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RESEARCH PAPER

α-Galactosylceramide suppresses murine eosinophil production through interferon-γ-dependent induction of NO synthase and CD95

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BACKGROUND AND PURPOSE

α-Galactosylceramide (α-GalCer), a pleiotropic immunomodulator with therapeutic potential in neoplastic, autoimmune and allergic diseases, activates invariant natural killer T-cells throughCD1-restricted receptors for α-GalCer on antigen-presenting cells, inducing cytokine secretion. However the haemopoietic effects of α-GalCer remain little explored.

EXPERIMENTAL APPROACH

α-GalCer-induced modulation of eosinophil production in IL-5-stimulated bone marrow cultures was examined in wild-type (BALB/c, C57BL/6) mice and their mutants lacking CD1, inducible NOS (iNOS), CD95 and IFN-γ, along with the effects of lymphocytes; IFN-γ; caspase and iNOS inhibitors; non-steroidal anti-inflammatory drugs (NSAIDs) and LTD_4; and dexamethasone.

KEY RESULTS

α-GalCer (10^{-6}–10^{-8}M) suppressed IL-5-stimulated eosinopoiesis by inducing apoptosis. α-GalCer pretreatment *in vivo* (100 μg·kg^{-1}, i.v.) suppressed colony formation by GM-CSF-stimulated bone marrow progenitors in semi-solid cultures. α-GalCer and dexamethasone synergistically promoted eosinophil maturation. Suppression of eosinophil production by α-GalCer was prevented by aminoguanidine and was undetectable in bone marrow lacking iNOS, CD95, CD28; or CD1d. Separation on Percoll gradients and depletion of CD3+ cells made bone marrow precursors unresponsive to α-GalCer. Responsiveness was restored with splenic lymphocytes. Experiments with (i) IFN-γ-deficient bone marrow, alone or co-cultured with spleen T-cells from wild-type, but not from CD1d-deficient, donors; (ii) IFN-γ neutralization; and (iii) recombinant IFN-γ, showed that these effects of α-GalCer were mediated by IFN-γ. Effects of α-GalCer on eosinophil production were blocked by LTD_4 and NSAIDs.

CONCLUSIONS AND IMPLICATIONS

α-GalCer activation of IFN-γ-secreting, CD1d-restricted lymphocytes induced iNOS-CD95-dependent apoptosis in developing eosinophils. This pathway is initiated by endogenous regulatory lymphocytes, antagonised by LTD_4, NSAIDs and aminoguanidine, and modified by dexamethasone.

Abbreviations

DEC, diethylcarbamazine; α-GalCer, α-galactosylceramide; iNKT cells, invariant natural killer T lymphocytes

Tables of Links

TARGETS
Enzymes
iNOS

LIGANDS	
Aminoguanidine	IFN-γ
Aspirin	IL-5
Dexamethasone	Indomethacin
GM-CSF	LTD_4

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

α-Galactosylceramide (α-GalCer), a natural immunomodulator (see Berkers and Ovaa, 2005; Molano and Porcelli, 2006), has therapeutic potential in malignant (Konishi *et al.*, 2004), autoimmune (Molano and Porcelli, 2006; Yamamura *et al.*, 2007) and allergic diseases (Hachem *et al.*, 2005; Matsuda *et al.*, 2005; Benoit *et al.*, 2007; Iwamura and Nakayama, 2007; Kim *et al.*, 2009; Umetsu and De Kruyff, 2010). α-GalCer activates natural killer T (NKT) cells, a specialized subset of innate regulatory/effector lymphocytes bearing α/β T-cell receptors, specific for glycolipid antigens and restricted by CD1 (CD1d in mice) (De Libero and Mori, 2012; Adams, 2014), which exert important immunoregulatory and effector functions, including rapid and intense secretion of cytokines (Yamamura *et al.*, 2007; Matsuda *et al.*, 2008; Bjordahl *et al.*, 2012; Adams, 2014). NKT cells comprise two immunologically distinct, but functionally similar, subpopulations (Matsuda *et al.*, 2008; Bjordahl *et al.*, 2012): '*semi-invariant*' (*iNKT*) cells, which recognize α-GalCer through CD1d-restricted α/β receptors bearing an 'invariant' rearrangement in their α chains, with a matching assortment of β chains, and *type II NKT* cells, which lack invariant rearrangements and do not recognize α-GalCer.

Many studies (summarized in Umetsu and De Kruyff, 2010) suggest that the *first* subset (iNKT cells) promotes the multi-step progression towards an asthma phenotype in humans and mice. Some human (Jyonouchi *et al.*, 2014) and experimental (Kim *et al.*, 2004; Chuang *et al.*, 2011) studies suggest that α-GalCer and/or iNKT cells increase eosinophilic inflammation, an important component of allergic reactions. By contrast, other studies show that iNKT cells suppress asthma through the secretion of IFN-γ, thereby reinforcing immunomodulation therapy (Hachem *et al.*, 2005; Iwamura and Nakayama, 2007; Fujita *et al.*, 2009; Bourgeois *et al.*, 2011). In murine lungs, eosinophil infiltrates are reduced by α-GalCer treatment (Benoit *et al.*, 2007) or by IL-33-activated iNKT cells (Bourgeois *et al.*, 2011).

However, it remains unclear whether bone marrow eosinopoiesis, which underlies chronic eosinophilic infiltrates in allergic disease, provides a separate target for regulation by α-GalCer. In mice, IFN-γ potently induces inducible NOS (iNOS) (Kleinert *et al.*, 2004; Rauch *et al.*, 2013), and NKT cells, producers of IFN-γ, are found in murine bone marrow and spleen (Matsuda *et al.*, 2008). These observations by others, along with our long-term interest on the extrinsic regulation of bone marrow eosinopoiesis by drugs and cytokines (Xavier-Elsas *et al.*, 2008; Queto *et al.*, 2010a,b), prompted us to examine the effects on bone marrow, of α-GalCer, iNKT cells and IFN-γ produced by iNKT cells.

We had previously characterized (Jones *et al.*, 2004; Gaspar-Elsas *et al.*, 2009; Queto *et al.*, 2010a) a proapoptotic mechanism for regulation of eosinophil production, dependent upon iNOS and the ligand for CD95/Fas (CD95L/FasL), which operates *in vivo* during airway allergen challenge of sensitized mice, and accounts for the therapeutic effect of diethylcarbamazine (DEC), a leukotriene synthesis inhibitor (Matthews and Murphy, 1982), in experimental asthma (Queto *et al.*, 2010a). Although activation of this mechanism by DEC and other drugs (de Luca *et al.*, 2013) prevents the induction of eosinophilia by allergen (Masid-de-Brito *et al.*, 2014b), its blockade by LTD_4 and non-steroidal anti-inflammatory drugs (NSAIDs) (Xavier-Elsas *et al.*, 2008) protects developing eosinophils from apoptosis. Furthermore, dexamethasone protects developing eosinophils from apoptosis by preventing expression of iNOS (de Luca *et al.*, 2013).

Here, we describe the suppression of bone marrow eosinophil production by IFN-γ-producing bone marrow and spleen lymphocytes, which are activated by α-GalCer, restricted by CD1d, dependent upon CD28 and counteracted by agonists that block the iNOS-CD95L pathway, including dexamethasone, LTD_4 and NSAIDs.

Methods

Animals, ethical aspects, in vivo *procedures*

All animal care and experimental procedures complied with and were approved by the institutional Animal Ethical Committees (CEUA No. L-010/04 and CEUA No. L-002/09). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 82 animals were used in the experiments described here.

Wild-type mice of the BALB/c and C57BL/6 (B6) strains and their mutants lacking iNOS (Laubach *et al.*, 1995), CD28 (Shahinian *et al.*, 1993), CD1 (Sonoda *et al.*, 1999), or IFN-γ (Dalton *et al.*, 2000), bred at CECAL-FIOCRUZ, Rio de Janeiro, Brazil, or lacking CD95 due to a spontaneous mutation in the BALB/c background (http://jaxmice.jax.org/strain/000485.html), bred at FMRP-USP, Brazil, were used at 6–8 weeks of age as a source of bone marrow and spleen cells. Naïve animals were used, unless otherwise indicated. Where indicated, mice were injected i.p. with 100 $\mu g \cdot kg^{-1}$ α-GalCer in a 400 μL volume of PBS containing 2% DMSO (controls received vehicle), once, 24 h before killing and bone marrow collection.

Bone marrow studies

Bone marrow cells were collected from both femurs of naïve mice, washed, counted in a haemocytometer, seeded at a density of 10^6 cells mL^{-1} of RPMI 1640 medium, 10% FCS and rmIL-5 (1 $ng \cdot mL^{-1}$; Gaspar-Elsas *et al.*, 1997) in 24-well clusters, and incubated at 37°C, 5% CO_2/95% air for 7 days. These conditions allow the addition of agonists and inhibitors at different times, unlike semi-solid culture, where proper mixing and diffusion of substances added after plating is restricted. Eosinopoiesis and its modulation by various agents were strictly dependent upon IL-5 (Gaspar-Elsas *et al.*, 1997; 2000a; 2009). Where indicated, cultures received α-GalCer (10^{-6}–10^{-12} M), together with indomethacin (10^{-7} M), aspirin (10^{-7} M) (Xavier-Elsas *et al.*, 2008; Queto *et al.*, 2010b), the iNOS inhibitor aminoguanidine (10^{-4} M; Queto *et al.*, 2010a) or the caspase inhibitor zVAD-fmk (20 μM; de Luca *et al.*, 2013). Each agonist or inhibitor was added only once, immediately after IL-5, at the beginning of the culture period. Where indicated, α-GalCer was added at various times after initiation of the culture to define the period during which it must act in order to suppress eosinophil production. Where indicated, cultures received dexamethasone (10^{-7} M) to enhance eosinophil precursor proliferation while keeping eosinophils cytologically immature and aggregated (Gaspar-Elsas *et al.*, 2009). To these cultures, as well as to IL-5 controls, α-GalCer was added to evaluate its ability to promote dissociation and terminal differentiation of eosinophils (Gaspar-Elsas *et al.*, 2009). Cells present in 7 day cultures were resuspended, collected, counted, cytocentrifuged and stained for eosinophil peroxidase (EPO; Gaspar-Elsas *et al.*, 2009). EPO+ cells were counted and photographed at 400× magnification, under oil. Eosinophil numbers were calculated from total and differential counts.

In another set of experiments, bone marrow from α-GalCer-injected and vehicle-injected control mice was used for semi-solid culture (Gaspar-Elsas *et al.*, 2000a) in 35 mm culture dishes, in triplicate. One mL of Iscove's modifed Dulbecco's medium 20% FCS, containing 2×10^5 bone marrow cells and GM-CSF (2 $ng \cdot mL^{-1}$ final concentration), was pre-incubated for 2 h with or without α-GalCer before mixing with agar to 0.3% final concentration and plating. Colonies (>50 cells) were scored at day 7 under the inverted microscope at low magnification. In additional controls, the frequency of eosinophil, granulocyte (G), macrophage (M) and granulocyte-macrophage (GM) colonies was determined on agar layers dried (50°C), mounted on microscope slides, stained for EPO and scored under high magnification (Gaspar-Elsas *et al.*, 1997; 2000a; Queto *et al.*, 2010b).

Where indicated, total bone marrow was separated on Percoll™ discontinuous (45%-60%-75%; Maximiano *et al.*, 2005) gradients to deplete mature cells, including eosinophils. The immature cells at the 45%-60% interface (the 'P2 layer') were collected, washed, counted and adjusted to 10^6 cells mL^{-1} medium with 10% FCS for (i) culture as above; (ii) further depletion with anti-CD3 immunobeads; or (iii) staining with anti-CD3 followed by flow cytometric analysis. In control experiments, the mature cells at the 60%-75% interface were collected and cultured as above. Alternatively, P2 layer cells were co-cultured with T lymphocytes from spleens of syngeneic wild-type (BALB/c in Figure 6C–F; B6 in Figure 7) or CD1-knockout (KO) (in Figure 7) donors.

For flow cytometry, 10^6 cells in Eppendorf tubes were centrifuged (500× *g*, 10 min, 20°C) and resuspended in 100 μL of serum-free medium, to which was added antibody to 0.25 $\mu g \cdot mL^{-1}$ final concentration, followed by a 30 min incubation in the dark, removal of excess antibody as above, fixation in 400 μL of 2% paraformaldehyde in PBS, and stored at 4°C in the dark before acquisition in a FACScalibur™ (Becton-Dickinson, San Diego, CA, USA), with an argon laser at 488 nm, and analysis by Cell Quest™ (Becton Dickinson). For immunomagnetic depletion of CD3+ cells, including iNKT (Li *et al.*, 2012), we used MidiMACS™ (Cat. No. 130-042-302; Miltenyi Biotec) and biotinylated anti-mouse CD3e antibody followed by streptavidin-coated microbeads. Where indicated, Annexin V staining of IL-5-differentiated eosinophils from 6 day cultures established in the absence and in the presence of α-GalCer was carried out with the TACS Annexin V-FITC Apoptosis Detection Kit from R&D Systems (Ref. No. 4830-01-K).

Preparation of spleen cells

Splenocyte suspensions were prepared by mincing the spleen with scissors and repeated passage through a 5 mL syringe (without needle) in media containing 1% FCS. Spleen mononuclear cells were filtered through nylon-wool columns according to Julius *et al.* (1973). Recovery was routinely 25–30% of the input mononuclear cells.

Data analysis

Data are presented as mean ± SEM throughout and were analysed with Systat for Windows version 4 software from Systat Inc. (Evanston, IL, USA). Comparisons of more than two means were carried out by factorial ANOVA with Tukey's HSD correction (as recommended in O'Neill and Wetheril, 1971). Where only two groups were compared, we used the two-tailed *t*-test with separate variances. $P < 0.05$ was taken to show significant differences between group means.

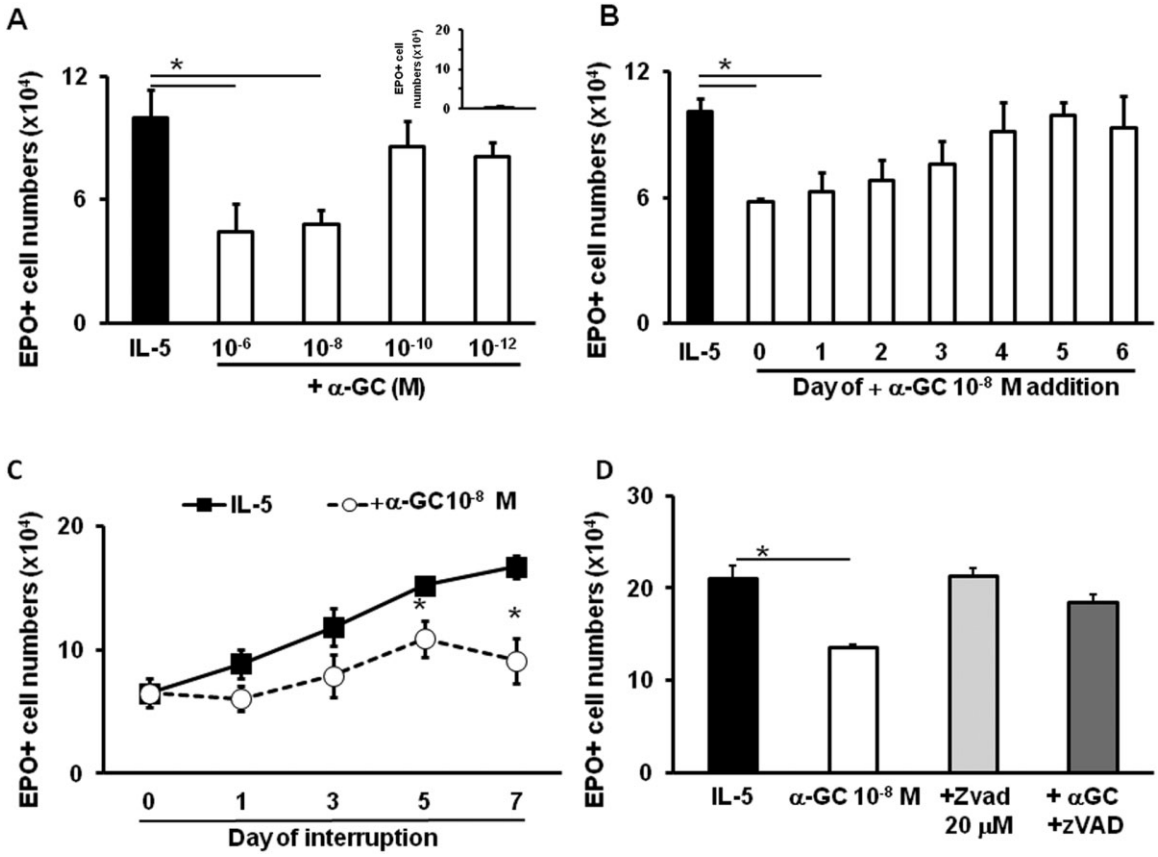

Figure 1

α-GalCer suppresses eosinopoiesis by activating a caspase-dependent mechanism. Bone marrow cultures were established from BALB/c donors in the presence of IL-5 (1 ng·mL^{-1}), alone or together with the indicated concentrations of α-GalCer, caspase inhibitor zVAD-fmk, or both; or α-GalCer alone (10^{-8} M; panel A, inset). All cultures were maintained for 7 days, except for panel (C). All agents were added at the beginning of the culture, except for panel (B). Data (mean + SEM) are the numbers of EPO+ cells recovered at the end of the culture. *$P \leq 0.05$, significantly different from the respective IL-5 controls; $n = 4$ (panels A and C); $n = 3$ (panels B and D).

Materials

Fetal calf serum (FCS) was from HyClone (Logan, UT, USA); culture media RPMI 1640 was from RHyClone, ThermoScientific (Waltham, MA, USA); recombinant murine cytokines (GM-CSF, IL-5) was from R&D Systems (Minneapolis, MN, USA); GalCer (Ref. No. ALX-306-027) was from Alexis (Lausen, Switzerland); dexamethasone 21-phosphate (D1159), indomethacin (I7378) and aspirin (A5376) were from Sigma (St. Louis, MO, USA); Z-VAD-fmk (Ref. No. V116) and aminoguanidine hydrochloride, a selective iNOS inhibitor (Ref. No. 396494), were from Sigma-Aldrich (St. Louis, MO, USA); and diaminobenzidine (DAB+) (Ref. No. K3467) solution was from Dako Cytomation (Dako Denmark A/S, Glostrup, Denmark). Purified rat anti-mouse IFN-γ (Ref. No. 18110D), recombinant murine IFN-γ (Ref. No. 193301U) and biotinylated anti-mouse CD3e antibody (Cat. No. 01082J) were from Pharmingen (Franklin Lakes, NJ, USA); streptavidin-coated microbeads (Order No. 481-02) was from Miltenyi Biotec (Bergisch Gladbach, Germany); purified PE-cyanine 5.5-conjugated hamster anti-mouse CD3e antibody (Ref. No. 35-003181, clone: 145-2C11, stock 0.2 mg·mL^{-1}) was from eBioscience (San Diego, CA, USA).

Results

α-GalCer suppresses eosinopoiesis by activating a caspase-dependent mechanism

α-GalCer dose-dependently suppressed eosinophil production in BALB/c bone marrow cultures (Figure 1A), as shown by the significantly decreased numbers of eosinophils recovered from 7 day cultures, established in the presence of IL-5 together with α-GalCer at 10^{-6}–10^{-8}M (but not at 10^{-10}–10^{-12} M), relative to the values with IL-5 alone. In the presence of α-GalCer alone (Figure 1A, inset), there was no eosinophil production. The effects of α-GalCer (10^{-8} M) depended upon the timing of its introduction in the culture, as significantly decreased eosinophil recovery, relative to IL-5 controls, was observed following the addition at days 0–1, but not at later times (Figure 1B). To define the kinetics of α-GalCer effects on IL-5-driven eosinophil differentiation, bone marrow cultures were established with rmIL-5 alone, or together with α-GalCer (10^{-8} M), and the EPO+ cell counts were determined at various times after initiation of the culture. As shown in Figure 1C, EPO+ cell numbers steadily

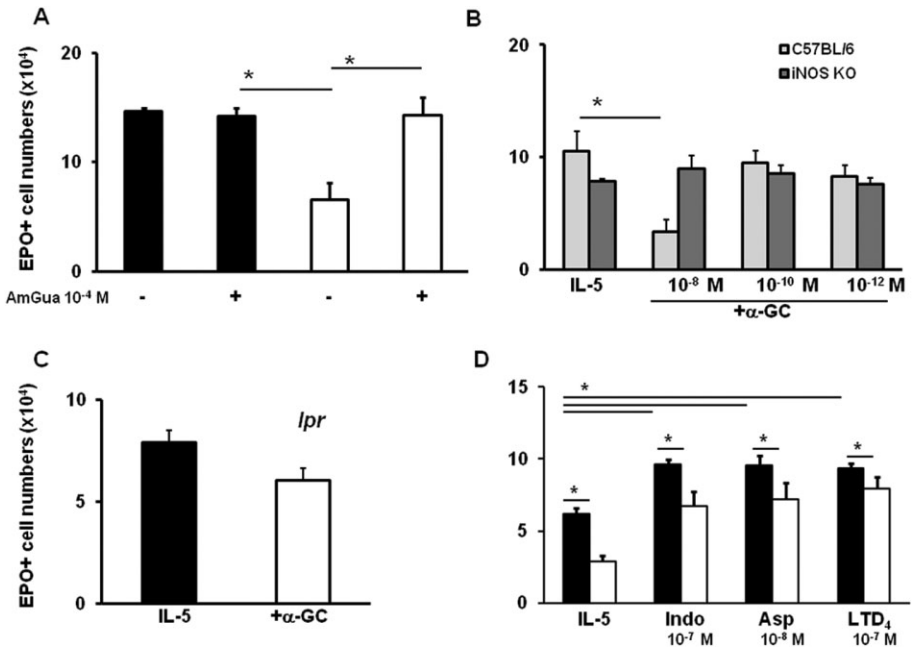

Figure 2

The effectiveness of α-GalCer depends on both iNOS and CD95. Bone marrow was collected from BALB/c (panels A and D) or from C57BL/6 wild-type controls and mutant iNOS-deficient mice (panel B) or from BALB/c *lpr* mutant mice, lacking CD95 (panel C). Cultures were established for 7 days in the presence of IL-5 (1 ng·mL^{-1}), alone or together with α-GalCer (10^{-8} M). Panel (A), where indicated (+), aminoguanidine (AmGua; 10^{-4} M); panel (D), as indicated, indomethacin (Indo, 10^{-7} M), aspirin (Asp, 10^{-8} M) or LTD_4 (10^{-7} M). $^{**}P \leq 0.05$, significantly different as indicated; $n = 3$ for all panels.

increased from day 1 to day 7 of culture, reflecting eosinophil differentiation from EPO– precursors. At days 5–7, EPO+ cell numbers were significantly reduced in cultures containing α-GalCer. The suppressive effect of α-GalCer on eosinopoiesis was abolished by the caspase inhibitor, zVAD-fmk (20 μM). zVAD-fmk had no effect by itself (Figure 1D). In separate flow cytometry control experiments, we evaluated Annexin V binding to cells in the granulocyte gate, in bone marrow cultures stimulated for 6 days by IL-5 alone (control) or together with α-GalCer (experimental). In these cultures, bone marrow neutrophils are reduced to 1% or less by day 6 (Gaspar-Elsas *et al.*, 2009), and the gated region contains essentially eosinophils. Cells staining positive for Annexin V binding were 3.1% in the controls, as opposed to 38% in α-GalCer-exposed cultures. This greater than 10-fold increase is consistent with an apoptotic mechanism.

Similarity of α-GalCer effects to those of PGE_2. The effects of α-GalCer in bone marrow culture resemble those previously described for PGE_2 in the same system (Gaspar-Elsas *et al.*, 2000b). PGE_2 acts through NO production by iNOS, followed by CD95L-mediated apoptosis (Jones *et al.*, 2004), and its effectiveness is abolished by NSAIDs (indomethacin and aspirin) and by the cysteinyl-leukotrienes (LTD_4, LTC_4 and LTE_4). Cysteinyl-leukotrienes are thought to mediate the actions of NSAIDs in this system because montelukast and MK571 (type I CysLT receptor antagonists) block NSAID effects (Xavier-Elsas *et al.*, 2008). For these reasons, we evaluated the possible role of NO, iNOS and CD95 in the actions of α-GalCer, as well as the ability of NSAIDs and LTD_4 to block these actions. Initially, we confirmed a requirement for iNOS in the effects of α-GalCer, as these were prevented by aminoguanidine, which had no effect in the absence of α-GalCer (Figure 2A). This is consistent (Figure 2B) with the difference in effectiveness of α-GalCer in cultures of iNOS-deficient bone marrow (which did not respond) and wild-type B6 controls (which responded as expected). Furthermore, bone marrow from CD95-deficient *lpr* mutants of the BALB/c background did not show a significant response ($P = 0.72$) to α-GalCer (Figure 2C), unlike that of wild-type controls of this background (Figure 2A). Finally, indomethacin, aspirin (Lintomen *et al.*, 2002) and LTD_4 (Xavier-Elsas *et al.*, 2008; Queto *et al.*, 2010b) showed comparable effectiveness in preventing the suppression of eosinopoiesis by α-GalCer (Figure 2D).

PGE_2 is able to synergically interact with dexamethasone to induce terminal differentiation of immature eosinophils, even though it does not suppress eosinopoiesis in the presence of dexamethasone (Gaspar-Elsas *et al.*, 2009). To determine whether the parallels between α-GalCer and PGE_2 would extend to this maturation-promoting effect, we established liquid bone marrow cultures in the presence of IL-5 together with dexamethasone (10^{-7} M), with or without α-GalCer (10^{-8} M). α-GalCer significantly decreased the numbers of eosinophils recovered from 7 day cultures, relative to IL-5 controls (Figure 3A). Dexamethasone increased significantly the total yield of EPO+ cells (Gaspar-Elsas *et al.*, 2009). α-GalCer lost its suppressive effect in the presence of dexamethasone. Figure 3B illustrates the representative aspects of the cultures: with IL-5 alone, eosinophils were

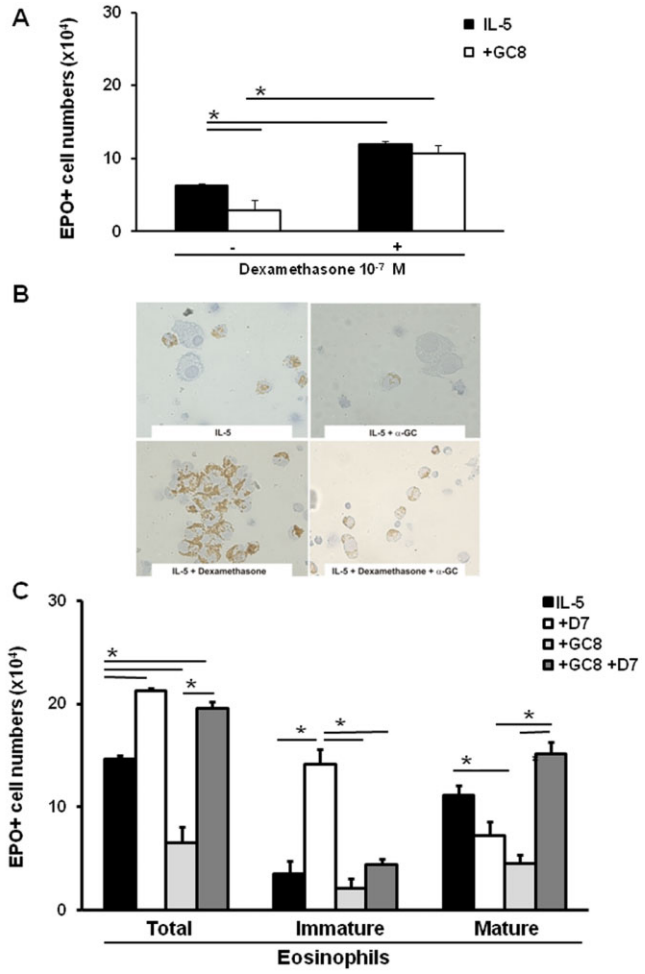

Figure 3

α-GalCer synergizes with dexamethasone to promote eosinophil maturation. Bone marrow cultures were established from BALB/c bone marrow for 7 days with IL-5 (1 ng·mL^{-1}), alone or together with dexamethasone (10^{-7} M, D7), α-GalCer (10^{-8} M, GC8) or both. Panels (A) and (C): Effects of dexamethasone, α-GalCer or both on total and differential EPO+ cell counts. Data are mean + SEM of the counts (panel A, total; panel C, differential) of EPO+ cells in 7 day cultures established in the indicated conditions. Panel (A): Cultures established with IL-5 alone or together with α-GalCer (10^{-8} M), were maintained without (–) or with (+) dexamethasone (10^{-7} M). Panel (B): Representative images of eosinophils (recognizable by EPO+, brown-stained, coarse cytoplasmic granules) in the indicated culture conditions. *Immature* eosinophils, a prominent feature of dexamethasone-exposed cultures, show variable size and nucleocytoplasmic ratios, with incomplete nuclear segmentation, and often form homotypic aggregates. *Mature* eosinophils are smaller, with regular size and donut-shaped nuclei, usually found as single cells. *Apoptotic* eosinophils, more frequent in α-GalCer-exposed cultures, are pyknotic and occasionally found attached to the surface of macrophages. Panel (C): Cultures established with IL-5 alone or together with dexamethasone (10^{-7} M) (+D7), α-GalCer 10^{-8} M (+GC8) or both (+GC8 + D7) were examined for the proportions of mature and immature eosinophils in the total EPO+ cell numbers. *$P \leq 0.01$, significantlydifferent as indicated; $n = 3$.

predominantly single cells with strong brown cytoplasmic staining, dispersed among EPO– cells (stromal cells and macrophages, which are IL-5-independent; Gaspar-Elsas *et al.*, 1997), visible after haematoxylin counterstaining (Figure 3B, upper left). Their morphology includes uniform size, doughnut-shaped nucleus and coarse, tightly packed, cytoplasmic granules (Gaspar-Elsas *et al.*, 2000a; 2009). Cultures established with α-GalCer have fewer EPO+ cells, with no change in cytological features (upper right), nor in the number of EPO– cells. By contrast, EPO+ cells in cultures stimulated by IL-5 and dexamethasone were found both as single cells and as large clusters of rather uniform appearance, containing EPO+ cells of various sizes, irregular shapes, incomplete nuclear segmentation and large cytoplasm with less densely packed brown granules (lower left). When present together, α-GalCer and dexamethasone interacted, so that apoptosis was prevented and eosinophil production brought back to the IL-5 control levels. This was accompanied by the absence of large clusters and presence of mature morphology (lower right). The effects of dexamethasone alone on eosinophil numbers could be accounted for by a significant increase in morphologically immature eosinophils (Figure 3C); those of α-GalCer alone by significant reduction in mature eosinophils. When both were present, there was a striking reduction in immature eosinophil numbers, accompanied by an equally significant increase in mature eosinophil numbers, relative to IL-5 plus dexamethasone controls. Overall, total eosinophil numbers were significantly increased by this synergic combination, relative to the IL-5 controls, but yielding mostly mature cells.

α-GalCer suppresses GM-CSF-stimulated progenitors in vivo *and* in vitro. To define whether the suppressive effects of α-GalCer were restricted to the eosinophil lineage, we further evaluated α-GalCer effects on the formation of myeloid colonies by bone marrow progenitors cultured in the presence of the multi-lineage haemopoietic factor, GM-CSF. In preliminary experiments with BALB/c mice (not shown), α-GalCer significantly decreased the total numbers of myeloid colonies in the same concentration range (10^{-6}–10^{-8}M), in which it suppressed eosinophil production in the liquid culture. This effect was accounted for by a rather uniform reduction of colony types of all lineages, not by exclusive or selective inhibition of eosinophil-containing colonies. We subsequently examined whether exposure to α-GalCer *in vivo* would affect subsequent colony formation *ex vivo*, by injecting B6 mice with α-GalCer or vehicle (see the Methods section) and collecting bone marrow 24 h later for the semi-solid culture. The ability to respond to GM-CSF by forming myeloid colonies, and the ability to respond to α-GalCer *in vitro*, in the absence or presence of α-GalCer pre-exposure *in vivo*, were evaluated. As shown in Figure 4, α-GalCer exposure *in vivo* significantly reduced colony formation *ex vivo* ($P = 0.03$ relative to the vehicle-injected control). Bone marrow exposure to α-GalCer *in vivo* did not prevent subsequent *in vitro* responses to α-GalCer *in vitro*, which were significant in both vehicle and α-GalCer-injected mice. The response to α-GalCer *in vitro* was blocked by aminoguanidine (not shown).

The effectiveness of α-GalCer requires IFN-γ production and signalling. The evidence for a critical involvement of iNOS in the suppression of eosinopoiesis prompted the evaluation of

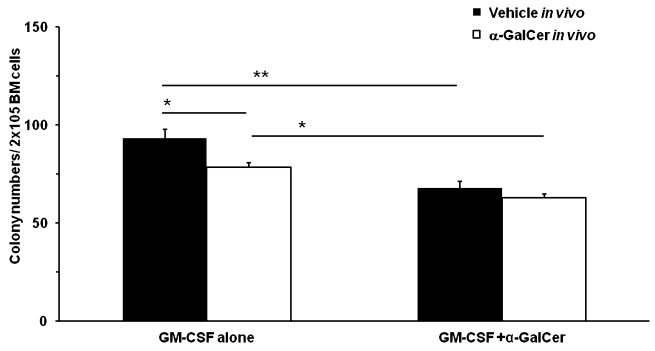

Figure 4

α-GalCer acts *in vivo* and *in vitro* to suppress colony formation. C57BL/6 mice were injected i.p. with α-GalCer 100 μg·kg^{-1} in a 400 μL volume of PBS containing 2% DMSO (vehicle), once, 24 h before killing and bone marrow collection Controls received vehicle in the same conditions Bone marrow cells (2×10^5 cells mL^{-1}, in Iscove's modifed Dulbecco's medium with 20% FCS) were pre-incubated for 2 h in the presence of GM-CSF (2 ng·mL^{-1} final concentration), alone (GM-CSF) or together with α-GalCer 10^{-8}M (GM-CSF+α-GalCer) before mixing with agar (0.3% final concentration) and plating in triplicate. Total myeloid colonies were scored at day 7 under an inverted microscope. Data are mean ± SEM ($n = 4$ for all groups). *$P \leq 0.05$, **$P < 0.01$, significantly different as indicated.

a role for IFN-γ in mediating this effect of α-GalCer because IFN-γ is both a product of activated iNKT cells and a strong inducer of iNOS. As shown in Figure 5A, α-GalCer had no significant effect in bone marrow cultures from IFN-γ-deficient mice. By contrast, a dose-dependent suppression of eosinophil production by α-GalCer (10^{-8}M, but not 10^{-10}–10^{-12}M) was observed in wild-type controls of the same background (B6) in identical conditions. This strongly suggests that the ability to produce IFN-γ underlies the effectiveness of α-GalCer in these conditions. We further examined whether α-GalCer (10^{-8}M) achieved maximal suppression of eosinophil production, or, alternatively, whether its effectiveness would be further increased by exogenously added IFN-γ. Addition of recombinant murine IFN-γ suppressed eosinophil production directly (Figure 5B) at 5 ng·mL^{-1} but not at a lower concentration (2 ng·mL^{-1}). At this ineffective concentration (2 ng·mL^{-1}), however, IFN-γ retained the ability to interact synergically with α-GalCer and significantly increased the suppressive activity of the latter (Figure 5B). Both sets of observations (IFN-γ alone vs. IFN-γ plus α-GalCer) suggest that the suppressive effect of α-GalCer is limited by the amount of IFN-γ it induces. Finally, independent evidence for mediation of α-GalCer effects by secondary induction of IFN-γ was obtained with the use of neutralizing IFN-γ-specific antibodies (Figure 5C). In bone marrow exposed to α-GalCer, we observed significant suppression of eosinophil production, relative to the controls, both without and with an irrelevant isotype control antibody. By contrast, no such effect was observed with α-GalCer if neutralizing antibody to IFN-γ was present, while the neutralizing antibody had no significant effect in the absence of α-GalCer.

α-GalCer requires bone marrow lymphocytes, CD1d and CD28. An indirect mode of action for α-GalCer in regulating bone marrow eosinophil production from naïve mice would require an endogenous lymphocyte population to be present in freshly harvested bone marrow, and to be activated *in vitro* by α-GalCer through CD1d (the restriction element) with an essential contribution of CD28 (the co-stimulation receptor). We evaluated these requirements with the use of CD1d- and CD28-deficient mice and depletion/reconstitution strategies (Figure 6). In the initial experiments, we examined the responses to α-GalCer in bone marrow from wild-type controls (B6) and from CD28-deficient (Figure 6A) or CD1d-deficient (Figure 6B) mutant mice. Neither mutant bone marrow responded to α-GalCer, in contrast to the wild-type controls, which showed a significant suppressive response to 10^{-8}M (but not to 10^{-9}–10^{-10}M) α-GalCer, as expected. We next used discontinuous Percoll gradients to deplete bone marrow of mature granulocytes and mononuclear cells, while retaining eosinophil precursors (Maximiano *et al.*, 2005). As illustrated in Figure 6C and D, unfractionated bone marrow responded to α-GalCer, while the fraction containing mature cells did not produce eosinophils (Figure 6C, inset); by contrast, the fraction depleted of mature cells produced eosinophils, but no longer responded to α-GalCer (compare two left columns in Figure 6D to each other and to Figure 6C). The response to α-GalCer could be reconstituted by exogenously added splenic lymphocytes (compare two right columns in Figure 6D to each other and to Figure 6C). The reconstituting lymphocyte population in Figure 6D, which amounts to only 10% of the cells in culture, does not produce eosinophils (not shown). This is consistent with the assumption that lymphocytes absent from the depleted culture can be replaced by splenic lymphocytes, a known source of iNKT cells. The separation of mature cells by the Percoll gradient fractionation effectively removes CD3+ cells from the bone marrow cells, as shown by flow cytometry (Figure 6E). A significant removal was achieved by gradient centrifugation alone, and a further step of immunomagnetic depletion with anti-CD3 beads resulted in no further removal. Use of these two-step purified cells for reconstituting bone marrow depleted of mature cells (Figure 6F) yielded results entirely consistent with those obtained without the supplementary step of anti-CD3 depletion (Figure 6D).

The combined results from Figures 5 and 6 prompted us to examine the role of IFN-γ in reconstitution of α-GalCer responses by splenic lymphocytes dependent upon CD1. As shown in Figure 7, this was done by co-culturing bone marrow cells from IFN-γ-deficient mice, depleted of mature cells as above, with spleen lymphocytes (purified using nylon wool) from wild-type donors or from CD1-KO negative controls. As expected, IFN-γ-KO bone marrow precursors produced eosinophils, but did not respond to α-GalCer. Addition of 10% lymphocytes from a wild-type donor did not significantly affect eosinophil production, but restored response to α-GalCer. By contrast, 10% lymphocytes from a CD1-KO donor failed to do so, even though mutant and wild-type mice differ only in the presence of iNKT cells. Further evidence that IFN-γ production underlies the ability of iNKT cells to suppress eosinophil production was provided by neutralization of their effect by a specific anti-IFN-γ antibody, but not by isotype-matched control antibody.

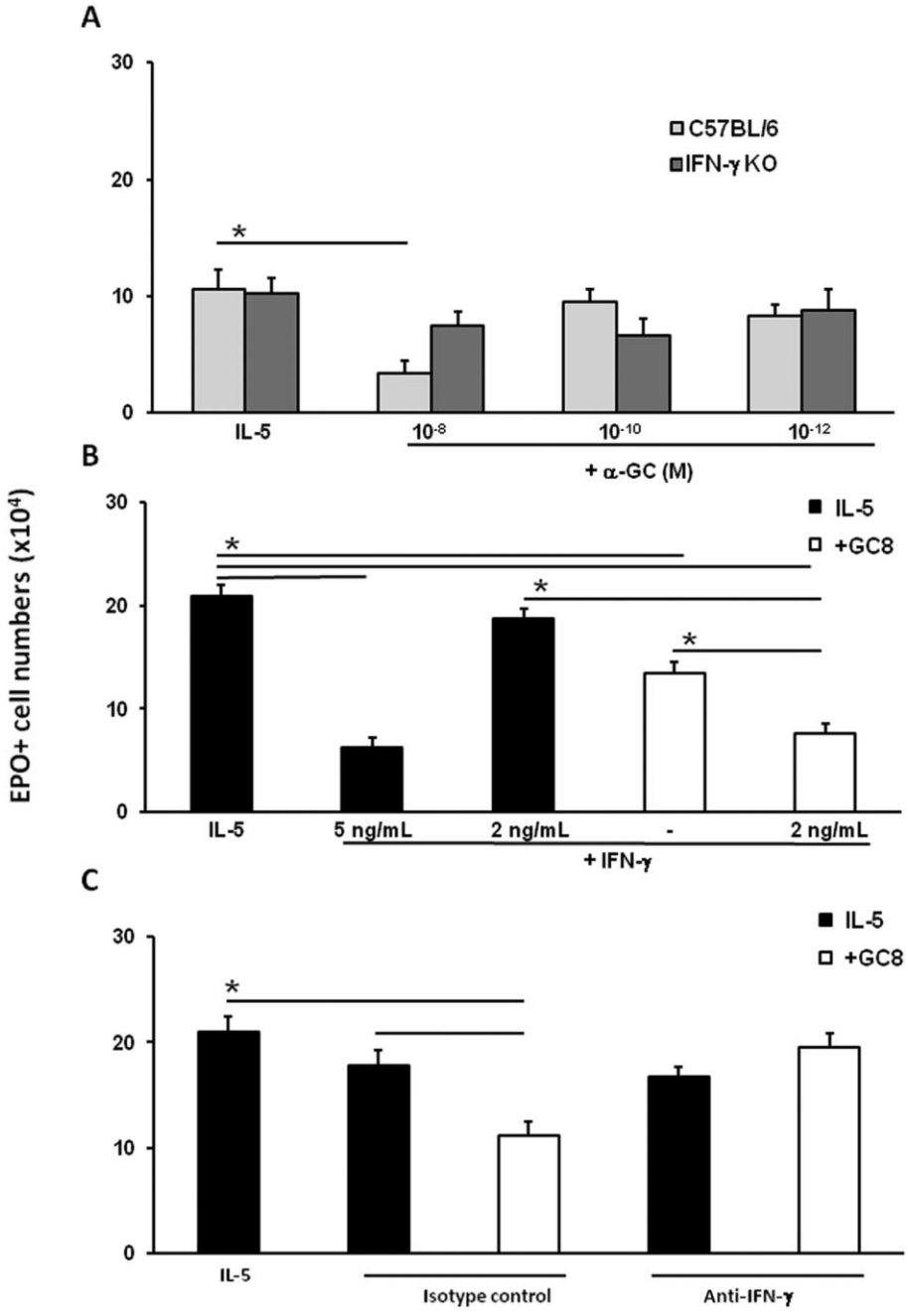

Figure 5

Contribution of IFN-γ to the effects of α-GalCer. Liquid bone marrow cultures were established from C57BL/6 wild-type controls and from IFN-γ-deficient mutants (IFN-γ-KO) (panel A), or from BALB/c mice (panels B and C), in the presence of IL-5 alone, or together with α-GalCer (+αGC), at the indicated concentrations. Where indicated, cultures also received: panel (B) (+IFN-γ): rmIFN-γ at the indicated concentrations or medium (–) as a control; panel (C): monoclonal neutralizing antibodies to an irrelevant antigen (isotype control) or to mIFN-γ. Data are mean ± SEM of the numbers of EPO+ recovered from cultures at day 7. *$P \leq 0.01$, significantly different as indicated; $n = 4$ (panel A); $n = 3$ (panels B and C).

Discussion

This study was prompted by reports of the participation of iNKT cells in normal (Liu *et al.*, 2005) and pathological (Umetsu and De Kruyff, 2010; Bjordahl *et al.*, 2012) immune responses, along with evidence that α-GalCer or iNKT cells may suppress eosinophilic inflammation in experimental models (Benoit *et al.*, 2007; Bourgeois *et al.*, 2011). Here, we report that α-GalCer suppressed eosinophil differentiation and colony formation by activating bone marrow-resident

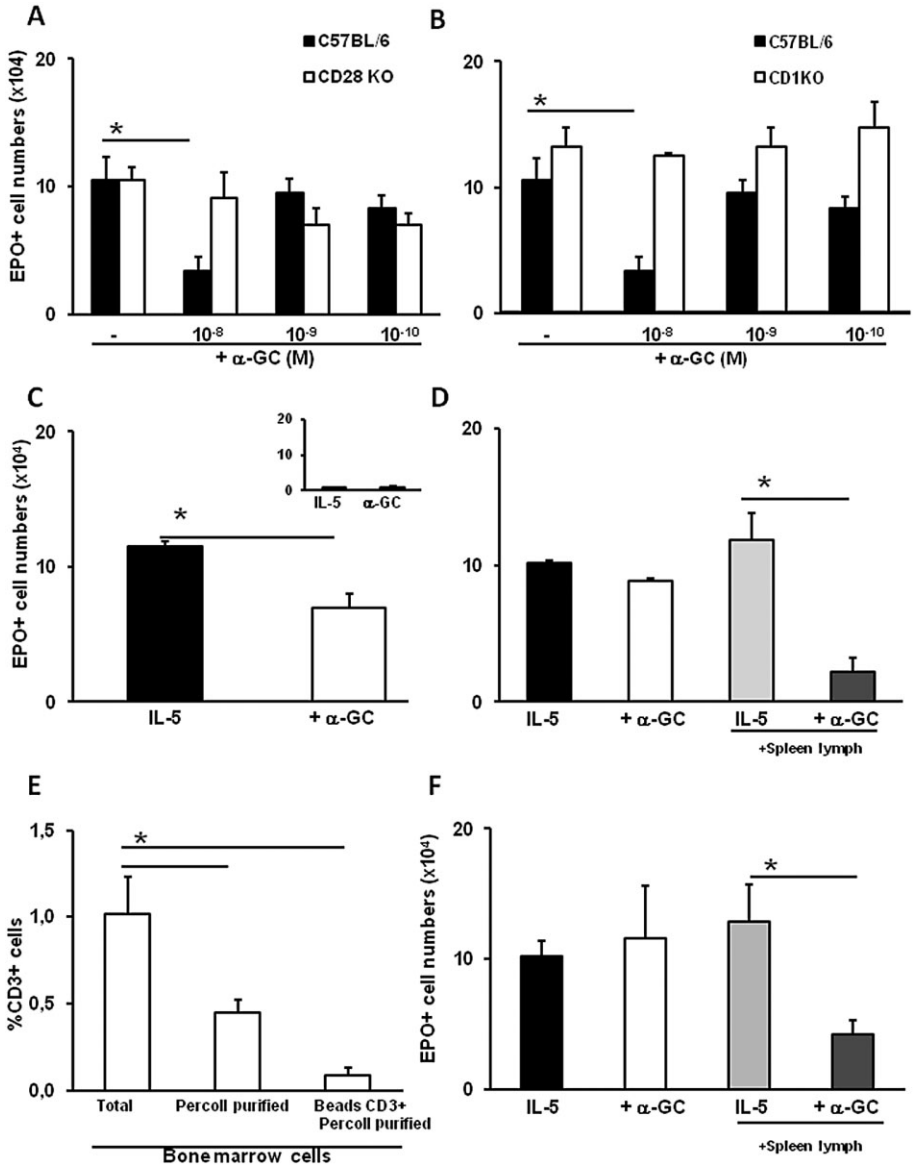

Figure 6

Responsiveness to α-GalCer in bone marrow requires CD1d, CD28 and lymphocytes. Liquid bone marrow cultures were established from C57BL/6 wild-type control (panels A and B) or from CD28-deficient (CD28KO) (panel A) or CD1-deficient (CD1KO) (panel B) mutant mice, or from BALB/c wild-type mice (panels C–F) in the presence of IL-5 alone, or together with α-GalCer (+α–GC), at the indicated concentrations for 7 days. Where indicated, the responses to α-GalCer of unfractionated (total) bone marrow cells were compared with those of Percoll-fractionated subpopulations consisting of mature granulocytes (panel C, inset) or of haemopoietic precursors depleted of mature cells by two different procedures (panels D and F). Where indicated, splenic T lymphocytes (spleen lymph) from syngeneic donors were added to the mature cell-depleted bone marrow cultures up to a 1:10 ratio (panels D and F, as indicated). Panels (A)–(F): Data (mean ± SEM) are numbers of EPO+ cells in 7 days of culture in the indicated conditions. Panel (E): Data (mean ± SEM) are percentage of bone marrow cells bearing CD3, a marker for conventional T and iNKT cells, before fractionation (total), following Percoll centrifugation (P2), and following further immunomagnetic depletion of CD3+ cells (beads CD3+ Percoll purified), as assayed by flow cytometry. *$P \leq 0.01$, significantly different as indicated; $n = 3$ for all panels.

iNKT cells. The underlying mechanism required iNOS and CD95, was mediated by IFN-γ, induces apoptosis and was blocked by dexamethasone and by the pro-allergic lipid mediator, LTD_4, as well as by NSAIDs (which induce cysteinyl-leukotrienes). Together, these observations: (i) show, for the first time, a regulatory role for iNKT cells in eosinophil production, which may be relevant to their reportedly beneficial effects in asthma models; (ii) establish the iNOS-CD95L pathway as inducible by innate cellular immunity; and (iii) substantially increase understanding of this pathway by highlighting its relationship to the pro-allergic effects of dexamethasone and cysteinyl-leukotrienes, and to recent

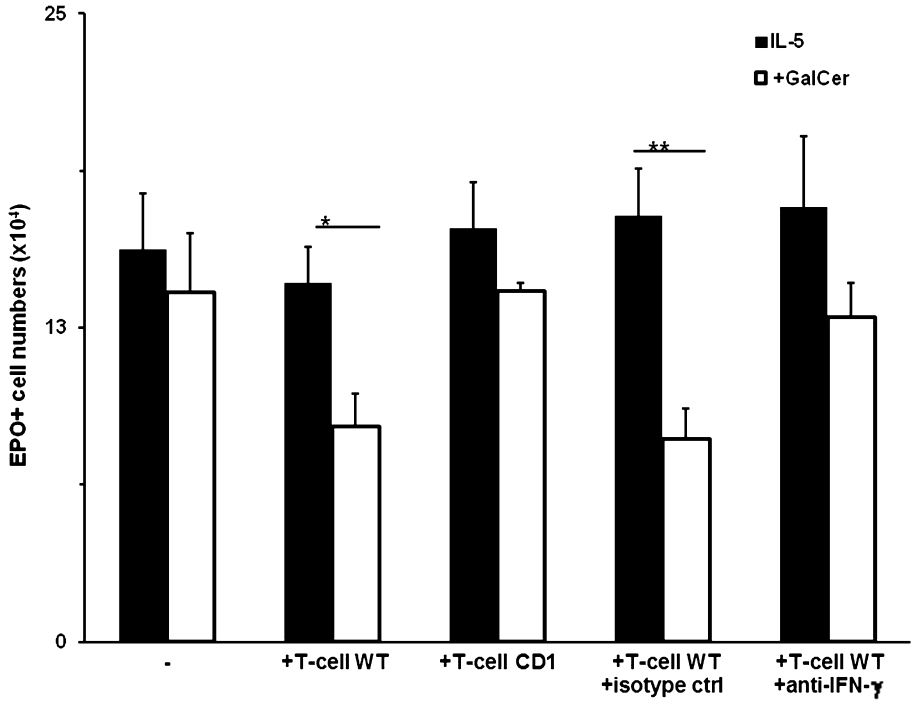

Figure 7

Reconstitution of responses to α-GalCer in IFN-γ-KO bone marrow cultures by CD1-dependent splenic T cells is mediated by IFN-γ. Bone marrow cells from IFN-γ-KO donor mice were collected after fractionation on Percoll gradients from the 60–75% Percoll interface (P2 layer), counted and adjusted to 10^6·cells mL^{-1}. Spleens were collected from C57BL/6 (WT) or CD1KO (CD1) donor mice, and mononuclear cell suspensions were prepared by Ficoll-Hypaque centrifugation. T lymphocytes (T-cell) were further purified on nylon wool columns. Cultures were established as in Figure 6, panel (F), with 1 ng·mL^{-1} IL-5, and in the absence or presence of 10^{-8}M α-GalCer (+GalCer) Cultures contained 10^6 P2 layer bone marrow cells alone (–), or co-cultured with 10^5 T lymphocytes (+T-cell WT, +T-cell CD1). Where indicated, the co-cultures received anti-IFN-γ neutralizing antibody (100 ng·mL^{-1}; +anti-IFN-γ), or an equivalent amount of isotype-matched irrelevant antibody as a control (+isotype ctrl). Data are mean + SEM of EPO+ cell numbers recovered at day 7. *$P \leq 0.05$, **$P < 0.01$, significant effect of α-GalCer; $n = 5$ for all means.

observations on the control of eosinopoiesis by regulatory lymphocytes (Xavier-Elsas *et al.*, 2013) and glucocorticoids (Masid-de-Brito *et al.*, 2014a).

The following specific points deserve detailed discussion and further experimental analyses.

Lineage-specific and non-specific effects of α-GalCer in bone marrow. The effects of α-GalCer on eosinophil production were characterized here to the point of establishing its critical requirements, as has been previously carried out for PGE_2 (de Luca *et al.*, 2013). α-GalCer is a powerful activator of the iNOS-CD95L pathway, initially described in bone marrow culture (Jones *et al.*, 2004) and has been shown to underlie the therapeutic actions of DEC *in vivo* in an asthma model (Queto *et al.*, 2010a). Importantly, α-GalCer is only known to act through activation of regulatory lymphocytes, unlike DEC, which has cellular and molecular targets both heterogeneous and ill-defined (Matthews and Murphy, 1982). The present study of α-GalCer actions demonstrated *indirect* activation of the pathway through a regulatory lymphocyte population, distinct from the ultimate target (eosinophil precursors). It further provided a well-characterized mechanism (IFN-γ-induced NOS), which has been extensively addressed in molecular studies (Rauch *et al.*, 2013). By contrast, there is less information on the molecular pathways linking PGE_2 (and especially DEC) to the expression and activation of iNOS. In addition, myeloid progenitors were suppressed in clonogenic assays. This strongly resembled the effects of PGE_2 on murine bone marrow (Gaspar-Elsas *et al.*, 2000b) and raises the question of whether the similarity extends to mechanism (activation of a specific iNOS-dependent pathway), as suggested by the dependence of α-GalCer on caspases and its ability to induce Annexin V staining of granulocytes in IL-5-stimulated 6 day cultures. The possible contribution of IFN-γ to multilineage suppression, as well as the possible neutropenia-inducing effect of α-GalCer *in vivo*, deserves further exploration, as multilineage suppression and neutropenia are more likely to be harmful than suppression of eosinopoiesis alone.

Modification of α-GalCer effects by dexamethasone. One important similarity between α-GalCer and PGE_2 in our study is the radical modification of their effects by dexamethasone present in the culture. Dexamethasone suppresses iNOS expression in these conditions (de Luca *et al.*, 2013) and hence, apoptosis is blocked, as it is by aminoguanidine or iNOS gene inactivation. However, α-GalCer remains able to interact with dexamethasone, promoting terminal maturation, as shown for PGE_2 (Gaspar-Elsas *et al.*, 2009). It is unclear whether activation of iNKT cells in the presence of

dexamethasone will lead to IFN-γ production, but a possible role of IFN-γ in the maturation effect must be evaluated in future experiments. A further issue is suggested by the similarity between the eosinophilia-promoting effects of dexamethasone in this study and those of endogenous glucocorticoids, which signal through the same receptors, in allergic inflammation models (Masid-de-Brito *et al.*, 2014a). One should consider the possibility that signalling by endogenous glucocorticoids might interfere with the ability of exogenous immunomodulators to suppress eosinophilia, another issue for future investigation.

Relationship to allergy. These results increase our understanding of the benefits of α-GalCer in allergy, about which consensus is lacking. Eosinophils are undisputably an important part of allergy and asthma pathogenesis (Rosenberg *et al.*, 2007), and the iNOS-CD95L pathway suppresses eosinophilia in inflammatory sites and in bone marrow when activated by DEC (Queto *et al.*, 2010a). Therefore, future studies of the benefits of α-GalCer in asthma or allergy models should consider whether suppressed eosinophilia and eosinopoiesis contribute to the observed benefit. The evidence that cysteinyl-leukotrienes antagonize the suppressive effects of α-GalCer raises the issue of whether part of their eosinophilia-promoting activity depends upon blocking the inhibitory effects of IFN-γ from various sources (including iNKT, which may be activated by self-antigens in the absence of α-GalCer; Molano and Porcelli, 2006). Another important finding of this study is that the effect of α-GalCer was converted from suppressive (anti-allergic) to stimulatory (pro-allergic) by interaction with dexamethasone. This agrees with previous studies on another activator of the iNOS-CD95L pathway (PGE_2) but is also consistent with the accumulating evidence that glucocorticoids, endogenous or synthetic, may interact with cytokines to enhance eosinophil production (Masid-de-Brito *et al.*, 2014a). It is unclear at present whether IFN-γ would interact with dexamethasone, or, alternatively, whether iNKT cells, in the presence of dexamethasone, would produce, instead of IFN-γ, another cytokine that enhances eosinophil maturation. This issue will certainly be addressed in future studies of this model.

Mediation of α-GalCer effects by IFN-γ. Some studies have reported beneficial effects in allergy and asthma models with α-GalCer, which were attributed to IFN-γ (Hachem *et al.*, 2005; Fujita *et al.*, 2009). Other studies showed exacerbation of experimental allergic disease models, not necessarily attributable to IFN-γ, which illustrates the risks of equating the effector cell (iNKT) with the mediator of one of its specific effects (IFN-γ). Our conclusion relies on the inability of α-GalCer to induce suppression of eosinopoiesis in bone marrow from IFN-γ-deficient mice, as well as on the ability of neutralizing anti-IFN-γ antibody to prevent this effect of α-GalCer in bone marrow from wild-type controls. Both approaches are reliable and, most importantly, independent of sensitivity of the assay, because by definition, IFN-γ-KO mice will have no IFN-γ, and in excess of neutralizing antibody, there should be full neutralization. Direct evidence that exogenous IFN-γ, as would be expected, duplicated the effects of α-GalCer, was also provided. Although some indication of the effective concentration range for exogenous IFN-γ was obtained, one should be cautious in assuming that α-GalCer-stimulated cells that reduce eosinopoiesis by a certain amount are secreting enough IFN-γ to achieve the same concentration as in the culture in which addition of exogenous IFN-γ elicited a comparable effect. Because iNKT cells may secrete IFN-γ in close proximity to their targets, they may achieve higher local concentrations than those reached in the total culture volume. Maintenance of a higher local concentration may also account for the ability of exogenous IFN-γ to boost the effect of α-GalCer. Of course, issues related to local concentration, diffusibility and turnover of IFN-γ would affect the interpretation of cytokine measurements, which is not the case with the intervention approaches (KO mice and neutralization) we have adopted.

Characterization of the regulatory/effector cell. One of the most important issues concerning this study is the nature of the regulatory/effector cell behind the effect of α-GalCer. Based upon previous studies (see the Introduction section), it is clear that α-GalCer is not recognized by conventional T lymphocytes, nor by type II NKT cells. Furthermore, iNKT cells are found in significant numbers in murine bone marrow and spleen and should be interchangeable between these sites. iNKT cells should be absent in bone marrow from CD1-deficient mice and, in wild-type mice, require not only ligand (α-GalCer) but co-stimulation as well, which was shown to be inadequate in CD28-deficient mice. We have provided experimental evidence for all of the above. Therefore, the criterion used was a combination of antigen specificity (α-GalCer), reliance on a specific type of antigen-presenting molecule (CD1d), dependence on CD28, residence in both bone marrow and spleen with comparable phenotype, expression of TCR (CD3+) production of IFN-γ. This view is supported by the evidence summarized in Figures 5–7. The IFN-γ-producing T-cell, as shown in co-culture experiments, is only found in spleen T-cells from CD1d+ wild-type donors. This approach allowed us to define unambiguously iNKT cell *function* on the basis of α-GalCer reactivity and restriction by CD1d, just as they can be defined by flow cytometry on the basis of *binding* of α-GalCer/CD1d fluorescent complexes (Matsuda *et al.*, 2008). We conclude that α-GalCer-activated iNKT cells are one of the regulatory lymphocyte subsets that can suppress eosinopoiesis in bone marrow, which differs from those previously described in an oral tolerization model (Xavier-Elsas *et al.*, 2013) because it is CD1d-restricted.

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Author contributions

M. I. G.-E. made the initial observations and carried out experiments with the help of T. Q., D. M.-B., B. M. V. and B. L. F. Q. C. provided indispensable reagents and animals, and insightful discussion of the data. P. X.-E. reviewed the experimental data and the literature on the subject and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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