Enforced RORγt expression in haematopoietic stem cells increases regulatory T cell number, which reduces immunoreactivity and attenuates hypersensitivity in vivo

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Summary

Background: The retinoic acid receptor-related orphan receptor γt (RORγt) is a key transcription factor involved in the generation of T-helper 17 (Th17) cells, which mediate tissue inflammation and autoimmunity. However, recent studies indicated that less than half of all RORγt+ Tαβ cells express IL-17, while the others are Foxp3+ Tαβ cells expressing IL-10. These observations raise questions regarding the role of RORγt in the early differentiation process of T cells from haematopoietic stem cells.

Methods: To examine the role of RORγt in T cell differentiation, mice were reconstituted with RORγt cDNA-transduced haematopoietic stem cells and the role of RORγt in T cell differentiation was studied in a mouse bone marrow transplantation model in vivo.

Results: While the number of Th17 cells increased with the reduction in Th1 cell number in transplanted mice, peripheral blood Foxp3+ Tαβ cell number also increased, which attenuated the severity of contact hypersensitivity on skin exposed to 2,4-dinitrofluorobenzene. The number of non-transduced Foxp3+ regulatory T cells (Treg cells) also increased in these mice.

Conclusion: These observations suggest that the enforced expression of RORγt in haematopoietic stem cells induces differentiation of Th17 cells and results in an increase in Foxp3+ Treg cell number to limit self-tissue damage. (Asian Pac J Allergy Immunol 2011;29:86-93)

Key words: RORγt, Th17, IL-17, Treg, Foxp3

Introduction

The T-helper 17 (Th17) lineage is a new subset of Th cells that predominantly produce interleukin-17 (IL-17) and play a key role in mediating tissue inflammation and autoimmunity ¹². Although IL-23 was initially considered to be the differentiation factor for Th17 cells ³⁴, recent studies indicate that transforming growth factor β (TGF-β) in combination with IL-6 induces Th17 differentiation from naïve CD4+ cells by regulating the chromatin remodelling of the IL-17 locus, whereas IL-23 is required for expansion and maintenance of Th17 cells ⁵⁶. However, TGF-β is an anti-inflammatory cytokine with pleiotropic functions, one of which is associated with the differentiation and maintenance of Foxp3+ regulatory T cells (Treg cells) ⁷⁸. TGF-β induces differentiation of Foxp3+ Treg cells from naïve CD4+ T cells and maintains their function. Paradoxically, TGF-β induces Foxp3+ Treg cells, whereas the addition of IL-6 inhibits Foxp3 expression followed by differentiation of Th17 cells, suggesting the existence of common precursor cells with the ability to differentiate into both Th17 and Treg cells in a cytokine-dependent manner ⁵.

RORγt, a member of the retinoic acid receptor-related orphan nuclear hormone receptor family, is considered to be a key transcription factor for Th17 cells because its expression is induced specifically during differentiation of Th17 cells by TGF-β and IL-6. Indeed, enforced expression of RORγt in naïve CD4+ T cells by retroviral vectors facilitated their differentiation of into cells expressing IL-17A and
IL-17F in the lamina propria\textsuperscript{10,11}. Furthermore, TGF-β alone was reported to be sufficient to induce expression of both RORγt and Foxp3, which are required for differentiation of Th17 and Treg cells, respectively\textsuperscript{12}. However, a recent study showed that not all of the cells expressing RORγt are Th17 cells, and more than half of the cells are Foxp3+ Tαβ cells that express IL-10 and function as Treg cells\textsuperscript{9}. These observations raise questions regarding the role of RORγt in the early differentiation process of T cells from haematopoietic stem cells (HSCs).

Therefore, we transduced murine HSCs with the RORγt gene using the retroviral vector GCDNsam and analysed the role of RORγt in T cell differentiation in a mouse bone marrow transplantation model \textit{in vivo}.

**Methods**

**Mice**

C57BL/6 (Ly5.1) mice were purchased from Nihon CLEA (Tokyo, Japan), and were maintained together with congenic mice (Ly5.2) at the University of Tsukuba Animal Research Center. All experiments using mice were approved by the Institutional Review Committee and performed in accordance with the guidelines of the University of Tsukuba.

**Retroviral vectors**

The full-length murine RORγt gene encoding a product of 495 amino acids was amplified by RT-PCR using cDNA prepared from the thymus of Ly5.1 mice as a template with the following primers:

\[ \text{Sense,} \quad \text{RORγt} + \text{cDNA} \]

The NotI–XhoI fragment containing the NGFR cDNA is inserted downstream of the internal ribosomal entry site (IRES). Sequences present in the vector are labelled as follows: MoMLV, Moloney murine leukaemia virus LTR; PCMV, PCC4 cell-passaged myeloproliferative sarcoma virus LTR; PBS, primer binding site; Ψ+, packaging signal. Expression of the RORγt gene was confirmed by RT-PCR using RORγt-transduced (R) and non-transduced Jurkat cells (wt) with or without reverse transcriptase (RT) (b). In contrast to non-transduced HSCs (c, left), more than 95% of the transduced cells expressed NGFR on the cell surface (c, right). Mice transplanted with non-transduced or RORγt-transduced HSCs survived for over 16 weeks and the chimaerism of transplanted cells was maintained at over 80% during the observation period (d).

**Figure 1.** Structure of the retroviral vector GCDN/RORγt/NGFR (a). The retroviral vector GCDNsam has the PCMV-derived LTR at the 3' LTR site with the intact splice donor (SD) and splice acceptor sequences. The NotI–XhoI fragment containing the NGFR cDNA is inserted downstream of the internal ribosomal entry site (IRES). Sequences present in the vector are labelled as follows: MoMLV, Moloney murine leukaemia virus LTR; PCMV, PCC4 cell-passaged myeloproliferative sarcoma virus LTR; PBS, primer binding site; Ψ+, packaging signal. Expression of the RORγt gene was confirmed by RT-PCR using RORγt-transduced (R) and non-transduced Jurkat cells (wt) with or without reverse transcriptase (RT) (b). In contrast to non-transduced HSCs (c, left), more than 95% of the transduced cells expressed NGFR on the cell surface (c, right). Mice transplanted with non-transduced or RORγt-transduced HSCs survived for over 16 weeks and the chimaerism of transplanted cells was maintained at over 80% during the observation period (d).
5'-GGCGCCATGAGACAAAAATTGAAGT-3', antisense, 5'-CACTTTGACAGCCCCCTCAG-3'. The PCR products were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA), and NotI–XhoI fragments containing the RORγt open reading frame digested from the vector were cloned into the corresponding sites of the retroviral vector pGCDNsam. A fragment of the truncated nerve growth factor receptor (tNGFR) cDNA was also inserted downstream of the RORγt gene together with the internal ribosome entry site (IRES) sequence to construct a vector expressing RORγt (DN/RORγt/NGFR) (Figure 1a). The vector was converted into the corresponding recombinant retrovirus by transduction into the packaging cell line 293gpg cells as reported previously. The virus titre was approximately 1×10^6 infectious units (IU)/mL on Jurkat cells.

**Cell culture**

The 293gpg cells were cultured in Dulbecco’s Modified Eagle’s Medium with 10% heat-inactivated foetal calf serum (FCS; Invitrogen), 2 mM l-glutamine, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulphate, 300 µg/mL G418, 2 µg/mL puromycin and 1 µg/mL tetracycline (D10/gpg). Jurkat cells and murine primary T cells were cultured in RPMI1640 with 10% FCS (HyClone, Logan, UT, USA), 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulphate (R10) and 50 µg/mL 2-mercaptoethanol (R10/2ME). All other culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Transduction into HSCs**

HSCs were obtained from Ly5.1 mouse bone marrow as c-KIT-positive/lineage marker-negative cells (KL cells) as described previously. KL cells (1–3×10^7) were cultured in StemPro34 (BRL, Rockville, MD, USA) in the presence of 50 ng/mL mouse stem cell factor (SCF; R&D Systems, Minneapolis, MN, USA), 100 ng/mL human thrombopoietin (100 ng/mL; Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL human Flt3-ligand (R&D Systems) in 24-well plates coated with fibronectin fragment CH296 (RetroNectin; Takara Bio, Tokyo, Japan) (Day 0). On days 1 and 2, aliquots of 50 µL of the concentrated virus supernatant were added to the cultures in which KL cells were pre-stimulated with the cytokines described above for 3 hours, and then half of the medium was replaced with fresh medium after 12 hours of incubation. On day 3, aliquots of 5×10^3 transduced cells were harvested and transplanted intravenously into Ly5.2 mice that were lethally irradiated with 550 cGy twice at 4-hour intervals.

**Cell surface analyses**

Mononuclear cells isolated from the peripheral blood of the recipient mice were stained with FITC-conjugated anti-Ly5.1 antibody and APC-conjugated anti-NGFR antibody (Miltenyi Biotech, Auburn, CA, USA) after blocking of Fc receptors with rat anti-mouse CD16/32 antibody (clone 93; Beckman Coulter, Fullerton, CA, USA). PE-conjugated antibodies for B220 (B cells), Gr-1 (granulocytes), and CD4 (T cells), and Cy5-conjugated antibodies for Mac-1 (myeloid) and CD8 (T cells) were used as other lineage markers. These antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA) and multicolour analyses were performed using a FACSCalibur flow cytometer (BD Pharmingen).

**Flow cytometric analyses of intracellular cytokine production**

Splenic CD4^+ T cells were isolated from the spleens of transplanted mice by depleting cells with CD8α, B220, Mac-1, Ter-119 and Gr-1 using magnetic beads (Dynabeads MyOne; Dynal Biotech, Oslo, Norway). Purified CD4^+ T cells suspended with R-10/2ME at 1×10^6 cells/mL were stimulated with PMA (50 ng/mL) plus ionomycin (500 ng/mL) and GolgiStop (BD Biosciences) for 4 hours and stained with FITC-conjugated anti-mouse CD4 antibody. Cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) followed by staining with PE-conjugated anti-mouse IL-17 antibody (BD Biosciences). For analysis of intracellular IL-5, IFN-γ and Foxp3 production, cells were suspended with R-10/2ME with PMA (50 ng/mL) plus ionomycin (500 ng/mL) and GolgiStop (BD Biosciences) for 4 hours and stained with APC-conjugated anti-mouse CD4 antibody. After fixation and permeabilization, cells were stained with FITC-conjugated anti-mouse IFN-γ, PE-conjugated anti-IL5, and PE-conjugated anti-Foxp3 antibodies (eBioscience, San Diego, CA, USA).

**Induction of contact hypersensitivity (CHS)**

CHS was induced using 2,4-dinitrofluorobenzene (DNFB) as described previously. Briefly, 50 µL of 0.5% DNFB diluted with acetone/olive oil was painted onto the shaved abdomen of the mice and 5 µL was applied to each footpad on days 0 and 1. On day 5, the mice were repainted with 20 µL of 0.3%
Figure 2. FACS analyses of the peripheral blood of transplanted mice at 16 weeks post-transplantation. Cells of each lineage, such as B cells (B220), granulocytes/macrophages (Gr-1/Mac1) or T cells (CD4/CD8), in mice transplanted with RORγt-transduced HSCs were reconstituted (lower panel in a) and the populations were comparable to those of control mice (upper panel in a). The frequency of CD4+ cells expressing IL-17 intracellularly was determined by FACS (b). The frequency of these cells in RORγt-BMT mice (right) was higher than that in controls (left) (**, p < 0.0001). The results shown are representative data from three independent experiments. The frequency of CD4+ showing intracellular Foxp3 expression was measured by FACS (c). The frequency of these cells in RORγt-BMT mice (right) was higher than that in controls (left) (*, p < 0.001). Expression of Foxp3 and RORγt in CD4+ cells in RORγt-BMT mice was analyzed by FACS (d). Intracellular cytokine staining for CD4+ T cells in control and RORγt-BMT mice. CD4+ T cells obtained from mice were cultured in medium (e, upper) supplemented with anti-IL-4 antibody plus IL-12 (e, middle: Th1 differentiation condition) or anti-IL-12 antibody plus IL-4 (e, lower: Th2 differentiation condition), and then analyzed by FACS for intracellular expression of IFN-γ and IL-5. *, p < 0.01. The results shown are representative data from three independent experiments.
DNFB on both sides of the right ear. As a control, the left ear was painted with 20 μL of acetone/olive oil only. The thickness of both ears was measured on days 1 to 6 and the swelling index was calculated as \([{(Tx–T0) \text{ right ear}}]–[{(Tx–T0) \text{ left ear}}]\), where \(Tx\) is the thickness of the ear on day \(X\) (mm). In CHS experiments after donor lymphocyte infusion (DLI), \(2\times10^6\) splenic CD4+ T cells from transplanted mice were adoptively transferred into recipient mice 6 days before induction of CHS.

**Histological analyses**

Ears were fixed with 10% formalin buffered with 0.01 M phosphate buffer (pH 7.2) and embedded in paraffin. The fixed ears were sliced into 3 μm sections and stained with haematoxylin and cosin for histological examination.

**Statistical analysis**

Statistical analyses were performed using the Mann-Whitney U test, and \(P<0.05\) was considered statistically significant.

**Results**

**Potential HSCs expressing RORγt to reconstitute bone marrow haematopoiesis**

Mouse RORγt cDNA was inserted into the retroviral vector pGCDNsam/ires-NGFR, referred to as DN/RORγt/NGFR (Figure 1a), and converted into the corresponding recombinant retroviruses by transduction into 293Gpg cells. The virus titre was approximately \(1\times10^7\) IU/mL as measured in Jurkat cells and the RORγt expression in the transduced cells was assessed by RT-PCR (Figure 1b). Following transduction of KL cells isolated from the bone marrow of Ly5.1 mice as HSCs with DN/RORγt/NGFR, more than 95% of the cells expressed NGFR on the surface membranes, suggesting that the RORγt cDNA was successfully transduced into murine HSCs (Figure 1c).

To assess the effects of RORγt on proliferation and differentiation of HSCs \(in vitro\), aliquots of \(5\times10^3\) of RORγt-transduced cells were transplanted intravenously into lethally irradiated mice. Unexpectedly, HSCs expressing RORγt reconstituted bone marrow haematopoiesis of the transplanted mice (RORγt-BMT) and retained high donor cell chimaerism for more than 16 weeks post-transplantation (Figure 1d). While the RORγt-transduced cells differentiated into three lineages \(i.e.,\) B cells, granulocytes/monocytes, and T cells) \(in vivo\) similar to untreated bone marrow cells transplanted into lethally irradiated mice (control-BMT) (Figure 2a), enforced expression of RORγt at the HSC level increased the population of Th17 cells showing intracellular expression of IL-17 compared with control mice (Figure 2b). However, the mice did not show any autoimmune disorders during the observation period of over 16 weeks, whereas mice transplanted with HSCs expressing IL-17 (IL17-BMT) died due to bone marrow failure within 14 days after transplantation (data not shown).

**Increased Treg cells in RORγt-BMT mice**

To account for the discrepancy between the phenotypes of RORγt-BMT and IL17-BMT mice, we next analysed other subsets of Th cells, such as Th1, Th2 and Treg, in RORγt-BMT mice. Consistent with the results reported previously 11, less naïve CD4+ cells in the spleens of RORγt-BMT mice differentiated into Th1 cells that showed intracellular IFNγ expression (Figure 2e, upper), while retaining the potential to differentiate into Th1 cells in the presence of IL-12 or Th2 cells in the presence of IL-4 (Figure 2e, middle and lower). In contrast, larger numbers of Treg cells with intracellular expression of Foxp3 were observed compared with wild-type controls (Figure 2c), suggesting that the enforced expression of RORγt at the HSC level induced differentiation of naïve CD4+ cells into Treg cells as well as Th17 cells in these mice.

**Suppression of immune responses by increased Treg cell number in RORγt-BMT mice**

To assess whether CD4+ cells expressing Foxp3 functioned substantially as Treg cells \(in vivo\), RORγt-BMT mice were challenged in the CHS assay using DNFB, which was used to evaluate delayed-type hypersensitivity (DTH) as a mouse model of contact dermatitis. DNFB was painted on the shaved abdomen and footpads of the mice on day 0 and again on the ears on day 5. The thickness of the swollen ears after the second challenge was measured as an index of CHS. After the DNFB challenge, the ears of control mice were swollen, which reached the maximum degree of swelling at 72 hours. In contrast, the ears of RORγt-BMT mice remained almost unaffected, and the swelling index was at most double that of the untreated control during the observation period (Figure 3, upper).

To examine whether the immune suppression was transferable to other mice by adoptive transfer of CD4+ T cells from RORγt-BMT mice, B6 mice were infused with splenic CD4+ T cells from either control or RORγt-BMT mice, followed by challenge...
with CHS (Figure 3, lower). Similar to RORγt-BMT mice, those adoptively transferred with CD4⁺ T cells from RORγt-BMT mice showed attenuated immune reactions against DNFB compared with controls. The attenuation of the immune response against DNFB was confirmed by histological analysis. Dermal oedema, infiltration of mononuclear cells, and vascular enlargement were observed in samples from control mice (Figure 4, upper), while these characteristic features, including infiltration of mononuclear cells into the lesion, were attenuated in samples from mice adoptively transferred with CD4⁺ T cells from RORγt-BMT mice (Figure 4, lower).

**Discussion**

A series of *in vivo* experiments in which HSCs expressing RORγt were transplanted into lethally irradiated mice were performed to evaluate the effects of RORγt on T cell development. In contrast to mice transplanted with HSCs expressing IL-17, which died within 14 days after transplantation, those with reconstituted bone marrow haematopoiesis survived without any autoimmune disorders during the observation period of over 16 weeks even though they had an increased number of Th17 cells showing intracellular expression of IL-17. Interestingly, the mice also had increased numbers of Treg cells expressing Foxp3 and an attenuated immune response against DNFB when challenged in the CHS assay. Furthermore, the observed immune suppression was transferable by adoptive cell transfer of CD4⁺ T cells from these mice into suitable recipients. As the fraction of Foxp3⁺ CD4⁺ T cells negative for RORγt and those positive for RORγt was increased (Figure 2d), the observed immune suppression may not be due to a direct effect of RORγt but to a reaction against Th17 cells, the numbers of which were increased by RORγt to maintain immune homeostasis.

The results of the present study raise several questions regarding the relationship between
Th17 and Treg cells. First, if RORγt is the key transcription factor in differentiation of Th17 cell, do HSCs transduced with the RORγt gene differentiate into Treg cells in vivo? Ivanov et al.11 demonstrated that RORγt was sufficient to induce expression of IL-17 in highly purified naïve CD4+ T cells retrovirally transduced with the RORγt gene in the absence of exogenous cytokines in vitro. However, not all the transduced cells differentiated into Th17 cells and approximately half did not express IL-17 in vitro, although it was unclear whether cells that did not express IL-17 were still naïve CD4+ T cells11. Furthermore, they also reported that RORγt+ Foxp3+ CD4+ T cells appeared when naïve CD4+ T cells were cultured in the presence of TGF-β16. Whether naïve CD4+ T cells co-expressing RORγt and Foxp3 differentiate into Th17 or Treg cells is dependent on the concentration of TGF-β in the local environment, with low and high concentrations favouring Th17 and Treg cell differentiation, respectively. In particular, expression of Foxp3 induced by TGF-β strongly inhibits the functions of RORγt, and therefore cells of the lamina propria T cells co-expressing the transcription factors produce less IL-17 than those expressing RORγt alone16. Consistent with the results of these in vitro experiments, we also observed the presence of Treg cells expressing RORγt driven by the retroviral vector. Therefore, RORγt may be necessary but not sufficient for generation of Th17 cells.

The second question is why the enforced expression of RORγt at the HSC level led to the increased number of Treg cells in the transplanted mice. As RORγt showed less ability to promote differentiation of naïve CD4+ T cells into Treg cells, the increased number of Treg cells observed in these mice should be due to causes other than RORγt, such as a rebound against the increase in Th17 cell number by RORγt, similar to the phenomenon known as self-control of Th1 cells in which IFN-γ-producing Th1 cells also produce IL-10 to maintain effective antimicrobial immune responses, while limiting self-tissue damage17. If this were the case, naïve CD4+ T cells derived from the transduced HSCs migrating into the recipient’s thymus would preferentially differentiate into Th17 cells under the influence of RORγt, which may be crucial for the host response. Similarly mice transplanted with HSCs expressing IL-17, and Treg cells would proliferate in response to the increase in number of Th17 cells because they proliferated regardless of RORγt expression (Figure 2d).

The third question with regard to the immune state of the transplanted mice concerns the fact that the mice showed little immune response against DNFB; they were apparently normal and survived without any immune disorders for over 16 weeks.

Figure 4. Histopathological characteristics of the swollen ears. At 24 hours after painting with DNFB. Sections of the swollen ears were stained with haematoxylin and eosin (magnification: ×100 and ×400). The characteristic features of inflammation, such as infiltration of mononuclear cells, dermal oedema and vascular enlargement, were observed in the sections of mice transplanted with CD4+ T cells from controls (upper panel), where as these features were markedly attenuated in samples from mice infused with CD4+ T cells from RORγt-BMT mice (lower).
post-transplantation. These observations suggest that the mice retained an adequate level of immunity against infection. Although further studies are required to assess the mechanism of immune protection against infectious agents and cancer cells, etc., the results of the present study suggested a closed interaction between Th17 and Treg cells. This has implications for suppression of Th17 cell functions, and may facilitate the development of new approaches for autoimmune diseases caused by Th17 cells.

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