Clinical Immunology 183 (2017) 91–98

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Retinoic acid induction of CD1d expression primes chronic lymphocytic leukemia B cells for killing by CD8$^+$ invariant natural killer T cells

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ARTICLE INFO

Article history:
Received 8 March 2017
Received in revised form 3 July 2017
accepted with revision 1 August 2017
Available online 03 August 2017

Keywords:
Chronic lymphocytic leukemia
Invariant natural killer T cells
CD1d
Retinoic acid
Cytotoxicity

ABSTRACT

Invariant natural killer T (iNKT) cells are cytotoxic T cells that respond to glycolipid antigens presented by CD1d. Therapeutic activation of iNKT cells with α-galactosylceramide (α-GalCer) can prevent and reverse tumor growth in mice and clinical trials involving α-GalCer-stimulated iNKT cells are ongoing in humans. B cells express CD1d, however, we show that CD1d expression is reduced on B cells from patients with chronic lymphocytic leukemia (CLL). B cells from CLL patients pulsed with α-GalCer failed to stimulate cytolytic degranulation by iNKT cell lines, but could present the more potent glycolipid analogue, 7DW8-5. Retinoic acid receptor-α (RAR-α) agonists induced CD1d expression by CLL B cells, restoring their ability to present α-GalCer to CD8α$^+$ iNKT cells, resulting in cytolytic degranulation. Thus, RAR-α agonists can augment the anti-tumor activities of iNKT cells against CLL cells *in vitro*. Their inclusion in iNKT cell-based therapies may benefit patients with CLL.

1. Introduction

Invariant natural killer T (iNKT) cells are a subset of innate T cells that express a semi-invariant T cell receptor (TCR) α-chain (Vα24Jα18 in humans and Vα14Jα18 in mice) that recognizes glycolipid antigens bound to the major histocompatibility complex-like molecule CD1d [1,2]. The Vα24Jα18 TCR recognizes a number of self [3,4] and microbial [5,6] glycosphingolipids, however, most of our understanding of iNKT cells comes from studies of murine and human iNKT cells stimulated with the xenogeneic glycolipid, α-galactosylceramide (α-GalCer). Upon activation with α-GalCer, iNKT cells kill target cells and secrete a diverse range of growth factors and cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), IL-5, IL-9, IL-10, IL-13, IL-17A and IL-22, allowing them to contribute to the activation of T cells [7–9], natural killer cells [9,10] and macrophages [11]. Activated iNKT cells can also interact directly with other cells of the immune system and can induce the maturation of dendritic cells (DC) into antigen-presenting cells (APC) [12,13] and of B cells into antibody-secreting plasma cells [14–17].

iNKT cells are thought to play a central role in immunity against tumors. Mice lacking CD1d or iNKT cells are predisposed to developing cancer [18] and therapeutic activation of iNKT cells in murine models can prevent and reverse tumor growth by activating NK cells and CD8$^+$ T cells [19,20]. iNKT cells can directly kill human tumor cell lines *in vitro* [21,22] with CD8$^+$ iNKT cells exhibiting superior cytotoxicity when compared to CD4$^+$ and CD4$^-$CD8$^-$ iNKT cells [8]. Numerical and functional iNKT cell deficiencies have been reported in a number of human cancers [23–27], and clinical trials involving the adoptive transfer of α-GalCer-pulsed autologous DC and/or *ex vivo* expanded iNKT cells are ongoing for a number of human cancer types [28–31].

Chronic lymphocytic leukemia (CLL) is a cancer type that is likely to benefit from iNKT cell-based immunotherapy. CLL is the most common leukemia in the western world and is characterised by the expansion of mature monoclonal B lymphocytes, which express CD5 and CD23 [32,33]. These cells can accumulate in the bone marrow affecting hematopoiesis and resulting in a plethora of secondary conditions including anemia, thrombocytopenia, lymphopenia, hypogammaglobulinemia, immune cell dysfunction, splenomegaly, and hepatomegaly. B cells express CD1d [14,17,34], suggesting that they can be primed for cytolysis by iNKT cells through the administration of glycolipid. However, similar to patients with solid tumors, iNKT cell frequencies have been shown to be reduced in patients with CLL [35]. Furthermore, CD1d expression may altered on CLL cells compared to healthy B cells [35–37].

Abbreviations: α-GalCer, α-Galactosylceramide; APC, antigen-presenting cell; ATRA, all-trans retinoic acid; CLL, chronic lymphocytic leukemia; DC, dendritic cell; DMSO, dimethylsulphoxide; IFN-γ, interferon-γ; IL, interleukin; iNKT, invariant natural killer T; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; RA, retinoic acid; RARα, retinoic acid receptor-α; TCR, T cell receptor; TNF-α, tumor necrosis factor-α.

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http://dx.doi.org/10.1016/j.clim.2017.08.002
1521-6616/

All trans retinoic acid (ATRA) is an active metabolite of vitamin A and is produced in the body to aid in cellular growth and development. Synthetically produced ATRA is being increasingly included in regimens for the treatment of various cancers and has become a first choice drug for the treatment of acute promyelocytic leukemia [38]. ATRA increases the sensitivity of CLL cells to fludarabine-induced apoptosis [39]. AM580 is a retinobenzoic acid derivative that was synthesized as a retinoic acid (RA) receptor alpha (RARα) agonist, and has been shown to act in a similar way to ATRA. RARα agonists such as ATRA and AM580 have been shown to upregulate CD1d expression on tonsillar B cells [40]. This led us to hypothesise that the use of RARα agonists may induce CD1d expression by CLL cells, which in combination with iNKT cell agonist glycolipids could sensitize CLL cells for lysis by iNKT cells. Here we have examined the numbers of circulating iNKT cells and their $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ (DN) subsets, as well as the expression of CD1d by B cells in peripheral blood from CLL patients. We also evaluated the ability of α-GalCer and the its potent glycolipid derivative 7DW8-5 to prime CLL cells for killing by iNKT cell subsets *in vitro*, and whether ATRA and AM508 can upregulate the expression of CD1d by CLL cells making them better targets for lysis by iNKT cells.

2. Materials and methods

2.1. Study design

Thirty-two patients diagnosed with CLL (21 male and 11 female), with a median age of 67 (range 45–87), were recruited from the Haematology Clinic at St. James's Hospital, Dublin. Twenty-three of the patients had stable disease and were given a clinical score of Binet A. Of the remaining patients, 3 were Binet B, 5 were Binet C and the clinical score of one patient was unknown. Twenty one of the patients, including all Binet A patients, had not undergone any previous treatment, while 11 patients had been treated (1 with Bendamustine; 1 with Campath; 3 with Chlorambucil; 3 with fludarabine, cyclophosphamide and Rituximab; 3 treatment unknown) but had discontinued treatment for at least one year prior to recruitment. Circulating iNKT and B cell numbers and phenotypes from 14 of these patients (median age 54; range 45–68) were compared with those from 12 age-matched healthy subjects (median age 55, range 48–66). The remaining CLL samples and anonymous buffy coat packs, kindly donated by the Irish Blood Transfusion Service, were used as sources of iNKT cells, B cells and feeder cells for iNKT cell expansion. The age-matched patient group consisted of 9 men and 5 women, of whom 10 had a clinical score of Binet A; 1 was Binet B, 2 were Binet C and the clinical score of one patient was unknown. Ten of the age-matched patients had never received treatment for CLL, whereas 2 received fludarabine, cyclophosphamide and Rituximab and the treatment was unknown for the remaining two patients. All patients had discontinued treatment for at least one year prior to recruitment. This study was granted ethical approval from the Research Ethics Committee of St. James's and Adelaide and Meath Hospitals incorporating the National Children's Hospital, Dublin. EDTA-anticoagulated venous blood samples (3.5 ml) were obtained from the subjects. Peripheral blood mononuclear cells (PBMCs) were isolated and stained for immunophenotyping, stimulated in culture for iNKT cell expansion, or cryopreserved for further use.

2.2. Immunophenotyping and flow cytometry

Freshly isolated PBMC were stained with fixable viability dye FVD eFluor®506 (eBioscience, Hatfield, UK) to exclude dead cells, followed by cell surface staining with flurochrome-conjugated monoclonal antibodies (mAbs) specific for human CD1d (clone 51.5), CD3, CD4, CD5 (clone UCHT2), CD8, CD14, CD19 and the complementarity-determining region 3 of the Vα24Jα18 TCR chain (clone 6B11), which is found on all iNKT cells. MAbs were purchased from BioLegand (San Diego, CA, USA) and Immunotools (Friesoythe, Germany). Cells were analysed using a CyAn ADP flow cytometer (Beckman Coulter, High Wycombe, UK) and FlowJo software (Treestar, Ashland, OR). Unstained, single stained, and fluorescence-minus-one controls were used in all experiments.

2.3. Expansion of iNKT cells from CLL and healthy donor PBMC

PBMCs from CLL patients and healthy controls were cultured in iNKT cell medium (RPMI 1640 containing 0.05 mM L-glutamine, 10% HyClone FCS, 1% penicillin-streptomycin, 25 mM HEPES, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture, and 1% essential amino acids mixture; Gibco-BRL, Paisley, UK and Thermo-Scientific, Logan, UT) supplemented with recombinant human IL-2 (100 IU/ml) and IL-7 (10 ng/ml) (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were stimulated with 100 ng/ml of α-GalCer (KRN7000; Funakoshi, Tokyo, Japan), and plated at 10^6 cells/ml in 96-well round bottom plates (Corning/MA, UK). Medium was replaced with fresh medium containing IL-2 and IL-7 every 2–3 days. After 20 days, iNKT cells were isolated from PBMCs using magnetic bead separation with anti-iNKT MicroBeads (Miltenyi Biotec). iNKT cells were then labelled with mAbs specific for CD3, and the Vα24 and Vβ11 TCR chains (Beckman Coulter) and sorted using a MoFlo™ XDP Cell Sorter (Beckman Coulter) to obtain $CD3^+V\alpha24^+V\beta11^+$ iNKT cells. iNKT cells were cultured for 30 days with α-GalCer (100 ng/ml) pulsed allogeneic irradiated (25 Gy) PBMCs from two donors at ratios of 1:200, in IL-2 and IL-7-supplemented iNKT cell medium. This method led to expansion of $>10^7$ iNKT cells with purities exceeding 98% from 3 ml of healthy donor blood.

2.4. Analysis of iNKT cell responses to glycolipid pulsed allogeneic B cells

$CD19^+$ B cells were purified from PBMC using CD19 MicroBeads (Miltenyi Biotec), to obtain B cells of >95% purity. $CD19^+$ cells were suspended in iNKT cell medium, and pulsed with appropriate amounts of α-GalCer or its more potent analogue 7DW8-5 (Funakoshi). α-GalCer and 7DW8-5 stocks in dimethylsulphoxide (DMSO) were heated to 80 °C for 2 min, sonicated for 10 min, and vortexed for 1 min before diluting in iNKT cell medium and again before adding to the cells. After 24 h, allogeneic iNKT cells were added to $CD19^+$ cells at 1:1 ratios, and the cytotoxic responses of iNKT cells were assessed by measuring cell-surface expression of the degranulation marker CD107a by flow cytometry. We have previously shown that iNKT cell-surface expression of CD107a correlates well with target cell death [8]. An anti-CD107a mAb (BioLegand) was added at the time the iNKT cells were added. After 1 h, 2 μM of monensin (BioLegand) was added and the cells were incubated for a further 3 h. Cells were then stained using FVD eFluor® 506, and mAbs specific for CD3, CD4, CD8, CD19 and Vα24Jα18. The percentage of total, $CD4^+$, $CD8^+$, and DN iNKT cells that expressed cell surface CD107a were determined by flow cytometry.

2.5. Treatment of $CD19^+$ cells with RARα agonists

Enriched $CD19^+$ cells or PBMC from patients and control subjects were suspended in iNKT cell medium and treated with 1–1000 nM ATRA or AM580 (Sigma-Aldrich, Poole, UK) for 24–72 h. CD1d expression was measured before and after treatment using flow cytometry. To test the effects of ATRA and AM580 treatment of B cells on their susceptibility to iNKT cell cytotoxicity, $CD19^+$ cells were treated with 100 nM of ATRA or AM580 for 48 h. Cells were then pulsed for 24 h with 1 μg/ml α-GalCer or 10 ng/ml 7DW8-5. $CD19^+$ cells were then co-cultured for 4 h at 1:1 ratios with allogeneic iNKT cells, and the cytotoxic response of iNKT cells was measured using a CD107a cytotoxicity assay.