Adoptive immunotherapy mediated by *ex vivo* expanded natural killer T cells against CD1d-expressing lymphoid neoplasms

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background
CD1d is a monomorphic antigen presentation molecule expressed in several hematologic malignancies. Alpha-galactosylceramide (α-GalCer) is a glycolipid that can be presented to cytotoxic CD1d-restricted T cells. These reagents represent a potentially powerful tool for cell mediated immunotherapy.

Design and Methods
We set up an experimental model to evaluate the use of adoptively transferred cytotoxic CD1d-restricted T cells and α-GalCer in the treatment of mice engrafted with CD1d$^+$ lymphoid neoplastic cells. To this end the C1R cell line was transfected with CD1c or CD1d molecules. In addition, upon retroviral infection firefly luciferase was expressed on C1R transfected cell lines allowing the evaluation of tumor growth in xenografted immunodeficient NOD/SCID mice.

Results
The C1R-CD1d cell line was highly susceptible to specific CD1d-restricted T cell cytotoxicity in the presence α-GalCer *in vitro*. After adoptive transfer of CD1d-restricted T cells and α-GalCer to mice engrafted with both C1R-CD1c and C1R-CD1d, a reduction in tumor growth was observed only in CD1d$^+$ masses. In addition, CD1d-restricted T-cell treatment plus α-GalCer eradicated small C1R-CD1d$^+$ nodules. Immunohistochemical analysis revealed that infiltrating NKT cells were mainly observed in CD1d nodules.

Conclusions
Our results indicate that *ex vivo* expanded cytotoxic CD1d-restricted T cells and α-GalCer may represent a new immunotherapeutic tool for treatment of CD1d$^+$ hematologic malignancies.

Key words: lymphoproliferative disorders, natural killer T cells, α-galactosylceramide, CD1d, CD1d-restricted T cells.

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Introduction

CD1d is a monomorphic glycoprotein belonging to the CD1 family of molecules involved in the presentation of glycolipids to CD1-restricted T cells. Beyond some hematopoietic cells, such as B lymphocytes, monocytes and immature cortical thymocytes,[1] CD1d is expressed on neoplastic cells of lymphoid and myeloid origin.[2-6] α-galactosylceramide (α-GalCer) is a glycolipid derived from a marine sponge and has the striking feature of being presented *via* CD1d to most CD1d-restricted T cells,[7] allowing considerable characterization of CD1d-restricted T cells. In humans, CD1d-restricted T cells show a highly limited T cell receptor (TCR) repertoire consisting of a TCR variable (V)α24 chain rearranged with the JαQ segment paired almost exclusively with TCR Vβ11 chains[8] and capable of expressing natural killer (NK) cell markers.[9] Because of these features, CD1d-restricted T cells are commonly termed invariant natural killer T (NKT) cells; we will refer to these cells as NKT throughout the paper. CD1d-restricted T cells other than Vα24/Vβ11 exist, although this T-cell sub-population is far less characterized.[10] These cells are defined type II NKT cells (as opposed to type I corresponding to invariant NKT cells) and are believed to have suppressive functions.[11]

NKT cells represent a T-cell population capable of mediating both the innate and adaptive immune responses that are sometimes involved in anti-tumor activity.[1] They have important regulatory functions in the immune system, mainly mediated by the secretion of both Th1 and Th2 cytokines.[12] The involvement of NKT cells has been observed in auto-immune diseases as well as microbial infections, where NKT cells seem to be implicated in the response against Gram-negative, LPS-negative α-protobacteria through recognition of glucuronyl and galacturonyl ceramides (found in the Sphingomonas cell wall)[13,14] or other yet uncharacterized bacterial glycolipid(s).[14] In other instances bacteria appear to trigger NKT recognition of the self lipid iGb3.[15]

The role of NKT cells in the anti-tumor immune response is thought to be mainly indirect as it can be played through the activation of NK cells and antigen specific $CD8^+$ T cells. Based on the expression of CD4 and CD8 molecules, subsets of NKT cells can be distinguished. Double negative ($CD4^-/CD8^-$) NKT cells are more likely to be cytotoxic and have a Th1 cytokine secretion profile as opposed to $CD4^+$ NKT cells which secrete the Th1 and/or Th2 set of cytokines.[16] However, NKT cells with cytotolytic activity can easily be expanded *in vitro* upon culturing in the presence of IL-2. These cells have been shown to be capable of killing $CD1d^+$ tumoral cells in several studies.[3-6]

We previously showed that the CD1d molecule is expressed on B-cell chronic lymphocytic leukemia (B-CLL) cells[3] and in a severe prognosis sub-group of childhood B-cell acute lymphoblastic leukemia (BCP-ALL) carrying the 11q23 rearrangement.[4] In addition, expression of CD1d has been demonstrated on myeloma[2,17] and myelo-monocytic leukemia cells.[5] $CD1d^+$ leukemic cells were found to be capable of α-GalCer presentation and susceptible to NKT cell mediated lysis. Thus, CD1d represents a potentially unique target molecule for adoptive immunotherapy, expressed on a variety of lympho- and myelo-proliferative disorders.

In the present study we generated a model to evaluate *in vivo* the efficacy of cytotoxic NKT cells for immunotherapy against $CD1d^+$ leukemic cells, and found that the adoptive transfer of NKT cells in the presence of α-GalCer is able to specifically reduce the growth of $CD1d^+$ leukemic cells and eradicate the neoplastic clone. We conclude that the use of cytotoxic NKT cells in conjunction with α-GalCer may be suitable for immunotherapy of hematologic neoplasia expressing CD1d.

Design and Methods

Cell culture

C1R lymphoblastoid cell line was maintained in RPMI 1640 (Invitrogen, Milan, Italy). Medium was supplemented with 10% heat-inactivated FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL, Invitrogen).

Isolation of NKTA cell line

CD1d-restricted T-cell lines (NKTA, NKTD and NKT-Fe cells) were obtained from healthy donors as described previously.[3] Briefly, monocyte-derived dendritic cells were used to stimulate autologous T lymphocytes in the presence of α-GalCer (KRN7000, 100 ng/mL, kindly provided by Kirin Brewery Co, Pharmaceutical Division, Tokyo, Japan), IL-2 (100 U/mL, Pepro Tech EC) and IL-7 (20 ng/mL, Pepro Tech EC). Consecutive sorting of TCR $V\alpha24^+$ and $TCR\beta11^+$ cells was achieved at day 14 and day 35 respectively using anti-mouse IgG conjugated microbeads (Miltenyi Biotec). NKT lines were periodically stimulated using heterologous PBMC and α-GalCer. NKTA cell line was composed predominately of $CD4^-/CD8^-$ T cells. The phenotype and the functional features were reported previously.[3,4] NKTD and NKT-Fe cell lines were composed mostly of $CD4^+$ T cells.

In vitro *cytotoxicity assay*

The cytolytic activity of NKT cells was determined by 4 h chromium-release assay. Briefly, target cells were labeled for 1 h with ^{51}Cr (Amersham Biosciences, Buckinghamshire, UK, 100 μCi/10^6 cells), washed twice with PBS, resuspended in RPMI 1640 with 10% FCS, and plated at 5×10^3 cells per well in 96-well U-bottom plates. Effector cells were harvested, washed, and incubated with target cells in triplicate at the E:T ratio indicated in the experiments. For blocking experiments the CD1d55 moAb was used (kindly provided by Dr. S. Porcelli, A. Einstein College of Medicine of Yeshiva University). Percent of cytotoxicity was calculated as follows: (experimental ^{51}Cr -spontaneous ^{51}Cr)/(100-spontaneous ^{51}Cr) x 100. Data are reported as mean SD.

Retroviral plasmid production

The pLXIH bicistronic retroviral vector was obtained from the pLXIN retroviral vector (Clontech) (previously modified inserting the restriction sites NotI, BglII, XhoI,

ClaI, SalI, BstxI, BamHI into the multiple cloning site) by replacing the Neomycin resistance gene (*neoR*) with the hygromycin B resistance (*hygro R*) gene. The firefly luciferase (ffluc) gene was obtained as a SalI/BglII fragment form pGL3-control vector (Promega), and cloned into the XhoI/BamHI sites of the retroviral pLXIH bicistronic vector to obtain the pL-Luc-IH vector. All plasmids were prepared by using Qiagen kits.

Generation of C1R transfectants

The lymphoblastoid cell line C1R was transfected with the expression vector pIRESneo vector (BD-Clontech, Milan, Italy) into which cDNAs encoding either CD1c and CD1d were inserted. To transfect C1R cell line the lipofectamine reagent (Invitrogen) was used. Cells growing after antibiotic selection (G418 at 300 μg/mL, Invitrogen) were analyzed by flow cytometry to confirm expression of CD1c and CD1d using moAb anti-CD1c (Beckman Coulter, Milan, Italy) and -CD1d (Pharmingen BD, Milan, Italy).

Retrovirus production and infection

Retroviruses were prepared by calcium phosphate transient transfection of HEK293 T cells by mixing five μg of pkat2ampac,[18] five μg of pVSV-G (BD-Clontech) and five micrograms of pL-Luc-IH. The retrovirus-containing supernatant was isolated 48 h post-transfection and used to infect C1R-CD1c and C1R-CD1d cells in the presence of 8 μg/mL Polybrene (Sigma-Aldrich, Milan, Italy). After 48 h, the transduced C1R cell lines (luc^+C1R-CD1c and luc^+C1R-CD1d) were selected with Hygromicin, 200 μg/mL (Sigma-Aldrich).

Xenograft tumor model

All animal experiments were authorized by the Ethical Committee of the Advanced Biotechnological Center of Genoa on behalf of the Italian Ministry of Health. NOD/LtSz-scid/scid (NOD/SCID) mice were bred and maintained under pathogen-free conditions at the National Cancer Research Institute's animal facility (IST, Genoa, Italy). The institutional and national guide for the care and use of laboratory animals was followed. Mice were sublethally irradiated with single dose 320 cGy (^{60}Co source; 99 cGy/min) one day prior to tumor cell injection. For tumor implantation, 2-4×10^{6} C1R cells and 1-4×10^{6} double transfectant luc^+C1R-CD1c or luc^+C1R-CD1d cells, resuspended in 200 μL PBS, were inoculated subcutaneously into two separate sites of the dorsal hemilateral thorax of 8- to 10- week-old (w-o) mice. By day 4 after injection, tumor growth was monitored by biophotonic imaging and tumor engraftment was defined as increasing tumor luc-mediated flux over at least two consecutive imaging sessions (3-day interval).

In vivo *imaging*

Anesthetized mice (plastic chamber filled with 2% isofluorane/air mixture) were imaged using a Xenogen IVIS 100 series system beginning approximately seven minutes after intraperitoneal injection of 400 μL (4.29 μg/mouse) of a freshly thawed aqueous solution of D-luciferin potassium salt (Xenogen, Alameda, CA, USA). Five sequential acquisitions were obtained in every case. Each animal was serially imaged at the same relative time point after D-luciferin injection. Imaging of animals treated with NKT cells was carried out after 48 h of NKT cell transfer. To quantify bioluminescence, circular regions of interest (ROI) were positioned to encircle luminescent tumor lesions and the integrated flux of photons emitted from C1R xenografts were quantified using the Living Image software program (Xenogen). The bioluminescence signal was measured as total photon flux normalized for exposure time and surface area and expressed in units of photons (p) per second per cm^2 per steradian (sr). For anatomic localization, a pseudocolor image representing light intensity (blue, least intense; red, most intense) was superimposed over a digital grayscale body-surface reference image. Nodules were defined as *small* if the ROI value was less than 4×10^{5} p/sec/cm^{2}/sr.

Immunohistochemical identification of natural killer T cells in tumoral nodules

Tumoral nodules were fixed with 2% paraformaldehyde and paraffin embedded. Five micrometer thick sections were treated in a microwave oven four times with citrate buffer (pH 6) for 5 min at 960 W. Sections were saturated with 10% BSA in PBS with 0.1% Triton X-100 and incubated overnight at 4°C in a humidified chamber with a mouse α-CD3 moAb (Santa Cruz Biotechnology, Inc, Santa Cruz. CA, USA). The reaction was developed after addition of a secondary goat anti-mouse antiserum (Southern Biotechnology Associates, Birmingham, AL, USA) according to the alkaline phosphatase-anti alkaline phosphatase technique, and stained with Fast Red TR (Dako, Glostrup, Denmark).

Results

C1R cell line expressing CD1d molecule is susceptible to cytotoxicity by natural killer T cells

Expression of the CD1d molecule in C1R cells was achieved upon transfection with an expression vector containing the CD1d molecule and selection of C1R cells in G418 containing medium. Growing C1R cells were further selected using magnetic bead sorting. As a control cell line, C1R cells transfected with an expression vector containing the CD1c molecule were selected. Expression of CD1d and CD1c molecules in the transfected C1R cells is shown in Figure 1.

To determine whether C1R-CD1d cells were susceptible to NKT cell lysis *in vitro*, we used NKTA, NKTD and NKT-Fe cell lines expanded from normal donors in the presence of IL-2. Cytotoxicity experiments showed that all NKT cell lines were capable of a highly efficient cytolysis of C1R-CD1d cells in the presence of 100 ng/mL of α-GalCer. NKTA-mediated cell lysis was as high as 100% at a 10:1 E:T ratio, but significant cytotoxicity was observed even at a 1:1 E:T ratio (Figure 2). Some, C1R-CD1d cytotoxicity was observed also in the absence of α-GalCer, although significantly reduced compared to that observed in the presence of α-GalCer. Conversely, the C1R-CD1c cell line was not susceptible

to NKT mediated cytolysis even in the presence of α-GalCer (Figure 3). Inhibition of NKT-mediated cytotoxicity was obtained after incubation of C1R-CD1d cells with an anti-CD1d moAb, but not with an irrelevant moAb such as anti-CD1c, further demonstrating that NKT cell line cytotoxicity was dependent on CD1d recognition (Figure 3).

Transfer of ex vivo *expanded natural killer T cells into NOD/SCID mice is feasible and safe with or without α-GalCer*

To investigate whether transfer of NKTA cell line was feasible and safe, 8-week-old sub-lethally irradiated NOD/SCID mice were injected with 1×10^7 (N=2) and 2×10^7 (N=2) NKTA cells only. None of the mice developed early or late pathological signs or symptoms attributable to NKTA adoptive transfer. When 2 μg of α-GalCer were co-injected with 2×10^7 NKTA cells (N=2) no adverse effects were observed.

C1R cells are capable of xenografting immunodeficient mice

From 2 to 4×10^6 C1R cells were subcutaneously inoculated into sublethally irradiated NOD/SCID mice, resulting in an 80-100% engraftment rate. Within 2-3 weeks after injection, palpable masses became apparent. Immunohistochemical analysis showed that the nodules were heavily infiltrated by human $CD19^+$ cells (*data not shown*). For instrumental evaluation of tumor growth, we expressed the ffluc gene in C1R-CD1c and C1R-CD1d cells upon retroviral infection and hygromicin selection. Cell lines were defined as luc^+C1R-CD1c and luc^+C1R-CD1d, respectively. When luc^+C1R cells were injected, the engraftment rate was basically equivalent to C1R cells and the tumor growth of luc^+C1R cells was easily monitored by the IVIS scan. Susceptibility to NKTA cytotoxicity of luc^+C1R cell lines, *in vitro*, was similar to parental cell lines (*data not shown*). Luc^+C1R-CD1c and luc^+C1R-CD1d cell lines were then used to assess therapeutic efficacy of CD1d-restricted T cells *in vivo*.

Adoptive transfer of natural killer T cells limits luc^+C1R-CD1d cell growth

To exclude non-specific activity of NKT cells, luc^+C1R-CD1c and -CD1d cells were both subcutaneously injected in the dorsal region to generate *chimeric* NOD/SCID mice. In addition, to better evaluate tumor growth mice were grafted with different numbers of luc^+C1R-CD1c and -CD1d cells (2×10^6 and 4×10^6 cells, respectively). Tumor engraftment was verified by obtaining 2 successive biophotonic measurements with increased ffluc mediated biophotonic tumor signal. Mice to be treated were selected on the basis of a sufficient difference in the CD1c and CD1d nodule ROI signals. After adoptive

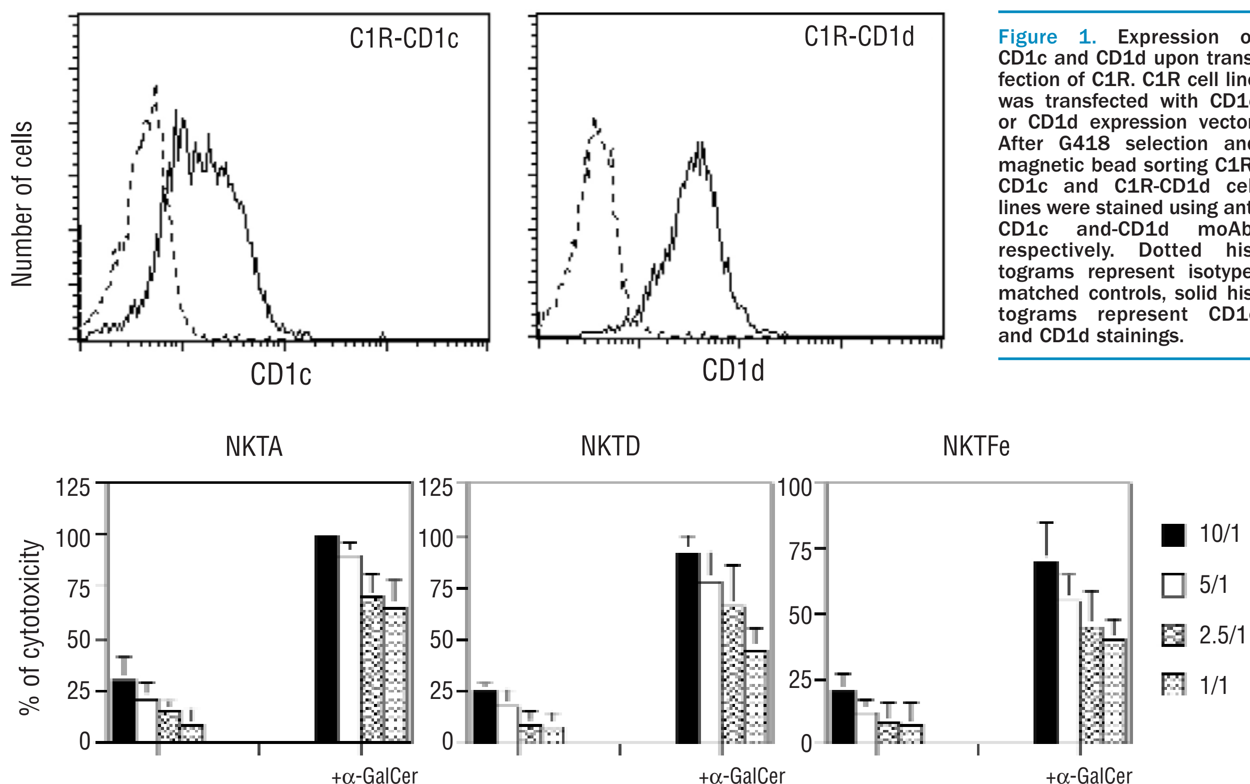

Figure 1. Expression of CD1c and CD1d upon transfection of C1R. C1R cell line was transfected with CD1c or CD1d expression vector. After G418 selection and magnetic bead sorting C1R-CD1c and C1R-CD1d cell lines were stained using anti CD1c and-CD1d moAb, respectively. Dotted histograms represent isotype-matched controls, solid histograms represent CD1c and CD1d stainings.

Figure 2. NKT-mediated cytotoxicity of C1R-CD1d cell line in the presence of α-GalCer. C1R-CD1d cell line was labeled with ^{51}Cr and then cultured for 4 h with NKTA, NKTD and NKT-Fe cell lines at the indicated E:T ratios. Alpha-GalCer was preincubated for 30 min before the addition of effector cells. Final concentration of α-GalCer was 100 ng/mL. In all instances, at least three separate experiments were performed.

transfer of 1×10^{7} NKTA cells by intravenous (i.v.) injection in conjunction with 2 μg of α-GalCer, only a marginal reduction of C1R-CD1d nodule growth compared to untreated animals was observed (Figure 4). However, when mice were treated with 2 consecutive administrations (24 h apart from each other) of 2×10^{7} NKTA cells and α-GalCer, the rate of tumor growth of luc+C1R-CD1d nodules was significantly reduced, as shown by bioluminescence imaging at 48 h after NKT therapy. Conversely, luc+C1R-CD1c nodules progressed in all mice despite treatment. This double treatment was repeated at day 3 and day 4, and again luc+C1R-CD1d nodule growth was significantly limited compared to untreated animals whereas no effect was observed on the growth of luc+C1R-CD1c nodules.

In order to evaluate whether NKT cells were capable of eradicating small luc+C1R-CD1d+ nodules, mice were grafted with 1×10^{6} luc+C1R-CD1d cells and 3×10^{6} luc+C1R-CD1c cells in two separate sites (Figure 5). Mice bearing small CD1d nodules, as defined on the basis of nodule ROI values (*see Design and Methods section*), were selected for treatment and a reduction of luc+C1R-CD1d nodules was recorded already after a single injection of ×10[7] NKTA cells and α-GalCer (Figure 5A). When animals were treated with 2 consecutive injections of 10[7] NKTA cells, small CD1d nodules completely disappeared from the bioluminescence imaging 72 h after adoptive therapy (Figure 5B). The complete tumor regression lasted over a period of 14 days without any additional NKT treatment, whereas luc+C1R-CD1c nodules continued to grow, as demonstrated by bioluminescence imaging.

To exclude the possibility that the α-GalCer could be capable of activating residual immunity in sub-lethally irradiated NOD/SCID mice we treated a group of mice with α-GalCer alone. Under this condition modification of the tumor mass growth rate was not observed (*Online Supplementary Figure S1*).

Identification of natural killer T cells in tumoral nodules

To investigate whether NKT cells could be identified in tumoral tissues, engrafted mice were sacrificed after 24 or 48 h from the inoculation of 10[7] NKT cells and α-GalCer. CD1c and CD1d nodules were removed and prepared for immunohistochemistry using a mouse

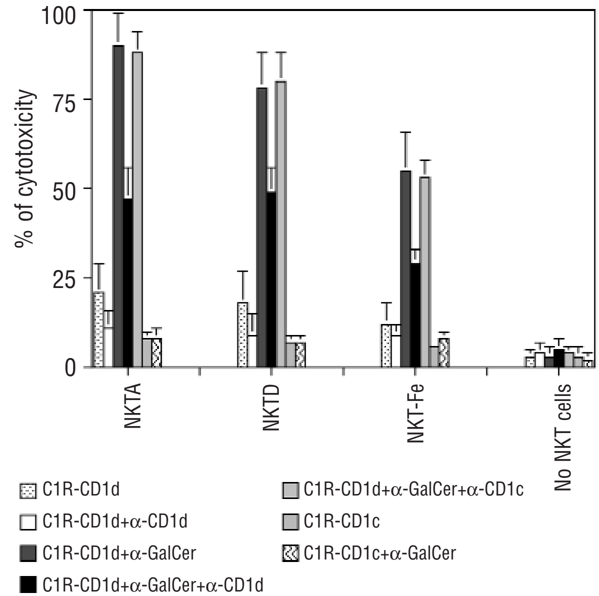

Figure 3. Specific recognition of C1R-CD1d cell line by NKT cell lines. [51]Cr labeled C1R-CD1c and CD1d cell lines were cultured for 4 h with NKT cell lines under the conditions indicated. Alpha-GalCer and anti CD1d moAb were preincubated with C1R cell lines for 3 min before the addition of NKT cell lines. Final concentration of α-GalCer and CD1d moAb was 100 ng/mL and 10 μg/mL, respectively. In all instances, at least three separate experiments were performed.

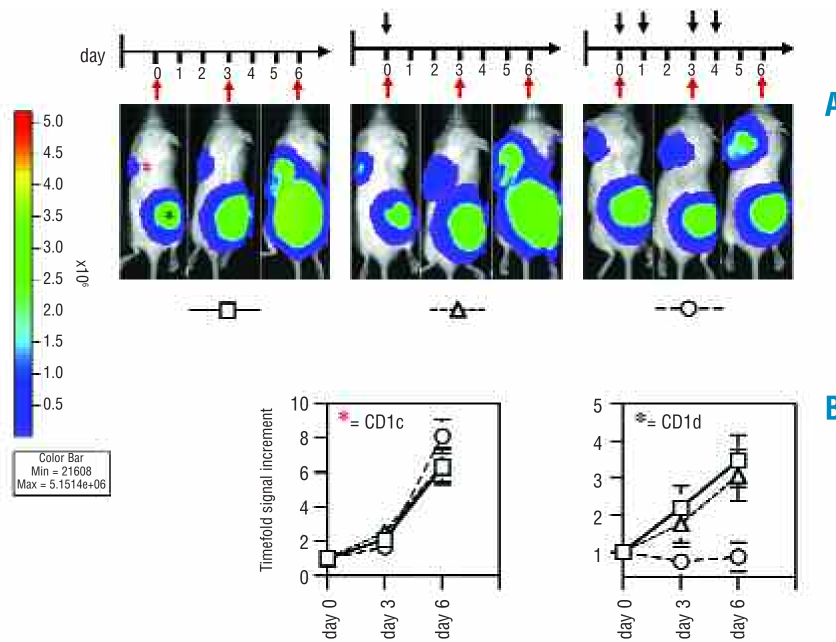

Figure 4. FfLuc+C1R cell engraftment of NOD/SCID mice and adoptive NKT cell therapy. (A) Pseudocolor images of mice serially imaged in dorsal position. The square represents untreated mice, the triangle represents mice treated with a single endovenous injection of 1×10^{7} NKTA cells and α-GalCer (executed at day 0) and the circle represents mice treated with two sets of two consecutive injections of 2×10^{7} NKTA cells and α-GalCer (executed at day 0 and day 1 than again at day 3 and day 4). Red arrows denote day of imaging, black arrows denote day of treatment. Administration of NKT cells and α-GalCer at day 0 and day 3 was carried out after imaging. (B) Representation of luc+C1R-CD1c (red asterisk) and luc+C1R-CD1d (black asterisk) nodule growth curves. Curve values indicate the timefold signal increment (relative growth) obtained through the ratio of the light intensity values recorded at day 3 and day 6 to value recorded at day 0. The data are representative of 3 experiments.

moAb CD3 to identify NKT cells. CD3 reactive cells were mostly observed in CD1d nodules that displayed entire fields of CD3 reactive cells (Figure 6). Conversely, CD1c nodules taken from the same mouse showed only rare and mostly solitaire CD3 positive cells (Figure 6). The frequency of CD3 reactive cells appears to be higher in sections obtained from nodules taken after 48 h from NKT inoculation compared to those taken after 24 h although a slight degree of experimental variability was observed. Finally, CD3 reactive cells were never observed in nodules derived from animals that received administration of α-GalCer only.

Discussion

Despite progress made in our understanding of the biology of lymphoproliferative disorders, their therapy is still challenging and many patients are failing to maintain clinical response. Tumor immunotherapy is an attractive tool that relies on the use of immune related molecules or cells to recognize and eradicate neoplastic cells.[19]

Immunotherapy of B-cell lymphoproliferative disorders has been limited by a relative paucity of suitable antigens to be used as common targets of the effector cells. Thus, clinical protocols may have to be tailored to each individual or rely upon complicated engineering of the effector cells in order to enable them to recognize antigens commonly expressed on B-cell neoplasms.[20,21] As a potential target for adoptive immunotherapy, the CD1d molecule exhibits very attractive features: i) it is monomorphic, ii) it binds to the synthetic lipid antigen α-GalCer which is capable of stimulating most CD1d-restricted T cells and is clinically approved, and iii) it is expressed on several lympho- and myelo-proliferative disorders and not on most non-hematopoietic cells.

On the other hand, NKT cells with cytotolytic activity can easily be expanded *in vitro* upon culturing in the presence of IL-2. These cells have been shown to be capable of killing CD1d$^+$ tumoral cells in several studies.[3-6]

We therefore evaluated whether NKT cells were suitable for use *in vivo* against CD1d$^+$ leukemic cells by setting up an experimental model using CD1d$^-$ and CD1d$^+$ leukemic target cells capable of being engrafted in immunodeficient mice.

We first demonstrated that C1R-CD1d cells were highly susceptible to *in vitro* NKT cell cytotoxicity upon incubation of target cells with α-GalCer. The killing of target cells was dependent upon CD1d expression as C1R-CD1c cells were not targeted even in the presence of α-GalCer. In addition, partial inhibition of the cytotoxicity of CD1d$^+$ target cells was observed via the use of an α-CD1d moAb (Figure 3). It is to be noted that inhibition of cytotoxicity is never complete using α-CD1d moAb in blocking experiments.[4,22] C1R-CD1d cell lysis occurred irrespective of the NKT cell sub-type (NKTD and NKT-Fe).

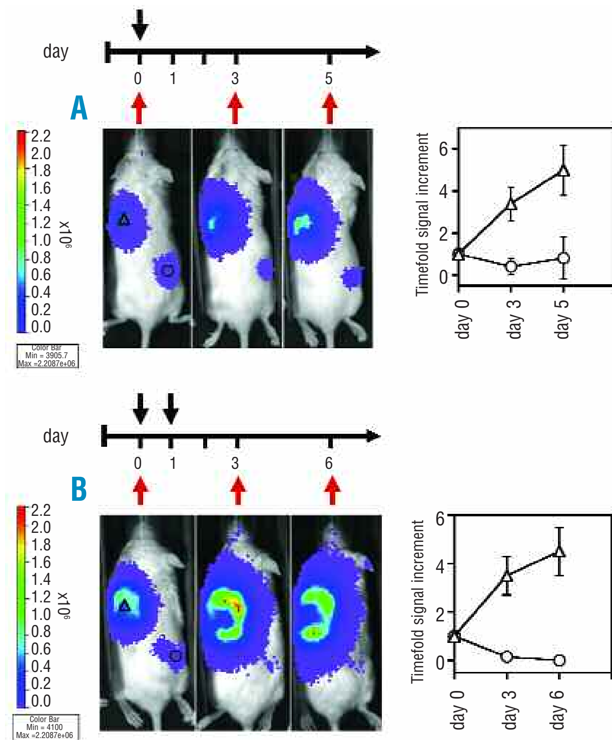

Figure 5. Adoptive therapy of NOD/SCID mice carrying small fluc$^+$C1R-CD1d nodules. Engrafted mice were selected on the basis of the C1R-CD1d nodule light intensity. (A) Mice were treated with 1x10^7 NKTA cells and α-GalCer at day 0. (B) Mice were treated with 1x10^7 NKTA cells and α-GalCer at day 0 and day 1. Red arrows denote day of imaging, black arrows denote day of treatment. On the right side the representation of luc$^+$C1R-CD1c (triangles) and luc$^+$C1R-CD1d (circles) nodule growth curves is reported. Curve values indicate the timefold signal increment obtained through the ratio of the light intensity values recorded at day 3, 5 (or 6) to value recorded at day 0. The data are representative of two (A) and three (B) experiments.

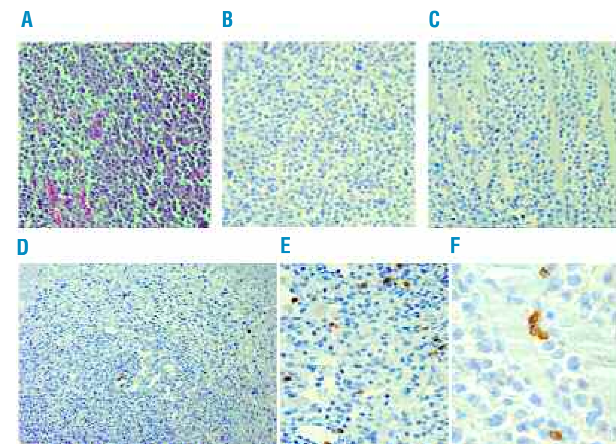

Figure 6. Immunohistochemical analyses of NKT cells in paraffin embedded sections derived from CD1d and CD1c nodules. (A) Hematoxylin-eosin stained section of a nodule showing the massive infiltration of lymphoid cells, (B) staining with an anti-CD3 moAb of a CD1d nodule derived from a mouse that received inoculation of α-GalCer only; no reactive cells are observed, (C) CD3 staining of CD1c nodule 48 h after the inoculation of 10^7 NKT cells and α-GalCer; positive cells are rarely observed; (D, E) and (F) CD3 staining of CD1d nodule 48 h after the inoculation of 10^7 NKT cells and α-GalCer; several fields show numerous positive cells. Original magnification of A, B, C and E 200 X; magnification of D 100 X; magnification of F 400 X.

C1R cell lines were then grafted in NOD/SCID mice to investigate the feasibility of NKT and α-GalCer adoptive transfer for immunotherapy *in vivo*.

Preliminary experiments performed on animals grafted with the luc+C1R-CD1d cell line showed that i.v. inoculation with NKT cells and α-GalCer results in a reduction in tumor size (*data not shown*). In order to exclude an involvement of residual host immunity, we performed all subsequent experiments using animals engrafted with both luc+C1R-CD1c and -CD1d cell lines. Mice were grafted with a different number of cells of the single cell lines to better evaluate growth of individual nodules. In a first set of experiments mice were grafted with 2×10^6 luc+C1R-CD1c and 4×10^6 luc+C1R-CD1d. While the C1R-CD1c nodule growth rate was not affected, a reduction of C1R-CD1d derived nodules was obtained after 2 consecutive i.v. inoculations of 2×10^7 NKT cells and α-GalCer. Treatments using fewer effector cells or single administrations were ineffective. Further experiments were then performed grafting mice with a smaller number of C1R-CD1d cells (1×10^6 cells). A single i.v. inoculation of 1×10^7 NKT cells and α-GalCer was able to reduce the tumor mass while two consecutive doses of 1×10^7 NKT and α-GalCer completely eradicated the nodule. Note that tumor size reduction was observed for about 48-72 h after NKT administration after which a new increase in nodule signal was observed (Figure 5A). This could be likely due to the absence of cytokine support in our experimental model such as IL-2 and IL-15, that are both capable of sustaining the cytotoxic activity of effector cells. The use of soluble factors in our experimental model is currently under investigation to evaluate the possibility of sustaining NKT cells' effector capability.

To further confirm the role of NKT cells in the tumor regression, we performed an immunohistochemical analysis to identify NKT cells infiltrating the lymphoid nodules and found that these cells were present in CD1d nodules and sporadically detected in CD1c nodules. We could not make a clear correlation between the observation of tissue necrotic areas and the presence of infiltrating NKT cells possibly because of the early occurrence of tissue necrosis within rapid growing tumoral nodules.

Involvement of NKT cells in anti-tumor response to B-cell lymphoma has been shown recently in a murine model.[23] However, our study is the first that explores *in vivo* the potential anti-tumoral action directly exerted by *in vitro* expanded NKT cells on neoplastic cells through interaction with the CD1d restriction molecule. Although NKT cells represent an apparently modest subpopulation of human peripheral blood cells (less than 1% of T cells), they can be efficiently expanded *in vitro* upon stimulation with α-GalCer.[24] Furthermore, an moAb capable of recognizing the invariant Vα24-JαQ chain of NKT TCR is now available that further speeds up purification and expansion of NKT cells.[25]

Administration of α-GalCer or NKT cells in clinical trials did not induce major side effects.[26] The use of α-GalCer or DC cells loaded with α-GalCer is aimed at boosting innate and adaptive anti-tumor immune responses. Indeed, T-cell activation, increased NK cell cytotoxicity and increased serum concentration of TNF-α, GM-CSF, IFN-γ and IL-12 were observed upon administration of α-GalCer[27] or α-GalCer loaded DC.[28] Non-small cell lung cancer patients treated with NKT cells showed an immunological response as assessed by the elevation of IFN-γ+ NK cells,[29] although no clinical response was observed. Thus, the use of NKT cells and α-GalCer in CD1d+ lymphoproliferative disorders could potentially combine direct and indirect NKT cell mediated immune responses against cancer cells.

Authorship and Disclosures

DB, AI, MC, NS, CT, ADS and AM designed and performed experiments; AD prepared firefly luciferase expressing C1R cell lines; AI, RC, GDR, FF, EC and FF designed the experiments, analyzed and interpreted the data, drafted and revised the manuscript; FF takes primary responsibility for the paper and created all figures.

The authors reported no potential conflicts of interest.

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