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Identification of a unique double-negative regulatory T-cell population

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Summary

Regulatory T (Treg) cells represent one of the main mechanisms of regulating self-reactive immune cells. Treg cells are thought to play a role in down-regulating immune responses to self or allogeneic antigens in the periphery. Although the function of Treg cells has been demonstrated in many experimental settings, the precise mechanisms and antigen specificity often remain unclear. In a hepatitis B e antigen–T-cell receptor (HBeAg-TCR) double transgenic mouse model, we observed a phenotypically unique (TCR^+ $CD4^-/CD8^-$ $CD25^{+/-}$ $GITR^{high}$ $PD\text{-}1^{high}$ $FoxP3^-$) HBeAg-specific population that demonstrates immune regulatory function. This HBeAg-specific double-negative regulatory cell population proliferates vigorously *in vitro*, in contrast to any other known regulatory population, in an interleukin-2-independent manner.

Keywords: double-negative regulatory T cells; hepatitis B e antigen; hepatitis B virus infection; tolerance

Introduction

The primary function of the immune system is to protect the self from pathogens. A highly effective and dynamic cellular network has evolved to signal the presence of pathogens and initiate a response that is specific for the invading pathogen while maintaining tolerance to self. Distinguishing between self and non-self is a fundamental property of the immune system and is accomplished by a variety of mechanisms. A function of regulatory T (Treg) cells is to prevent self-reactive immune cells from damaging self. The Treg cells, particularly $CD4^+$ $CD25^+$ conventional Treg (cTreg) cells, are thought to play a role in down-regulating immune responses to self or allogeneic antigens in the periphery.[1–4] Although the function of Treg cells has been shown in a number of *in vivo* models of autoimmunity and transplantation, the precise mechanism and antigen specificity often remains unclear.[5]

In 1971 it was first suggested that Treg cells had the ability to transfer antigen-specific tolerance to naive animals.[6] Even though a role for regulatory cells during an immune response was widely accepted, the existence of Treg cells was controversial until a specific surface marker was described by Sakaguchi *et al.*[7] Conventional Treg cells constitutively express a variety of cell markers, such as CD4, CD25, $CD45RB^{low}$, CD62 ligand (CD62L), CD103, as well as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR).[7–14] Although cTreg cells express $CD4^+$ $CD25^+$, CD25 is not a specific marker for cTreg cells. Other cell markers (i.e. CTLA-4, GITR and CD103) are also not exclusive markers for Treg cells, because in most cases they are up-regulated on effector T cells upon activation. The transcription factor Forkhead box P3 (FoxP3) is predominantly expressed on Treg cells and appears to be expressed at the thymic $CD4^+/CD8^+$ stage.[15–18] In contrast to the cell surface markers mentioned above, FoxP3 is not observed in non-Treg cells upon activation or differentiation into T helper type 1/type 2 cells, nor in natural killer T cells. Furthermore, retroviral gene transfer of FoxP3 converts naive T cells to a regulatory T-cell phenotype similar to cTreg cells.[17] However, some Treg cell populations (i.e. Tr1 and Th3) do not express FoxP3. Taken together, FoxP3 is also not an

Abbreviations: APC, antigen-presenting cell; CD62L, CD62 ligand; CTLA-4, cytotoxic T-lymphocyte antigen-4; DC, dendritic cell; DN, double-negative; FoxP3, Forkhead box P3; GITR, glucocorticoid-induced tumour necrosis factor receptor-related protein ; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IL-2, interleukin-2; IFN, interferon; mAb, monoclonal antibody; MΦ, macrophage; TCR, T-cell receptor; Tg, transgenic; Treg cell, regulatory T cell.

exclusive marker for, but rather is a specific control gene for the development and function of cTreg cells. To determine a possible therapeutic application for the use of Treg cells, it is extremely important to know the detailed phenotype of a Treg cell population.

Recently, Zhang *et al.*[19] and others reported the presence of MHC class I restricted $CD4^-$ $CD8^-$ double-negative (DN) T cells with a unique phenotype for a Treg cell population (i.e. TCR^+ $CD4^-$ $CD8^-$ $CD25^+$ $CD28^-$). The DN T cells comprise 1–3% of peripheral T lymphocytes in the mouse.[20–22] The DN Treg cells isolated from mice that have permanently accepted allografts or xenografts can specifically suppress and kill syngeneic anti-donor $CD4^+$ and $CD8^+$ T cells *in vitro*.[20,23–25] Upon expansion *in vitro* with allogeneic donor lymphocytes, the DN Treg cells can specifically suppress proliferation of syngeneic $CD4^+$ and $CD8^+$ T cells *in vitro* and prolong donor-specific allogeneic skin graft survival when infused into syngeneic naive mice. Recent studies suggest that this immune suppressive function is mediated by suppression of antigen-presenting cell (APC) function.[26,27] Also, adoptively transferred DN Treg cells augment recipient Treg cell accumulation and enhance long-term cardiac allograft survival.[28]

We have produced a number of T-cell receptor (TCR) transgenic (Tg) mice specific for the hepatitis B core (HBcAg) and precore (HBeAg) antigens, which share significant amino acid homology.[29] When the TCR-Tg lineage 7/16-5 is bred with Tg mice that secrete HBeAg in the serum, the resulting double-Tg (dbl-Tg) mice demonstrate T-cell tolerance.[29,30] The degree and nature of tolerance is dependent on the nature of the hepatitis B virus (HBV) antigen. For example, in HBeAg × 7/16-5 dbl-Tg mice, *in vitro* interleukin-2 (IL-2) production is significantly reduced compared with 7/16-5 single TCR-Tg mice and the dbl-Tg mice do not spontaneously produce anti-HBe antibodies *in vivo*. In contrast, in 7/16-5 TCR-Tg mice bred with HBcAg-Tg mice, which express the HBcAg intracellularly in the liver, the resulting dbl-Tg mice undergo spontaneous anti-HBc seroconversion between 4 and 6 weeks of age and are significantly less tolerant at the T-cell level than HBeAg-expressing dbl-Tg mice.[30] Furthermore, in triple-Tg mice expressing the 7/16-5 TCR, secreted HBeAg, and intracellular HBcAg spontaneous anti-HBc seroconversion is suppressed. These studies demonstrated that the secreted HBeAg functions as a tolerogen in a TCR-Tg system in which the intracellular HBcAg is an immunogen and the presence of HBeAg as a serum protein can regulate the immune response to the HBcAg.[30,31] Because HBeAg-specific tolerance is not mediated by clonal deletion of 7/16-5 TCR-Tg T cells in HBeAg × 7/16-5 dbl-Tg mice,[30] we explored the possibility that HBeAg-specific Treg cells may be present.

In this study, we demonstrate an HBeAg-specific Treg cell population in the TCR × HBeAg-dbl-Tg mouse model that possesses a unique DN phenotype (i.e. TCR^+ $CD4^-$ $CD8^-$ $CD25^{+/-}$ $GITR^{high}$ PD-1^{high} $FoxP3^-$). Most strikingly, these HBeAg-specific DN T cells exhibit extremely efficient regulatory function compared with other Treg cells *in vitro*. As a result of its vigorous proliferation *in vitro*, suppressive effects and unique phenotype, the HBeAg-specific DN T-cell population described herein may represent a distinct Treg cell subset.

Materials and methods

Transgenic mice

The 7/16-5 transgenic TCR ($V\beta11^+$-$V\alpha5^+$) is specific for residues 120–140 of HBc/HBeAgs, is restricted by the $I\text{-}A^b$ MHC class II molecule, is expressed on 53% of $CD4^+$ T cells,[29,30] and is uniquely expressed on a high proportion of $CD8^+$ T cells (unpublished data). Transgenic mice engineered to express relatively high levels of HBeAg in the serum (4–10 μg/ml) and HBcAg in the liver (0·2–2 μg/mg protein) through the use of the liver-specific major urinary protein promoter have been described.[32,33] All Tg mice were bred onto a C57BL/10 background. The mice designated as HBcAg or HBeAg-Tg were hemizygous for the transgenes, as were the 7/16-5 TCR-Tg mice. Ovalbumin-specific OT-II Tg mice, MHC class I knockout (KO) mice, and TCR α-chain KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal care was performed according to the National Institutes of Health standards as set forth in the Guide for the Care and Use of Laboratory Animals.

Recombinant proteins and synthetic peptides

Recombinant HBcAg of the *ayw* subtype was produced in *Escherichia coli* and purified as described elsewhere.[34] A recombinant HBeAg corresponding in sequence to serum-derived HBeAg encompassing the 10 precore amino acids remaining after cleavage of the precursor and residues 1–149 of HBcAg was produced as described previously.[34] The presence of the 10 precore amino acids prevents particle assembly, and HBeAg is recognized efficiently by HBeAg-specific monoclonal antibodies (mAbs) but displays little HBc antigenicity. Peptides were synthesized by the simultaneous multiple peptide synthesis method.[35] The HBe/HBcAg-derived synthetic peptide representing the recognition site for the 7/16-5 TCR was designated from the N-terminus of HBcAg: 120–140, VSFGVWIRTPPAYRPPNAPIL. OVA (323–339) peptides were purchased from Anaspec (Fremont, CA).

Antibodies and reagents

The following antibodies were all purchased from eBioscience (San Diego, CA): Fluorescence- or biotin-labelled

anti-CD4, anti-CD8, anti-Vβ11, anti-CD25, anti-CD11c, anti-CD11b, anti-CD49b, anti-B220, anti-GITR, anti-FAS, anti-FASL, anti-IL-15R, anti-CTLA-4, anti-PD-1 and Foxp3 intracellular staining. Cell separation apparatus and reagents used were purchased from Miltenyi Biotech (Auburn, CA).

Cell preparation

Five- to 10-week-old HBeAg × 7/16-5 TCR dbl-Tg mice were used as a DN T-cell source. Spleen cells were cultured in Dulbecco's modified Eagle's medium supplemented with nutrients, 4% fetal calf serum, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. Single cell suspensions from spleen or cultured cells were labelled with biotinylated anti-CD4, anti-CD8, anti-B220, anti-CD11c, anti-CD11b and Gr1 for negative depletion. Cells were then magnetized with streptavidin-Microbeads (Miltenyi Biotech), and passed through the LS column to collect the flow through as DN T cells. For cultured cells, dead cell removal was performed before negative depletion using the dead cell removal kit from Miltenyi Biotech. Briefly, 4-day-cultured splenocytes from HBeAg × 7/16-5 dbl-Tg were harvested and labelled with propidium iodide, and subsequently mixed with anti-propidium iodide-Microbeads. Propidium iodide-labelled dead cells were subsequently removed by magnetic selection. B cells were purified by positive selection with B220-microbeads. For DN progenitor depletion, cells were positively selected by biotinylated anti-CD4, anti-CD8, anti-B220, anti-CD11c, anti-CD11b and anti-Gr1 and were subsequently magnetized with streptavidin-Microbeads to exclude DN T-cell progenitors.

Cell culture

For antigen-specific stimulation, 4×10^5 splenocytes from 7/16-5 TCR Tg mice or HBeAg × 7/16-5 dbl-Tg mice were cultured in 4% fetal calf serum supplemented with Dulbecco's modified Eagle's medium in the presence of truncated $HBcAg_{149}$, or the HBcAg-derived peptide p120–140 at concentrations of 0·2–2 μg/ml. Cells were placed in flat-bottom 96-well-plates for 1, 2, 3 or 4 days for further analysis. At day 2 and day 4, supernatants were collected for cytokine analysis. For the DN T-cell suppression assay, 4×10^5/well of naive 7/16-5 TCR-Tg splenocytes were used as target cells and 4-day cultured HBeAg-specific DN T cells were separated by negative enrichment as described above, and were added to the culture at given numbers.

Flow cytometry analysis

To analyse T-cell activation, antigen-specific T cells (V$\beta11^+$ CD4 T cells) were stained for CD25 as well as CD69 at given time points. For the surface staining, cells were incubated in 3% fetal bovine serum in PBS containing 10 μg/ml 2.4G2 to block FcR binding followed by the addition of fluorochrome-conjugated antibodies. All fluorochrome-conjugated antibodies were obtained from eBioscience as described above. For the detection of CTLA-4, we performed intracellular staining and surface staining because of the nature of CTLA-4 expression. Foxp3 intracellular staining was performed using a commercially available kit from eBioscience. Flow cytometry was performed on an LSRII flow cytometer (Becton Dickinson, CA) available through the CCMI at the Salk Institute (La Jolla, San Diego, CA). Data were analysed using FlowJo software (Tree Star, Ashland, OR).

In vitro *cytokine analysis and proliferation assay*

Spleen cells from either unprimed or primed TCR-Tg, TCR × antigen dbl-Tg or wild-type mice were cultured (4×10^5/well) with various concentrations of a series of antigens. Culture supernatants were harvested at 48 hr for IL-2 determination and at 96 hr for interferon-γ (IFN-γ) determination. Cytokines were measured by two-site ELISA with pairs of cytokine-specific mAbs. One unlabelled mAb was adsorbed to the microtitre plate well and used as a capture antibody, and the other labelled mAb served as the probe. For the multicytokine assay, we have used Multiflex Biomarker Immunoassay (Millipore, Billerica, MA). To determine T-cell proliferation, TCR-Tg spleen T cells were labelled *in vitro* with the intracellular dye carboxyfluorescein succinimidyl ester (CFSE) by using the Vybrant CFSE SE tracer kit (Molecular Probes, Eugene, OR.). Carboxyfluorescein succinimidyl ester (CFSE; 0·5 mM) was added to the cell suspensions, and the mixture was incubated for 10 min at 37°. The labelling reaction was stopped by repetitive washing with ice-cold RPMI-1640 medium containing 10% fetal calf serum. Labelled cells (4×10^6/ml) were cultured with various concentrations of antigen for 2, 3 or 4 days. Cultured cells were harvested, washed and labelled with anti-TCR Vβ11 antibodies. The number of cell divisions was determined by dilution of the intracellular dye CFSE in TCR V$\beta11^+$ gated T cells by flow cytometry. In some experiments, Bodipy-FL (Invitrogen, Carlsbad, CA) was used for the cell proliferation assay instead of CFSE.

Results

HBeAg is tolerogenic in HBeAg × 7/16-5 TCR-dbl-Tg mice

To measure the effect of HBeAg on effector T-cell activation *in vitro*, we cultured splenocytes from naive 7/16-5 TCR-Tg and 7/16-5 × HBeAg dbl-Tg mice in the presence of 1 μg/ml of the HBeAg-derived peptide p120–140 and

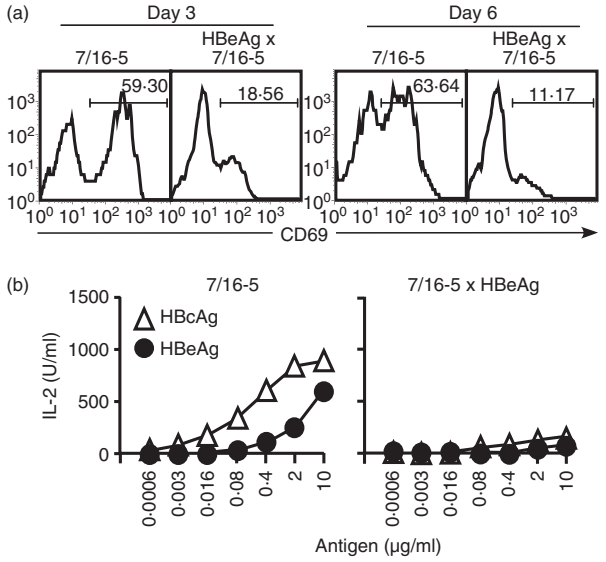

Figure 1. Evidence of T-cell tolerance in *in vitro* cultures of splenocytes from naive hepatitis B virus e antigen (HBeAg) × 7/16-5 T-cell receptor (TCR) double transgenic (dbl-Tg) mice. Splenocytes from 7/16-5 TCR-Tg mice and HBeAg × 7/16-5 dbl-Tg mice were collected and (a) 4×10^5 splenocytes/well were cultured in the presence of 1 μg/ml of the HBeAg-derived peptide 120–140 and CD69 expression was analysed by FACS at day 3 and day 6. (b) Supernatants of pooled splenocytes from 7/16-5-Tg and HBeAg × 7/16-5 dbl-Tg mice cultured with various concentrations of the HBc/HBe antigens were collected at day 2 and interleukin-2 (IL-2) was measured by ELISA. The data shown are representative of three independent experiments.

analysed CD69 expression by FACS. There was a dramatic difference between T-cell activation in 7/16-5 TCR-Tg mice versus 7/16-5 × HBeAg dbl-Tg mice. A high percentage (59·30%) of T cells derived from 7/16-5 TCR-Tg mice expressed CD69 after 3 days in culture, which was sustained after 6 days. In contrast, only 18·56% of T cells derived from HBeAg × 7/16-5 dbl-Tg mice expressed CD69 at 3 days of culture and the expression of CD69 remained low (11·17%) at day 6 (Fig. 1a). Similarly, *in vitro* IL-2 production by 7/16-5 × HBeAg dbl-Tg splenic T cells cultured with either HBeAg or HBcAg was significantly reduced compared with T cells from 7/16-5 single TCR-Tg mice (Fig. 1b). It is notable that the tolerance exhibited in 7/16-5 × HBeAg dbl-Tg mice is not the result of clonal deletion of 7/16-5 TCR-Tg T cells either in the thymus or in the spleen (data not shown,[30]).

Expansion of an HBeAg-specific TCR$^+$, CD4$^-$/CD8$^-$ (DN) population *in vitro*

CFSE-labelled splenocytes from 7/16-5 TCR-Tg and 7/16-5 × HBeAg dbl-Tg mice and 7/16-5 × HBcAg dbl-Tg mice were cultured *in vitro* in the presence of HBeAg peptide p120–140 to examine CD4$^+$ and CD8$^+$ effector cell proliferation. As shown in Fig. 2, after 4 days in culture both CD4$^+$ and CD8$^+$ Vβ11$^+$ TCR-Tg T cells from 7/16-5 mice and 7/16-5 × HBc dbl-Tg mice proliferated. In contrast, the vast majority of the proliferating Vβ11$^+$ TCR-Tg T cells from 7/16-5 × HBeAg dbl-Tg mice were neither CD4$^+$ cells nor CD8$^+$ cells (Fig. 2, middle column) and represented a DN, Vβ11$^+$ population. Interestingly, this predominant Vβ11$^+$/DN T-cell population can be expanded only from 7/16-5 TCR-Tg mice also expressing the HBeAg, but not from HBcAg-expressing 7/16-5 TCR-dbl-Tg mice or single 7/16-5 TCR-Tg mice (Fig. 2). This indicates the absolute requirement for the presence of HBeAg *in vivo* for the development of HBeAg-specific DN T cells in the TCR-Tg model. To determine if the proliferation of DN T cells was MHC class II restricted, we added anti-MHC class II and anti-MHC class I antibodies in the culture compared with an isotype control. Anti-MHC class II antibodies (anti-I-A^b) completely inhibit the proliferation of DN T cells, whereas anti-MHC class I antibodies had no effect (data not shown). Therefore, DN T cells proliferate in an MHC class II-restricted manner.

DN T cells are a phenotypically distinct population

We next examined the cell surface markers of DN T cells. Cells were harvested from a 4-day spleen culture of 7/16-5 × HBeAg dbl-Tg mice, then negatively depleted of CD4$^+$, CD8$^+$, B220$^+$, CD11c$^+$ and Gr-1$^+$ cells. The majority of cells were harvested as flow through, and these cells were collected as purified DN T cells. As expected from the FACS analysis, approximately 50% of total cells harvested were DN T cells. The subsequent FACS analysis revealed that the Vβ11$^+$ DN T cells were Thy-1.2$^+$ (data not shown), B220$^-$, PD-1$^+$, GITR^{high} and CD25^{low} (Fig. 3a), and CD49b (DX-5)$^-$ (data not shown). Interestingly, the CD25 expression on DN T cells was very low, but PD-1, which is known as an inhibitory co-stimulatory molecule, was highly expressed (51·49%). Therefore, autocrine consumption of IL-2 in the culture environment may not be the mechanism driving the proliferation of DN T cells. A DN Treg cell phenotype has been reported previously;[19,21,36] however, the previously reported DN Treg cells highly expressed CD25 and produced IL-2 and IFN-γ, whereas the HBeAg-specific, Vβ11$^+$, DN T cells have low expression of CD25 and no detectable IL-2 and IFN-γ production after *in vitro* activation (see below and Fig. 4). In addition to this unique phenotype, HBeAg-specific DN T cells proliferate *in vitro* very efficiently compared with the anergic status of most Treg cells *in vitro* (see Fig. 2).

CTLA-4 is often expressed by cTreg cells and may play an important role in the suppressive function of Treg cells.[14,37–39] However, HBeAg-specific Vβ11$^+$ DN T cells do not express CTLA-4 (data not shown). Conventional Treg cells also express FoxP3 in the cytoplasm, which can

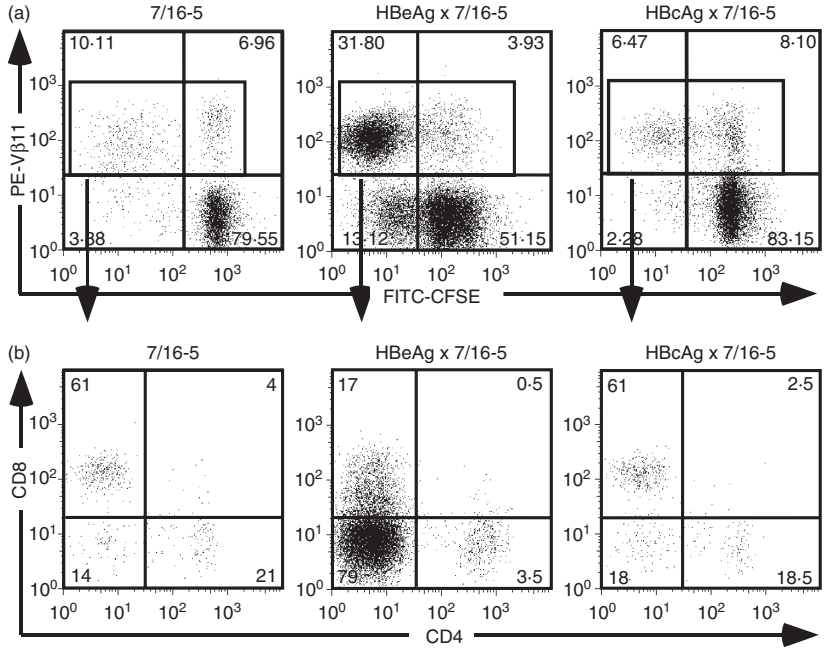

Figure 2. Efficient proliferation of hepatitis B virus e antigen (HBeAg) -specific double-negative (DN) T cells. (a) Splenocytes from 7/16-5 transgenic (Tg), HBeAg × 7/16-5 double (dbl) -Tg and HBcAg × 7/16-5 dbl-Tg mice were harvested and labelled with CFSE then cultured in the presence of 1 μg/ml of HBeAg peptide 120–140 for 4 days. (b) Proliferating T-cell receptor (TCR) -Tg ($V\beta11^+$) T cells were gated and further analysed for CD4 and CD8 expression. Data shown are representative of more than three independent experiments.

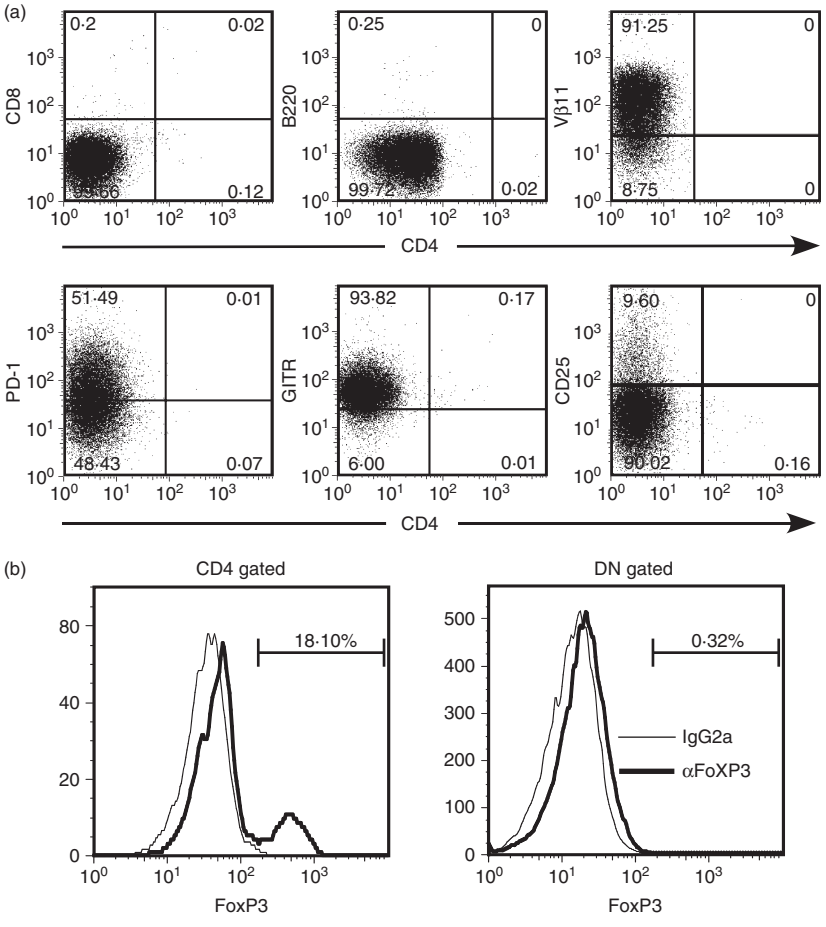

Figure 3. Surface phenotype of hepatitis B virus e antigen (HBeAg) -specific double-negative (DN) T cells. (a) After 4 days of culture with 1 μg/ml HBeAg peptide 120–140, splenocytes from HBeAg × 7/16-5 dbl-Tg mice were collected and incubated with biotinylated anti-CD4, -CD8, -B220, -CD11c, -CD11b and -Gr-1 followed by incubation with streptavidin-Microbeads (Miltenyi Biotec) for negative depletion. The indicated cell surface markers were analysed on the purified HBeAg-specific DN T-cell population by FACS, and revealed > 98% purity. (b) Intracellular staining was performed for FoxP3 detection. CD4 cells and DN cells were gated respectively and FoxP3-positive cells were compared with the isotype control.

represent a specific marker for cTreg cells. FoxP3 can also be involved in the generation of Treg cells as shown in an FoxP3 expression model *in vitro*.[17] To investigate the expression of FoxP3 in DN cells, intracellular FACS staining was performed, however, no detectable FoxP3 was observed in HBeAg-specific, $V\beta11^+$ DN T cells (Fig. 3b).

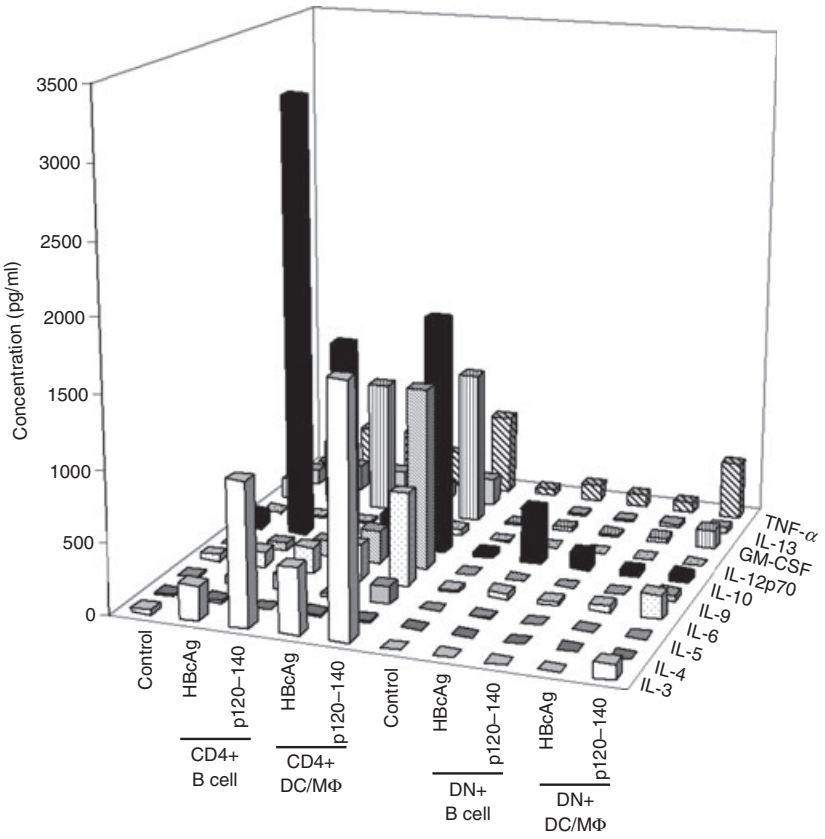

Figure 4. Cytokine profile of hepatitis B virus e antigen (HBeAg) -specific double-negative (DN) T cells. HBeAg-specific DN T-cell progenitor cells were harvested from HBeAg × 7/16-5 double transgenic (dbl-Tg) mice as described in the Materials and methods. HBeAg-specific DN T-cell progenitor cells were cultured with B cells or dendritic cell/ macrophages (DC/MΦ) in the presence of HBcAg or p120–140 for 4 days. Supernatants were analysed for the indicated cytokines using Multiflex Biomarker Immunoassay (Millipore, MA). HBeAg-specific $CD4^+$ T cells from 7/16-5-Tg mice were cultured identically for comparison.

Cytokine profile of HBeAg-specific DN cells at day 4 of culture

Because cytokines other than IL-2 may be involved in the proliferation of T cells, we have examined the cytokine production profile of *in vitro* cultured HBeAg-specific DN T cells, using the Multiflex Biomarker Immunoassay (Fig. 4). After 4 days of culture with p120–140, DN T cells derived from 7/16-5 × HBeAg dbl-Tg mice were compared with 7/16-5 TCR-Tg-derived CD4 T cells. Although a variety of cytokines were produced by 7/16-5 $CD4^+$ T cells after *in vitro* culture with both HBcAg and p120–140 peptide presented by either B cells or dendritic cell (DC)/macrophage (MΦ) APCs, no significant production of cytokines was detected in the culture of HBeAg-specific DN T cells.

DN cells inhibit the cytokine production of HBeAg-specific and HBeAg-non-specific effector cells

Because HBeAg-specific DN T cells predominate in a 4-day culture and are only observed in HBeAg-expressing dbl-Tg mice, we examined the possibility that the DN T cells possessed regulatory activity. In previous unpublished experiments, total spleen cells from 7/16-5 × HBeAg dbl-Tg mice inhibited the HBeAg-specific production of cytokines by 7/16-5 effector cells, whereas, fractionated $CD4^+$, $CD8^+$ or both did not inhibit the activation of effector cells. Therefore, we fractionated the DN T cells from 4-day HBeAg-specific cultures and co-cultured the DN, $V\beta11^+$ T cells with 7/16-5 effector T cells in the presence of p120–140 and measured antigen-specific expansion and cytokine (i.e. IL-2 and IFN-γ) production by the 7/16-5 T cells. As shown in Fig. 5(a), the cytokine production of the 7/16-5 effector T cells was dramatically suppressed by the DN T cells, and the proliferation of the $CD4^+$, $V\beta11^+$ effector T cells was also inhibited even at an effector cell : Treg cell ratio as low as 32 : 1. This is a very low ratio of Treg cells to effector cells and indicates potent regulatory activity by the DN T cells. Further studies will be needed to clarify the precise mechanism of suppression. These data indicate that the DN T cells are HBeAg-specific, highly proliferative and effective suppressors, which defines a unique population of HBeAg-induced Treg cells in 7/16-5 × HBeAg dbl-Tg mice.

To investigate whether this suppression by DN T cells is only specific for the 7/16-5 Tg-TCR, we investigated the inhibitory effect of DN T cells on a polyclonal HBeAg-specific T-cell population. We immunized B10 mice with 20 μg HBeAg to prime polyclonal HBeAg-specific T cells, and harvested spleen cells after 10 days and restimulated the spleen cells in the presence of HBeAg and the indicated numbers of DN T cells. As shown in Fig. 5(b), even at a 10 : 1 effector : DN T-cell ratio, IL-2 production was effectively suppressed indicating that the Treg cell activity is functional for a polyclonal HBeAg-specific $CD4^+$ T-cell

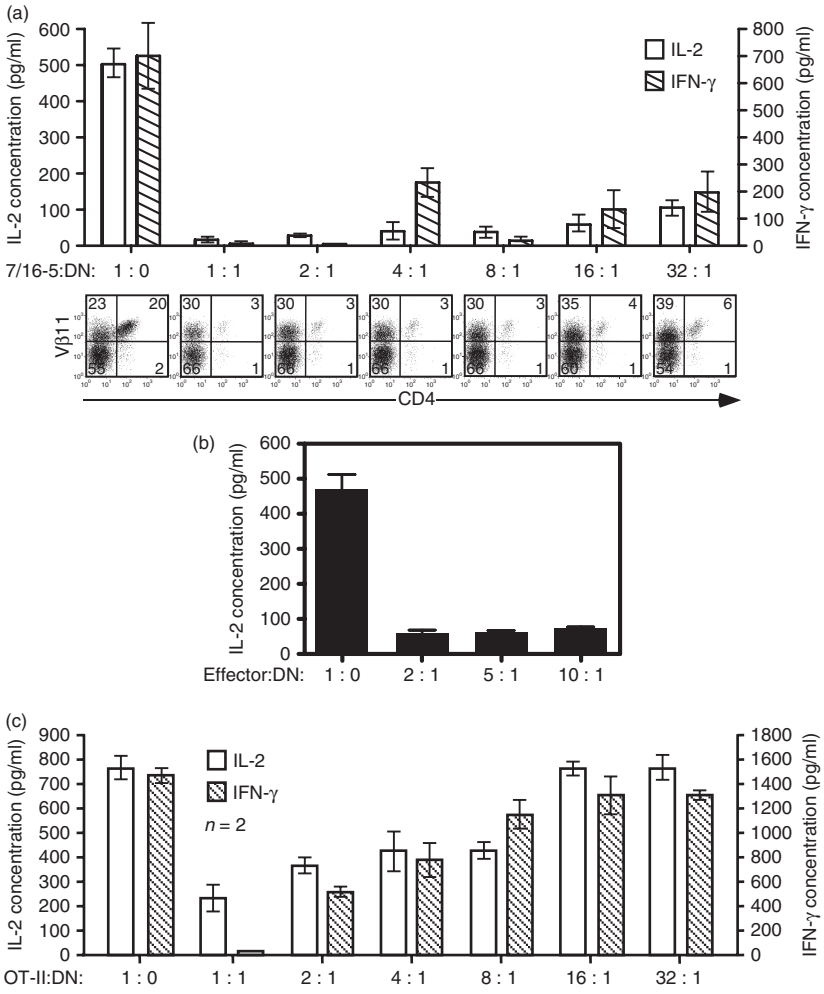

Figure 5. The hepatitis B virus e antigen (HBeAg) -specific double-negative (DN) T-cell population inhibits cytokine production and proliferation of HBeAg-specific $CD4^+$ effector T cells. (a) After 4 days of culture with p120–140, DN T cells were purified by negative selection and added to naïve 7/16-5-transgenic (Tg) spleen cells (effector T-cell culture) at the indicated ratios and p120–140 was added. Supernatants from cultured wells were collected at day 2 and day 4 for interleukin-2 (IL-2) and interferon-γ (IFN-γ) analysis, respectively. Cytokines were measured by ELISA. Remaining cells were analysed by FACS for CD4 and Vβ11 as an indicator of effector cell proliferation. (b) Spleen cells from HBeAg-immunized B10 wild-type (WT) mice were collected and cultured *in vitro* in the presence of 5 μg/ml of HBeAg and varying numbers of DN T cells. Supernatants were collected at day 2 for IL-2 analysis. (c) Spleen cells from ovalbumin-specific OT-II mice were collected and cultured with class II-restricted ovalbumin (323–339) peptide and *in vitro* activated DN T cells as described in (a). Supernatants from cultured wells were collected at day 2 and day 4 for IL-2 and IFN-γ analysis, respectively. Cytokines were measured by ELISA. Data are expressed as mean value ± SD from three independent experiments.

response, and is not restricted to 7/16-5 Tg-TCR-bearing effector cells. Furthermore, to confirm whether this inhibitory effect is HBeAg specific or not, we investigated the inhibitory effect of DN T cells on cytokine production in an unrelated MHC class II-restricted TCR-Tg lineage, OT-II (Fig. 5c). The DN T cells activated *in vitro* inhibited the production of IL-2 from OT-II effector cells at a ratio of 8 : 1 (effector cell : regulatory cell) at day 2. Similar inhibitory effects were observed in IFN-γ production at day 4. Therefore, while activation of the DN T cells is HBeAg-specific, once activated the suppressive function is non-specific. However, it is also notable that the inhibitory effect of DN T cells in an antigen-specific setting is superior to non-specific inhibition.

The DN T-cell population originates from DN progenitor T cells

As a result of the vigorous HBeAg-specific proliferative property of the DN T-cell population during *in vitro* culture, it is possible that the DN cells are derived from HBeAg-specific $CD4^+$, $CD8^+$ or from an independent DN progenitor population. To determine the origin of the DN T-cell population, depletion of T-cell subpopulations from total spleen of HBeAg × 7/16-5 dbl-Tg mice was performed and the remaining cells were cultured *in vitro* with p120–140 for 4 days and compared with total spleen cells. As shown in Fig. 6, $CD4^+$ and $CD8^+$ T-cell depletion from total spleen did not affect the generation of the DN T-cell population in the culture (i.e. 40–48%). However, negative depletion of DN T cells before culture prevented the generation of the HBeAg-specific DN T-cell population in the 4-day culture (i.e. 8%). Hence, HBeAg-specific DN T cells exist in the periphery and are not generated from $CD4^+$ or $CD8^+$ T cells in the periphery. It is notable that without DN T cells, $CD4^+$ T cells from HBeAg × 7/16-5 dbl-Tg mice demonstrate robust proliferation and cytokine production *in vitro* (data not shown).

The DN T cells expand *in vivo* in the presence of high concentrations of exogenous p120–140

The frequency of V$\beta11^+$ DN T cells in thymus and spleen *ex vivo* was measured. The V$\beta11^+$ DN T cells in thymus

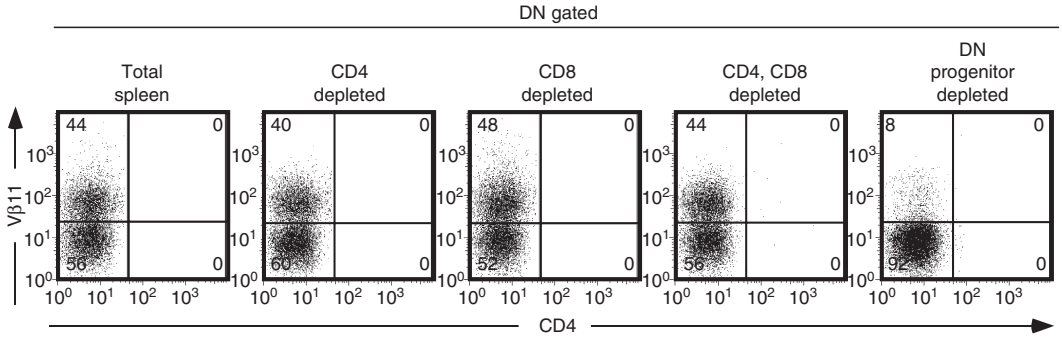

Figure 6. The hepatitis B virus e antigen (HBeAg) -specific double-negative (DN) T-cell population originates from DN progenitor T cells. HBeAg × 7/16-5 double transgenic (dbl-Tg) splenocytes were harvested and prepared as single cell suspensions. The $CD4^+$, $CD8^+$ or both $CD4^+$/$CD8^+$ T cells were depleted, respectively, using magnetic beads, and flow through from each preparation was cultured. For the progenitor DN-depleted culture, positively purified $CD4^+$ and $CD8^+$ cells were cultured with antigen-presenting cells. The HBeAg-specific DN progenitor cells were depleted as described in the Materials and methods. Each preparation of cells was cultured in the presence of 2 μg/ml of p120–140 for 4 days. Cells were gated for DN ($CD4^-$ and $CD8^-$) and further analysed for Vβ11 positivity.

of 7/16-5 × HBeAg dbl-Tg mice were present at a slightly higher frequency (5% higher) than in the thymus of 7/16-5 × HBcAg dbl-Tg mice. There was also a 20% higher frequency of V$\beta11^+$ DN T cells in the *ex vivo* spleens of 7/16-5 × HBeAg compared with the spleens of 7/16-5 single TCR-Tg mice (data not shown). However, in absolute terms DN V$\beta11^+$ T cells are present in low frequency *in situ* in HBeAg × 7/16-5 dbl-Tg mice (i.e. 3–5%) and require antigen stimulation for expansion. It is not clear if DN T cells can proliferate and be activated *in vivo*. To determine the capability of DN T cells to expand *in vivo*, we injected HBeAg-derived p120–140 (250 μg) into 7/16-5 × HBeAg-dbl Tg mice. As shown in Fig. 7, at least a twofold increase in the DN T-cell frequency *in vivo* was observed 1 and 2 weeks after injection, whereas in control, 7/16-5 mice no evidence of expansion of DN T cells occurred. Although HBeAg-specific Treg cells appear quiescent *in vivo* in HBeAg × 7/16-5 dbl-Tg mice, these cells are capable of being activated *in vivo*, in this case by exogenous antigen. The ability to activate DN T cells *in vivo* will permit further studies of their *in vivo* function.

MHC class I molecules and mature $CD8^+$ T cells and/or endogenous TCR α-chains do not directly affect the presence of DN T cells in the periphery

To further pursue the origins of DN T cells, we bred 7/16-5 × HBeAg dbl-Tg mice onto MHC class I KO, and TCR α-chain KO backgrounds. Because the 7/16-5 TCR is surprisingly expressed on $CD8^+$ as well as $CD4^+$ T cells in the thymus and the periphery, it was important to determine if expression of CD8 was necessary for selection of the DN T-cell population. The absence of MHC class I molecules and mature $CD8^+$ T cells did not affect the presence of DN T cells in the periphery (Fig. 8a). Furthermore, because it is possible that endogenous TCR α-chains are necessary for DN T-cell selection or function and because we cannot monitor the frequency of the TCR-Tg Vα5 chain by FACS (mAb against Vα5 is not commercially available), we also bred 7/16-5 × HBeAg dbl-Tg mice on to a TCR α-chain KO background. The absence of endogenous TCR α-chains did not affect the presence of DN T cells in the periphery (Fig. 8b). This demonstrates that TCR-Tg Vα5 is sufficient to confer the DN T-cell phenotype.

DN T cells require IL-15 for proliferation that may be produced by DC/MΦ

To examine the APC requirement for DN T-cell expansion, DN progenitor T cells from 7/16-5 × HBeAg dbl-Tg mice were fractionated, and cultured with different APC populations in the presence of HBeAg peptide p120–140. As shown in Fig. 9(a), fractionated B cells do not support the proliferation or survival of DN T cells even with a relatively high concentration of antigen. This is surprising because B cells are the primary APCs for HBcAg and present p120–140 and HBc/HBeAgs efficiently to HBc/HBeAg-specific $CD4^+$ T cells.[40,41] To examine non-B-cell APC function, an APC fraction from B-cell KO (μMT) mice was used for co-culture with DN T-cell progenitors. Non-B cells supported the proliferation of DN T cells efficiently even at low concentrations (0·2 μg/ml) of p120–140 peptide (Fig. 9a). It was also interesting that APCs from μMT mice support the survival of DN T cells even in the absence of antigen. It appeared that in the induction phase of DN T-cell expansion, specific soluble factors or surface co-stimulatory molecules from DC or MΦ, but not from B cells specifically support the survival and proliferation of DN T cells.

Although IL-2 is not necessary for the proliferation of DN T cells, there are several other soluble factors involved

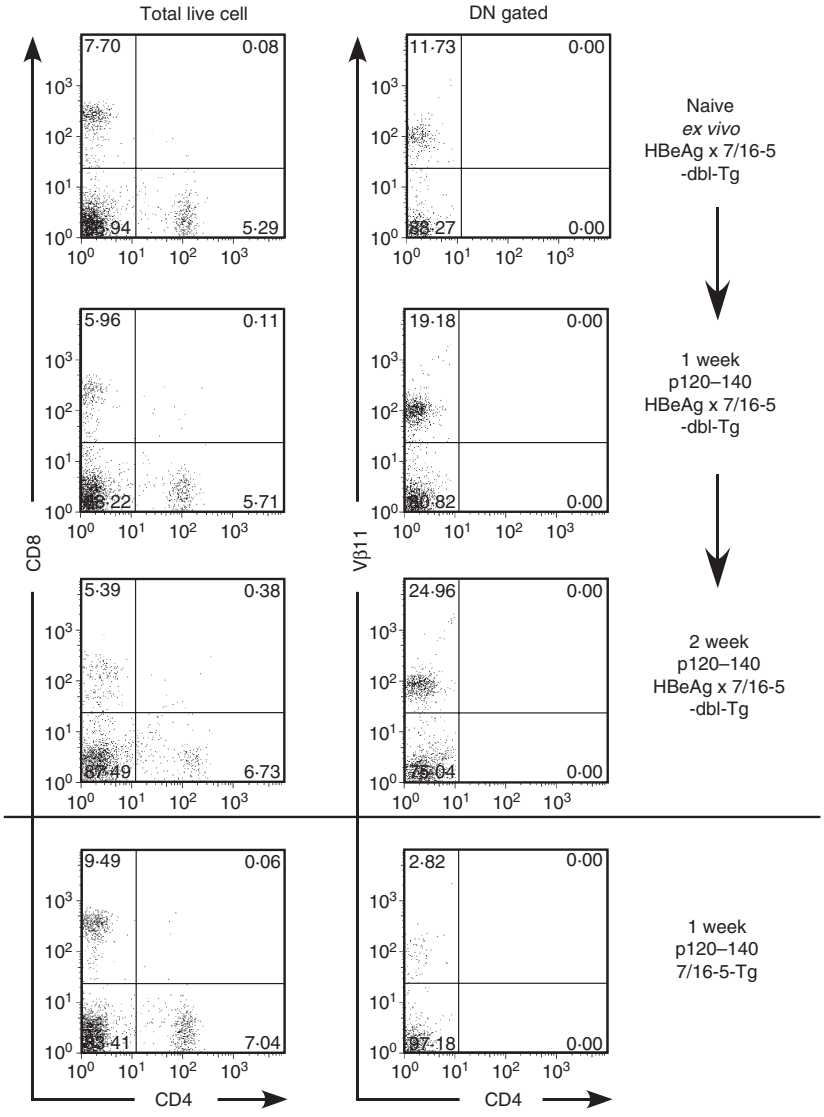

Figure 7. Injection of of hepatitis B virus e antigen (HBeAg) -derived p120–140 peptide triggers *in vivo* expansion of double-negative (DN) T cells. HBeAg × 7/16-5 double transgenic (dbl-Tg) mice were injected with 250 μg of HBeAg-derived p120–140 at the indicated times. One week and 2 weeks after peptide injection, mice were killed and splenocytes were analysed for the DN T-cell population by FACS analysis. The parallel experiment was performed in 7/16-5 Tg mice (bottom panel).

in the proliferation of T cells. Notably, IL-7 and IL-15 are prominent candidates for the induction of IL-2-independent proliferation. Interleukin-7 is known as a regulator of proliferation of T cells, in IL-2-dependent and IL-2-independent circumstances.[42] Both IL-15 and IL-7 are also known to mediate homeostatic proliferation of naive T cells.[42,43] Additionally, IL-15 is produced by DCs and can have IL-2-like function. To test the effect of IL-7 and IL-15 on the proliferation of DN T cells, we cultured purified DN progenitor cells with different APCs in the presence of antigen and cytokines. As observed in the previous experiment, DC/MΦ supported the proliferation of DN T-cell progenitor cells, whereas B cells did not, even at the higher concentration of antigen. However, when exogenous IL-15 was added to the B-cell APC culture, it rescued the proliferation of DN T-cell progenitor cells in the presence of p120–140 (Fig. 9b). This result suggests that IL-15 produced by DC/MΦ may play an important role in the proliferation of DN T cells. The DN T cells express high levels of IL-15R on their surface, whereas DN gated splenocytes from control mice do not express IL-15R (Fig. 10). IL-7 showed no significant induction of proliferation (data not shown).

Discussion

Herein, we demonstrated that Vβ11$^+$/Vα5$^+$ DN T cells derived from TCR × HBeAg dbl-Tg mice represent a unique population that possesses a distinctive cell surface marker phenotype, (i.e. TCR$^+$, Thy-1.2$^+$, CD4$^-$, CD8$^-$, CD25^{low}, GITR$^+$, PD-1$^+$, FoxP3$^-$). Furthermore, our data directly show that this DN T-cell population possesses suppressive function against effector T cells specific for the same HBeAg specificity as well as non-specific T cells. In contrast to cTreg cells, the Vβ11$^+$ DN T cells defined in this model system possess a vigorous proliferative

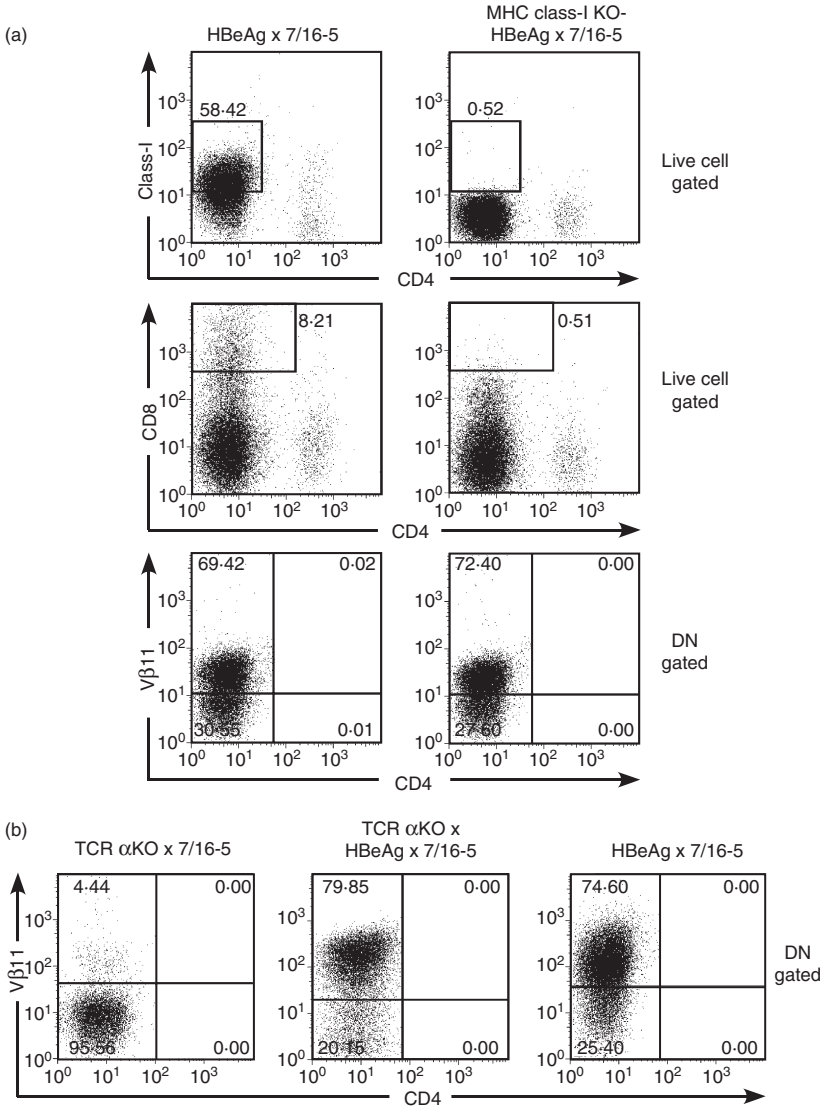

Figure 8. Absence of MHC class I molecules and mature $CD8^+$ T cells and/or endogenous T-cell receptor (TCR) α-chains does not affect the presence of double-negative (DN) T cells in the periphery. (a) Splenocytes from hepatitis B virus e antigen (HBeAg) × 7/16-5 dbl-Tg mice and MHC class I knockout (KO) × HBeAg × 7/16-5 mice were cultured in the presence of HBeAg-derived peptide p120–140 for 4 days. Cells were collected and analysed for class I, CD8 and Vβ11 expression from the DN gated population by FACS. (b) Splenocytes from TCR α-chain KO × HBeAg × 7/16-5 mice and TCR α-chain KO × 7/16-5 and HBeAg × 7/16-5 mice were cultured in the presence of HBeAg-derived peptide p120–140 for 4 days. Cells were collected and analysed for Vβ11 from the DN gated population by FACS.

capacity upon *in vitro* antigenic stimulation and represent as much as 70% (it varies between 50 and 70%) of the cells remaining after 4 days of *in vitro* culture. Those characteristics are unique and a similar Treg cell population has not been previously reported. We therefore refer to this unique population as DN Treg cells. Considering that this DN Treg cell population is only observed in TCR-Tg mice, which also express the secreted HBeAg and their strong suppressive effect, HBeAg-specific DN Treg cells may play a role in tolerance induction by HBeAg in the murine model system. We do not know if an identical DN Treg cell population may exist in chronically infected humans; however, in the mouse model the HBeAg, but not the HBcAg, has the potential to elicit Treg cells *in vivo*. Therefore, the induction of HBeAg-specific Treg cells may be added to the repertoire of mechanisms by which the secreted HBeAg mediates T-cell tolerance. Recent publications have suggested that Treg cells may contribute to impaired immune function in an HBV-Tg mouse model [44] and in chronic HBV patients.[45–47] Furthermore, in one study, in which the T-cell response to HBcAg was studied, an increase in Treg cell frequency and function was observed in HBeAg-positive compared with HBeAg-negative patients, suggesting a role for HBeAg.[46] The previous studies of Treg cells in either an HBV-Tg mouse model or HBV patients have concentrated exclusively on $CD25^+$ Treg cells or cTreg cells. The HBeAg-specific DN Treg cells observed in the 7/16-5 × HBeAg dbl-Tg mouse model may serve as a useful tool to study the functional characteristics of HBeAg-specific Treg cells in general such as clonal expansion and mechanisms of suppression, which may have implications for viral persistence during natural HBV infection. However, to relate the presence and function of DN Treg cells to T-cell tolerance and chronicity in HBV infection will require further studies.

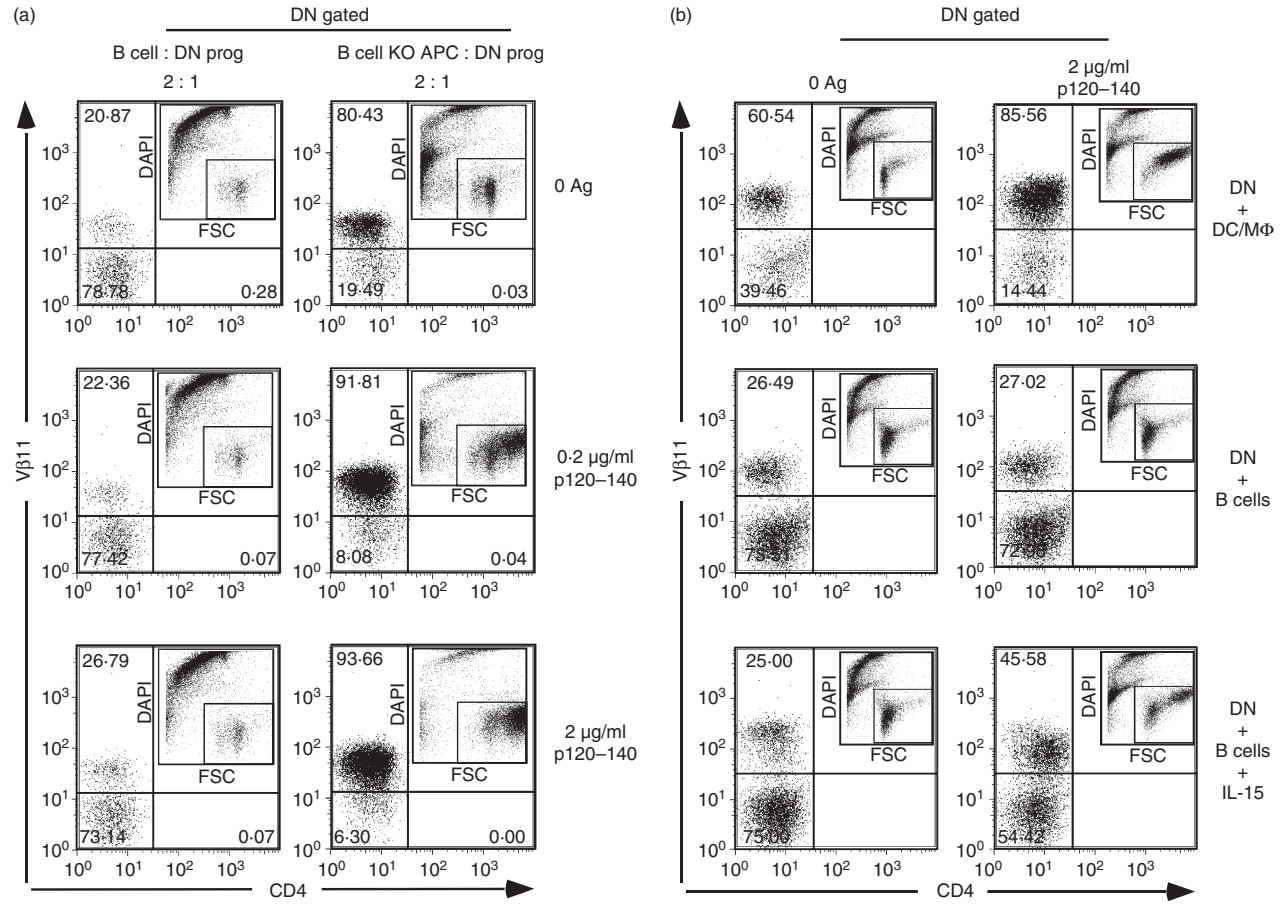

Figure 9. Non-B cells act as antigen-presenting cells (APCs) for hepatitis B virus e antigen (HBeAg) -specific double-negative (DN) T cells, and interleukin-15 (IL-15) is crucial for proliferation. (a) HBeAg-specific DN T cells were purified by negative depletion as described and cultured with purified B cells from B10 mice or T-cell-depleted splenocytes from B-cell knockout (KO) mice in the presence of HBeAg-specific peptide p120–140 at the indicated concentrations. Fluorescently labelled cells were collected by flow cytometry and gated as DN ($CD4^-$ and $CD8^-$) and analysed for Vβ11 positivity. The ancestry plot indicates the activation of cells. (b) Recombinant IL-15 (5 μg/ml) was added to HBeAg-specific DN T cells cultured with B-cell APCs, which do not support proliferation, and compared with dendritic cells/macrophages (DC/MΦ) APCs. The ancestry plot indicates the activation of cells. Data shown are representative of multiple independent experiments.

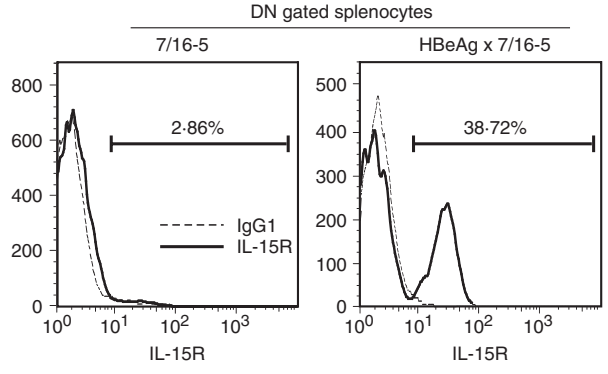

Figure 10. Double-negative (DN) T cells express high levels of interleukin-15 receptor (IL-15R); 4×10^5 splenocytes/well from 7/16-5 and hepatitis B virus e antigen (HBeAg) × 7/16-5 were cultured in 96-well plates for 72 hr with 2 μg/ml of the HBeAg-derived peptide 120–140, and cells were harvested and analysed for surface expression of IL-15R by FACS.

In contrast to anergic cTreg cells that lack efficient *in vitro* expansion, HBeAg-specific DN Treg cells proliferate vigorously *in vitro*, suggesting that this DN Treg cell population may be a useful tool to elucidate the proliferative potential of Treg cells in general. The mechanism by which HBeAg-specific DN Treg cells proliferate is not yet clear; however, preliminary data suggest that IL-15 produced by DC/MΦ plays an important role in the proliferation of DN Treg cells. There may be other possible factors that promote the proliferation of DN Treg cells in combination with IL-15, possibly other cytokines or co-stimulatory molecules that deliver signals to DN Treg cells. This is the subject of ongoing investigations.

The function of Treg cells has been described, both *in vitro* and *in vivo*. It has been proposed that Treg cells function as modulators of autoimmune responses because of their suppressive effect on autoreactive lymphocytes.

Furthermore, this suppressive function can be transferred by injecting Treg cells into autoimmune animal model systems.[7] The Treg cells have also been shown to function in many non-autoimmune models such as graft-versus-host disease and allergy.[48–51] In contrast, Treg cells can interrupt the activation of effector T cells responding to tumour cells and infectious pathogens.[46] However, clinical applications using Treg cell suppressive function have been limited because of the hypoproliferative property and polyclonal nature of Treg cells. *In vitro* studies using cTreg cells show that only a relatively high ratio of Treg : effector cells can suppress the effector cells (i.e. 5 : 1 to 1 : 1). As a result of this inefficient *in vitro* suppression, the therapeutic potential of Treg cells has been critically limited. However, HBeAg-specific DN Treg cells demonstrate superior suppressive effects on effector cells at effector cell : Treg cell ratios as low as 32 : 1 (see Fig. 5).

The multiple mechanisms of suppression used by Treg cells is an ongoing subject of research and remains somewhat controversial. The suppressive effects of cTreg cells *in vitro* have been reported mostly on $CD4^+$ and $CD8^+$ effector cells, but have also been found to act directly on APCs and natural killer cells.[52–56] Inhibitory cytokines, IL-10 and transforming growth factor(TGF)-β are known to be produced by cTreg cells and thought to be a part of the mechanism of Treg cells.[57,58] According to our preliminary data in a transwell system, IL-10 and TGF-β are not candidates as the primary mediators of suppression demonstrated by HBeAg-specific DN Treg cells (data not shown). Another report showed that the regulatory function of Treg cells is serine protease granzyme-B (GZ-B)-dependent using GZ-$B^{-/-}$ mice.[59] Other suppressive mechanisms have been suggested to function via cell–cell contact. CTLA-4, FAS–FASL, GITR and CD103 have also been suggested to play a role in the function of Treg cells. Recently, the inhibitory function of Treg cells has been demonstrated to be mediated through the exoenzymes CD73/CD39.[60–62] Interestingly, a high frequency of HBeAg-specific DN Treg cells are $CD73^+/CD39^+$ after activation (Fig. 11). We are investigating whether this pathway may explain the efficient immunoregulation mediated by HBeAg-specific DN Treg cells.

It is likely that HBeAg-specific DN Treg cells result from positive selection in the thymus or as a result of continuous exposure to the HBeAg in the periphery but are not derived from $CD4^+$ or $CD8^+$ HBeAg-specific T cells in the periphery. It is interesting that the 7/16-5 TCR is expressed on $CD8^+$ T cells as well as $CD4^+$ T cells although both $CD4^+$ and $CD8^+$ T cells are specific for p120–140 in the context of MHC class II molecules ($I\text{-}A^b$). It is possible that the 7/16-5 TCR may also recognize a self-peptide in the context of MHC class I molecules in the thymus with sufficient affinity to be selected on MHC class I. To address this question, we bred 7/16-5 × HBeAg dbl-Tg mice on a MHC class I negative

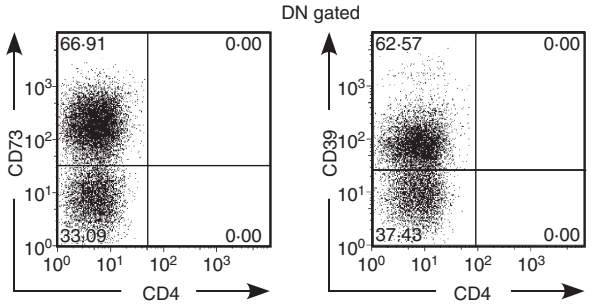

Figure 11. Double-negative (DN) T cells express high levels of ectoenzyme CD73 and CD39 4×10^5 splenocytes/well from hepatitis B virus e antigen (HBeAg) × 7/16-5 were cultured in 96-well plates for 72 hr with 2 μg/ml of HBeAg-derived peptide 120–140, and cells were harvested and analysed for surface expression of CD73 and CD39 by FACS.

background. While HBeAg × 7/16-5 dbl-Tg mice on a MHC class I KO background do not produce mature $CD8^+$ T cells in the periphery, HBeAg-specific DN T cells are produced, and are, therefore, not dependent on MHC class I or CD8 expression. Endogenous TCR-α chains also do not affect the presence of DN T cells in the periphery.

At present, we have no direct evidence to address whether this DN Treg cell population is unique to this model or not. The frequency of this population is low *in situ* in 7/16-5 × HBeAg dbl-Tg mice and their presence in other systems may be difficult to detect. The 7/16-5 × HBeAg dbl-Tg mice may be a useful model for low-affinity self-reactive T cells that escape deletion in the thymus and are quiescent in the periphery until activated (i.e. tissue injury, mimicked here by high concentrations of peptide *in vitro* or *in vivo*). Most dbl-Tg mice are models of high-affinity self-reactive T cells, which are largely deleted *in vivo*. It is anticipated that further characterization of this low-affinity DN Treg cell population may yield a phenotypic marker that would allow identification in other systems.

Recent publications have suggested that Treg cells may contribute to impaired immune function in an HBV-Tg mouse model [44] and in patients with chronic HBV.[45–47] Furthermore, in one study, in which the T-cell response to HBcAg was studied, an increase in Treg cell frequency and function was observed in HBeAg-positive patients compared with HBeAg-negative patients, suggesting a role for HBeAg.[46] Previous studies of Treg cells in either an HBV-Tg mouse model or HBV-infected patients have concentrated exclusively on $CD25^+$ Treg cells or cTreg cells. The HBeAg-specific DN Treg cells observed in the 7/16-5 × HBeAg dbl-Tg mouse model may serve as a useful tool to study functional characteristics of HBeAg-specific Treg cells in general such as clonal expansion and mechanisms of suppression, which may have implications for viral persistence during natural HBV infection.

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Disclosures

The authors have no conflicts of interests to declare.

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