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Association of CD45^{dim}VLA-4^{+} cells with the NKT cell lineage and their selective expression of IL-13, IP-15, and CCR3 transcripts

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Abstract

Introduction: Estrogen (E2) was shown to prevent experimental autoimmune encephalomyelitis (EAE) and to produce a novel population of regulatory CD45^{dim}VLA-4^{+} cells. Although their appearance was dependent upon an elevated hormonal level, E2 was not required for their production, as they also were induced by immunization with *Mycobacterium tuberculosis* as a component of complete Freund's adjuvant.
Materials and Methods: Molecular techniques, including ribonuclease protection assays and quantitative RT-PCR, were used to provide further characterization of CD45^{dim}VLA-4^{+} cells. Moreover, we determined the developmental requirements of the CD45^{dim}VLA-4^{+} cells using genetically modified mice and extensive flow cytometry analysis.
Results: Characterization of CD45^{dim}VLA-4^{+} mRNA profile revealed highly elevated levels of CD16, CD44, CCR3, IP-15, and IL-13 transcripts compared with their CD45^{high}VLA-4^{+} counterparts. Furthermore, we found up-regulation of anti-apoptotic *bcl-w* and *bcl-xl* genes and transcripts encoding the TCRα and CD8α homodimer. The production of CD45^{dim}VLA-4^{+} cells was evident in nude mice and in MHC class II- and β2-microglobulin, but not in CD1-deficient mice, suggesting a crucial role for CD1 in their induction.
Conclusions: These findings suggest that CD45^{dim}VLA-4^{+} cells might resemble natural killer T cells and imply possible roles for IL-13 and IP-15 in the protective function of CD45^{dim}VLA-4^{+} cells. A better understanding of how these cells, also occurring naturally during pregnancy, suppress the harmful immune response of EAE may lead to novel therapeutic approaches to combat multiple sclerosis and other autoimmune diseases.

Key words: autoimmunity, EAE/MS, regulatory cells, adhesion molecules, chemokines/cytokines, pregnancy tolerance.

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INTRODUCTION

A disruption of the balance between pathogenic and protective/regulatory cells is a key event in the induction, development, and progression of autoimmune diseases such as multiple sclerosis (MS) [1, 2]. Recently there has been renewed interest in the regulatory cells that influence the developmental course of these autoimmune diseases [17, 18, 27]. The best known of these regulatory cells are CD4^{+}CD25^{+} T cells, which have been shown to inhibit several autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) [14], which is an animal model for MS, and type 1 diabetes in rats and mice [9]. In the past few years, researchers have also discovered several new populations of regulatory T cells with previously unknown phenotypes, including those found in the murine vaginal tissue [31] and female genital tracts [12].

Recently we demonstrated that treatment with several related estrogenic compounds confers complete protection against EAE and led to the induction of a novel subpopulation of regulatory cells in the spleen and central nervous system (CNS). These previously uncharacterized cells, which also occur naturally in pregnant mice, display a low-level expression of CD45 (CD45^{dim}) and no detectable expression of cell surface markers related to T cell receptor (TCR) signaling, including CD3 and TCR. However, these cells retained expression of VLA-4, an extracellular protein involved in cellular migration. Injection of purified CD45^{dim}VLA-4^{+} cells conferred protection from spontaneous EAE and suppressed antigen-specific proliferation of primed lymphocytes [19].

While we have documented the presence and the suppressive function of CD45^{dim}VLA-4^{+} cells, little is known about their cellular lineage and developmental fate. Because these cells do not seem to express surface markers beyond CD45 and VLA-4, normally found on conventional, defined immune cell populations, the CD45^{dim}VLA-4^{+} cells appear to constitute a developmentally immature population of cells. In the current study we provide evidence suggesting that these cells may be precursors of natural killer (NK) T cells with expression of CCR3 transcript and a potential ability to produce IL-13 and IP-15 cytokines. Better definition of the origin of this subpopulation of cells and the role of IL-13, IP-15, and the anti-apoptotic molecule *bcl-xl* in the survival of CD45^{dim}VLA-4^{+} cells will lead to an understanding of the role of estrogenic hormones in protection against EAE and in tolerance induction in general.

MATERIALS AND METHODS

Mice

Female C57BL/6, B6.Cg-Foxn1nu/J (Nu/nu$^{-/-}$), B6.129P2-B2m^{tm1Unc}/J (β2-m$^{-/-}$), B6.129S2-C2^{tatm1Ccum}/J (MHCII$^{-/-}$), C;129S-CD1^{tm1Gru}/J (CD1$^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) at 8 weeks of age. The mice were housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional animal care committee guidelines.

Induction of CD45^{dim}VLA-4^{+} cells

CD45^{dim}VLA-4^{+} cells were generated in mice using 60-day-release 2.5 mg 17β-estradiol (E2) pellets (Innovative Research of America, Sarasota, FL). For kinetic studies, 21-day-release pellets were used. Additionally, and only for kinetic study purposes, C57BL/6 mice were immunized according to our standard procedure previously described [19] to boost the production of CD45^{dim}VLA-4^{+} cells. Pellets were implanted subcutaneously in the scapular region behind the neck using a 12-gauge trochar as described by the manufacturer (Innovative Research of America). The serum concentration of estrogen was determined by radioimmune assay. Based on information provided by the manufacturer, the serum concentration of E2 achieved following implantation of a 2.5 mg E2 pellets should be between 1.5 and 2.0 ng/ml (1/5 pregnancy level).

Induction of CD45^{dim}VLA-4^{+} cells by complete Freund's adjuvant challenge

C57BL/6 mice were immunized with complete Freund's adjuvant (CFA) to induce the production of CD45^{dim}VLA-4^{+} cells. The control mice were challenged with incomplete Freund's adjuvant (IFA) only. One week after CFA injection, spleens were isolated and assayed for the presence of CD45^{dim}VLA-4^{+} cells.

Sorting of CD45^{dim}VLA-4^{+} cells by FACS

Spleens from experimental mice were harvested and single cell suspensions were obtained by mechanical disruption. To obtain CD45^{dim}VLA-4^{+} and CD45^{high}VLA-4^{+} cell subpopulations, splenocytes were stained with anti-CD45-FITC and anti-VLA-4 PE monoclonal antibody (PharMingen, San Diego, CA) on ice for 30 min, washed, and sorted using the Vantage SE (Becton--Dickinson, Sunnyvale, CA). Forward and side scatter and staining with 7-AAD was used to gate on lymphocytes and to exclude dead cells.

RNA isolation and RT-PCR

Total RNA was isolated from spleens using the RNeasy mini kit protocol (Qiagen, Valencia, CA) and then converted to cDNA using oligo dT, random hexamers, and Superscript RT II enzyme (Invitrogen, Grand Island, NY, USA). Real-time PCR was performed using Quantitect SYBR Green PCR master mix (Qiagen) and primers (synthesized by ABI). Reactions were conducted on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) to detect the following genes: CD1, CD2, CD3, CD4, CD5, CD7, CD8a, CD11b, CD16, CD28, CD38, CD40, CD44, CD45, CD45R, CD62L, CD90, CD117, NK1.1, T-bet, FcR, LFA-1, C3, STAT-1, IP-15, CtsC, H2A, Ly5, TNFR, CXCR1, CXCR2, CXCR3, MIP-1β, CD25, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7R, IL-10, IL-13, IL-16, IFN-γ, TNF-α, TGF-β1, TGF-β3, Foxp3, RANTES, IP-10, MIP-2, MCP-1, MIP-1a, CCR1-8 but not CCR4, and L32.

Sequences for the presented genes were *L32*: (F: GGA AAC CCA GAG GCA TTG AC; R: TCA GGA TCT GGC CCT TGA AC); *CD16*: (F: ACT GGA CCC CCC ATG GAT; R: GGG TCC CTT CGC ACA TCA) *CD44*: (F: CTG GCA CTG GCT CTG ATT CTT; R: TCT GCC CAC ACC TTC TCC TAC T); *CCR3*: F: GGG CAC CAC CCT GTG AAA; R: TGG AGG CAG GAG CCA TGA; *IP-15*: F: CAG CAA CAT CTA TGA GGT CTT TCT G; R: TTC CGC TGG ACA CCT TCT TC; *IL-13*: F: ACT GCT CAG CTA CAC AAA GCA ACT; and R: TGA GAT GCC CAG GGA TGG T.

RNase protection assay

Total RNA was extracted from CD45^{dim}VLA-4^{+} and CD45^{high}VLA-4^{+} cells using the STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA). A broad range of mRNA species, including those for the cell surface antigens TCRδ, TCRα, CD3ε, CD4, CD8α, CD8β, CD19, F4/80, and CD45 and the apoptosis-related molecules *bcl-w*, *bfl1*, *bcl-X*, *bak*, *bax*, *bcl-2*, and *bad* were determined using the RiboQuant RPA kit (PharMingen, Inc., San Diego, CA, USA) according to the

manufacturer's instructions. The sample loading was performed on 2–10 μg total RNA hybridized with probes labeled with [^{32}P] UTP. After digestion of ssRNA, the RNA pellet was solubilized and resolved on a 5% sequencing gel. The gels were analyzed using a phosphor imager (Bio-Rad Laboratories, Hercules, CA, USA) and the experimental signal normalized to L32 using Quantity One software (Bio-Rad Laboratories).

Statistical analyses

Differences in incidence between two cell-populations were assessed by Fisher's exact test. The Student's *t*-test was used for unmatched data for analysis of significance. Values of $p \leq 0.05$ were considered significant.

RESULTS

CD45^{dim}VLA-4$^+$ cells preferentially express CD16, CD44, CCR3, IP-15, and IL-13 transcripts

While the presence and the suppressive function of CD45^{dim}VLA-4$^+$ cells were documented previously [19], their cellular origin and the mechanisms by which they regulate the autoimmune response remain unclear. Despite several attempts to phenotypically characterize CD45^{dim}VLA-4$^+$ cells, we were unable to detect any other (beyond CD45 and VLA-4) surface markers using flow cytometry. We thus continued our characterization by studying the expression of gene transcripts using the RT-PCR and RPA techniques. Our broad screening of transcripts (see Materials and Methods) revealed prominent expression of five genes, i.e. those for CD16, CD44, CCR3, IL-13, and IP-15, in the CD45^{dim}VLA-4$^+$ population (Fig. 1). The other tested genes were not expressed or were expressed to varied levels in the CD45^{dim}VLA-4$^+$ population. The transcriptional profile suggests that a large fraction of CD45^{dim}VLA-4$^+$ cells might constitute precursors of the NK/T cell lineage that may express the CCR3 chemokine receptor and produce IL-13 and IP-15 cytokines.

CD45^{dim}VLA-4$^+$ cells express CD8α and TCRα transcripts

Using the RPA, we screened the CD45^{dim}VLA-4$^+$ cells for the presence of mRNA species encoding several cell surface antigens (see Materials and Methods). As shown in Fig. 2A and B, we found that CD45^{dim}VLA-4$^+$ cells express TCRα, a marker of T cell commitment. Although the message levels of TCRα were markedly lower than in their CD45^{high}VLA-4$^+$ counterparts, this result indicates that CD45^{dim}VLA-4$^+$ cells might include immature T cells committed to the αβ lineage. CD45^{high}VLA-4$^+$ cells also express message-encoding CD8α, which forms an unusual co-receptor, the CD8α homodimer (Fig. 2A, B). This co-receptor is characteristic of T cells that mature in a thymus-independent manner. Compared with CD45^{high}VLA-4$^+$ cells, CD45^{dim}VLA-4$^+$ cells show predominance in expressing CD8α co-receptor. The presence of both of these messages suggests that CD45^{dim}VLA-4$^+$ cells are precursors for a type of regulatory T cell that matures outside of the thymus. Additionally, CD45^{dim}VLA-4$^+$ cells express

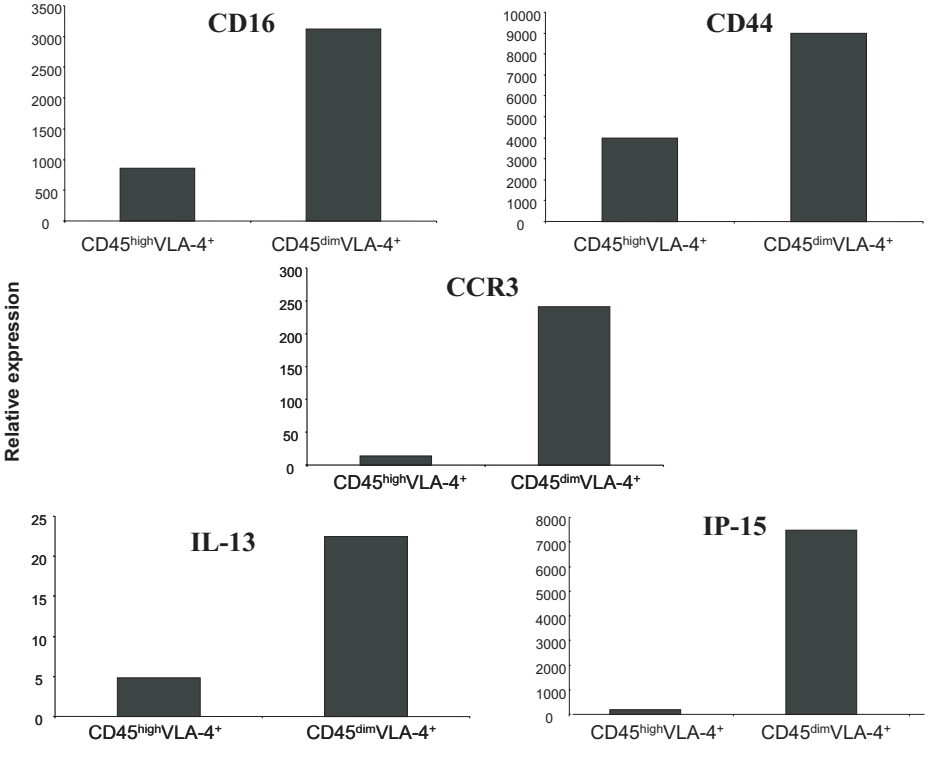

Fig. 1. Expression of mRNA encoding CD16, CD44, CCR3, IL-13, and IP-15 in CD45^{dim} VLA-4$^+$ cells and CD45^{high} VLA-4$^+$ cells. Cells were isolated and sorted and mRNA levels were quantified by RT-PCR as described in Materials and Methods. Expression of CD16, CD44, CCR3, IL-13 and IP-15 is presented. Quantification of individual signals compared with the housekeeping gene L32 in both CD45^{dim}VLA-4$^+$ cells and CD45^{high}VLA-4$^+$ cell populations is presented as relative expression. The data presented are representative from a total of three experiments.

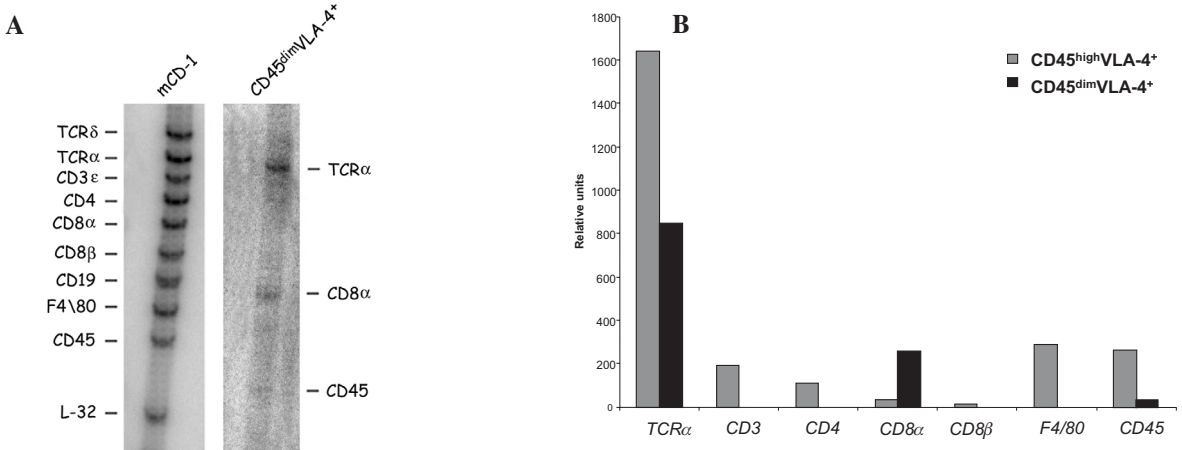

Fig. 2. Expression of mRNA encoding cell surface markers in $CD45^{dim}VLA\text{-}4^+$ cells and $CD45^{high}VLA\text{-}4^+$ cells. Cells were isolated and sorted and mRNA levels were quantified by RPA as described in Materials and Methods. **A** – phosphorimaging revealed that $CD45^{dim}VLA\text{-}4^+$ cells selectively expressed transcripts for TCRα, CD8α and CD45; **B** – quantification of individual RPA bands compared with the housekeeping gene L32 in both $CD45^{dim}VLA\text{-}4^+$ cells and $CD45^{high}VLA\text{-}4^+$ cell populations. The data presented are representative from a total of three experiments.

low levels of transcripts for CD45, which is in correlation with the *dim* expression of this molecule on the cell surface (Fig. 2A, B).

Extrathymic origin of $CD45^{dim}VLA\text{-}4^+$ cells and CD1-dependent generation of $CD45^{dim}VLA\text{-}4^+$ cells

To support the hypothesis that the development of $CD45^{dim}VLA\text{-}4^+$ cells is independent of the thymus and to determine if MHC class I, class II, or CD1 molecules are required for their development, we investigated the estrogen-induced production of $CD45^{dim}VLA\text{-}4^+$ cells in *nu/nu*, MHC class II-, β2-microglobulin-, and CD1-deficient mice. $CD45^{dim}VLA\text{-}4^+$ cells were found in *nu/nu*, MHC class II-, β2-m mouse strains to the same extent as in normal wild-type mice, creating 18–29% of

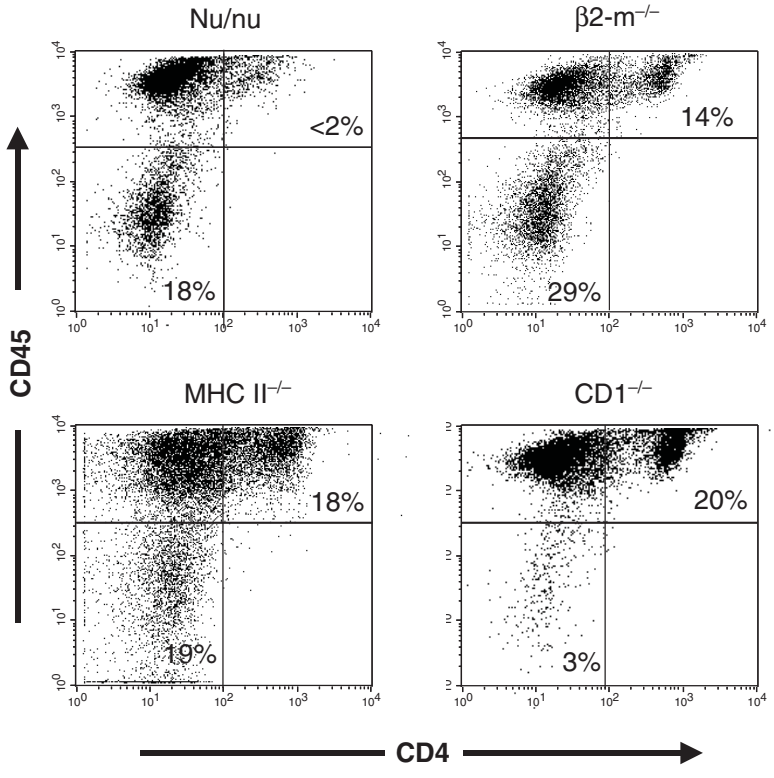

Fig. 3. $CD45^{dim}VLA\text{-}4^+$ cells may be of extrathymic origin. Induction of $CD45^{dim}VLA\text{-}4^+$ cells was studied in nude, MHC class $II^{-/-}$, $\beta2\text{-}m^{-/-}$, and $CD1^{-/-}$ mice. Surprisingly, $CD45^{dim}VLA\text{-}4^+$ cells were not found in CD1-deficient mice, suggesting that the induction of these cells might be dependent on CD1 molecule. The data presented are representative from a total of three experiments.

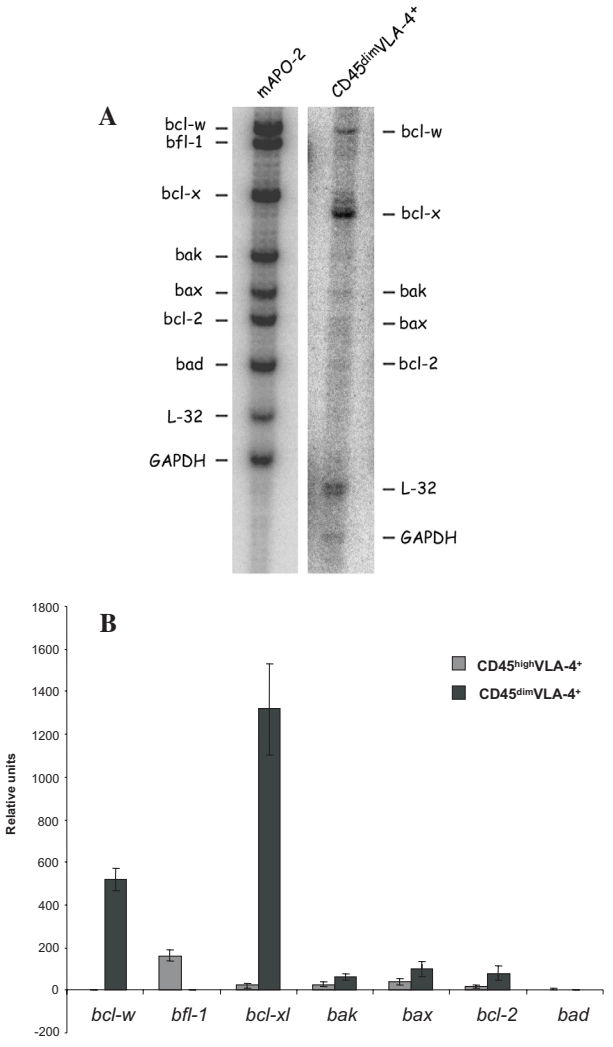

Fig. 4. Expression of mRNA encoding apoptosis-related molecules in $CD45^{dim}VLA\text{-}4^+$ cells and $CD45^{high}VLA\text{-}4^+$ cells. **A –** phosphorimaging revealed that transcripts for anti-apoptotic molecules *bcl-w, bcl-xl* are highly expressed in $CD45^{dim}VLA\text{-}4^+$ cells; **B –** quantification of individual RPA bands compared with the housekeeping gene L32 in $CD45^{dim}VLA\text{-}4^+$ cells and $CD45^{high}VLA\text{-}4^+$ cells populations. The data presented are representative from a total of three experiments.

the total of splenocyte population (Fig. 3). This strongly suggests that $CD45^{dim}VLA\text{-}4^+$ cells were extrathymically derived and could develop in the absence of MHC class I, class II molecules. Unexpectedly, $CD45^{dim}VLA\text{-}4^+$ cells were virtually undetectable in mice deficient in the CD1 molecule, comprising about 3% of the total spleen population (Fig. 3). This result suggests an important role of CD1 molecule in the induction and the development of $CD45^{dim}VLA\text{-}4^+$ cells and suggests their NK/T cell lineage commitment.

$CD45^{dim}VLA\text{-}4^+$ cells predominantly express bcl-w and bcl-xl genes

To protect against cell death, lymphocytes express a separate set of proteins that inhibit programmed cell death, or apoptosis. Using the RPA, we screened $CD45^{dim}VLA\text{-}4^+$ cells for the presence of several transcripts related to apoptotic molecules. Cells were isolated using FACS sorting and frozen until assay. We found that two anti-apoptotic molecules, *bcl-w* and *bcl-xl*, were highly and preferentially expressed in $CD45^{dim}VLA\text{-}4^+$ cells (Fig. 4). Another anti-apoptotic gene, *bcl-2,* was slightly upregulated in $CD45^{dim}VLA\text{-}4^+$ cells, but the difference did not reach significance; in contrast, *bfl-1* was exclusively expressed by $CD45^{high}VLA\text{-}4^+$ cells. The death-promoting genes *bax* or *bad* were equally expressed by both studied populations, although at a low level. Interestingly, the expressions of *bcl-w* and *bcl-xl* in $CD45^{dim}VLA\text{-}4^+$ cells were dramatically higher than in their $CD45^{high}VLA\text{-}4^+$ counterparts, which displayed very low signal for these genes.

The induction of $CD45^{dim}VLA\text{-}4^+$ cells follows increased serum estrogen levels

The kinetic studies presented in Fig. 5 show that $CD45^{dim}VLA\text{-}4^+$ cells appear within 72 h after estrogen treatment in naïve mice. By using 21-day-release pellets, we set out to determine if the continued presence of estrogen was required for the maintenance of $CD45^{dim}VLA\text{-}4^+$. Naïve mice were implanted with estrogen (E2) pellets and immunized with 35-55 MOG-specific peptide on day 7 post implantation, as described in Materials and Methods. Immunized and E2-treated mice did not develop EAE, in striking contrast to their untreated counterparts which served as controls for E2 protection from EAE (data not shown). For the assay, mice selected at random were sacrificed and their splenocytes were isolated and stained for the presence of $CD45^{dim}VLA\text{-}4^+$ cells after 24, 48, and 72 h post implantation, 9 days post implantation (2 days post immunization), 21 days post implantation (14 days post immunization), which was the last day of estrogen release, 23 days post implantation (16 days post immunization), and 28 days post implantation (21 days post immunization). $CD45^{dim}VLA\text{-}4^+$ cells were detectable 72 h after E2 treatment, constituting 13% of the total splenocytes. In our previous studies we observed that immunization increased the number of $CD45^{dim}VLA\text{-}4^+$ cells. In the current study we found that the number of $CD45^{dim}VLA\text{-}4^+$ cells started increasing 48 h post immunization, eventually comprising 33% of total splenocyte population 14 days post immunization. The drop in high hormonal levels provided by the E2 pellet in the serum was accompanied by a significant drop in the percentage of $CD45^{dim}VLA\text{-}4^+$ cells in the spleen, decreasing to 18% on day 2 after pellet expiration. One week after expiration of the E2 pellets, the proportion of $CD45^{dim}VLA\text{-}4^+$ cells in spleen was no higher than 6%.

$CD45^{dim}VLA\text{-}4^+$ cells can be induced by CFA

Previously we observed that immunization with 35–55 MOG peptide in CFA (*Mycobacterium tuberculo-*

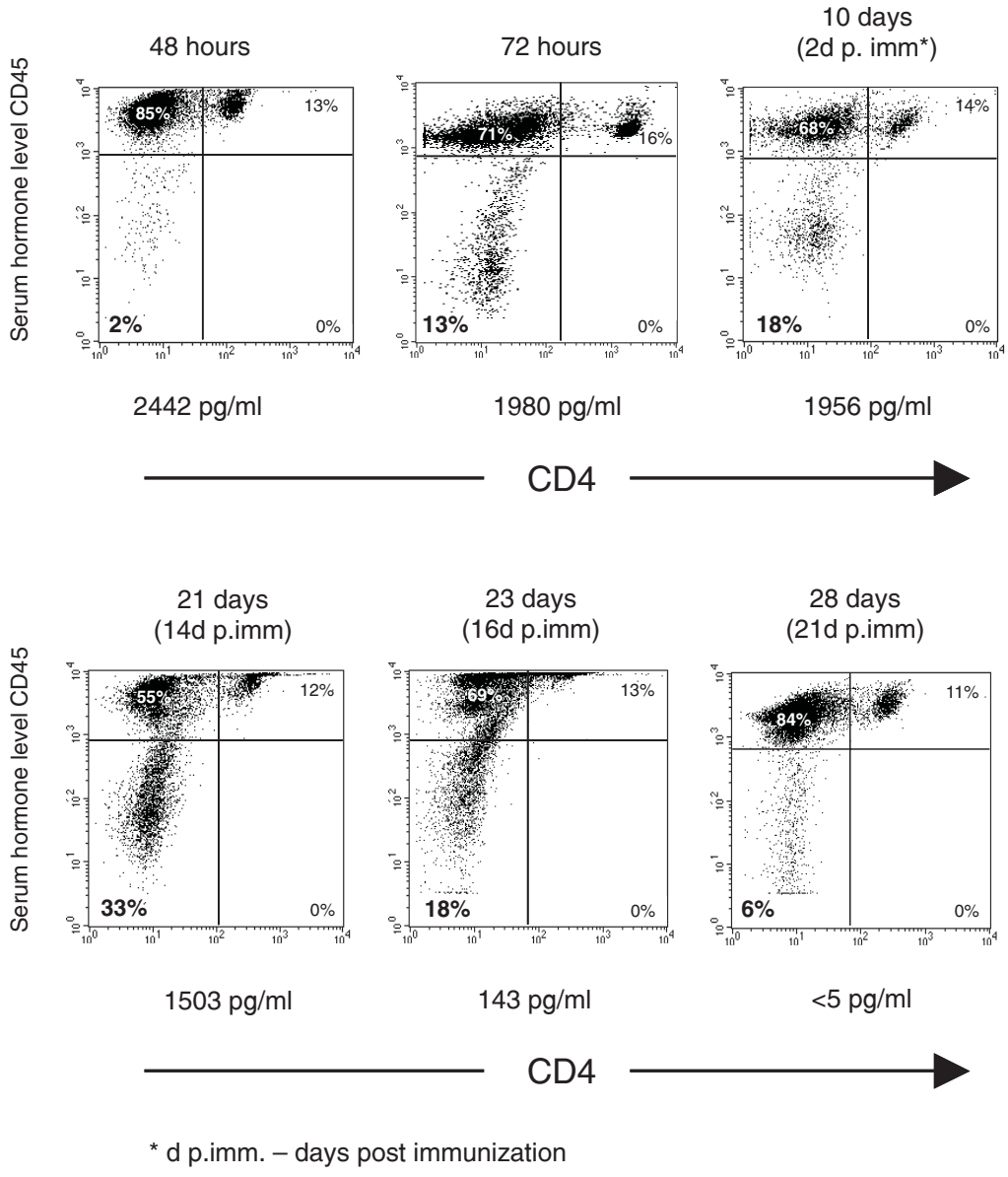

Fig. 5. Kinetics of the production of CD45^{dim}VLA-4^{+} cells as a function of serum hormone level provided by 2.5-mg E2 pellets. Naïve C57BL/6 mice were implanted with 21-day-release E2 pellets 7 days before immunization. The production of CD45^{dim}VLA-4^{+} cells was determined at the indicated times. At 72 h post implantation the number of CD45^{dim}VLA-4^{+} cells reached up to 13% and increased significantly at day 14 post immunization up to 33%. Note the reduction in the percentage of CD45^{dim}VLA-4^{+} cells two days after expiration of the pellet (18%), which was accompanied by a significant drop in the E2 serum level. The data presented are representative from a total of three experiments.

sis in IFA) in combination with E2 treatment increased the number of CD45^{dim}VLA-4^{+} cells compared with mice treated with E2 only [19]. We found that this increase was caused by the *M. tuberculosis* component present in CFA, but not the peptide or IFA alone. CFA can generate CD45^{dim}VLA-4^{+} cells to levels comparable with those observed in mice treated with E2 only, reaching 20–30% of the total splenocyte population. The combined treatment with both CFA and E2 resulted in the induction of a larger percentage of CD45^{dim}VLA-4^{+} cells, reaching up to 50% of the total spleen cell population (Fig. 6). IFA--challenged mice served as negative controls.

DISCUSSION

To prevent attack of host tissues, the immune system uses several different mechanisms, including anergy, ignorance, deletion, and immune suppression. Clonal deletion provides central tolerance, while the other mechanisms provide peripheral tolerance. In order to develop improved treatments for autoimmune diseases including MS, it is imperative to develop a better understanding of immune suppression by regulatory T cells. Because regulatory cells express a variety of phenotypes, it is becoming increasingly clear that they are better

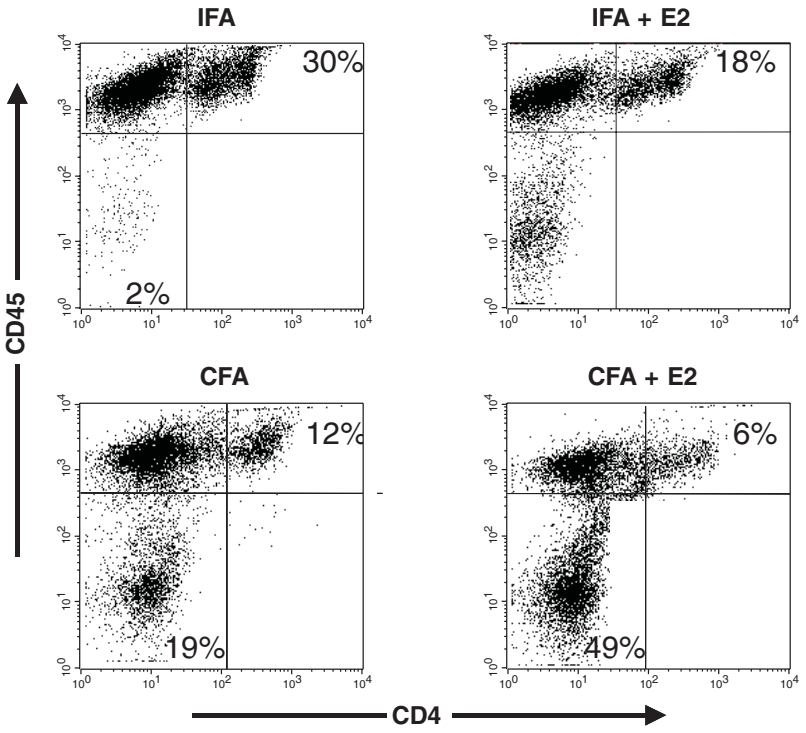

Fig. 6. Treatment with Complete Freund's Adjuvant (CFA) alone induced the production of $CD45^{dim}VLA\text{-}4^{+}$ cells. Mice were immunized with CFA and/or treated with E2 pellets. IFA-challenged mice served as controls. One week later, donor spleens were checked for the presence of $CD45^{dim}VLA\text{-}4^{+}$ cells. The data presented are representative from a total of three experiments.

characterized by their suppressive function rather than phenotype. Recently we showed that treatment strategies using several estrogen-related compounds confer complete protection from EAE and collagen-induced arthritis, which serve as animal models for human MS and rheumatoid arthritis, respectively. Although the protective mechanisms are unknown, each of these treatments resulted in the appearance in the spleen and CNS a population of $CD45^{dim}VLA\text{-}4^{+}$ cells, which also occurs naturally in pregnant mice [19]. Injection of purified $CD45^{dim}VLA\text{-}4^{+}$ cells conferred protection from spontaneous EAE and suppressed antigen-specific proliferation of primed lymphocytes. $CD45^{dim}$ cells have lost the expression of many cell surface markers related to TCR signaling and other functions, but retain expression of VLA-4, thus creating a novel population of regulatory cells.

The majority of T cells develop in the thymus and exhibit well-characterized phenotypic changes associated with their maturation. However, there are distinct T cell populations that mature extrathymically, including intestinal intraepithelial lymphocytes (IELs) [22]. IELs of the small intestine contain a complex mixture of T cells that includes both TCRαβ- and TCRγδ-bearing populations. Those lymphocytes can be distinguished from conventional thymically derived T cells in that they express an unusual co-receptor, a CD8α homodimer [10, 15], and mature extrathymically within the gut epithelium [24, 25]. Immature IELs can rearrange their TCR genes and express pre-TCRs (pre-Tα), a marker of T cell commitment. Pre-Tα transcript is expressed by immature $CD4^{-}CD8^{-}$ thymocytes, but not by TCRγδ thymocytes, splenic NK cells, or other nonlymphoid tissue [6]. Interestingly, we were able to detect transcripts for both CD8α and TCRs in our $CD45^{dim}VLA\text{-}4^{+}$ cells, but not other mature thymic T cell markers, suggesting that $CD45^{dim}VLA\text{-}4^{+}$ cells may represent precursors committed to the TCRαβ lineage that matures extrathymically. The fact that the $CD45^{dim}VLA\text{-}4^{+}$ cells were generated in MHC class $II^{-/-}$, $\beta2\text{-}m^{-/-}$, as well as in nude mice, which lack a thymus, further supports the idea that these cells may develop extrathymically and do not need MHC class I or MHC class II for their differentiation. In this context, it is important that non-classical MHC-like molecules, in particular CD1, are important for extrathymic T cell differentiation and antigen presentation to NKT cells. Of note, we were unable to generate $CD45^{dim}VLA\text{-}4^{+}$ cells in $CD1^{-/-}$ mice. Thus, $CD45^{dim}VLA\text{-}4^{+}$ cells might resemble NKT precursor cells in that they require the CD1 molecule for normal development. Furthermore, the idea that $CD45^{dim}VLA\text{-}4^{+}$ cells might be committed to the NK lineage was supported by a strong expression of transcripts encoding CD16 and CD44, molecules, which serve as cell surface receptors on NK cells.

The $CD45^{dim}VLA\text{-}4^{+}$ population expressed a high level of the IL-13 transcript. IL-13 is one of the Th2 cytokines produced by activated $CD4^{+}$ T-helper cells and was shown to play a significant role in suppressing EAE [33]. It has been reported that NKT cells are another substantial source of IL-13 cytokine production [28]. Recently, non-classical CD1d-restricted NK T cells

have been reported to produce IL-13 and mount an atypical Th2 response in ulcerative colitis [8]. The potential of $CD45^{dim}VLA\text{-}4^+$ cells to produce IL-13 might represent one of the possible mechanisms used by these cells to prevent EAE. Interestingly $CD45^{dim}VLA\text{-}4^+$ cells were also characterized by selective expression of CCR3 chemokine receptor transcript, which is known to be a key feature of Th2 cells [5]. Exclusive expression of transcripts for IL-13 and CCR3 in $CD45^{dim}VLA\text{-}4^+$ cells suggests their resemblance to regulatory cells within the Th2 subset. The IP-15 transcript was also found to be highly transcribed in $CD45^{dim}VLA\text{-}4^+$ cells. Our previous study using the Affymetrix microarray system revealed that expression of this gene was increased 10-fold in splenocytes isolated from estrogen-treated mice [20]. Others found that this gene was induced in mice during pregnancy, and might be critical in the regulation of proteins involved in establishing and maintaining pregnancy [13]. Of note, in our previous study we showed that $CD45^{dim}VLA\text{-}4^+$ cells were induced during pregnancy, reaching the highest number in the third trimester [19]. Thus we speculate that an alternative function of $CD45^{dim}VLA\text{-}4^+$ cells could be linked to tolerance established in pregnancy. The elucidation of the roles of IL-13 and IP-15 in the protective mechanisms of $CD45^{dim}VLA\text{-}4^+$ cells will be a focus of future investigations.

While the importance of apoptosis in controlling immune homeostasis is clear, it is of equal importance for some cells, especially those with effector/suppressive functions, to remain viable for long periods of time. It is well known that $CD4^+CD25^+$ regulatory T cells express GITR, TNFR-2, OX40, and 4-1BB, marker proteins that can prolong survival by increasing expression of anti-apoptotic genes such as *bcl-xl* [11, 21, 26]. *Bcl-xl* is a well-characterized member of the *bcl-2* family of anti-apoptotic genes. *Bcl-xl* has been shown to inhibit *bax*/*bid*-induced apoptosis by blocking the release of mitochondrial cytochrome c that occurs in response to a variety of apoptotic stimuli [7, 29, 30, 32]. The anti-apoptotic gene *bcl-xl* has been shown to promote survival of cells of the erythroid, myeloid, and lymphoid lineages. Expression of *bcl-xl* was found to play an essential role in preventing neuronal cell death in embryonic and adult neurons of the CNS [4, 23]. Our results show a strong signal for *bcl-xl* expression in $CD45^{dim}VLA\text{-}4^+$ cells. One of the future goals is to clarify the role of *bcl-xl* as a survival factor in the $CD45^{dim}VLA\text{-}4^+$ cell population.

Kinetic studies *in vivo* suggest that $CD45^{dim}VLA\text{-}4^+$ cells arise 72 h after estrogen treatment and diminish in number when the exogenous hormone is cleared from the body. It is important to point out that $CD45^{dim}VLA\text{-}4^+$ cells are unstable under *in vitro* conditions, suggesting that they need an *in vivo* environment to thrive. Because of this unusual characteristic, additional manipulations of these cells for testing by ELISA and ELISPOT characterization are essentially impossible at this time. The results of the present study also suggest that the $CD45^{dim}VLA\text{-}4^+$ cells could be involved in protection against bacterial infections. We found that injection of *M. tuberculosis* stimulates the production of $CD45^{dim}VLA\text{-}4^+$ cells to a similar extent as E2 treatment. It is well known that NKT cells can be expanded by the glycolipid extracts from the cell walls of *M. tuberculosis* [3]. Further investigation will be required to understand the significance of this finding.

The data reported here suggest that $CD45^{dim}VLA\text{-}4^+$ cells include cells that exhibit some, but not all, attributes characteristic of NKT cells. It is likely that the $CD45^{dim}VLA\text{-}4^+$ cell population represents partially differentiated or even undifferentiated intermediates along the NKT lineage pathway. However, we have not excluded the possibility that this may be a heterogeneous population, including dendritic cells or their precursors. As stated before, $CD45^{dim}VLA\text{-}4^+$ cells cannot be characterized phenotypically using any commercially available antibodies (including those recognizing NK1.1, DX5, CD44, TCRa, or CD8a). However, messages encoding several known NK cell markers, including CD44, CD16, TCRa, and CD8a, were detected by RT-PCR and/or RPA. We are aware that the absence of a phenotypic characterization will stimulate controversy, but at this point we propose the hypothesis that the $CD45^{dim}VLA\text{-}4^+$ cells form a previously unknown population, possibly with unusual markers that have yet to be defined.

Many questions remain to be answered about the origin and developmental fate of the $CD45^{dim}VLA\text{-}4^+$ cell population. For instance, the nature of the signals that drives the appearance of $CD45^{dim}VLA\text{-}4^+$ cells is not clear. It is conceivable that $CD45^{dim}VLA\text{-}4^+$ cells may mature via an unidentified mechanism, similar to that exhibited by $TCR\gamma\delta^+$ T cells, which drive the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition of the host thymocytes upon injection into SCID hosts [16]. Further study will be focused on the requirements for the normal differentiation of this lymphocyte population. A better understanding of the progenitors, the role, and the significance of $CD45^{dim}VLA\text{-}4^+$ cells, also occurring naturally during pregnancy, may open new avenues of investigation in immunology. Our work may lead to new therapeutic approaches in autoimmune diseases and lead to a better understanding of the regulation of immune tolerance.

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