions were found to be significantly deregulated in the microarray data, the genes were analyzed for functional interconnections using Ingenuity Pathways Analysis software (Ingenuity Systems Inc., Redwood City, CA, USA).

Statistical methods

Results were compared using the Student's *t*-test. A *p* value <0.05 was considered significant.

Results

CIK cells derive from CD3⁺CD8⁺CD56⁻ T cells and have a limited lifespan

We generated CIK cells from PBMCs from normal donors stimulated with interferon- γ , OKT3 followed by IL-2. These culture conditions led to expansion of total and CIK cells with maximal yields reached between days 21 and 35, followed by a decline, suggesting exhaustion of the culture (Figure 1A). Overall, starting from 15×10^6 PBMC, we reached a mean peak of 584×10^6 total cells (varying from 250×10^6 to 1200×10^6), which is a mean 38.8-fold expansion. At the peak of culture, CD3⁺CD56⁺ CIK cells represented the predominant population (mean: 67%; range, 49-85%), as exemplified in the growth curve shown in Figure 1A.

In order to analyze the proliferative potential of CD3⁺CD56⁻ T cells and CD3⁺CD56⁺ CIK cells, they were purified from day-11 cultures, stained with CFSE, and then returned to culture. After another 6, 8, and 11 days, FACS analysis of the cells clearly showed that only the CD3⁺CD56⁻ population has significant proliferative activity (Figure 1B). These data suggest that CD56⁻ T cells are responsible for cellular expansion in CIK cultures and may terminally differentiate into CIK cells during culture.

Analysis of the differentiation potential of different subsets revealed that CD3⁺CD56⁻ cells, sorted at the beginning of culture, expanded and gave rise to CD3⁺CD56⁺ cells in 21-day cultures to a similar extent as unsorted populations (71% vs 72% CIK cells at day 21, and with 45 compared to 90-fold expansion in total cell number)

(Figure 1C). In contrast, sorted CD3⁺CD56⁺ cells maintained their phenotype mostly unchanged during culture (94% CD3⁺CD56⁺ cells at day 21 vs 98% at day 0), but did not expand (Figure 1C). In addition, we have performed CD56-depletion experiments at the start of culture and analyzed growth rate and appearance of CD3⁺CD56⁺ CIK during culture, comparing either nondepleted or CD56-depleted populations. CD56-depleted and nondepleted PBMCs grew to a similar extent in CIK culture conditions and the percentage of CD3⁺CD56⁺ cell after 21 days of culture were equivalent for the two cultures (data not shown). We also investigated whether CD3⁺CD56⁻ cells present throughout culture were able to generate CIK cells. For this purpose, CD3⁺CD56⁻ cells were purified from either 11 or 21 days of culture and returned to CIK culture conditions for an additional 15 to 21 days. CD3⁺CD56⁺ cell were efficiently generated in both cases, reaching 51% and 72%, respectively (data not shown). Expansion fold of total cells was 2.7 and 4.2, respectively (data not shown), showing that CD3⁺CD56⁻ cells isolated during culture still maintained a proliferative capacity.

We then investigated the relative contribution of different T-cell subsets to CIK cell differentiation. CD8 and CD4 single-positive, double-negative, and double-positive cells were FACS-sorted and expanded in vitro in CIK conditions (Figure 1D). Results demonstrate that CD8⁺ single-positive cells could expand in vitro 17-fold and give rise to CD3⁺CD56⁺ cells. In contrast, CD4⁺ single-positive cells proliferated to some extent (sevenfold expansion of total cells) but remained mainly CD56-negative (0.5 % CD56⁺ cells) and double-positive and double-negative populations did not expand (0.1- and

1.6-fold expansion, respectively) (Figure 1D) in agreement with that previously demonstrated by Lu et al.¹⁸

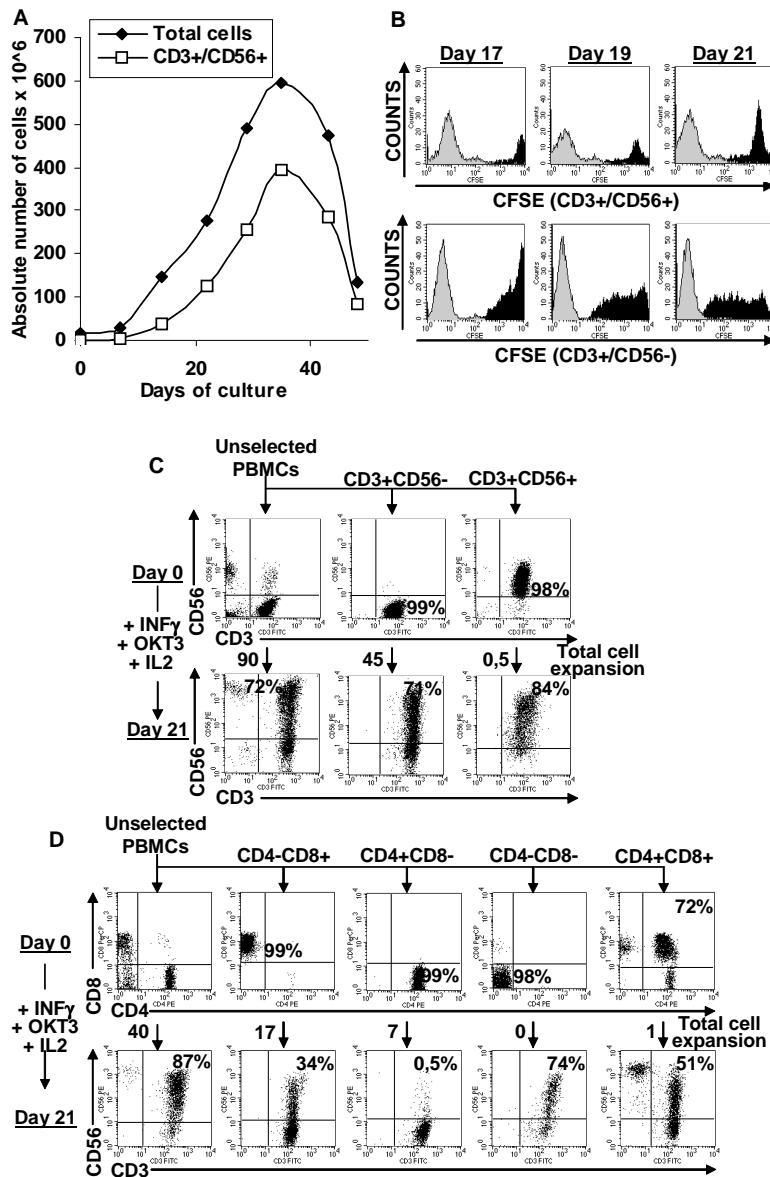


Figure 1. Cytokine-induced killer (CIK) cells derive from CD3+CD56+ CD8+ T cells. (A) CIK cells were generated in vitro and CD3+CD56+ cells were counted at different time points. Data shown are the mean absolute

number of cells \pm standard deviation from 3 separate experiments, representative of at least 10 experiments performed using 10 different peripheral blood mononuclear cell (PBMC) donors. (B) After 11 days of CIK culture, CD3 $^{+}$ CD56 $^{+}$ (upper panels) and CD3 $^{+}$ CD56 $^{-}$ cells (lower panels) were FACS sorted, labeled with carboxy fluorescein succinimidyl ester (CFSE) and then returned to culture. Flow cytometry analysis of both populations was performed after another 6, 8, and 11 days of culture (black curves). Unlabeled control cells are shown in grey. One representative experiment is shown. (C) Freshly isolated PBMCs were left unselected or FACS sorted into CD3 $^{+}$ CD56 $^{-}$ or CD3 $^{+}$ CD56 $^{+}$ populations. All three populations were then cultured in CIK conditions for 21 days and then labeled with CD3-fluorescein isothiocyanate (FITC) and CD56-phycoerythrin (PE) antibodies. Percentages of CIK cells obtained are shown in each dot plot. The fold expansion of total leukocytes (i.e., total leukocytes at end of culture/total leukocytes at the beginning of culture) is shown. Results are representative of three independent experiments using three different PBMCs donors. (D) CD4 $^{+}$ CD8 $^{+}$, CD4 $^{+}$ CD8 $^{-}$, CD4 $^{-}$ CD8 $^{+}$, and CD4 $^{-}$ CD8 $^{-}$ cells were FACS sorted from PBMC and cultured in CIK conditions for 21 days. Cells were then counted and stained with CD3-FITC and CD56-PE antibodies. The percentages of CIK cells obtained are shown in each dot plot. The fold expansion of total leukocytes is shown below for each population. Results are representative of three independent experiments using three different PBMCs donors.

Characterization of circulating compared to in vitro generated CD3 $^{+}$ CD56 $^{+}$ cells

Presence of circulating CD3 $^{+}$ CD56 $^{+}$ in the blood was described many years ago and varies in different donors from 1% to 11% of mononuclear cells (mean: 4.7%; data not shown). Their phenotypic and functional properties were compared to those of in vitro-

generated CIK cells. As shown in Figure 2A, the two cell populations were mostly CD8⁺ and CD4- and showed comparable $\alpha\beta$ and $\gamma\delta$ distribution. In vitro-generated CIK cells were strongly positive for NKG2D, with a mean of 85% (Figure 2A). In contrast, circulating CD3⁺CD56⁺ were only 6.5% NKG2D-positive. The other NK receptors tested (NKp30, NKp44, NKp46) were not expressed in circulating CD3⁺CD56⁺ cells and barely detectable in in vitro-generated CIK cells (Figure 2A). Similarly, both populations were mostly negative for CD16. Finally, CD56 was much more intensely expressed on in vitro-generated CIK cells (mean fluorescence intensity MFI: 710) than on circulating CD3⁺CD56⁺ cells (MFI: 135) (Figure 2B). For comparison, a similar analysis was performed with the “bona fide” NK cells (CD3-CD56⁺) showing that the culture-induced CD56 expression was even higher on NK cells (Figure 2C).

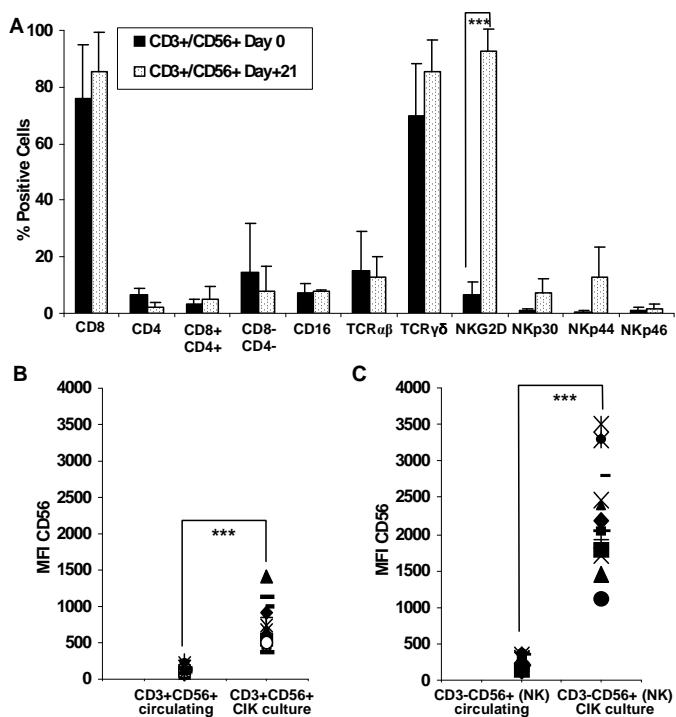


Figure 2. In vitro expanded cytokine-induced killer (CIK) cells differ from circulating CD3⁺CD56⁺ cells by their high NKG2D and CD56 expression.

(A) Circulating CD3⁺CD56⁺ cells present in freshly isolated peripheral blood mononuclear cells or in 21 days CIK cultures were analyzed for expression of T and natural killer (NK) cell markers by flow cytometry in triple or quadruple fluorescence. Data shown are the mean percent expression ± standard deviation from four separate experiments. (**p < 0.001 for CIK vs circulating CD3⁺CD56⁺ cells). (B) Mean fluorescence intensity (MFI) of CD56 expression in circulating CD3⁺CD56⁺ cells and in vitro expanded CIK cells is shown. Data represent at least 16 separate experiments (**p < 0.001 for circulating CD3⁺CD56⁺ cells vs expanded CIK cells). (C) For comparison, the MFI of CD56 expression in circulating NK (CD3-CD56⁺) cells and in vitro-expanded NK cells is shown. Data are from at least 12 separate experiments. (**p < 0.001 for circulating CD3-CD56⁺ NK cells vs expanded NK cells).

We next analyzed clonality of the TCR in both circulating CD3⁺CD56⁺ cells and CIK cells. Interestingly, we observed that circulating CD3⁺CD56⁺ cells are often oligoclonal for the V β repertoire. Indeed, among eight samples, five showed one to three V β families overrepresented with respect to the normal range (Figure 3A). For example, sample 8 had 55% V β 3 and 10% V β 11, suggesting that 65% of the CD3⁺CD56⁺ cells are oligoclonal. In only three samples, all V β subfamilies staining fell within the expected range (Figure 3A). In contrast, analysis of the CD3⁺CD56⁻ populations in freshly isolated PBMCs showed the expected pattern of V β repertoire, demonstrating polyclonality of this cell subset (data not shown).

When in vitro-generated CD3⁺CD56⁺ were similarly analyzed, they were repeatedly found to express polyclonal V β proteins, which fell within the normal range for peripheral blood T cells (six samples

analyzed)(data not shown). Furthermore, when the same sample (sample 8) was analyzed for V β repertoire in circulating CD3 $^+$ CD56 $^+$ cells or CD3 $^+$ CD56 $^+$ CIK cells at the end of culture, an oligoclonal pattern was found in circulating CD3 $^+$ CD56 $^+$ cells and polyclonal pattern in CIK cells obtained from the same donor (Figure 3B).

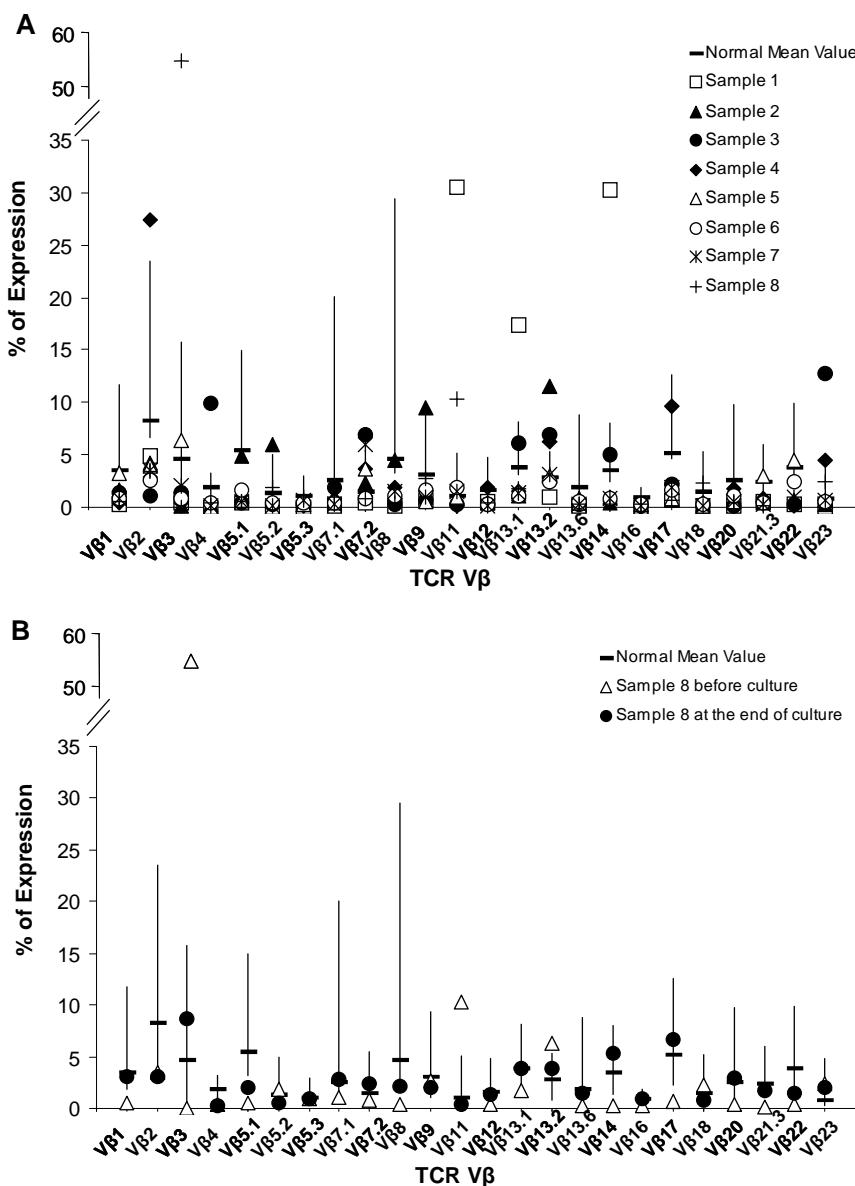


Figure 3. V β repertoire analysis of circulating CD3 $^{+}$ CD56 $^{+}$ and cytokine-induced killer (CIK) cells. Peripheral blood mononuclear cells (PBMCs) or 21-day CIK cultures were labeled with CD3-peridinin chlorophyll and CD56-allophycocyanin antibodies and with the indicated V β subfamily-specific antibodies conjugated to either a fluorescein isothiocyanate and phycoerythrin fluorescent markers and analyzed with FACS by quadruple immunofluorescence. (A) Percentage of expression of each V β subfamily in circulating CD3 $^{+}$ CD56 $^{+}$ cells is shown, each sample being indicated by a different symbol. Vertical bars indicate the normal range of V β subfamily expression in a large panel of normal PBMCs, as given by the manufacturer. Normal mean values of V β subfamily expression are also indicated as horizontal bars. Any percentage outside this normal range is considered an outlier. (B) Percentages of V β subfamilies expression in CD3 $^{+}$ CD56 $^{+}$ at the start and at the end of CIK culture in sample 8 are shown. TCR = T-cell receptor.

The skewed T-cell repertoire of circulating CD3 $^{+}$ CD56 $^{+}$ cells suggests they may represent immune-experienced T cells present in the adult. In agreement with this hypothesis, in 12 cord blood samples studied, CD3 $^{+}$ CD56 $^{+}$ cells were virtually undetectable (mean: 0.5%), whereas it was a mean of 4.7% in 45 adult PBMCs studied ($p < 0.0001$) (data not shown). Furthermore, most of them belong to effector memory (EM) ($34.5\% \pm 20.5\%$), RA $^{+}$ effector memory (EMRA) ($33.4\% \pm 26.9\%$) or central memory ($17.2\% \pm 14.3\%$) subsets. For comparison, we show the same distribution analysis also for the CD3 $^{+}$ CD56 $^{-}$ T cells from the same eight donors: Naïve (N) ($41.9\% \pm 19.9\%$) or central memory ($21.6\% \pm 17.1\%$) represent the vast majority of cells, while EM ($23.6\% \pm 15.8\%$) or EMRA ($12.9\% \pm 11.5\%$) are less

represented (Table 1). This distribution does not differ significantly from the one described for the CD3⁺CD56⁺ population.

	Circulating CD3 ⁺ CD56 ⁺	Circulating CD3 ⁺ CD56 ⁻
Naïve (CD62L⁺/CD45RA⁺)	14.8 ± 12.1*	41.9 ± 19.9*
CM (CD62L⁺/CD45RA⁻)	17.2 ± 14.3**	21.6 ± 17.1**
EM (CD62L⁻/CD45RA⁻)	34.5 ± 20.5***	23.6 ± 15.8***
EMRA (CD62L⁻/CD45RA⁺)	33.4 ± 26.9****	12.9 ± 11.5****

Table 1. Immune subsets in circulating CD3⁺CD56⁺ cells vs CD3⁺CD56⁻ cells

Mean percent values (± standard deviation) are reported (n = 8).

CM = central memory; EM = effector memory; EMRA = RA⁺ effector memory.

*p = 0.03

**p = 0.43

***p = 0.65

****p = 0.05

(p values was determined according to Student's *t*-test).

We then compared the natural cytotoxicity of circulating CD3⁺CD56⁺ and in vitro-generated CIK cells against cell line K562. Purity of the immunoselected CD3⁺CD56⁺ cells was 97% and 99%, respectively (Figure 4A and B). CIK cell lysed up to 50% of K562 target cells at a 30:1 effector-to-target ratio. In contrast, circulating CD3⁺CD56⁺ cells were very poorly cytotoxic, also upon in vitro activation with IL-2 (Figure 4C).

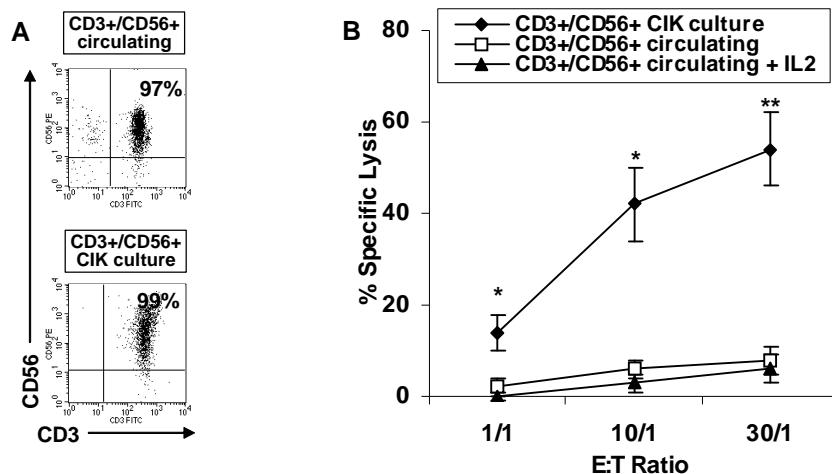


Figure 4. Cytokine-induced killer (CIK) cells show higher cytotoxic activity towards the K562 leukemia cell line compared to activated circulating CD3+CD56+ cells. CD3+CD56+ cells purified by immunoaffinity either from freshly isolated peripheral blood mononuclear cells or from 21-day CIK cultures were assayed for cytotoxicity against the K562 leukemia cell line. Purified circulating CD3+CD56+ cells were activated in vitro by incubation overnight with 250 IU/mL of IL-2 prior to use. (A) Dot plot of the purified circulating CD3+CD56+ cells. Percentage of purity is shown. (B) Dot plot of the purified CD3+CD56+ cells from day-21 CIK cultures. Percentage of purity is shown. (C) Cytotoxicity was assessed after 4 hours at the indicated effector-to-target ratio. Data are mean \pm standard deviation of three different experiments (*p < 0.01 and **p < 0.005 for CIK vs activated circulating CD3+CD56+ cells).

Phenotypic analysis of CIK cells compared to NK cells and T cells present in CIK cultures

At the end of CIK cell expansion, at least three different subpopulations can still be distinguished: CD3+CD56+ CIK cells (ranging from 40% to 80%), CD3+CD56- T cells (ranging from 20% to

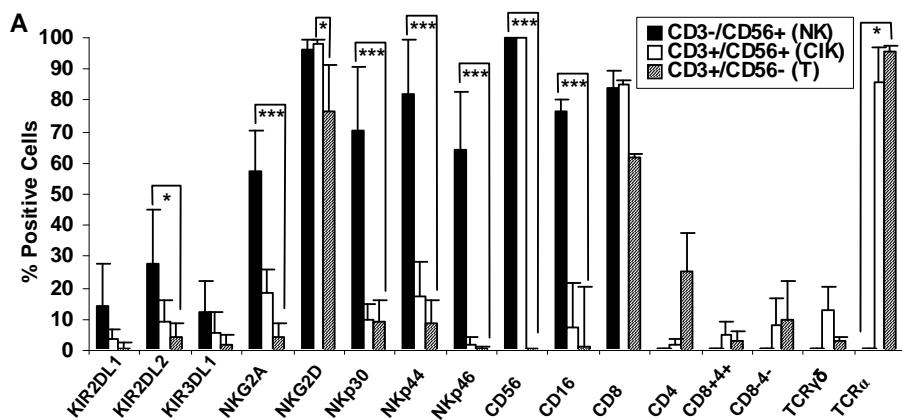
60%), and few CD3-CD56⁺ bona fide NK cells (ranging from 1% to 10%). The phenotype of these three populations was compared. As shown previously, CD3⁺CD56⁺ CIK cells express high levels of NKG2D (mean: 98%; MFI: 251) and low, although detectable, levels of the activating receptors NKp44 (18%), NKp30 (10%). These cells do not express significant levels of KIR (KIR2DL1, KIR2DL2, KIR3DL1) (< 10%) or NKG2A (18%) (Figure 5A). As expected, cultured NK cells highly expressed all activating receptors (57%-96%), including NKG2D (mean: 96%; MFI: 214) and to lower levels the KIR receptors (12%-28%) (Figure 5A). Finally, the CD3⁺CD56⁻ cells present in the CIK cultures expressed NKG2D (mean: 76%; MFI: 220), but they did not express to a significant extent the other activating or inhibitory NK receptors (<10%) (Figure 5A). Furthermore, CD16 was highly expressed only on bona fide NK cells at the end of culture, but was mostly negative on CIK and CD3⁺CD56⁻ T cells (Figure 5A). Expression of T-lineage markers was also analyzed on the same populations. TCR (mostly $\alpha\beta$) was expressed on both T and CIK populations, but not on NK cells, as expected. CD8 was present on all populations, whereas CD4 expression was present only on T cells (Figure 5A). Finally, > 80% of the CIK cells at the end of culture showed the large granular lymphocyte (LGL) morphology in cytocentrifuge preparations, while < 30% LGL were detected in the CD3⁺CD56⁻ T-cell population at the end of culture (data not shown).

Gene profile of CIK cells compared to CD3⁺CD56⁻ T and NK cells

The gene transcript repertoire of these populations was also analyzed using microarrays carrying 1076 immune-related genes and

a validated assay. Results showed that the gene expression profile of CIK cells was more similar to that of CD56⁻ T cells, with only 50 differently expressed genes (4.7%), of which 35 were upregulated and 15 downmodulated in CIK compared to T cells. More differences were observed between CIK and NK cells with 115 modified genes (10.7%) of which 73 were upregulated and 42 downmodulated (Figure 5B and Suppl. Tables 1 and 2).

The differential expression of several genes detected by microarray was verified at the protein level by immunophenotyping. The following proteins were confirmed to be differentially expressed in CIK vs NK cells: CD16, CXCR4, CD27, CD62L, and CD94 (Figure 5C), in agreement with the microarray data (Suppl. Table 2). Smaller differences of CD16, CXCR4, and CD62L also detected in microarray between CIK and T cells were also confirmed by immunophenotyping. Clearly, however, differences in these five surface proteins analyzed were more marked between CIK and NK cells than between CIK and T cells (Figure 5C).



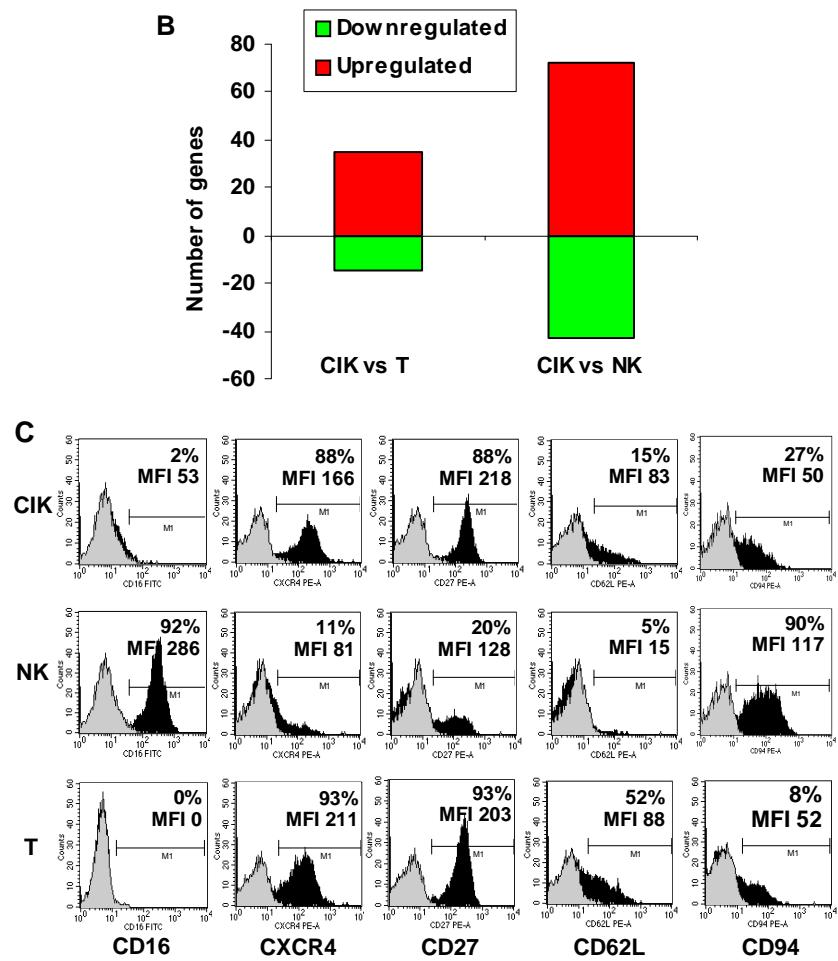


Figure 5. Phenotypic and genotypic comparison of cytokine-induced killer (CIK), T, and natural killer (NK) cells at the end of the culture. (A) The 21-day expanded CIK cultures were analyzed for expression of T- and NK-cell markers by flow cytometry in triple or quadruple fluorescence. Percentage positive cells for the indicated marker in the CD3-CD56⁺ NK cells (black bars), CD3⁺CD56⁺ CIK cells (white bars), or CD3⁺CD56⁻ T subpopulations (striped bars) are shown as mean values and standard deviations of nine independent experiments. (*p < 0.01 and ***p < 0.001 for CIK vs NK or T cells). (B) Gene profiling of CIK, T, and NK cells from CIK culture. A 21-day expanded CIK culture was sorted into three cellular subsets CD3-CD56⁺ NK

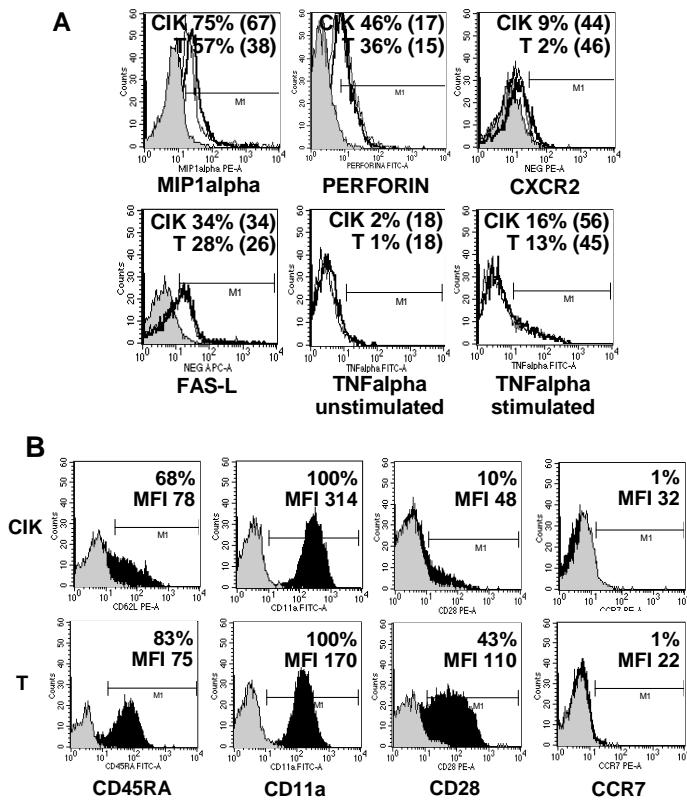
cells, CD3⁺CD56⁺ CIK cells, and CD3⁺CD56⁻ T subpopulations, RNA extracted from each population and analyzed by microarray, using a panel of 1076 immune-related probes. Schematic representation of the number of genes that are either upregulated (red) or downregulated (green) in the CIK compared to T-cell subset or in the CIK compared to the NK subsets. (C) Expression of CD16, CXCR4, CD27, CD62L, and CD94 in CIK (upper panels), NK (medium panels), and CD56⁻ T cells (lower panels) is shown. Background staining using an isotype control antibody is shown in each histogram (grey curve). The numbers in each panel refer to the percentages of positive cells and the mean fluorescence intensity (MFI) values.

Further differences noted between T and CIK cells in the microarray analysis, in particular MIP1 α , perforin, CXCR2, Fas ligand (FASL) and tumor necrosis factor (TNF)- α expression, were also confirmed by immunophenotyping, as shown by the histogram overlay of Figure 6A. All these proteins were expressed by both CIK and T cells, albeit CIK cells showed slightly higher expression levels, presumably in agreement with microarray data (Figure 6A). More interesting is the observation that all these genes expressed by CIK and T cells are consistent with an inflammatory phenotype, as also suggested by the finding that 19 of the 35 (54%) upregulated genes are comprised in the genes network centered around the TNF gene (indicated in red or green in Suppl. Table 2).

Low levels of expression of CD62L in CIK and T cells suggest further exploring the phenotype of these populations to evaluate their naïve/memory distribution. CIK and T cells were both CD45RA⁺ and CCR7⁻ with an overlapping immune subsets distribution (Figure 6B and data not shown). By all these

considerations, both CIK and T cells could be classified as T-EMRA. The very recent subdistribution of T-EMRA led us to investigate the CD27, CD28, and CD11a markers and results clearly showed that CIK and T cells are CD27⁺ (Fig. 5C) and CD11a⁺, but differ for the CD28 staining, which is much weaker on CIK cells (Fig. 6B). Therefore, the CD45RA, CCR7, CD27, and CD28 expression, in addition to perforin, FASL and MIP1 α , almost exactly overlaps with the described EMRA CD27 SP subgroup of fully differentiated memory T cells.

Finally, we have tested the natural cytotoxic activity of immunoselected CIK and T populations from end cultures and show that only CIK cells were significantly cytotoxic against K562 (Fig. 6C) similarly to that previously observed by Lu et al.^{1,9}



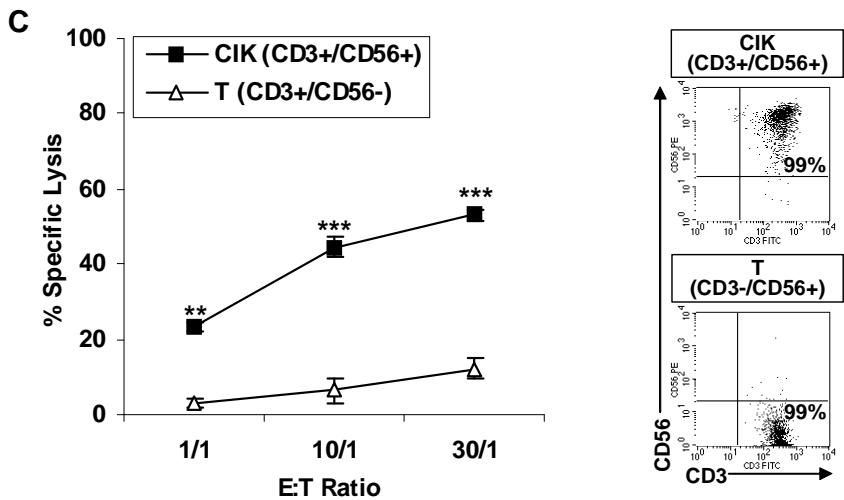


Figure 6. Phenotypic and functional comparison between CD3⁺CD56⁻ T and CD3⁺CD56⁺ cytokine-induced killer (CIK) cells at the end of culture.

(A) CIK and CD56⁻ T cells express genes relevant for an inflammatory phenotype. Expression of macrophage inflammatory protein 1 α (MIP-1 α), perforin, CXCR2, Fas ligand (FASL), and tumor necrosis factor- α (TNF α) (white curves) and control isotype antibody (grey curves) is shown for CIK (solid line) and CD56⁻ T cells (dotted line). TNF α was analyzed with or without Staphylococcal enterotoxin B stimulation. Percentage positivity and mean fluorescence intensity (MFI; in the parentheses) are shown in each plot. Results are representative of at least three experiments. (B) Immune subset identification of CIK (upper histograms) and CD56⁻ T cells (lower histograms). Staining for CD45RA, CD11a, CD28, and CCR7 (black curves) or isotype control (gray curves) by CIK and CD56⁻ T cells is shown. Results are representative of at least three experiments. (C) Cytotoxic activity of CIK and CD56⁻ T cells. CIK and CD56⁻ T cells purified at the end of culture were tested against cell line K562 at the indicated effector-to-target (E:T) ratios. Data are mean \pm standard deviation of five different experiments (**p < 0.005 and ***p < 0.001 for CIK vs T cells). Dot plots of the purified CIK (upper) and T cells (lower) and percent purity are shown on the right.

Discussion

We have demonstrated that CD3⁺CD56⁺ CIK cells derive from proliferating CD56⁻ T cells and not from the few CD3⁺CD56⁺ present in the starting culture. Indeed CD3⁺CD56⁺ cells were shown to be nonproliferating and could not, therefore, expand in culture. In contrast CD3⁺CD56⁻ T cells, isolated both at the beginning of culture or at different times during expansion, could proliferate and give rise to CD3⁺CD56⁺ cells. Mainly the CD3⁺CD56⁻CD8⁺ T-cells subset could proliferate in vitro in CIK culture conditions and give rise to CIK cells as previously supported by Lu et al.^{1,9}. Also the CD3⁺CD4⁺ T cells subset could proliferate to a minor extent under these conditions, but do not significantly differentiate to CIK cells. Therefore, one note of caution should be made with respect to previously published CIK expansion folds, which were calculated by dividing the final vs starting numbers of CD3⁺CD56⁺ (1,6).

CIK cells express the TCR, with similar proportion of $\alpha\beta$ chains compared to $\gamma\delta$ as peripheral blood T cells and they express it in a polyclonal fashion, like normal T cells, as shown by V β repertoire analysis. In contrast, circulating CD3⁺CD56⁺ cells generally display a skewed V β repertoire, suggesting overrepresentation of few clones. Interestingly, PBMCs containing circulating CD3⁺CD56⁺ with strongly skewed V β repertoire gave rise to fully polyclonal CIK cells, supporting that CIK derive from the polyclonal CD3⁺CD56⁻ cells rather than from the oligoclonal CD3⁺CD56⁺ cells present at the beginning of culture.

Complete phenotypic analysis confirmed that CIK cells are more related to T cells than NK cells. Indeed, except for NKG2D, CD56, and LGL morphology²⁰, which are not specific NK-cell markers, CIK cells do not significantly express NK-cell markers, such as KIR receptors, CD16 and NKG2A. CIK cells only express NKp44 and NKp30, but to rather low levels (10%-20%) compared to NK cells. These data support the notion that expression of the natural cytotoxicity receptors NKp30, NKp44, and NKp46 are indeed not as NK-specific as originally reported²¹⁻²³.

Gene expression microarray analysis has revealed that CIK cells share a larger number of genes with CD56⁻ T cells present at the end of culture rather than with NK cells. Interestingly, most of the genes upregulated in CIK cells as compared to T cells were tightly linked with each other, being part of the gene network centered on the TNF gene. Moreover, CIK cells express many genes shared by the “inflammatory” memory T cells and, indeed, they almost exactly overlap with the recently described T-EMRA CD27 single positive subtype (CD8⁺, CD45RA⁺, CCR7⁻, CD62L weak, CD11a⁺, CD27⁺, CD28⁻, MIP1 α ⁺, perforin⁺, FASL⁺)²⁴. It is clear that OKT3, interferon- γ , and IL-2 are sufficient to stably induce this “activated-memory”-like phenotype both to antigen-experienced (this paper) or -naïve (our previous data on cord blood-derived CIK cells)¹⁹, although the question remains if this phenotype simply reflects the expression induction of few markers in these culture conditions or rather the true induction of a “memory program”.

The careful comparison of CIK with CD3⁺CD56⁻ T from the same cultures shows that the CD28, CD11a, NK cytotoxicity and

proliferation, in addition to CD56 expression, can distinguish these two populations. The described differences may all be reconciled with the hypothesis that CIK cells may represent an even more advanced stage of differentiation of CD3⁺CD56⁻ T cells.

The CIK culture conditions result in a strong upregulation of CD56 on both the NK and CIK cells. Indeed, CD56 is the best marker, together with CD28, to distinguish between T and CIK cells during culture, and we confirm that expression of CD56 is accompanied by the acquisition of cytotoxicity²⁵, even if its functional role is still unknown²⁶. Nonetheless, CD56 has been repeatedly invoked as a crucial marker for T cytotoxic cells^{27,28}.

Interestingly, Slavin and colleagues were the first to use intentionally mismatched donor lymphocytes activated in vitro with rhIL-2 for cancer immunotherapy by selection of activated CD56-positive cells, indicating that such cells could be safely administered without causing graft-vs-host disease despite major histocompatibility complex disparity of human leukocyte antigen (HLA) class I and class II.

CIK cells differ from circulating CD3⁺CD56⁺ cells functionally, since they are cytotoxic for NK-cell targets, such as K562 cells and are polyclonal. The cytotoxic activity of CIK cells may be due to their higher expression of NKG2D with respect to circulating CD3⁺CD56⁺ cells. Indeed, NKG2D, together with the DAP10 molecule, has been shown to play a role in the mechanism of cytotoxicity of CIK and T cells^{7,29-31}. Nonetheless, it is intriguing that at the end of culture, CD56⁻ T cells, which are also NKG2D-positive, are not cytotoxic against K562.

The origin of circulating CD3⁺CD56⁺ is still unclear. These cells have been described already, many years ago^{32,33}. Here we have confirmed that these cells are often oligoclonal²⁷ suggesting they may arise in vivo following activation of T cells by antigenic challenge. Such a hypothesis is supported by our observations that cord blood does not contain significant numbers of circulating CD3⁺CD56⁺ cells, whereas most adult peripheral blood samples do. Their function is unclear but have been described as abundant in the liver^{34,35} and gut³⁶.

In addition, we believe CIK cell are different from the NK-T population^{37,38} because the latter bear few invariant α and β chains³⁹. CIK cells also differ from T-NK effector memory (originally identified as T γ), which have a LGL morphology and are CD16⁺, as reviewed recently²⁰.

Based on previous reports on unrestricted anti-tumoral cytotoxic activity of human CIK cells in vitro^{24,9,40,41} and in vivo^{1,2,8,10,42} CIK cells have been used for adoptive therapy in cancer patients. Once in vivo CIK cells should not be able to migrate to the lymph nodes due to the lack of CD62L (L-selectin) and CCR7⁴³, but rather be able to cross the inflamed endothelium of bone marrow and of other organs using CD49d (α 4 integrin) and CD11a (β 2 integrin), as well as CXCR4, as discussed previously (this article and⁴¹). The available in vivo data so far indicate strong tumor localization for human and mouse CIK cells in different experimental models^{8,42,44}. Interestingly, cells positive for CD3 and NKG2D have been described in melanoma lesions⁴⁵ and CD3⁺CD56⁺ cells have been found in the ascitic fluids from advanced ovarian cancer patients. It is therefore tempting to

speculate that CIK cells should home to tumors and express a strong local cytotoxic effect, also in consideration of the strong expression of the CD27 molecule, because a correlation has been suggested between total number of administered CD27⁺ tumor infiltrating lymphocytes and clinical response in melanoma patients⁴⁶.

Moreover, the nonrestricted anti-tumoral cytotoxicity (NK-like), the limited acute graft-vs-host disease reactivity in vitro (in animal models and in patients even in allogeneic HLA-matched combinations) in conjunction with the apparent lack of KIR expression may suggest their use even in the context of HLA-mismatched haploidentical bone marrow transplantation⁴⁷⁻⁴⁹.

Overall, these data suggest that CIK cells are CD8 terminally differentiated activated EMRA lymphocytes. We cannot affirm that CIK cells recapitulate in vitro what occurs in vivo following antigenic stimulation leading to circulating CD3⁺CD56⁺ cells, even if it is tempting to speculate so. The full ontogenic and functional characterization of CIK cells described here is of importance in view of the ongoing Phase II studies using these cells as therapeutic agents in cancer patients.

Supplementary Table 1. Genes differentially expressed in CIK vs T cells

Molecule	Type	Fold Change
CR2	transmembrane receptor	7.92
FASLG	cytokine	4.40
TNF	cytokine	2.90
PLCG2	enzyme	2.81
IL8RB	G-protein coupled receptor	2.72
CCL4	cytokine	2.70
CX3CL1	cytokine	2.63
IL12RB2	transmembrane receptor	2.63
GZMB	peptidase	2.55
CCL3	cytokine	2.49
CX3CR1	G-protein coupled receptor	2.41
SKIV2L	translation regulator	2.33
PIK3R1	kinase	2.18
PRF1	other	2.17
PTGDR	G-protein coupled receptor	2.16
KLRD1	transmembrane receptor	2.12
THBS3	other	2.10
CCL3	cytokine	2.04
CCL5	cytokine	2.02
XCL1	cytokine	1.98
CHUK	kinase	1.98
PLCG1	enzyme	1.95
COL7A1	other	1.91
PTPN7	phosphatase	1.89
LAG3	transmembrane receptor	1.88
FCGR3A	transmembrane receptor	1.86
HLA-DQA1	transmembrane receptor	1.86
MADD	other	1.85
LMO4	transcription regulator	1.83
CD160	transmembrane receptor	1.82
IFNB1	cytokine	1.79
IRF5	transcription regulator	1.77
GATA3	transcription regulator	1.75
NFIL3	transcription regulator	1.74
IFNAR2	transmembrane receptor	1.71
SEMA7A	other	-1.72
LGALS3	other	-1.72
FLT3LG	cytokine	-1.85
CD79A	transmembrane receptor	-1.92
TCF12	transcription regulator	-1.92
CXCR4	G-protein coupled receptor	-2.00
RPS7	other	-2.00
IL5RA	transmembrane receptor	-2.04
BST1	enzyme	-2.04

Mean coefficient of variation was 19%

Genes indicated in bold belong to the tumor necrosis factor hub

Supplementary Table 2. Genes differentially expressed in CIK versus NK cells

Molecules	Type	Fold Change
CD3D	transmembrane receptor	21.89
CD1A	other	13.81
CD5	transmembrane receptor	13.03
CD6	transmembrane receptor	7.93
CD27	transmembrane receptor	7.48
SLAMF1	transmembrane receptor	5.98
CD8A	other	5.74
CCR7	G-protein coupled receptor	5.63
DPP4	peptidase	4.12
TIMP1	other	4.09
STAT1	transcription regulator	3.87
JAK1	kinase	3.58
EBI2	G-protein coupled receptor	3.58
LAMA1	other	3.47
CD46	other	3.35
BAK1	other	3.33
IL7R	transmembrane receptor	3.27
MYD88	other	3.27
IFNGR1	transmembrane receptor	3.08
FLT3LG	cytokine	3.04
CXCR3	G-protein coupled receptor	2.91
ITGA4	other	2.76
CXCR4	G-protein coupled receptor	2.74
AP1G2	transporter	2.59
PTP4A3	phosphatase	2.58
ICOS	other	2.57
TNF	cytokine	2.52
CDH5	other	2.37
CISH	other	2.35
SEMA7A	other	2.24
LTB	cytokine	2.18
ITGA6	other	2.18
INPP4B	phosphatase	2.17
BCL2L1	other	2.13
SOS1	other	2.11
CD82	other	2.11
LGALS3	other	2.08
CCR5	G-protein coupled receptor	2.00

INPP5D	phosphatase	2.00
TNFSF8	cytokine	1.99
TCF12	transcription regulator	1.96
ITGA5	other	1.96
CHUK	kinase	1.95
CDC42BPA	kinase	1.94
PTPRC	phosphatase	1.94
CD3E	transmembrane receptor	1.93
IL5RA	transmembrane receptor	1.93
IKZF1	transcription regulator	1.93
CCNG2	other	1.93
XBP1	transcription regulator	1.92
MCL1	transporter	1.92
MADD	other	1.91
GATA3	transcription regulator	1.90
VEGFC	growth factor	1.89
HSPD1	other	1.88
SELL	other	1.87
BST1	enzyme	1.86
TAP1	transporter	1.86
IL12RB2	transmembrane receptor	1.82
CCND3	other	1.82
TNFRSF25	transmembrane receptor	1.81
CD7	other	1.81
CD40LG	cytokine	1.80
CSF3R	transmembrane receptor	1.79
FAS	transmembrane receptor	1.79
IL12RB1	transmembrane receptor	1.79
CD74	transmembrane receptor	1.76
MAP2K1	kinase	1.75
UBE2G1	enzyme	1.75
PRKCB1	kinase	1.74
BCL2L1	other	1.74
CX3CR1	G-protein coupled receptor	1.72
TNFSF12	cytokine	1.71
TBX21	transcription regulator	-1.72
BAX	other	-1.75
CD151	other	-1.79
XCL1	cytokine	-1.85

YY1	transcription regulator	-1.85
FYN	kinase	-1.92
GSTM1	enzyme	-1.92
BTG1	transcription regulator	-1.96
NFKBIA	other	-1.96
COL9A2	other	-2.08
CCL3	cytokine	-2.22
FGFR1	kinase	-2.22
PTPN6	phosphatase	-2.27
CCL4	cytokine	-2.38
IRF5	transcription regulator	-2.38
MGST2	enzyme	-2.38
CD63	other	-2.38
CFLAR	other	-2.38
WSB1	other	-2.38
UVRAG	other	-2.44
CSF2	cytokine	-2.50
YES1	kinase	-2.56
PLCG1	enzyme	-2.70
PRF1	other	-2.70
KLRD1	transmembrane receptor	-2.78
PIK3R1	kinase	-2.78
INHBC	growth factor	-2.78
SHC1	other	-2.86
GZMB	peptidase	-2.94
MATK	kinase	-3.03
IL8RB	G-protein coupled receptor	-3.03
FGR	kinase	-3.13
CCL3	cytokine	-3.23
CX3CL1	cytokine	-3.33
FCGR3A	transmembrane receptor	-3.70
PTGDR	G-protein coupled receptor	-4.00
LYN	kinase	-4.17
CR2	transmembrane receptor	-4.35
MMP21	peptidase	-4.35
HTRA1	peptidase	-7.14
CD160	transmembrane receptor	-8.33
PLCG2	enzyme	-8.33

Mean coefficient of variation was 14%

Genes indicated in bold belong to the tumor necrosis factor hub

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Chapter 3

Dual functional capability of CD3⁺CD56⁺ CIK cells, a T cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity

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Submitted

Abstract

CD3⁺CD56⁺ cytokine-induced killer (CIK) cells display a potent cytolytic activity. The adhesion molecule LFA-1 plays a crucial role in binding as well as in cytolytic activity of CIK cells against tumor target cells expressing the corresponding ligands. CIK cells express activating NK receptors including NKG2D, DNAM-1 and low levels of NKp30. Cell Signalling not only through TCR/CD3, but also through NKG2D, DNAM-1 and NKp30, leads to CIK cell activation resulting in granule exocytosis, cytokine secretion and cytotoxicity. Antibody blocking experiments revealed that DNAM-1, NKG2D and NKp30 are involved in tumor cell recognition and killing. Anti-CMV specific CIK cells could be expanded in standard CIK cultures and mediate both specific, MHC-restricted recognition and TCR-independent NK-like cytolytic activity against leukemic cell lines or fresh leukemic blasts. Antibody blocking of LFA-1 and DNAM-1 led to significant reduction of both CTL and NK cell functions, while blocking of NKG2D and NKp30 only inhibited NK-like cytotoxicity. Their dual effector function suggests that CIK cells, when used in a clinical setting, may control both neoplastic relapses and viral infections, two frequently associated complications in transplanted patients.

Introduction

Cytokine-induced killer (CIK) cells are ex vivo activated lymphocytes that can be obtained in large numbers within 3 weeks of culture from either human peripheral blood or bone marrow, or cord blood mononuclear cells by the sequential addition of interferon- γ (IFN- γ), anti-CD3 antibody (OKT3), and high doses of recombinant human interleukin-2 (rhIL-2)¹⁻⁴. CIK represent an heterogeneous cell population including a large majority of CD3⁺CD56⁺ cells and minor fractions of typical T cells (CD3⁺CD56⁻) and NK cells (CD3⁻CD56⁺)^{5,6}. CIK cells can lyse a broad array of tumor targets, including acute myeloid leukemia (AML), chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL) by a non MHC-restricted, natural killer (NK)-like mechanism^{3,6}. Interestingly, in mice, CIK display negligible alloreactivity and cause minimal graft-versus-host disease as compared to allogeneic splenocytes, when infused after allogeneic bone marrow transplantation in murine models^{7,8}. CIK cells express several chemokine receptors and can migrate to the site of tumors after intravenous administration, as shown by models in vivo⁹⁻¹³. Since CIK cells could be produced by a simple approach and displayed antitumor activity in vitro, they appeared to be suitable candidates for cell therapy in solid and hematopoietic tumor treatment. Indeed, both autologous and allogeneic CIK cells have been used in phase I and II clinical trials for the treatment of different tumor types. In these trials, they displayed limited toxicity, while evidence has been obtained that they exert antitumor activity¹⁴⁻²⁰.

The molecular structures that account for tumor recognition and killing by CIK cells are only partially understood. Previous observations suggested a possible involvement of the NKG2D and LFA-1 molecules²¹⁻²³, however, little is known on the role of other activating NK receptors including DNAM-1, NKp30, NKp44, NKp46, as well as on the involvement of TCR/CD3 complex in the cytolytic activity of CIK cells.

Our previous studies clarified that CD3⁺CD56⁺ CIK cells have phenotypic characteristics typical of terminally differentiated CD8⁺ effector memory T cells (T_{EMRA}; CCR7-, CD45RA⁺, CD62L^{low}, CD11a⁺, CD27⁺, CD28⁺). We also showed that CIK cells originate in vitro from CD56-CD8⁺ T cell progenitors which strongly expand upon culture in the presence of IL-2 and acquire CD56 antigen⁵. In addition, CIK cells share several characteristics with NK cells, such as the large granular lymphocyte morphology, the capacity to kill the HLA class I-negative cell line K562, and the surface expression of high densities of CD56 and NKG2D. Different from NK cells, however, CIK cells express low densities of NKp30, whereas they do not express NKp44 and NKp46, and the inhibitory killer immunoglobulin-like receptors, NKG2A and CD94⁵.

In the present study, we investigated in detail the receptors involved in the NK-like cytolytic activity of CIK cells and analyzed whether they have retained also their ability to elicit specific cytolytic effector T cell function.

Methods

Cells

The human cell lines BJAB (B lymphoma), KARPAS422 (B lymphoma), MOLT4 (T cell leukemia), RAJI (B cell leukemia) and K562 (erythroleukemia) were maintained in RPMI-1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Euroclone, Wetherby, West Yorkshire, U.K.), 2 mM L-glutamine (Euroclone) and 110 µM gentamycin (PHT Pharma, Milano, Italy), hereafter called complete medium.

Leukemic cells, obtained by Ficoll-Hypaque density centrifugation of peripheral blood samples, were used for cytolytic assays after thawing.

Phytohemagglutinin-induced blasts (PHA-blasts) were obtained by stimulating peripheral blood lymphocytes with 1 µg/mL PHA (Sigma-Aldrich, St Louis, MO, USA) and 120 U/mL rhIL2 (Proleukin; Chiron, Emeryville, CA, USA).

Generation of CIK cells

CIK cells were prepared as previously described⁵. Briefly, the CD56-depleted fractions separated from peripheral blood mononuclear cells (PBMCs) by negative selection using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD56 microbeads (Miltenyi), were cultured in serum-free X-VIVO-15 medium (BioWhittaker, Walkersville, MD, USA) with 1000 U/mL IFN-γ (Gammakine; Boehringer Ingelheim, Vienna, Austria) added on day 0, 50 ng/mL anti-CD3 (OKT-3, Janssen-Cilag S.p.a., Italy) added on day 1, and 500 U/mL rhIL-2 included in the medium from day 1

onwards. Expansion was performed for 21 to 28 days. At the end of the expansion, CD56⁺ CIK cells were immunoselected with anti-CD56 microbeads according to the manufacturer's instructions.

Anti-CMV specific CIK cells were generated, as described above, starting from peripheral blood lymphocytes obtained from selected HLA-A*0201 CMV-seropositive healthy donors. Anti-CMV specific lymphocytes were identified by flow cytometry after staining with PE-conjugated HLA-A*0201/pp65₄₉₅₋₅₀₃ tetramer (Immunotech Laboratories, Beckman Coulter, Marseille, France) and FITC-conjugated anti-CD8 antibody (Becton Dickinson, San Jose, CA, USA). Anti-CMV specific cells were isolated at the end of the expansion, using cognate PE-conjugated tetramers followed by separation with anti-PE antibody-coated microbeads (Miltenyi) and MACS (Miltenyi). After sorting, tetramer-positive cells were resuspended in X-VIVO-15 medium containing 500 U/mL rhIL-2 and irradiated allogeneic feeder cells, and cultured for 2 weeks. At the end of culture, CD56⁺ CMV-specific CIK cells were immunoselected.

mAbs and Flow Cytometry Analysis

To characterize the surface expression of the activating receptor ligands on the tumor cell lines, a panel of mAbs was used: anti-ULBP1 (170818 clone), anti-ULBP2 (165903 clone), anti-ULBP3 (166510 clone), and anti-MICA/B (159207 clone) (R&D Systems, Minneapolis, MN). Anti-Nectin-2 (L14 clone) and anti-PVR (L95 clone) were previously described²⁴. The adhesion molecules CD54 (ICAM-1), CD102 (ICAM-2), and CD50 (ICAM-3) were detected with

PE of FITC-conjugated antibodies clones HA58, CBR-1C2/2 and TU41 respectively (BD Biosciences Pharmingen, San Diego, CA).

CIK cells were characterized using FITC-conjugated anti-CD3 (SK7 clone) and PE-conjugated anti-CD56 (NCAM16.2 clone) mAbs (Becton Dickinson). To analyze the expression of activating receptors on CIK cells, the following mAbs were used: anti-NKG2D (BAT221 clone), anti-NKp30 (A76 clone), anti-NKp46 (BAB281 clone), anti-NKp44 (KS38 clone), and anti-DNAM-1 (F22 clone)^{24,25}. CD11a (LFA-1) was detected with FITC-conjugated antibody clone G-25.2 (Becton Dickinson).

For indirect immunofluorescence staining, cells were stained with the primary mAb, followed by PE-conjugated goat anti-mouse IgG1 (Invitrogen, Paisley, UK) or FITC-conjugated goat anti-mouse Ig second reagent (Becton Dickinson). A FACScan flow cytometer device (Becton Dickinson) was used to analyze the samples.

CFSE and PKH26 Staining

Target cells were washed and resuspended in 1 mL of phosphate buffer solution (PBS) with 1% bovine serum albumin at a final concentration of 5×10^6 /mL, then labeled for 10 min at 37°C with green fluorescent dye carboxyfluorescein succinimidyl ester (CFSE, 2 µM, Sigma-Aldrich). Quench-staining was performed on ice for 5 min by adding 5 volumes of ice-cold complete RPMI. Cells were then washed three times with ice-cold PBS with 1% bovine serum albumin and cultured under appropriate conditions.

For PKH-26 staining of CIK cells, 10×10^6 cells were washed in PBS and the pellet was resuspended in 500 µL of diluent C (Sigma-

Aldrich). Lipophilic red fluorescent dye PKH-26 (Sigma-Aldrich) was diluted at a final concentration of 4 μ M in 500 μ L of diluent C and the solution was rapidly added to cells and incubated for 5 min at room temperature with occasional agitation. 1 mL of FBS was then added, and after 1 min of incubation at room temperature, cells were washed three times in complete culture medium.

Cell Conjugation assay

A total of 5×10^5 PKH26-labeled CIK cells and 5×10^5 CFSE-labeled target cells were resuspended in 200 μ L of complete medium containing or lacking 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) and mixed in a 12- x 75-mm polystyrene tube (Becton Dickinson Labware, Franklin Lakes, NJ). The effector-target cell mixture was centrifuged at 1,000 rpm for 1 min and incubated at 37°C for the indicated time points. The cells were then gently resuspended and analyzed by flow cytometry. The conjugation ratio was calculated as the portion of FITC/PE double positive events within the PE-positive events. For antibody-blocking experiments, 5×10^5 PKH26-labeled CIK cells were preincubated with 10 μ g/mL blocking anti-LFA1 mAb for 30 min and then the conjugation ratio was detected by flow cytometry as described.

Calcein release cytotoxicity assay

Cell lysis was evaluated using the calcein release assay as previously described². Briefly, target cells were labeled for 30 minutes at 37°C with 3.5 μ M calcein-acetoxyethyl ester (calcein-AM, Fluka, Sigma-Aldrich Company, Ayrshire, UK). After washing, labeled target cells were distributed in 96-well plates at 5×10^3 per well. CIK

cells were added at different effector-to-target (E:T) ratios. In some experiment, the cytotoxicity assay was performed in presence of 1 mM EDTA. After 4 hours, the cells were sedimented by centrifugation, 100 µL supernatant were collected and calcein release was determined using a fluorescence microplate reader (GENios, TECAN, Austria GmbH, Salzburg, Austria) with excitation at 485nm and emission at 535nm. The percentage of specific calcein release was calculated using the following formula: percent specific lysis = (test release minus spontaneous release) times 100 divided by (maximal release minus spontaneous release). Maximal lysis was achieved with 1% Triton X-100.

In some experiments, autologous or recipient PHA- blasts pulsed with pp65₄₉₅₋₅₀₃ or a HLA-A*0201-binding control peptide (Primm, Milano, Italy) were used as target cells. Peptide-pulsed cells were incubated with 10 µg/mL peptide for 16 hours at 37°C, prior to labelling with calcein-AM.

For masking experiments, anti-NKp30 (F252 clone), anti-DNAM-1 (F5 clone), anti-NKG2D (BAT221 clone) and anti-HLA-I (W6/32 clone) were used²⁶. Masking of LFA1 was performed with TS1-22 hybridoma supernatant (a kind gift of Dr. P. Allavena, Istituto Clinico Humanitas, Milano, Italy). CIK cells were preincubated 15 minutes at RT with 10 µg/mL of mAbs or surnatant and, after washing, used in the cytolytic assay tested.

Redirected cytotoxicity assay

For redirected killing assay, FcγR-positive cell line P815 (murine mastocytoma) was labeled with calcein-AM as described

above. Anti-CD3 (OKT3 clone), anti-NKG2D (BAT221 clone), anti-NKp30 (A76 clone), anti-DNAM-1 (F22 clone), anti-NKp46 (BAB281 clone), or isotope-matched control antibodies were added at saturating concentration and incubated for 15 minutes. The P815 target cells were then used as target in the calcein release cytotoxicity assay as described.

Degranulation assay

Degranulation assay was performed as previously described^{27,28}. Briefly, CIK and P815 cells loaded with anti-CD3, anti-NKG2D, anti-NKp30, anti-DNAM-1, anti-NKp46, or IgG1 isotype control mAb were plated at E:T ratio of 1:1 and incubated for 2 h at 37°C in the presence of CD107a-PE mAb (BD Pharmingen). CIK cell degranulation was assessed by cell surface staining for the lysosomal markers CD107a by flow cytometry.

Statistical analysis

The data were analysed using paired or unpaired Student *t* tests, as appropriate. (*: p<0.05; **: p< 0.01; ***: p< 0.001).

Results

Natural cytotoxicity of CD3⁺CD56⁺ CIK correlates with the binding rate to tumor targets and is LFA-dependent

In the standard protocol for CIK cells expansion, cultures are initiated starting from unselected PBMCs and performed as previously described by us⁵ and others¹. In the resulting bulk cultures three subpopulations can be distinguished: CD3⁺CD56⁺ terminally differentiated CIK cells, responsible for the HLA-unrestricted antitumor activity, CD3⁺CD56⁻ early effector T cells, and a minor fraction of CD3⁺CD56⁺ NK cells⁵. To directly assess the functional activity of CD3⁺CD56⁺ CIK cells derived from purified CD3⁺CD56⁻ cells (i.e. the putative peripheral blood precursors of CD3⁺CD56⁺ CIK cells), PBMCs were depleted of CD56-positive cells, and then cultured by applying the standard expansion protocol. Final yields and expansion folds were not changed as compared to CIK cultures started from unmanipulated PBMCs (data not shown). At the end of the culture, CD3⁺CD56⁺ CIK cells were purified by CD56 positive selection (mean purity: 95%, range: 88% to 99% in 5 independent experiments, data not shown). The surface phenotype of such CIK cells did not differ from that previously described for CIK cells obtained from unselected PBMCs and is consistent with $\alpha\beta$ CD8⁺ T EMRA cells (⁵, and data not shown). All the following experiments were performed by using such purified CD3⁺CD56⁺ CIK cells to directly test their antitumor function.

In order to investigate whether cytotoxicity of CIK cells requires effector-target cell contact and involves conjugates

formation, PKH26-labeled CIK and CFSE-labeled BJAB were mixed and incubated at 37 °C for different time intervals. The cells were then analyzed by flow cytometry. Figure 1A shows a representative experiment of 3 performed. $34\% \pm 3\%$ of CIK cells were able to bind target after 5 minutes while a slow progressive decline was observed at later intervals ($24\% \pm 2\%$ after 1 hour) (Figure 1A and data not shown). To investigate whether binding to target cells is necessary to CIK-mediated cytotoxicity, experiments were performed in which conjugate formation was blocked by adding EDTA, a calcium chelator that prevents cell adhesion. Binding of CIK to BJAB cells was significantly inhibited by EDTA at all time points (average reduction of binding rate: $77\% \pm 3\%$; Figure 1A and data not shown). Similarly, the cytolytic activity of CIK cells pretreated with EDTA against BJAB cells was significantly reduced by $79\% \pm 7\%$ at all effector-to-target (E:T) ratios tested ($p < 0.05$; Figure 1B).

The BJAB and KARPAS422 human B lymphoma cell lines displayed different sensitivities to CIK-mediated cytotoxicity, of $35\% \pm 0.6\%$ and $22\% \pm 4\%$ specific lysis at 10:1 E:T ratio, respectively ($n = 3$; $p < 0.05$; Figure 1D). Notably, CIK-BJAB conjugates were significantly more abundant than those formed with KARPAS422 cells during the 60 min incubation period ($n = 3$; $p < 0.05$; Figure 1C). These results suggest that CIK cell binding to target and formation of the cellular conjugates are prerequisite to and may correlate with CIK-mediated cytotoxicity.

Cell surface adhesion molecules, such as lymphocyte function-associated antigen (LFA)-1, are known to participate in effector-target recognition and stable conjugate formation. CIK cells

expressed LFA-1 at high levels. Moreover, the most susceptible target BJAB expressed all the major LFA-1 ligands (ICAM-1, -2, and -3) (Figure 1E). In contrast, KARPAS422 did not express ICAM-1 and only low levels of ICAM-2 and -3 (data not shown). In addition, while CIK-target cell conjugate formation and cytotoxicity against BJAB were strongly inhibited by anti-LFA-1 mAb (respectively 76% ± 2% and 68% ± 6%; n=3; p<0.05; Figure 1F), the cytotoxicity against KARPAS422 was only marginally affected (data not shown). These results confirm that LFA-1 has a crucial role in binding as well as in cytotoxicity of CIK cells, but additional molecules may also be involved in target cell recognition by CIK cells.

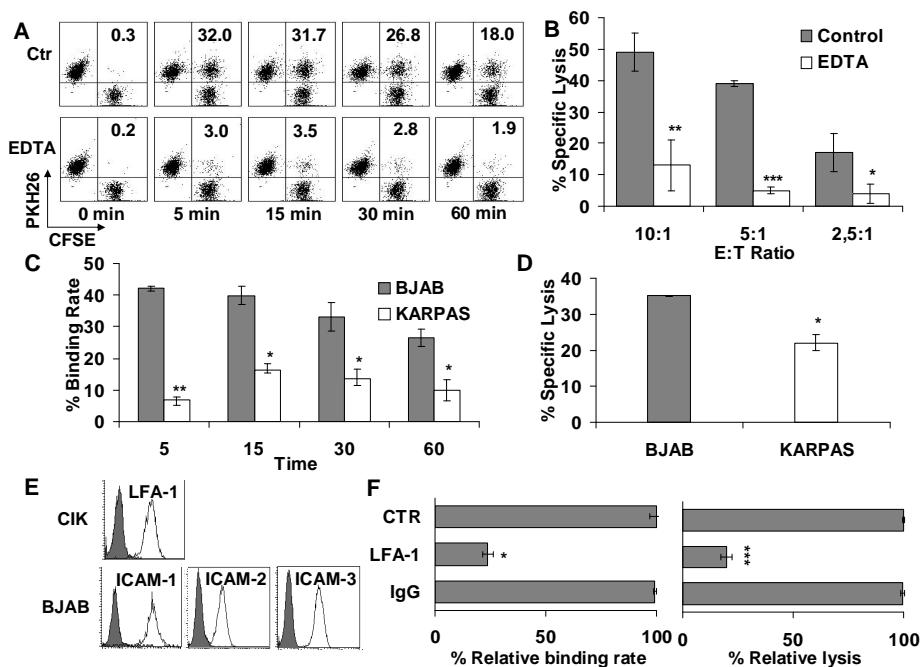


Figure 1. Natural cytotoxicity of CD3⁺CD56⁺ CIK correlates with binding to tumor targets and is LFA-1 dependent. (A) PKH26-labeled CIK and CFSE-labeled BJAB were mixed and incubated at 37 °C for the indicated times with or

without 1 mM EDTA and analyzed by flow cytometry. Percentage of binding (values indicated in the upper right quadrants) is calculated as the portion of CFSE/PKH-26 double positive events within the PKH-26 positive events. One representative example is shown. (B) Cytotoxicity of CIK cells against BJAB in presence (white bars) or absence (grey bars) of 1 mM EDTA was evaluated at the indicated effector-to-target ratios. Data were collected from three independent experiments and analyzed by Student *t*-test; *, p<0.05; **, p<0.01; ***, p<0.005. (C) CIK cells binding to BJAB or KARPAS422 at different times was analyzed by flow cytometry. Data were obtained from at least three independent experiments and analyzed by Student *t* test; *: p<0.05; **: p<0.01. (D) Cytotoxicity of CIK cells against BJAB or KARPAS422 was evaluated by Calcein-release assay. Data were obtained from at least three independent experiments and analyzed by Student *t*-test; *, p<0.05. (E) Expression of LFA-1 and their ligands (ICAM-1, -2, and -3) was determined respectively on CIK cells and BJAB target by flow cytometry. Background staining using an isotype control antibody is shown in each histogram (grey curve). (F) The functional role of LFA-1 in CIK binding to BJAB (left panel) and in cytotoxicity (right panel) was evaluated. For binding assays, PKH-26-labeled CIK cells were exposed to saturating concentrations of blocking antibody anti-LFA-1, control IgG or medium alone (CTR) for 30 min and then incubated with CFSE-labelled BJAB for 15 min. For cytotoxicity assays, CIK cells were preincubated with saturating concentration of anti-LFA-1 or mouse IgG1 for 15 min and then tested in a Calcein-release assay against BJAB. Data are the mean percentage lysis obtained with respect to untreated controls. Data were obtained from at least three independent experiments and analyzed by Student *t*-test; *: p<0.05; **: p<0.01; ***, p<0.005 compared with control.

Functional role of CD3 and activating NK receptors in CIK cells

We next attempted to identify the receptors mediating non-HLA restricted tumor cell killing by CIK cells. To investigate this point, we analyzed the main receptors involved in NK cell triggering in the process of tumor cell lysis. These include NKp46, NKp30,

NKp44, NKG2D and DNAX accessory molecule-1 (DNAM-1). As previously reported by our⁵ and other groups²¹, CIK cells express NKG2D at high density. The NCRs NKp44 and NKp46 were not or very poorly expressed, while NKp30 was present at low densities (Figure 2A and⁵). On the other hand, DNAM-1 was found to be highly expressed (Figure 2A).

We investigated the functional status of the various NK receptors expressed on CIK cells by a reverse Ab-dependent cellular cytotoxicity assay. As shown in Figure 2B, robust redirected lysis was obtained by crosslinking of NKG2D and DNAM-1, although less robust than the one observed by anti-CD3 mAb. NKp30 crosslinking also led to redirected cytolysis, albeit to a lesser extent. On the contrary, anti-NKp46 mAb did not induce target cell lysis (Figure 2B). We also tested cytotoxic granule release following receptor engagement, using a mAb directed against the lysosomal-associated membrane protein-1 (LAMP-1, CD107a)^{27,28}. Consistently with cytotoxicity data, CD3 ligation resulted in an increase in the percentage of cells undergoing degranulation ($p < 0.005$) (Figure 3C). Ligation of DNAM-1, NKG2D and partially NKp30 also resulted in some degranulation (Figure 2C). Finally, also the production of IFN- γ and tumor necrosis factor (TNF)- α was investigated following receptor engagement. Crosslinking of CD3 resulted in IFN- γ and TNF- α production ($p < 0.05$). A weaker but significant cytokine secretion was detected also after stimulation of NKp30 and NKG2D (data not shown).

Altogether, these results demonstrate that signals delivered not only through TCR, but also through activating NK receptors can

lead to CIK cell activation resulting in granule exocytosis, cytokine secretion and cytotoxicity.

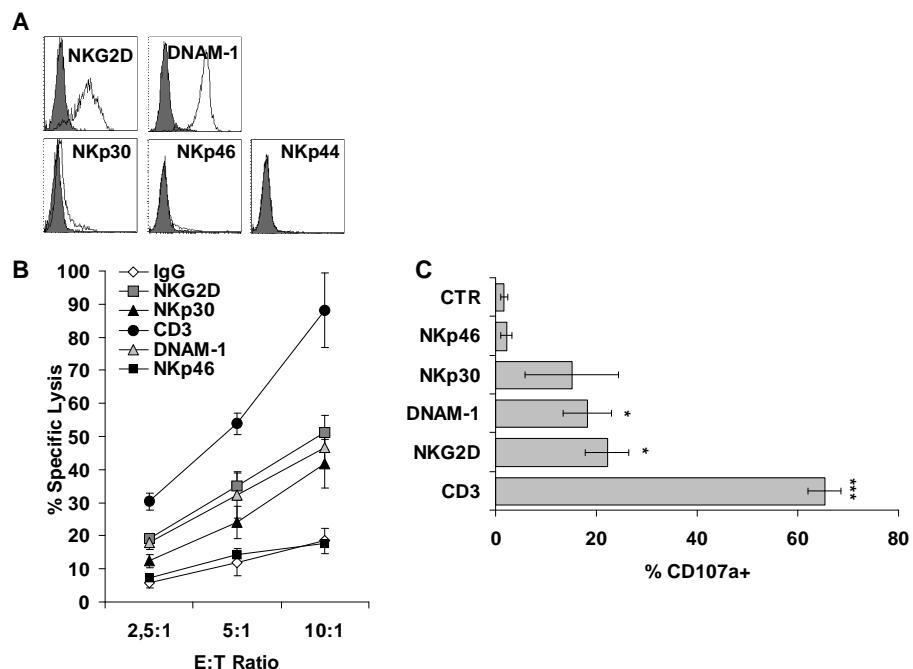


Figure 2. Functional role of CD3/TCR and NK cytotoxic activating receptors on CIK cells. (A) Expression of activating receptors NKG2D, DNAM-1, NKp30, NKp46, and NKp44 on CIK cells was analyzed by flow cytometry. Grey profiles represent isotype controls. (B) Redirected killing assay was performed Calcein release assays using CIK cells treated with indicated agonist mAbs and calcein-labeled P815 cells. The data were obtained from three independent experiments. (C) CIK cells were triggered with P815 cells loaded with different mAbs, and then assayed for degranulation by cell surface staining for the lysosomal markers CD107a. The data were obtained from at least three independent experiments and analyzed by Student *t*-test; *: *p*<0.05; **, *p*<0.005 compared with control.

Effect of mAb-mediated blocking of NKG2D, DNAM-1 and NKp30 on CIK mediated target cell lysis

In order to identify the triggering molecules that are actually involved in the induction of CIK-mediated lysis of tumor targets, we first analyzed the ligands of activating NK receptors expressed on target cells.

Thus, we assessed the expression of ligands of NKG2D (MICA/B and ULBPs) and DNAM-1 (Nectin-2 and PVR) by the use of specific mAb and FACS analysis. Figure 3 shows two targets similarly susceptible to CIK-mediated lysis that differ for the expression of these ligands. While, RAJI cells were virtually negative for all ligands tested, MOLT4 were positive for PVR and ULBP-1 and -2 (Figures 3A and B).

Analysis of the effect of antibody blocking of the activating receptors expressed by CIK cells was consistent with data on ligand expression. Indeed, masking of DNAM-1 had no effect on CIK-mediated lysis of RAJI, while lysis of MOLT4 was partially inhibited. Similarly, NKG2D-masking resulted in a sharp inhibition of lysis of MOLT4 only. Interestingly, mAb-mediated blocking of NKp30 consistently resulted in a significant inhibition of killing of both targets implying that RAJI and MOLT4 express NKp30 ligands²⁹ (Figures 3C and D).

Taken together, these data indicate that NKG2D, DNAM-1 and NKp30 may be involved in CIK-mediated killing of target cells upon recognition of their specific ligands.

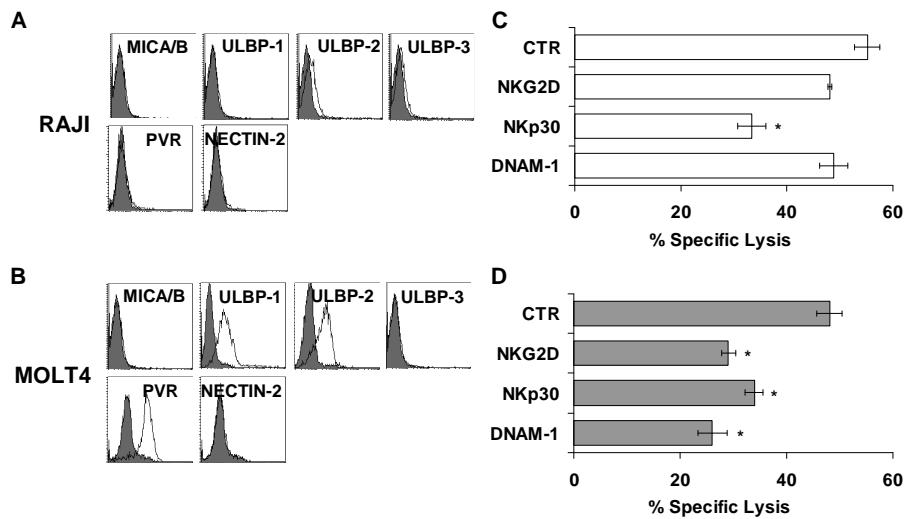


Figure 3. Direct role of activating receptors in CIK-mediated lysis of tumor targets (A, B) RAJI (A) and BJAB (B) were analyzed for expression of MICA/B and ULBP-1, -2, and -3 (NKG2D ligands) and PVR and Nectin-2 (DNAM-1 ligands) by flow cytometry. Grey profiles represent isotype control. (C, D) Blocking of activating receptors NKG2D, NKp30 and DNAM-1 in CIK cells cytotoxicity against RAJI (C) and MOLT4 (D) targets. CIK cells were preincubated with saturating concentrations of anti-NKG2D, anti-NKp30, anti DNAM-1 and cytotoxic activity measured in Calcein-release assays. The data were obtained from three independent experiments and analyzed by Student's t test, *, p<0.05; ***, p<0.005 compared with control.

CIK cells retain TCR-mediated antigen specificity

The data above suggested that CIK cells may maintain a functional TCR/CD3 complex and acquire NK-like cytolytic functions through the expression of activating NK receptors, including NKG2D, NKp30 and DNAM-1. Therefore, we asked

whether TCR expressed by CIK cells could mediate antigen specific binding and function. To this end, CIK expansion was induced in PBMCs from CMV-seropositive healthy donors. As exemplified in the case of the HLA-A*0201 donor shown in Fig 4A, the presence of tetramer positive CD8⁺ T cells was maintained after the expansion of CIK cultures. In five separate experiments the percentage of CMV tetramer⁺ T cells did not significantly change from the beginning (average percentage of tetramer⁺ CD8⁺ cells: 1.7%; range, 0.1%–3.5%) to the end of the culture (average percentage of tetramer⁺ CD8⁺ cells: 1.8%; range, 0.3%–4.2%) (data not shown). More importantly, in these experiments, CMV tetramer⁺ T cells expanded by 36.7 folds (range, 5.4–59.9), with similar efficiency with respect to the total CD8⁺ T cells (mean 34.5, range, 26.9–44.9) (Table 1). This allowed us to obtain a mean absolute number of 42.4×10^6 CMV tetramer⁺ T cells starting from PBMCs obtained from 15 ml peripheral blood (Table 1).

At the end of cultures, the CMV tetramer⁺ T cells were mostly CD3⁺CD56⁺ CIK cells (mean: 75%; range, 40%–88%). Most of them showed an effector memory (T_{EM} , CD62L-CD45RA⁻; mean: 40%; range, 17%–68%) or a terminal effector memory RA⁺ (T_{EMRA} , CD62L-CD45RA⁺; mean: 54%; range, 29%–87%) phenotype (data not shown).

Sample	Absolute number of cells (Day 0)			Absolute number of cells recovered at the end of the expansion protocol (Day +21)		
	TNC x 10 ⁶	CD8 ⁺ x 10 ⁶	CMV tetramer ⁺ CD8 ⁺ x 10 ⁶	TNC x 10 ⁶	CD8 ⁺ x 10 ⁶	CMV tetramer ⁺ CD8 ⁺ x 10 ⁶
#1	15	6.6	0.9	220	177.4	53.9
#2	45	14.2	8.3	480	419.9	45.1
#3	12	3.5	1.6	240	157.2	84.7
#4	10	2.9	0.9	180	120.2	12.7
#5	35	7.8	0.3	510	233.0	15.4
Mean ± sd	23.4 ± 15.7	7.0 ± 4.5	2.4 ± 3.3	326 ± 156.1	221.5 ± 118.1	42.4 ± 29.7
Fold Increase (range)				11.4 (6.7-14.7)	34.5 (26.9-44.9)	36.7 (5.4-59.9)

Table 1

Cell expansion obtained from CMV-seropositive donors

TNC: total nucleated cells

To directly test if indeed antigen-specific CD8⁺ T cells expanded in CIK culture conditions, had maintained TCR-dependent cytolytic activity, we analyzed purified, antigen-specific CD3⁺CD56⁺ CIK cells. In some experiments, as exemplified in Figure 4A, after 21 days of culture, CMV tetramer⁺ T cells were positively selected using anti-PE paramagnetic beads (average percentage of purity: 85%; range, 75%-94%; n= 3; data not shown) and then cultured for 2 weeks in the presence of irradiated allogeneic feeder and rhIL-2. At the end of the culture, CD3⁺CD56⁺ CIK cells were isolated by immunomagnetic selection (average percentage: 85%; range 78%-91%; n= 3; data not shown) and used for functional experiments.

As shown in Figure 4B, CMV-specific CIK cells showed a strong cytolytic activity against CMV-pulsed autologous T-PHA blasts (average lysis: 63% ± 3%; n= 3) at E:T 10:1. No lysis was observed against PHA-blasts pulsed with EBV peptides (average lysis: 1% ± 4%). No killing was detected against autologous unpulsed (average lysis: 4% ± 2%) or allogeneic T-PHA blasts (average lysis: 8% ± 3%).

We next analyzed whether CMV tetramer⁺ CIK cells could mediate NK-like MHC-unrestricted cytotoxicity against a variety of malignant cell lines, including K562 (eritroleukemia), RAJI (B-cell leukemia), and MOLT4 (T-cell leukemia). As shown in Figure 4C, CMV tetramer⁺ CIK cells efficiently killed all malignant cell lines tested with 58% to 82% specific lysis at 30:1 E:T ratio. Perhaps more importantly, CMV tetramer⁺ CIK cells were able to lyse freshly isolated allogeneic leukemic blasts (Figure 4D).

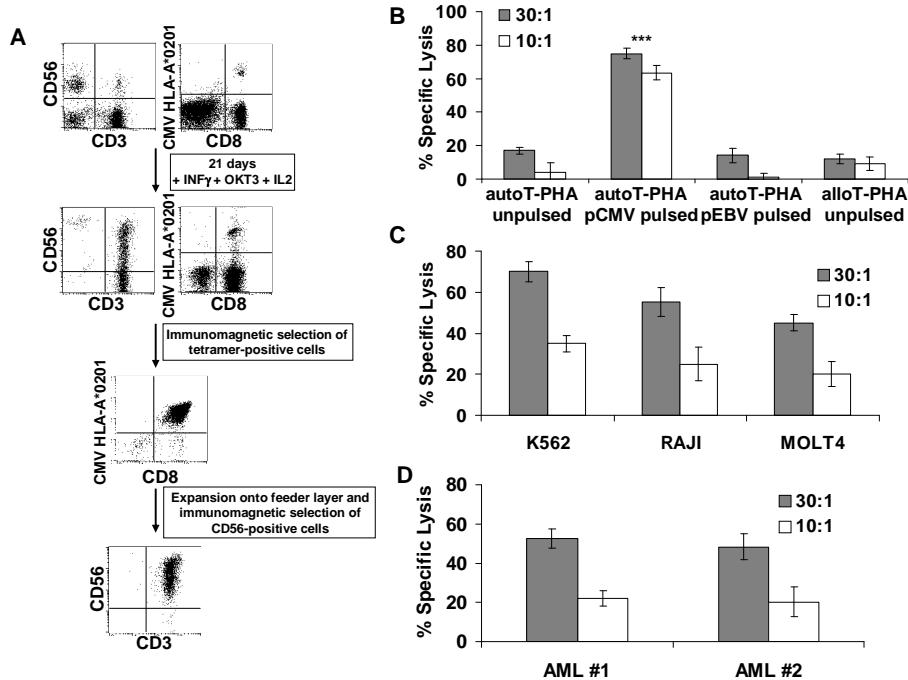


Figure 4. CMV-specific CIK cells maintain their antigen specificity and acquire HLA-unrestricted cytotoxicity. (A) Peripheral blood lymphocytes from HLA-A*0201 seropositive donors were expanded for 21 days in presence of IFN- γ , OKT3 and IL-2 and the percentage of anti-CMV specific cells measured at the end of culture by tetramer binding. CMV-specific CIK cells were isolated by immunomagnetic selection and expanded onto irradiated allogeneic feeder layer for further 2 weeks. At the end of the culture, CD56 $^{+}$ CMV-specific CIK cells were immunoselected. The dot plots of CD3-CD56 and HLA-A*0201/pp65₄₉₅₋₅₀₃ tetramer-CD8 staining, obtained during a representative experiment, are shown. (B) Cytotoxic activity of expanded CMV-specific CIK cells was determined on unpulsed, CMV-pulsed, EBV-pulsed autologous T-PHA blasts and on unpulsed allogeneic T-PHA blasts in Calcein-release assays. The data were obtained from three independent experiments and analyzed by Student's *t* test; ***, p<0.005. (C, D) MHC-unrestricted cytotoxic activity of expanded CMV-specific CIK cells

against a panel of malignant cell lines (panel C) or against freshly isolated myeloid leukemic cells (panel D) was determined in Calcein-release assays. The results show the mean values \pm SD of three independent experiments.

Along this line, CMV tetramer⁺ CIK cells displayed an expression profile of NKG2D, NKp30, DNAM-1 and LFA-1 similar to that of unselected CIK cells (data not shown). Therefore, we further tested the functional capability of all these receptors in the context of both TCR-dependent (antigen-specific) and TCR-independent cytotoxicity. K562 and CMV-pulsed T-PHA blasts were assessed for the expression of ligands recognized by NKG2D, DNAM-1 and LFA-1. As shown in Figure 5, the K562 cells expressed MICA/B, ULBP-2, ULBP-3, PVR, Nectin-2, ICAM-1 and ICAM-2 but not ICAM-3 (Figure 5C), whereas CMV-pulsed autologous T-PHA blasts expressed ULBP-2 and -3, and low levels of PVR and Nectin-2 (Figure 5A). Calcein-release assays were performed in the presence and absence of anti-LFA-1, anti-DNAM-1, anti-NKG2D and anti-NKp30 blocking mAbs. Importantly, the addition of blocking mAb directed against LFA-1 and DNAM-1 induced a strong inhibition of lysis of both targets, possibly interfering with binding of the effector to the target cells (Figures 5B and D). More importantly, anti-NKG2D and anti-NKp30 blocking mAbs affected the non MHC-restricted cytotoxicity but had no effect on TCR-dependent killing of CMV-pulsed autologous T-PHA blasts (Fig 5B and D), suggesting a role in NK-like cytotoxicity only. It is of note that mAb-mediated masking of HLA-class I molecules inhibited lysis of CMV-pulsed autologous T-PHA blasts but not K562 (Figure 5C).

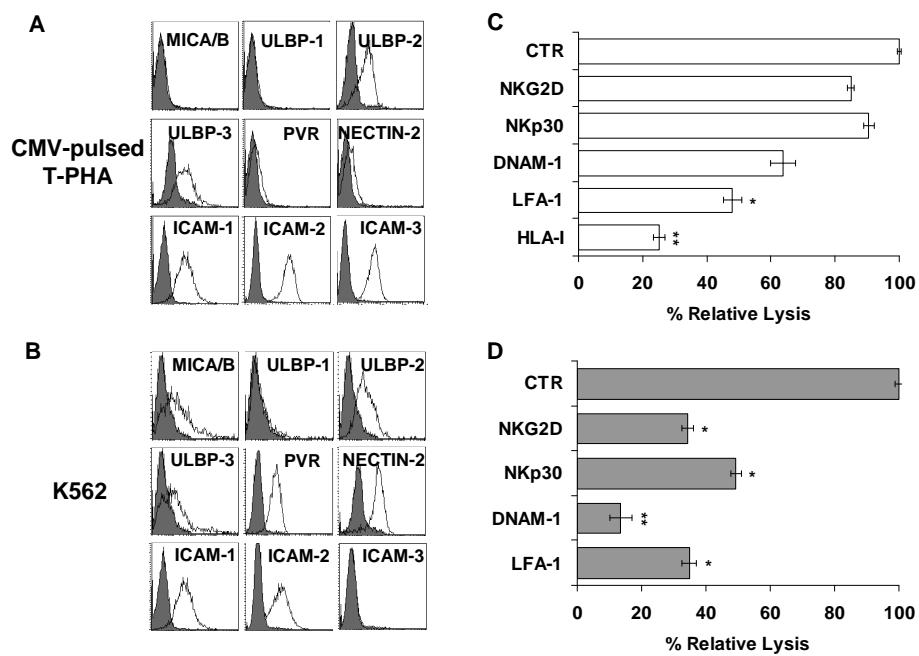


Figure 5. NKP30, NKG2D, LFA-1 and DNAM-1 are differently involved in antigen-specific and HLA-unrestricted cytotoxicity exerted by CMV-specific CIK cells. (A, B) CMV-pulsed autologous T-PHA blasts (A) and K562 cell line (B) were analyzed for expression of MICA/B and ULBP-1, -2, and -3 (NKG2D ligands) and PVR and Nectin-2 (DNAM-1 ligands).by flow cytometry. Grey profiles represent isotype control. (C, D) Blocking of activating receptors NKG2D, NKP30 and DNAM-1 in CIK cells cytotoxicity against CMV-pulsed autologous T-PHA blasts (C) and K562 cell line (D) targets. CIK cells preincubated with saturating concentrations of the indicated or control mAbs were used in Calcein-release assays. The results shown are the mean percentage cytotoxic activity of treated compared to untreated CIK cells. The data were obtained from three independent experiments and analyzed by Student *t*-test. *, p<0.05; **, p<0.01 compared with control.

Discussion

In this report we analyzed CD3⁺CD56⁺ CIK cells obtained from PBMCs after in vitro expansion for 3 weeks by the sequential addition of IFN- γ , OKT3 and IL-2. We show that CIK cells are characterized by dual function acting both as CD8⁺ specific effector T and NK-like cells. Thus they represent a particularly useful tool, in adoptive immunotherapy, to treat not only cancer but also associated life threatening viral infections.

A number of reports on CIK cells, including clinical trials of adoptively-transferred CIK cells infusion, used bulk-expanded CIK cultures. Within bulk cultures two main subpopulations can be distinguished, one coexpressing the CD3 and CD56 molecules, characterized by a phenotype typical of terminally differentiated effector cells, a potent non-MHC-restricted cytotoxicity and low proliferative capacity. The other cell fraction, displaying a CD3⁺CD56⁻ phenotype, is composed of effector T cells with reduced cytotoxicity but higher capability of proliferating. The bulk culture also comprises a small CD3-CD56⁺ classical NK cell subset⁵. In the present study, we characterized in detail the CD3⁺CD56⁺ CIK cell fraction, in terms of their molecular mechanism of activation and cytolytic function.

CD3⁺CD56⁺ CIK cells are capable of rapid binding to lymphoma target cells, such as BJAB. Preventing cell adhesion with EDTA sharply reduces cytolytic activity of CIK cells against BJAB. A direct correlation exists between degree of binding and sensitivity to CIK-mediated cytotoxicity in different cell lines. In this context, a

significant inhibition of CIK mediated cytotoxicity was obtained by blocking the cell surface adhesion molecule LFA-1^{23,29,30}. While it is clear the crucial role of LFA-1 in CIK cells for functional binding and cytotoxicity against target cells such as BJAB, which express high amounts of ICAM-1, -2, and -3 (the major LFA-1 ligands), in the case of other target cells such as KARPAS422, it is likely that other surface molecules are involved in binding.

CIK cells express high levels of CD3 and respond to TCR/CD3-mediated stimulation by degranulating, killing P815 cells loaded with anti-CD3 mAb and producing cytokines such as TNF- α and IFN- γ mAb. This is in agreement with our previous observations that CIK cells are characterized by a phenotypic profile typical of terminally differentiated effector CD8+ T cells which originate in vitro from CD56-CD8+ T cell progenitors. In addition, CIK cells express NKG2D, DNAM-1 and, at lower surface densities, NKp30. In contrast, NKp44 and NKp46 are virtually absent. Using redirected killing, degranulation assays and cytokine secretion we could show that NKG2D, DNAM-1 and NKp30 expressed by CIK cells are functional. Remarkably, CD3 triggering induced higher cytotoxicity in redirected killing and higher degranulation and cytokine production following receptor triggering by activating mAb, as compared with NKG2D, DNAM-1 and NKp30.

In spite of various studies on CIK cells, the molecular mechanism by which they kill tumor cells was not clearly identified. Although the sensitivity of MOLT4 and RAJI cell lines to the cytolytic activity of CIK cells did not appear to correlate with the surface expression of the ligands of DNAM-1 and NKG2D, the inhibitory

effect exerted by anti-DNAM1 or anti-NKG2D blocking antibodies in CIK-mediated lysis of MOLT4 suggested a role of these receptors at least in the case of targets (such as MOLT4) expressing the specific ligands. A partial inhibition of lysis of both targets could be achieved by mAb-mediated blocking of NKp30.

NKG2D has a significant role in triggering IL-2-activated NK cells and its ligation induces calcium flux, cytokine release, and cytotoxicity. NKG2D is also expressed on $\gamma\delta$ T cells and CD8 $^{+}$ TCR $\alpha\beta$ T cells^{31,32}. In contrast to NK cells, crosslinking of NKG2D in antigen-specific CTL clones did not induce calcium flux or cytokine production or cytotoxicity. However, NKG2D signaling has been shown to augment cytotoxic and proliferative responses of T cells to antigen stimulation, thus qualifying NKG2D as a T-cell costimulatory molecule³²⁻³⁴. A major role for NKG2D molecule in CIK cell function has already been proposed^{21,22}. Studies of antibodies blocking NKG2D molecules, RNA interfering and redirected cytolysis indicated that the antitumor cytotoxic activity of CIK cells is exerted through NKG2D rather than TCR engagement^{21,22}.

In the present study we provide evidence that also DNAM-1 and NKp30 play a role in CIK cell-mediated antitumor activity. We show that NKp30, although expressed at relatively low density, is involved in the recognition and killing of lymphoma targets. Notably, a functional role of NKp30 in T cells has been described so far only in the case of IL-15 long term cultured cord blood lymphocytes³⁵. The expression of NKp30 in CIK cells is intriguing also in light of the reciprocal activation between CIK and dendritic cells³⁶ and of the

crucial role of NKp30 in the interaction between NK cells and dendritic cells^{37,38}.

Another relevant information provided by our study is the demonstration that antigen-specific CD8⁺ T cells displaying the CD3⁺CD56⁺ CIK phenotype can be expanded in CIK cultures. We show that these cells are characterized by a dual function. Indeed, they can both specifically kill autologous cells loaded with CMV peptides and lyse tumor cell lines and freshly isolated leukemic blasts. Cytotoxicity mediated by CIK cells is non-MHC restricted and the addition of anti-MHC class I antibody to target cells had no inhibitory effect³⁰. Moreover, CIK cells killed with similar efficiency allogeneic and autologous leukemic cells⁴. Killer inhibitory receptors have not been detected in CIK cells⁵. Interestingly, anti-NKG2D and anti-NKp30 blocking mAbs inhibit the CIK-mediated killing of the HLA-class I negative K562 while no effect was detected on TCR-dependent killing of CMV-pulsed autologous T-PHA blasts. This data suggests that these receptors are only involved in NK-like cytotoxicity. Notably, previous reports suggested that, in T cells, NKG2D plays a role as costimulating molecule rather than as a true receptor. In contrast blocking mAbs against LFA-1 and DNAM-1 induced a significant inhibition of lysis of both target types, most likely by interfering with binding between effector and target cells.

On the basis of the data presented, we can hypothesize that antigen-specific T cells may, in particular inflammatory conditions, here mimicked by IFN- γ and IL-2 stimulation, acquire additional NK-like functions, similar to those already suggested for a population of T cells expanded in the presence of IL-15³⁹⁻⁴¹. Indeed, if this was the

case, antigen-specific T “CIK” cells may home to antigen sites, such as the lung in case of CMV infection, and there exploit also their NK-like potential by contributing to kill infected cells and cytokines release.

The information that CIK cells retain the CTL function, here tested only for anti-CMV specific memory, adds further interest to their potential clinical application. In bone marrow transplanted patients the recovery of functional specific T cells is particularly delayed and most patients suffer from severe infections^{42,43}. In this respect, CIK cells may thus represent a “one coin-two sides” chances of treating leukemia relapses and transferring the donor T memory to cope with most frequent and possibly fatal infections (ie, CMV, Epstein Barr virus, Aspergillus, etc). Indeed the absolute numbers of CMV-specific CIK cells which can be obtained from a small amount of peripheral blood, are fully compatible with the idea of obtaining a clinically effective anti-CMV CTL (less than 1×10^5 in most reports^{44,45}) in standard GMP approved CIK cultures.

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Chapter 4

Enhanced killing of human B-cell lymphoma targets by combined use of cytokine-induced killer cell (CIK) cultures and anti-CD20 antibodies

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Abstract

We have investigated combining adoptive immunotherapy with cytokine-induced killer (CIK) cells and anti-CD20 monoclonal antibodies (mAb) GA101 or rituximab to optimize B-cell non-Hodgkin lymphoma (B-NHL) therapy. CIK cultures alone demonstrated significant cytotoxic activity against B-NHL cell lines or freshly isolated samples in either an autologous or allogeneic combination. This natural cytotoxicity (NC) was mainly due to the predominating CD3⁺CD56⁺ CIK population (40%-75%) present in the cultures. The addition of anti-CD20 mAb GA101 or rituximab further increased cytotoxicity by 35% and 15%, respectively. This enhancement was mainly due to antibody-dependent cytotoxicity (ADCC) mediated by the 1%-10% NK cells contaminating CIK cultures. The addition of human serum (HS) inhibited NK-cell activation induced by rituximab, but not activation induced by GA101. Overall lysis in presence of serum, even of a resistant B-NHL cell line, was significantly increased by 100 µg/mL of rituximab, but even more so by GA101, with respect to CIK cultures alone. This was due to the combined action of complement-mediated cytotoxicity (CDC), ADCC and CIK-mediated NC. These data suggest that rituximab, and even more so GA101, could be used *in vivo* to enhance CIK therapeutic activity in B-NHL.

Introduction

Cytokine-induced killer (CIK) cells are activated T cells with natural killer (NK) properties that can be expanded in vitro in presence of recombinant human interleukin-2 (rhIL-2) starting from peripheral blood mononuclear cells stimulated by interferon- γ and anti-CD3 antibody¹. They express CD3 and CD56 as well as the NKG2D antigen and show major histocompatibility complex (MHC)-unrestricted cytotoxicity toward neoplastic but not normal targets²⁻⁵. CIK cells express several chemokine receptors, and in vivo models suggest that they can migrate to the site of tumors after intravenous administration⁶⁻¹⁰, there carrying out their cytotoxic potential and helping to control tumor growth.

The ease of CIK cell production in vitro and the cells' antitumor potential have made them suitable candidates for cell therapy programs in solid and hematopoietic tumor treatment. Indeed, both autologous and allogeneic CIK cells have been used in phase I and II clinical trials for the treatment of different tumor types. In these trials, they have shown limited toxicity in vivo, and evidence of antitumor activity has been seen¹¹⁻¹⁸. CIK cells have shown cytotoxic activity in vitro and in vivo against hematopoietic neoplastic cells, including AML (acute myeloid leukemia), CML (chronic myelogenous leukaemia), and CLL (chronic lymphocytic leukaemia)^{9,19-22}. Their cytotoxicity against B-NHL (B-cell non-Hodgkin lymphoma), however, has not been fully investigated in vitro, although in vivo murine models suggest they may be active^{1,23}. Other biologic treatments available for B-NHL are the anti-CD20

antibodies, for instance, rituximab, and a new-generation glycoengineered type II GA101 antibody. These antibodies are thought to act through immune-mediated mechanisms²⁴⁻²⁶. Rituximab induces complement-mediated cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and phagocytosis^{24,27}. In contrast, GA101, which is glycoengineered and defucosylated in its Fc portion, has diminished CDC, but enhanced ADCC compared with rituximab²⁵. ADCC mediated by NK cells is very effectively induced by GA101, because these cells express the Fc γ RIIIA receptor (CD16A), and GA101 binds with higher affinity to this receptor regardless of Fc γ RIIIA polymorphism at residue 158^{25,28}. Furthermore, recent data suggest that GA101 binding to CD16A is not inhibited by complement, in contrast to that of rituximab²⁹.

In this report, we investigate whether CIK cells alone have cytotoxic potential against B-NHL targets in vitro and whether addition of rituximab or GA101 could increase the killing activity of CIK cell cultures toward these cellular targets. We show that indeed both antibodies enhance killing, and that GA101 has superior cytotoxicity compared with rituximab.

Methods

Cells

Diffuse large B-cell lymphoma (SU-DHL4, WSU-NHL), follicular lymphoma (KARPAS 422, DOHH2) and Burkitt lymphoma (BJAB, DAUDI, RAJI, NAMALWA) cell lines were maintained in a combination of RPMI-1640 medium (Cambrex Bio Science) supplemented with 10% fetal bovine serum (Euroclone), 2 mM L-glutamine (Euroclone) and 110 µM gentamycin (PHT Pharma), hereafter called complete medium.

To generate Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL), 5×10^6 peripheral blood mononuclear cells (PBMCs) from normal donors were exposed overnight to B95-8 virus supernatant and 1 µg/mL cyclosporin A and expanded in complete medium.

PBMCs were acquired from B-NHL patients. Waldenström macroglobulinemia, follicular lymphoma, and mantle cell lymphoma cells were obtained by Ficoll Hypaque (Lympholyte-H; Cedarlane) gradient centrifugation after informed consent. CD19⁺ cells were immunoselected by passage through a separation column (MS MidiMacs; Miltenyi Biotec) with anti-CD19 microbeads (Miltenyi Biotec) according to the manufacturer instructions. Positive fractions of more than 90% CD19⁺ and CD20⁺ were cryopreserved and subsequently thawed to be used in the experiments, and negative fractions were used to generate CIK cultures.

Generation of CIK cells

CIK cells were prepared as described previously³⁰. Briefly, PBMCs were cultured in serum-free X-VIVO 15 medium (BioWhittaker) with 1000 U/mL interferon- γ (IFN- γ ; Gammakine; Boehringer Ingelheim) added on day 0, 50 ng/mL anti-CD3 (OKT-3; Janssen-Cilag SpA, Italy) added on day 1, and 500 U/mL rhIL-2 (Proleukin; Chiron BV) included in the medium from day 1 onwards. Expansion was performed for 21 to 28 days. In some experiments, NK cells were removed at the beginning of the culture by CD56 immunodepletion.

Calcein release cytotoxicity assay

Cell lysis was evaluated using the calcein release assay as previously described³¹. Briefly, lymphoma cell lines or patient derived lymphoma cells were labeled for 30 minutes at 37°C with 3.5 μ M calcein-acetoxymethyl ester (Calcein-AM; Fluka, Sigma-Aldrich Company). After washing, labeled target cells were distributed in 96-well plates at 5×10^3 cells per well. Cells of CIK culture were harvested, washed, and added at different effector-to-target (E/T) ratios in the presence or absence of different concentrations of rituximab, GA101, or the control irrelevant antibody trastuzumab (Roche). After 4 hours, the cells were sedimented by centrifugation, 100 μ L of supernatant were collected and calcein release was determined using a fluorescence microplate reader (GENios; TECAN, Austria GmbH) with excitation at 485 nm and emission at 535 nm. The percentage of specific Calcein release was calculated using the following formula: percent specific lysis = (test release minus

spontaneous release) times 100 divided by (maximal release minus spontaneous release). Maximal lysis was achieved with 1% Triton X-100. In some experiments, CD5⁺ CIK cell immunoselected at term of the expansion were used as effector cells.

FACS cytotoxicity assay

Cytotoxicity against the WSU-NHL cell line was measured using carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytotoxicity assays as previously described³². Target cells were labeled with 5 µM CFSE (Molecular Probes Europe). 20 × 10³ labeled target cells were plated with 60×10⁴ CIK cells (30:1 E/T ratio) in presence or absence of mAbs or 20% human serum (HS). After 4 hours of co-culture, fluorescence-activated cell sorting (FACS) analysis was performed. Cell death was measured as a decrease in CFSE⁺/7-aminoactinomycin D (7-AAD)⁻ population. In some experiments, a fixed volume of calibration beads was added to each sample before FACS analysis to measure the decrease in absolute number of CD19⁺/7AAD⁻. Equivalent results were obtained by measuring the relative or absolute decrease in WSU-NHL cells (data not shown). Analyses were performed using a FACScan instrument (BD Biosciences).

CD107a mobilization assay

NK-cell activation and degranulation were evaluated using the lysosomal marker CD107a as previously described³³. CIK cultures from normal donors or lymphoma patients were incubated with lymphoma cell lines or autologous primary lymphoma cells at a 1:1 effector-target (E/T) ratio in the presence of increasing concentrations

of rituximab, GA101, and trastuzumab. After 3 hours, cells were washed with phosphate buffer solution (PBS) and stained with phycoerythrin anti-CD107a (BD Pharmingen), peridinin-chlorophyll-protein complex-conjugated anti-CD3, and allophycocyanin-conjugated anti-CD56 (BD Biosciences) to gate the different populations: CD3-CD56⁺ (NK), CD3⁺CD56⁺ (CIK) and CD3⁺CD56⁻ (T). Percent of CD107a positive cells for each population was evaluated using a FACSCantoI instrument (BD Biosciences). In some experiments, incubations were performed in presence of 20% HS with or without 200 µg/mL blocking anti-C5 mAb eculizumab (Soliris; Alexion Pharmaceuticals), 20% heat inactivated HS, or 400 µg/mL intravenous immunoglobulin (IVIG).

Immunofluorescence analyses

For complement deposition measurement, lymphoma cells were incubated in complete medium supplemented with 20% HS and increasing concentrations of mAbs for 1h at 37°C and then stained with Alexa488-labeled anti-C3 mAbs 1H8 specific for C3b/iC3b/C3dg and 7C12 specific for C3b/iC3b (a kind gift of Prof R.P. Taylor, University of Virginia School of Medicine, Charlottesville, VA).

Statistical analysis

The data were analysed using paired or unpaired Student *t* tests, as appropriate.

Results

CIK cultures are cytotoxic for B lymphoma cells

To investigate whether cells of CIK culture were cytotoxic against B-NHL targets, B-NHL target cell lines derived from patients with diffuse large B-cell lymphomas (DLBCLs), follicular lymphomas (FLs) or Burkitt lymphomas (BLs) were put in contact with cells of CIK culture at different E/T ratios and target cell killing measured after 4 hours by calcein release assays. As shown in Figure 1A, the cells of CIK cultures quite efficiently killed all B-NHL target cell lines tested, with 20% to 52% specific cell death observed in different cell lines at the 30:1 E/T ratio. In several experiments, SU-DHL4 was consistently the most responsive and WSU-NHL the least responsive cell line, respectively (Figure 1A and data not shown).

To determine whether allogeneic or autologous CIK cells differed in activity against transformed B-cell targets, we generated CIK cells and EBV-immortalized B-cell lines from several healthy donors. We then measured the cytotoxic potential of these CIK cultures against EBV-LCL in either the autologous or allogeneic combination (ie, derived from an unrelated donor). As shown in Figure 1B, both autologous and allogeneic EBV-LCL targets were killed by CIK cells to a similar extent, with 26% to 27% killing observed at the 30:1 ratio in both cases.

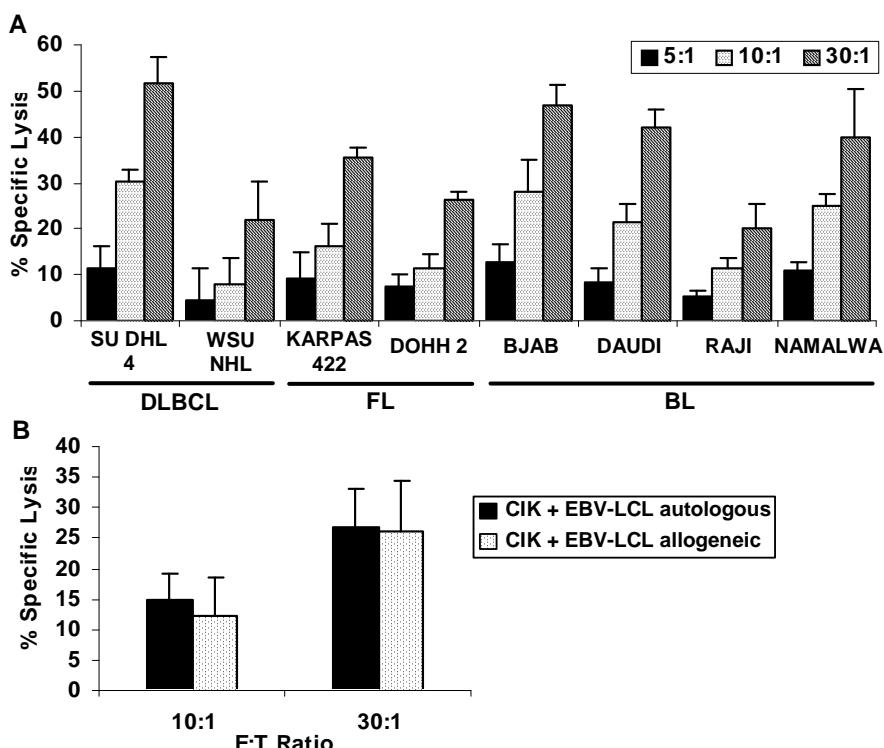


Figure 1. Cytotoxic activity of CIK cultures against malignant human B-cell lines. (A) Cytotoxicity of CIK against B lymphoma cell lines. A panel of diffuse large B-cell lymphoma (SU-DHL4 and WSU-NHL), follicular lymphoma (KARPAS422 and DOHH2) and Burkitt lymphoma (BJAB, DAUDI, RAJI and NAMALWA) cell lines was used as the target. Calcein-AM labeled target cells were co-incubated for 4 hours with CIK effector cells at effector-to-target ratios of 30:1, 10:1, and 5:1; the mean percentage of specific lysis \pm SD from 3 independent experiments is shown. (B) Cytotoxicity of CIK cells against autologous and allogeneic lymphoblastoid B-cell lines. Three CIK effector cultures derived from normal donors, each against an autologous and allogeneic EBV-transformed B-cell line (*EBV-LCL*), were tested in standard 4 hours calcein-AM release assay at effector-to-target ratios of 30:1 and 10:1. Data are expressed as mean percentage \pm SD of 3 independent experiments.

We then investigated whether freshly isolated primary B lymphoma targets could be killed by CIK cells. Primary neoplastic cells purified from 3 B-NHL patients were lysed by 22% to 27% at a 30:1 E/T ratio by CIK cells cultured from healthy normal donors (Figure 2A). CIK cultures were also generated from CD19-depleted peripheral blood mononuclear cells from the same patients and were equally cytotoxic against autologous or allogeneic primary lymphoma cells (Figure 2B).

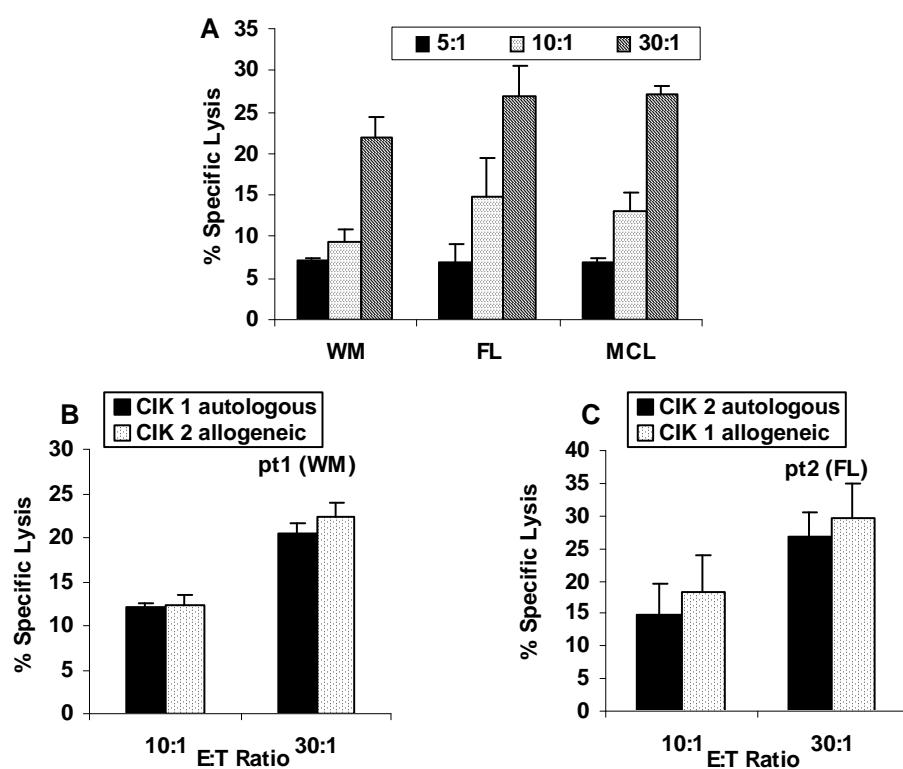


Figure 2. Expanded CIK cells from lymphoma patients present comparable cytotoxicity against autologous or allogeneic freshly isolated lymphoma cells.

(A) CIK cultures from healthy normal donors were used as effectors. The patient-derived purified lymphoma cells were labeled with calcein-AM and incubated 4 hours with CIK cells at effector-to-target ratios of 30:1, 10:1, and 5:1. The mean percentage of specific lysis \pm SD from 3 independent experiments is shown. (B) CIK cells were expanded from PBMCs from 2 patients (with Waldenström macroglobulinemia and follicular lymphoma, respectively) and were used as effector cells against their own purified blasts as targets (patient 1 target, left panel; patient 2 target, right panel), testing them in both the autologous combination (CIK1 versus patient 1 or CIK2 vs patient 2, black columns) or allogeneic combination (CIK1 vs patient 2 and CIK2 vs patient 1, stippled columns), at the indicated effector-to-target ratios. Data are expressed as mean percentage \pm SD of 3 independent experiments.

We conclude that autologous or allogeneic CIK cell cultures have significant cytotoxic activity against both established cell lines and primary freshly isolated B lymphoma cells in vitro.

B lymphoma target cell killing by CIK cultures is enhanced by anti-CD20 antibodies, a process mediated by the NK-cell subpopulation

We investigated whether the addition of anti-CD20 monoclonal antibodies rituximab or GA101 could enhance CIK cell culture-mediated cytotoxicity. As shown in Figure 3A, rituximab increased BJAB cell killing by 8.6%, 14%, and 15% at 5:1, 10:1, and 30:1 E/T ratios, respectively. This was statistically significant ($p < .05$). Much more strikingly, GA101 anti-CD20 antibodies enhanced target cell killing by 32%, 49%, and 39% more than controls without antibodies, at the same E/T ratios ($p < .05$). The effect of GA101 was

significantly greater than that of rituximab ($p < .01$). These data show that anti-CD20 mAbs can enhance the cytotoxic activity of CIK cell cultures against B-NHL cells. Importantly, this additivity is specific, because the addition of the irrelevant antibody trastuzumab, which binds to the Her2 antigen absent on B-NHL cells, did not significantly modify the cytotoxicity (Figure 3A).

CIK cultures at the end of in vitro expansion contained a mean of $47\% \pm 8\%$ (range 40%-75%) CD3⁺CD56⁺ “true” CIK cells, $49\% \pm 9\%$ (22%-59%) CD3⁺CD56⁻ cells and $4\% \pm 3\%$ (1%-10%) CD3⁻CD56⁺ NK cells (data not shown and³⁰). We and others have demonstrated that the CD3⁺CD56⁺ population has NK-like natural cytotoxic activity against tumor cells, whereas the CD3⁺CD56⁻ fraction represents CIK precursors^{23,30}. Because NK cells are CD16 (Fc γ RIII)-positive whereas CIK cells do not express significant levels of all Fc γ Rs (CD16, CD32 and CD64)³⁰, we considered whether the enhancement of cytotoxicity by rituximab and GA101 mAbs was due to CIK cells themselves or rather to the NK cells present in CIK cultures. CIK cultures were therefore depleted of NK cells by CD5 immunoselection at the end of the expansion and cytotoxicity experiments repeated in the presence or absence of anti-CD20 mAbs. As shown in Figure 3B, NK-cell depletion did not abolish the basal natural cytotoxicity of CIK cell cultures against the B lymphoma target, with 22% lysis observed at a 10:1 E/T ratio (Figure 3B, black bars). However, in these conditions, no significant enhancement of killing could be observed by either rituximab or GA101. These data suggest that basal natural cytotoxicity toward B lymphoma targets is predominantly mediated by CD3⁺CD56⁺ “true” CIK cells, whereas enhanced killing in presence

of anti-CD20 mAbs is mediated by the small fraction of NK cells present in CIK cultures.

NK and CD8⁺ T cell-mediated cytotoxicity is accompanied by the release of cytotoxic granules, which can be followed by the appearance of CD107a on the surface of the NK cells³³ and is used as a surrogate marker of NK-mediated ADCC³⁴. We therefore directly identified the cell population degranulating in the presence of B lymphoma target cells, rituximab, and GA101 antibodies by measuring CD107a induction in the different subpopulations present in CIK cultures, using triple immunofluorescence analysis. As shown in Figure 3C, addition to CIK cultures of rituximab or GA101, together with BJAB targets, led to CD107a induction on CD3-CD56⁺ NK cells by nearly 20% and 30% more than controls, respectively. In contrast, CD107a was induced much more weakly, and by only 5%-7% above background levels, on CD3⁺CD56⁺ CIK cells, and not at all on CD3⁺CD56⁻ T cells. These data confirm that antibody-mediated cytotoxicity in CIK cell cultures is due mostly to the activated NK cells present in the final cell product that degranulate on addition of anti-CD20 antibodies. Finally, the data show that degranulation is more efficient with GA101 compared with rituximab (28 % ± 6% vs 19% ± 5%, respectively; p< .005).

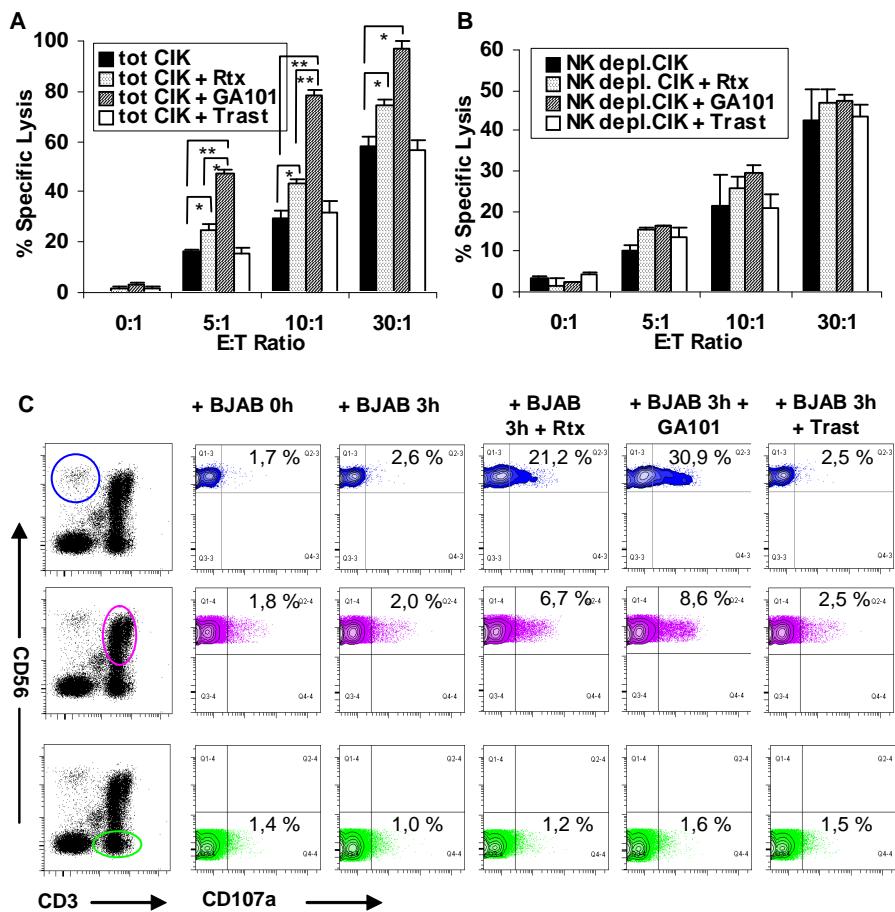


Figure 3. Cytotoxicity of CIK cultures against B lymphoma cell line BJAB was increased in presence of anti-CD20 monoclonal antibody. (A- B) Cytotoxicity of CIK cells against B lymphoma cell line BJAB in the presence of mAbs. Target cells were labeled with calcein-AM and incubated 4 hours with effector cells at effector-to-target ratios of 30:1, 10:1, 5:1, and 0:1. The killing assay was done with no added antibody (black columns), 1 µg/mL rituximab (stippled columns), 1 µg/mL GA101 (striped columns), or 1 µg/mL trastuzumab control mAb (open columns). Total CIK cultures (A) and NK-depleted CIK cultures (B) were used as effector cells. Data are expressed as mean percentage ± SD of 3 independent experiments. (C) CD107a mobilization in CIK cell cultures on interaction with BJAB. CIK

cultures and BJAB were mixed at a 1:1 ratio (0 hours) or co-incubated for 3 hours in presence of 10 µg/mL of rituximab, GA101, or the irrelevant antibody trastuzumab. Cells were harvested and stained with phycoerytrin anti-CD107a, peridinin-chlorophyll-protein complex-conjugated anti-CD3, and allophycocyanin-conjugated anti-CD56 mAbs. To determine the percentage of CD107a⁺ NK, CIK, and T lymphocytes, gates defining respectively, CD3-CD56⁺, CD3⁺CD56⁺, and CD3⁺CD56⁻ lymphocytes were carried out (left dot plots). The percentage of CD107⁺ lymphocytes for each gate was evaluated under different conditions (right dot plots). One representative of 3 experiments is shown.

Similar experiments were performed using primary lymphoma cells as targets. Three independent experiments were performed on CIK cell cultures expanded from the peripheral blood of the patients and tested against the autologous freshly purified tumor cells. Addition of anti-CD20 monoclonal antibodies increased CIK-mediated cytotoxicity. Again, more killing was observed with the addition of GA101 compared with rituximab, with approximately 34% versus 16% increased lysis, respectively, at both the 30:1 or 10:1 E/T ratios ($p < .05$; Figure 4A). This correlated with higher CD107a induction on CD3-CD56⁺ NK cells by GA101 compared with rituximab (24% vs 14%, $p < .05$, Figure 4B).

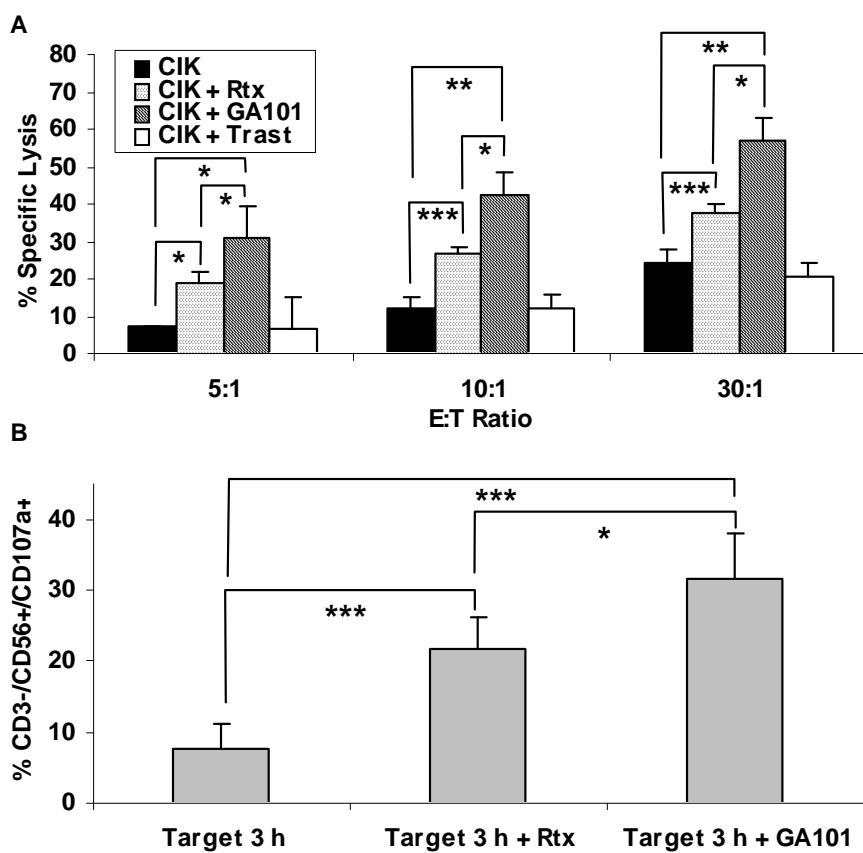


Figure 4. Cytotoxicity of expanded CIK cells from lymphoma patients against autologous lymphoma cell in presence of anti-CD20 antibody. (A) Cytotoxicity of patient-derived CIK cultures against autologous lymphoma cells in the presence of mAbs. Target cells were labeled with calcein-AM and incubated for 4 hours with effector cells at effector-to-target ratios of 30:1, 10:1, and 5:1. The killing assay was done with no added antibody (black columns), 1 µg/mL rituximab (stippled columns), 1 µg/mL GA101 (striped columns), or 1 µg/mL trastuzumab (open columns). Data are expressed as mean percentage \pm SD of 3 independent experiments. (B) CD107a mobilization: 3 expanded CIK cultures from lymphoma patients and each autologous patient-derived lymphoma's cells were mixed at a 1:1 ratio for 3

hours with 1 µg/mL of rituximab or GA101. Surface CD107a marker expression was determined using flow cytometry with gating on CD3-CD56⁺ NK lymphocytes. Mean percentage of CD107a⁺CD3-CD56⁺ cells and SDs are shown (n = 3).

Effect of complement activation on anti-CD20 antibody-mediated degranulation

Complement activation *in vivo* is the most rapid effect of rituximab administration in patients²⁷. *In vitro*, complement activation by rituximab has been shown to inhibit ADCC³⁶, presumably because deposited C3 fragments interfere with Fc γ R binding. We therefore wished to investigate the effect of complement on the activation of NK cells present in the CIK cultures. For this purpose, we performed degranulation assays in presence of BJAB target, 10 or 100 µg/mL rituximab or GA101, and one of the following: complete human serum (HS), heat-inactivated HS, human immunoglobulins (IVIG) or HS and the blocking anti-C5 antibody eculizumab. Addition of HS inhibited NK-cell degranulation induced by 10 or 100 µg/mL rituximab by 65% and 70%, respectively (Figure 5A). Inhibition of NK-cell activation by HS was due to complement activation, because heat inactivation (striped bars) abrogated it completely. In contrast, human immunoglobulins (gray bars) had no effect, confirming lack of inhibitory effect due to competition by excess serum immunoglobulins. Finally, blocking complement activation downstream of C5 by the anti-C5 antibody eculizumab (open bars) did not modify the inhibitory effect of HS, showing that a step upstream from C5 was involved in the complement-mediated

inhibition of NK-cell degranulation by rituximab and serum (Figure 5A).

Interestingly, in contrast to what was observed with rituximab, GA101-induced NK-cell degranulation (that is, ADCC) was not at all affected by HS, whether heat inactivated or not (Figure 5A). Similarly, excess human immunoglobulins or the presence of eculizumab did not modify degranulation (Figure 5A). Together, these data show that activation by rituximab of NK cells in CIK cultures is inhibited by complement, whereas that of GA101 is not. This was true of concentrations of 10 and 100 µg/mL GA101. This lack of inhibition of GA101-mediated NK-cell degranulation may have been due to the lower C3 activation reported for this type II CD20 antibody compared with the type I CD20 antibody rituximab, or to a higher-affinity binding of GA101 to CD16. To test this hypothesis, we verified C3 deposition induced by GA101. We observed equivalent C3b/iC3b and C3dg deposition with 100 µg/mL rituximab or GA101 by FACS analysis (Figure 5B). We conclude that lack of inhibition by GA101 and serum is not due to lack of C3 deposition.

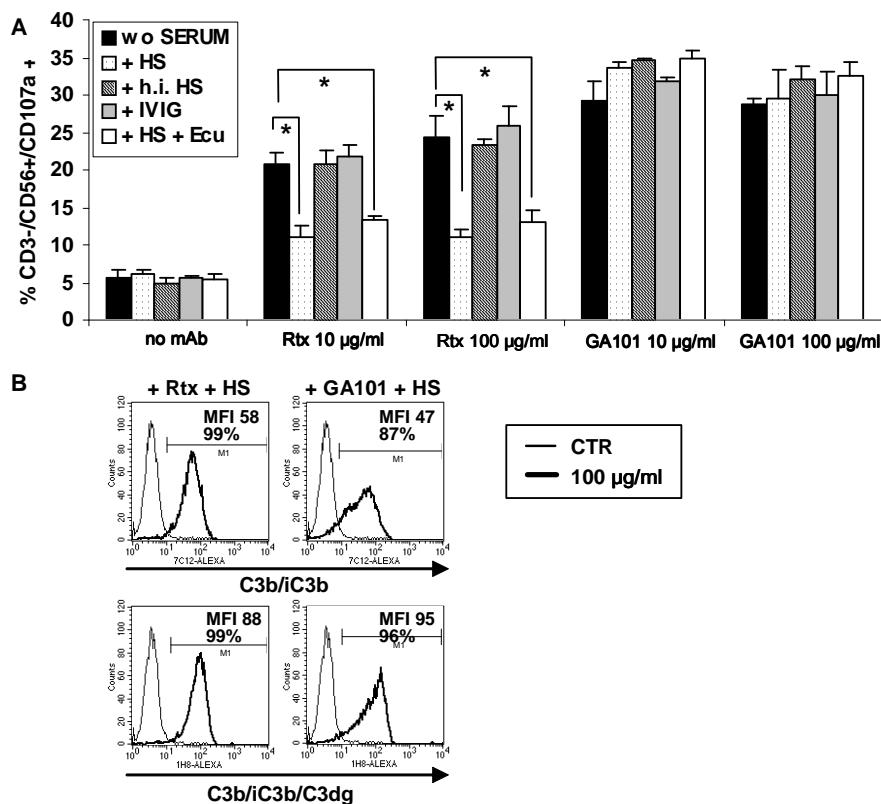


Figure 5. Complement inhibits rituximab-mediated but not GA101-mediated NK-cell degranulation. (A) Expanded CIK cultures from normal donors and BJAB were mixed at 1:1 ratio for 3 hours with 10 or 100 µg/mL of rituximab or GA101 in complete medium (black columns), in the presence of 20% human serum (HS) with (open columns) or without (stippled columns) 200 µg/mL blocking anti-C5 mAb eculizumab, heat inactivated HS (striped columns) or 400 µg/mL IVIG (gray columns). Surface CD107a marker expression was determined using flow cytometry with gating on CD3-CD56⁺ NK lymphocytes. Mean percentage of CD107a⁺CD3-CD56⁺ cells and SDs are shown (n = 2). (B) C3b/iC3b (top panels) or C3b/iC3b/C3dg deposition (bottom panels) on BJAB cell line was measured by direct

immunofluorescence after addition of 100 µg/mL rituximab (left) or GA101 (right).

Cell-killing by CIK cultures and anti-CD20 mAbs in presence of complement

All the data in the previous paragraph indicate that anti-CD20 mAbs activate the NK cells present in the CIK cultures and may therefore increase the cytotoxic potential of the entire cultures through induction of ADCC. On the other hand, complement activation in the presence of serum impairs NK-cell activation, and should therefore also impair ADCC by rituximab, but not GA101. We wanted therefore to measure directly global cell lysis induced by CIK cultures in the presence of serum and rituximab or GA101. In these conditions, overall target cell lysis may include CIK cell-mediated natural cytotoxicity (NC) and NK-mediated and mAb-mediated ADCC, as well as mAb-mediated and complement-mediated cytotoxicity (CDC). For these experiments, we chose the WSU-NHL cell line, which is partially resistant to rituximab and GA101 and is not very responsive to CIK-mediated cytotoxicity (Figure 1A and data not shown).

We first investigated CDC only, that is, lysis in the presence of HS without addition of CIK cells. We observed that rituximab induced CDC at both 10 and 100 µg/mL (mean 27% and 37% target lysis, respectively; Figure 6A). In contrast, significant CDC with GA101 was observed only at the higher 100 µg/mL concentration (27%; Figure 6A). Cell lysis correlated with C3b/iC3b deposition,

which was observed with 10 µg/mL rituximab (57%) and increased with 100 µg/mL of this antibody (75%), and was observed at levels of 4% and 68%, respectively, with the same doses of GA101 (Figure 6B). We then investigated the effect on cell lysis of adding CIK cells to the same targets in the presence or absence of antibodies and HS. Addition of CIK alone at a 30:1 E/T ratio led to a mean of 36% lysis from natural cytotoxicity; this was not significantly modified by HS (Figure 6C). Further addition of 10 or 100 µg/mL rituximab in the absence of serum increased lysis to 49% and 53%, respectively (Figure 6C). This increase was greater when GA101 was used (62% and 70% lysis at 10 and 100 µg/mL, respectively), presumably because of ADCC; this observation agrees with the higher NK-cell activation seen with GA101 than with rituximab (Figure 5A). The further addition of serum did not significantly modify the amount of cell lysis with either antibody at 10 µg/mL (55% vs 49% for rituximab and 63% vs 62% for GA101; Figure 6C). However, with antibody concentrations of 100 µg/mL, serum significantly increased lysis, which reached 73% for rituximab and 87% for GA101. Based also on the CDC (Figure 6A) and NK-cell activation observations, these data suggest that in the presence of HS, killing by rituximab is mainly due to the combined effect of NC and CDC, whereas killing by GA101 is the additive effect of NC, CDC, and ADCC.

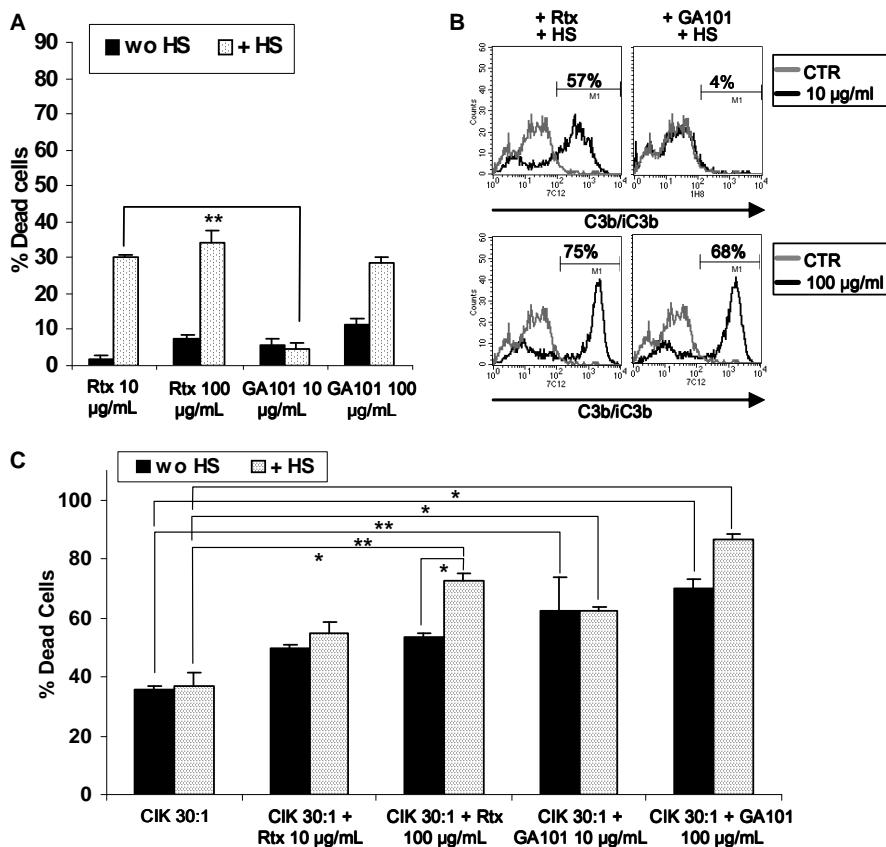


Figure 6. Cell killing by CIK cultures and anti-CD20 mAbs in presence of human serum. (A) CDC of WSU-NHL cell line in the presence of 10 or 100 $\mu\text{g/mL}$ rituximab or GA101 and 20% HS. (B) Deposition of C3b/iC3b on WSU-NHL cell line was measured by direct immunofluorescence after addition of 10 $\mu\text{g/mL}$ (top panels) or 100 $\mu\text{g/mL}$ (bottom panels) rituximab (left) or GA101 (right). (C) CFSE-labeled WSU-NHL cells were incubated for 4 hours with 10 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$ of rituximab or GA101 and CIK cells at effector-to-target ratios of 30:1 in the presence (grey bars) or absence (black bars) of 20% HS. Cell death was measured as a decrease in CFSE⁺ / 7-AAD⁻ cells relative to untreated control. The results are the mean and SD of duplicate wells and are representative of at least 2 independent experiments.

Discussion

In this report, we investigated the cytotoxic capacity of CIK cultures against B-NHL targets in presence or absence of anti-CD20 antibodies and human serum. We show that CIK cultures alone have significant cytotoxic activity *in vitro* against B lymphoma cell lines and freshly isolated samples from patients in either the autologous and allogeneic combination. Most of this cytotoxicity can be attributed to the CD3⁺CD56⁺ CIK cell subpopulation, which kills target cells by an MHC-unrestricted, NK-like mechanism (natural cytotoxicity)²⁹. Furthermore, rituximab, and more markedly, GA101, enhance this effect via ADCC. Finally, for GA101, an additive effect of complement-mediated lysis was observed, leading to very high killing also of resistant lymphoma targets.

The basal cytotoxic activity of CIK cells against both autologous and allogeneic B-cell lymphoma, both from cell lines and patients samples, is of interest because CIK cultures are easily expanded in clinically useful numbers in good manufacturing practice conditions. Furthermore, in previous experiences, these cells, in an allogeneic or autologous setting, have been shown to be relatively safe *in vivo* and to have antitumor activity *in vitro* and *in vivo* against either solid tumors or hematopoietic neoplasms, including AML, CLL, and HD¹¹⁻¹⁸.

Cytotoxicity in the absence of anti-CD20 antibodies was shown to be mediated predominantly by CD3⁺CD56⁺ bone fide CIK cells, which represent 40%-70% of cells at the end of culture. In contrast, killing enhancement mediated by anti-CD20 antibodies in

the absence of serum was mediated by the NK-cell fraction present in CIK cultures, as shown by its absence from NK-depleted CIK cultures and correlation with CD107a surface induction on NK cells only. This fraction represents only 5%-10% of total cells in final CIK culture products. We have previously demonstrated that the third population present in culture, the CD3⁺CD56⁻ cells, are proliferating CIK precursor cells without cytotoxic potential³⁰.

Interestingly, the activation of NK cells in CIK cultures, measured both as CD107a expression as well as target cell lysis, was more effective using GA101 than rituximab. Indeed our data demonstrate that activation by the GA101 antibody of even a small subpopulation within CIK cultures can approximately double the cytotoxic potential of these cells. Cytotoxicity was enhanced by 15%-20% from doses of 10 or 100 µg/mL rituximab, and by 26%-34% from the same doses of GA101. The presumed reason is that the NK cells present in CIK cultures are preactivated because they are cultured in presence of rh-IL2. Such cells are known to be very active in mediating ADCC³⁵. Furthermore, GA101 itself is a very strong activator of NK cells, because it has an approximately 10-fold higher affinity for CD16 than rituximab due to the glyconeengineering of its Fc-portion^{25,28}. From the present data, we conclude that GA101 induces stronger NK-cell activation and ADCC than does rituximab.

We also show that not only is the type II CD20 antibody GA101 more effective than rituximab in activating NK cells, but simultaneous complement activation did not inhibit this activation, as occurs with rituximab antibody-induced inhibition. Enhancement of NK-cell activation by GA101 was fully maintained even in the

presence of human serum and complement activation. This was true for low and high concentrations of GA101, up to 100 µg/mL; at the latter concentration, GA101 is nearly as efficient as rituximab at activating complement and C3 deposition. In contrast, activation of NK cells by rituximab at all doses was inhibited by serum. This inhibition was presumably due to complement C3b deposition, because it was not observed with heat-inactivated serum, nor with serum in the presence of the blocking C5 antibody eculizumab, which blocks the complement cascade downstream from C3. Finally, we showed that excess immunoglobulin is not responsible for the inhibitory effect of serum on rituximab-mediated NK-cell activation. Together, these data suggest that C3 deposition blocks rituximab-induced but not GA101-induced NK-cell activation and ADCC, as previously suggested using resting peripheral blood NK cells^{29,36,37}.

Measurement of global target cell lysis showed that addition of serum and rituximab to CIK led to a significant increase in lysis with 100 µg/mL antibody. Lysis in this case was presumably the sum of natural cytotoxicity by CIK cells (36% lysis with CIK alone) and CDC induced by this antibody (37% with antibody and serum alone), leading to the 73% global lysis observed in the presence of CIK, antibody and serum. ADCC is unlikely to play a role in this case because of the demonstrated inhibition of NK-cell activation by complement activation (summarized in Figure 7). GA101 also increased lysis of target cells in presence of serum when added at doses of 100 µg/mL. Killing in this case was probably due to natural cytotoxicity combined with both ADCC and CDC, because GA101-mediated NK-cell activation is not blocked by complement. Thus, the

87% observed overall target cell killing at this mAb concentration may be the approximately additive effect of 36% NC by CIK cells, 34% ADCC by NK cells, and 27% CDC. GA101 may therefore have the advantage over rituximab of more effective ADCC and a potentially additive effect of CDC, ADCC, and NC (Figure 7).

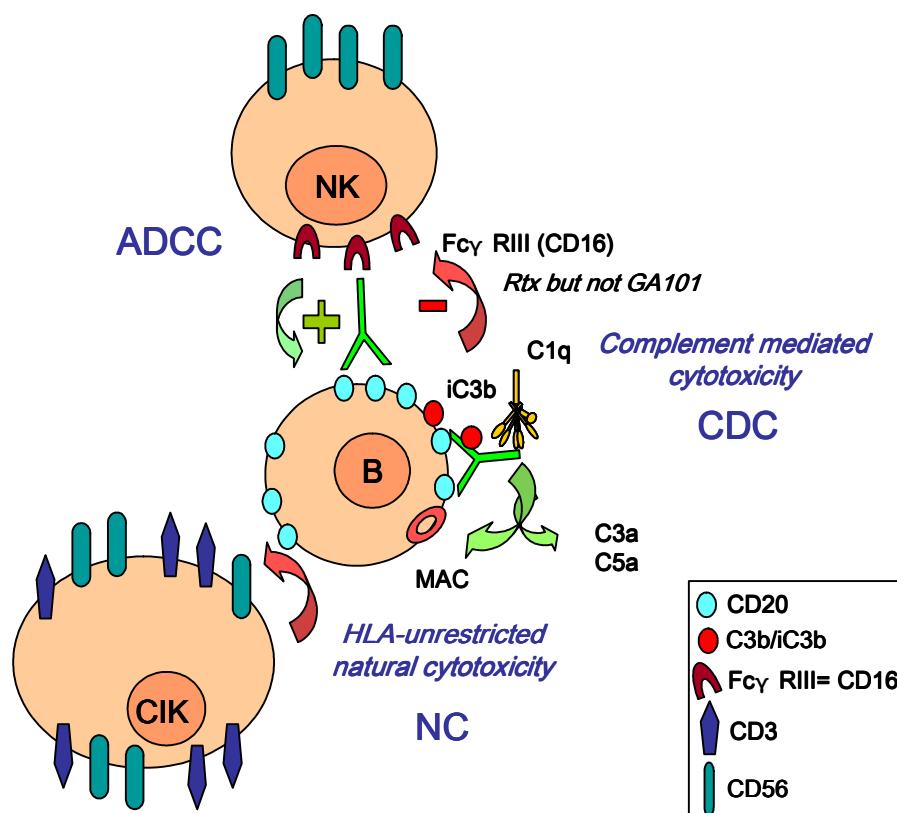


Figure 7. Multiple mechanisms of B lymphoma cell killing by CIK cultures in the presence of anti-CD20 mAbs and human serum. CIK cells kill B lymphoma target by an HLA-unrestricted mechanism (natural cytotoxicity, NC). In the presence of anti-CD20 mAbs, complement-dependent cytotoxicity (CDC) may take place, as well as antibody-dependent cellular cytotoxicity (ADCC), by activated NK cells present in CIK cultures. NK activation and ADCC induced by rituximab, but not by

GA101 coated target cells, are inhibited by complement fragments deposited on the antibody.

Together, these data suggest that CIK cultures could be used to treat B-NHL patients in both an autologous or allogeneic setting. Furthermore, rituximab, and even more so GA101, could be used to enhance the antitumor activity of CIK cells. Clearly, the potential in vivo toxicity of combination treatments of CIK cultures and anti-CD20 mAbs, in particular toxicity from the release of complement anaphylatoxins or cytokines, will need to be investigated before clinical use in humans³⁸. We have generated CIK cultures from CD19-depleted peripheral blood mononuclear cells from B-NHL patients, and no significant differences were observed in terms of expansion and phenotype compared with CIK preparations from normal donors (data not shown). We have also attempted to expand CIK cells in clinically useful numbers in good manufacturing practice conditions starting from unmanipulated leukapheresis sample of one B-NHL patient in remission and demonstrated that this is feasible. Furthermore, we could show that contaminating lymphoma cells present at the start are not detectable by flow cytometry at the end of culture (G.M. Borleri, unpublished observations, February 2010). Thus, CIK cells could be used for the treatment of B-NHL at relapse, either in an autologous or allogeneic context.

We conclude that autologous CIK cells may be combined with third-generation anti-CD20 GA101 antibodies to offer novel immunotherapy protocols for the treatment of chemotherapy-resistant and rituximab-resistant B-cell lymphoma patients.

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Chapter 5

5.1 Summary

Cytokine-induced killer (CIK) cells are a heterogeneous population of lymphocytes, enriched in CD3+CD56+ “bona fide” CIK cells subset, obtained in vitro within 21 days from mononuclear cells under the influence of cytokines (IFN- γ , anti-CD3 antibody OKT3, and rhIL-2)¹⁻⁴. CIK cells show potent MHC-unrestricted cytotoxicity against a variety of tumor cells, in particular hematological neoplasms^{3,5}, rapid and specific homing to tumor site following in vivo injection^{6,7}, and minimal tendency to induce graft-versus-host disease^{8,9}. The ease of CIK cells production in vitro and their antitumor potential have made them suitable candidates for cell therapy programs in solid and hematopoietic tumors treatment.

Despite both autologous and allogeneic CIK cells have already been used in phase I and II clinical trials for the treatment of different tumor types, little is yet known about the mechanism of tumor recognition and the unique features of the various cell subsets present in CIK cultures. Moreover, there are some hematological tumors, such as B-cell lymphomas, B-ALL and B-CLL that are only partially susceptible to CIK-mediated lysis. The aims of my PhD thesis project were to better characterize cell subsets of CIK culture and to perform a preclinical evaluation about the possibility of combining CIK cells

and anti-CD20 monoclonal antibodies to optimize their therapeutical efficacy against B-cell lymphoma.

In the first part of this work we have characterized the ontogenetic and functional properties of the predominant CD3⁺CD56⁺ "bona fide" CIK subset in relation to the others present in bulk culture. Indeed, the heterogeneous bulk CIK culture consists of over 90% CD3⁺ cells, of which at least 40% coexpresses CD56 and the remaining cells are CD3⁺CD56⁻. There is also a relatively small subset of CD3-CD56⁺ NK cells.

CD3⁺CD56⁺ cells obtained in culture are generated from the CD3⁺CD56⁻ subset and not from the few CD3⁺CD56⁺ cells present in the starting peripheral blood mononuclear cells. Indeed, circulating CD3⁺CD56⁺ cells are shown to be nonproliferating and cannot therefore expand in culture. In contrast, CD3⁺CD56⁻ cells isolated at the beginning of the culture strongly expand and acquire the expression of CD56 in the presence of IL-2, and so give rise to CD3⁺CD56⁺ cells. They still maintain their proliferative and differentiative capacity during culture. It seems that CD3⁺CD56⁺ CIK cells are preferentially generated from CD3⁺CD8⁺CD4⁻ precursors and, to a lesser extent, from CD4⁺CD8⁺ and CD4-CD8⁻ T cells. These observations are in agreement with what previously demonstrated in a early paper of Lu et al⁵.

Circulating CD3⁺CD56⁺ cells, contrary to in vitro expanded CD3⁺CD56⁺ cells, are poorly cytotoxic for HLA-class I negative K562 cell line also upon IL-2 stimulation and express lower levels of CD56 and NKG2D. Circulating CD3⁺CD56⁺ cells show often a skewed

TCRV β repertoire. In contrast, at the end of culture, the expanded CD3+CD56+ and CD3+CD56- subsets express a polyclonal TCRV β repertoire in a Gaussian distribution. CIK cells share several characteristics with NK cells, such as the large granular lymphocyte morphology, the capacity to kill K562 cell line, and the expression at high density of CD56, DNAM-1 and NKG2D molecules. Contrary to NK cells however, CIK cells express at low density the activating natural cytotoxicity receptor (NCR) NKp30, whereas they do not express the other NCRs, NKp44 and NKp46, and the inhibitory killer immunoglobulin-like receptors, NKG2A and CD94. Gene expression profile confirms that CD3+CD56+ cells are more related to the CD3+CD56- compared to the CD3-CD56+ subset present in culture. The CD3+CD56+ subset kills K562 target more efficiently than its CD3+CD56- counterpart. Hence, the CD3+CD56+ subset, generated following repeated stimulations, would represent a relatively more terminally differentiated effector T-cell population. We showed that this cell subset has the phenotype CD45RA $^{+/-}$ CD62L $^{\text{lo}}$ CCR7-CD27 $^{+/-}$ CD28 $^{\text{lo}}$. Such late stage effector T cells are known to possess potent cytotoxicity but have low proliferative capacity¹⁰. On the other hand, the CD3+CD56- cell subset represents early effector T cells that have reduced cytotoxicity but higher capacity to proliferate. These CD45RA $^{+}$ CD62L $^{+}$ CCR7-CD27 $^{+}$ CD28 $^{+}$ early effector T cells, expressing a higher level of CD62L, have the potential to home to lymph nodes and potentially persist in vivo after adoptive transfer¹¹.

We have tried to clarify which receptors are involved in tumor recognition and killing by CD3+CD56+ CIK cells. Cytotoxicity of CD3+CD56+ CIK cells requires effector-target cell contact and

involves conjugated formation. The adhesion molecule LFA-1 plays a crucial role in binding as well as in cytolytic activity of CIK cells against tumor target cells expressing the corresponding ligands.

CD3+CD56+ CIK cells, which originate in vitro from CD56-CD8+ T cell progenitors, respond to TRC/CD3-mediated stimulation by degranulating and killing P815 cells loaded with anti-CD3 mAb. In addition, also activating NK receptors NKG2D, DNAM-1 and NKp30 expressed by CD3+CD56+ CIK cells are functional. Antibody blocking experiments revealed that DNAM-1, NKG2D and NKp30 are actually involved in CIK-mediated killing of tumor target cells upon recognition of their specific ligand.

Another relevant information provided by our study is the demonstration that anti-CMV specific CD8+ T cells can be expanded in standard CIK cultures, acquire the CD3+CD56+ CIK phenotype and are characterized by a dual function. Indeed, they can mediate both specific MHC-restricted recognition of CMV-pulsed autologous T-PHA blasts and TCR-independent, NK-like cytolytic activity against leukemic targets. Antibody blocking of LFA-1 and DNAM-1 led to significant reduction of both CTL and NK cell functions, while blocking of NKG2D and NKp30 only inhibited NK-like cytotoxicity.

The information that CIK cells retain the CTL function (here tested only for anti-CMV specific memory) adds further interest to their potential clinical application. In bone marrow transplanted patients the recovery of functional specific T cells is particularly delayed and most patients suffer from severe infections^{12,13}. In this respect, CIK cells may thus represent a “one coin-two sides” chances of treating leukemia relapses and transferring the donor T memory to cope with

most frequent and possibly fatal infections (ie, CMV, Epstein Barr virus, Aspergillus, etc). Indeed the absolute numbers of CMV-specific CIK cells, which can be obtained from a small amount of peripheral blood during the standard GMP approved CIK ex vivo expansion, are fully compatible with the idea of obtaining a clinically effective anti-CMV CTL (less than 1×10^5 in most reports^{14,15}).

The second part of my project is focused on the possibility to increase the antitumor activity of CIK cells by a combined use with antibodies. For this purpose, we have investigated the cytotoxic activity of CIK cultures against B-NHL targets in vitro in presence or absence of type I and type II anti-CD20 antibodies and human serum. CIK cultures possess moderate cytotoxic activity in vitro against B lymphoma cell lines and freshly isolated autologous and allogeneic lymphoma samples. This cytotoxicity is mainly due to the CD3⁺CD56⁺ subpopulation.

The addition of anti-CD20 chimeric type-I rituximab mAb, but more significantly of a new generation type-II GA101 mAb, enhances this effect via ADCC, by activation of CD3-CD56⁺ NK subpopulation present in CIK cultures.

The activation by anti-CD20 mAbs of even a small NK subpopulation within CIK cultures (5%-10% of total cells) can significantly increase the cytotoxic potential of these cells. The presumed reason is that the NK cells present in CIK culture are strongly activated because they are cultured for long-term in presence of high dose of IL-2. Such cells are known to be very active in mediating ADCC¹⁶.

Furthermore, GA101 is a stronger activator of NK cells compared to rituximab, because it has an approximately 10-fold higher affinity for Fc γ RIIIa than rituximab due to the glycoengineering of its Fcportion¹⁷⁻¹⁹.

ADCC is usually studied in the absence of human serum through the quantitation of cell lysis by the addition of effector cells to mAbs and target cells. However, the use of this cytotoxicity assay makes it difficult to assess how complement, which is present under physiologic conditions, can impact on effector-cell activation induced by mAb-coated target cells. There are many evidences that CDC and ADCC are not completely independent mechanisms.

We have demonstrated that in the presence of serum, NK cells activation by rituximab-coated target cells was blocked. Similarly, Wang SY et al. observed an inhibitory effect of serum on resting peripheral blood NK-cell activation induced by rituximab-coated target cells^{20,21}. The observation that the inhibitory properties of serum are heat labile suggests that the inhibition is due to complement activity. In particular, blocking mAb anti-C5 had no effect on the inhibition, suggesting that a step of complement cascade upstream from C5 was involved. C3b deposition onto the target bound-rituximab is presumably the key component in the observed inhibitory effect because it is likely to sterically hinder the interaction between the Fc portion of rituximab and Fc γ RIIIa on NK cells. This finding is consistent with the known structures of these molecules^{22,23}. In contrast to the results with rituximab, we have found that serum has no effect on the ability of GA101-coated target cells to activate NK cells present in CIK cultures.

Rituximab and GA101 have been shown to vary in their ability to induce complement fixation and mediate CDC¹⁷⁻¹⁹. Indeed, type I (rituximab-like) mAbs are able to evoke stronger complement activation and CDC compared to type II (GA101-like) mAbs²⁴.

A concentration of 10 µg/mL rituximab is sufficient to activate C3 deposition and induce CDC and blocking of NK activation. This is consistent with both CDC and blockade of NK activation being dependent on initiation of complement cascade.

In the case of GA101, higher concentrations of mAb are needed to activate complement and to induce CDC. At 100 µg/mL GA101 is nearly as efficient as rituximab at activating C3 deposition, but did not inhibit NK cells activation. This can be due to the enhanced affinity of GA101 Fc for FcγR on NK cells.

Measurement of lysis of a resistant B-HNL target in the presence of effector CIK cells, complement and mAb can allow assessing the role of each mechanism in physiologic conditions. The addition of serum and rituximab to CIK cells led to a 50% of increase in lysis with 100 µg/mL mAb. Lysis in this case was presumably the sum of natural cytotoxicity by CIK cells and CDC induced by rituximab. ADCC is unlikely to play a role in this case because of the demonstrated inhibition of NK cell activation by complement deposition. GA101 also increased lysis of target of an additional 20% in the presence of serum and CIK cells when added at the same dose. In this case, killing was probably due to natural cytotoxicity combined with both CDC and ADCC, because GA101-mediated NK cell activation is not blocked by complement. GA101 may therefore have the advantage

over rituximab of more effective ADCC and a potentially additive effect of CDC, ADCC and natural cytotoxicity.

The results described have a potential clinical interest. B lymphoma patients with chemotherapy/rituximab resistant disease represent a minor but important group of patients. We believe that complete immunotherapy protocols may be offered to these patients by combining CIK cell with third-generation anti-CD20 monoclonal antibody GA101. Toxicity of this treatment would be probably minimal because several clinical studies, including lymphoma patients, have demonstrated no toxicity with large number of CIK cells²⁵⁻²⁷. Similarly GA101 has so far showed marginal toxicity ²⁸. The proposed combination should nonetheless be further tested *in vivo* in animal models before clinical application.

5.2 Conclusions and future perspectives

Although hematopoietic cell transplantation can be a curative therapy for a variety of hematological malignancies, disease recurrence remains a major problem. The use of donor leukocyte infusions (DLI) has been a fundamental step to improve treatment, particularly in CML and has served to demonstrate the effectiveness of the graft versus leukemia (GVL) effect in controlling relapse. However DLI is gravated by concomitant graft versus host disease (GVHD) and is also poorly effective in several neoplastic diseases, such as AML, ALL and myeloma. These problems have therefore led to the search for procedures allowing the selection of leukocyte subpopulations with intact GVL and reduced GVH and with applicability to different neoplasias. NK cells have potent antitumor cytotoxicity which is under the control of both activating and inhibitory molecules, but their full potential in allogeneic combinations has yet to be fully understood. Furthermore, the difficulty of expanding NK cells in vitro and the likely requirements of high doses of IL-2 coadministration with its associated toxicity, have so far severely restricted their use in the clinic. Specific cytotoxic T lymphocytes (CTL) against allogeneic leukemia cells may also represent an alternative, but the identification of the exact conditions to reproducibly obtain leukemia reactive T cells in vitro are far from being applicable in large studies.

One much more simple, reproducible and rapid methodology which has recently began to be explored is represented by cytokine-induced killer (CIK) cells to be used for treating relapsed leukemia patients.

Interestingly, this therapeutic opportunity may be offered in several different clinical settings. An autologous use has been extensively validated in numerous phase I clinical studies, including also hematological neoplasms^{25,27}. In the allogeneic setting, the safety of allogeneic (donor's leukapheresis derived) CIK cells in patients relapsing after allogeneic hematopoietic stem cell transplantation has been shown. Allogeneic CIK cells appeared to be endowed with a reduced propensity to cause GVHD, compared with conventional T cells, making an appealing and promising alternative to classic DLI²⁹. Finally, CIK cells can be expanded in sufficient numbers to cover the needs of a clinical protocol for adult patients also from the washouts of the cord blood unit bags left over at the end of the transplant^{2,30}. Relatively low toxicity and some activity were observed in the first group of patients treated with cord blood-derived CIK cells³⁰. This could represent a promising immunotherapy program for cord blood transplanted patients at relapse who could not otherwise benefit from donor lymphocytes infusions.

The observations collected in this PhD thesis suggest that expanded bulk CIK cell culture may represent an ideal cell population for adoptive immunotherapy of haematological malignancies. The heterogeneous bulk CIK culture consists of over 90% CD3⁺ cells, of which the majority coexpress CD56 and the remaining cells are CD3⁺CD56⁻. We have clarified that:

1- CD3⁺CD56⁺ CIK cells are terminally differentiated non dividing lymphocytes which could deliver potent MHC-unrestricted cytotoxicity for the immediate destruction of tumor cells.

2- The other less cytotoxic CD3⁺CD56⁻ cell subset represents a progenitor reservoir that could proliferate and differentiate into CD3⁺CD56⁺ CIK cells. It is still a matter of speculation whether the "maintainance" in vivo of the cytotoxic activity of the total cell culture may be dependent upon the presence of this proliferating reservoir of precursors.

3- CD3⁺CD56⁺ CIK cells are T cells which have acquired the natural cytotoxic potential of NK cells. The information that CD3⁺CD56⁺ CIK cells, in addition to their well-known HLA-unrelated antitumor activity, retain the CTL function (here tested only for anti-CMV specific memory) adds further interest to their potential clinical application. Indeed they can represent a particularly useful tool, in adoptive immunotherapy, to treat both neoplastic relapses and viral infections, two frequently associated complications in transplanted patients.

This PhD thesis suggests also new therapeutic applications of CIK cell cultures for the treatment of hematological neoplasias with a potential clinical interest. The combined use of CIK cells with anti-CD20 monoclonal antibodies rituximab and GA101 to a greater degree could represent a novel immunotherapy protocol for the treatment of B lymphoma patients with chemotherapy/rituximab resistant disease, that are a minor but important group of patients.

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