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Developing Lymph Nodes Collect CD4$^+$CD3$^-$ LTβ$^+$ Cells That Can Differentiate to APC, NK Cells, and Follicular Cells but Not T or B Cells

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Summary

For a brief period during fetal lymph node organogenesis in mice, lymph node postcapillary high endothelial venules surprisingly express the Peyer's patch addressin MAdCAM-1. This expression allows initial seeding of this incipient structure by two unusual lymphocyte populations selectively expressing the Peyer's patch homing receptor integrin α4β7: CD4$^+$CD3$^-$ oligolineage progenitors and TCR γδ$^+$ T cells. We show here that CD4$^+$CD3$^-$ cells are lineage-restricted progenitors that express surface lymphotoxin-β (LTβ) and the chemokine receptor BLR1 and that can become natural killer cells, dendritic antigen-presenting cells, and follicular cells of unknown outcome, but these cells do not become T or B lymphocytes. Since the necessity of lymphotoxin in lymphoid organ development has been shown, we propose that the novel subset of CD4$^+$CD3$^-$LTβ$^+$ fetal cells is instrumental in the development of lymphoid tissue architecture.

Introduction

Lymphocyte homing to lymph nodes is mediated by a set of adhesion molecules expressed on endothelial cells (addressins) and their ligands (homing receptors) on lymphocytes. Lymphocytes in the bloodstream enter lymph nodes by adhering to and migrating through the high endothelial venules (HEV), specialized postcapillary endothelial cells that express adhesion molecules (Gowans and Knight, 1964; Kraal and Mebius, 1996). Because of differential expression of adhesion molecules on HEV in lymph nodes at different locations—namely peripheral node addressin (PNAd) on HEV in peripheral lymph nodes (PLN), and PNAd and MAdCAM-1 on HEV in mesenteric lymph nodes (MLN)—lymphocytes are directed to different sites depending on their homing receptor expression profile.

The tissue distribution of these adhesion molecules involved in directing the lymphocyte flow to different lymph nodes in adult mice is different from the distribution in fetal and perinatal stages, when the first colonization of lymph nodes occurs (Mebius et al., 1996). MAdCAM-1 and α4β7 are the main adhesion molecules that direct the colonization of both PLN and MLN in late fetal and early neonatal life. Beginning at 24–48 hr after birth, PNAd expression begins on the luminal side of LN HEV, allowing L-selectin–expressing cells in the bloodstream to bind to and transmigrate through the endothelial cell barrier in a L-selectin/PNAd–dependent fashion. Before that time, cell traffic into the nodes is directed mainly by the MAdCAM-1/α4β7 receptor/ligand pair, even though the majority of nucleated blood cells are L-selectin positive (Mebius et al., 1996). Interestingly, cells expressing α4β7 are infrequent in peripheral blood of fetal mice, while the majority of the cells in the lymph nodes are α4β7$^+$, suggesting a highly selective entrance mechanism for the first cells that colonize the lymph node, although proliferation of these cells within the lymph node cannot be excluded. One population of cells that express α4β7 and that enter lymph nodes before birth consists of CD4$^+$ cells that lack expression of CD3 (Mebius et al., 1996), first described by Kelly and Scollay (1992). The unusual phenotype of these cells demanded their further characterization.

Results

CD4$^+$CD3$^-$ Cells Are a Unique Subset of Cells That Express the Integrin α4β7 Homing Receptor and That Arise in Fetal Spleen and Selectively Enter Fetal Lymph Node before Birth

We have observed an unexpected and exclusive expression of MAdCAM-1 on HEV in PLN and MLN of fetal and newborn mice until 24 hr after birth. During this interval, a unique cell population of CD4$^+$CD3$^-$ cells enters the lymph nodes using α4β7, a ligand for MAdCAM-1 (Mebius et al., 1996). Since a specific adhesion mechanism is in place for this cell population to colonize lymph nodes, we chose to study these cells in more detail.

At first, fetal peripheral blood was analyzed to determine the frequency of these cells. To our surprise the frequency of CD4$^+$CD3$^-$ cells in peripheral blood was low at all time points studied (14-, 15-, 17-, and 18-day-old fetuses) (Figure 1A). The peak in the percentage of CD4$^+$CD3$^-$ cells in peripheral blood occurred at 15.5 days postconception, but the total percentage of CD4$^+$CD3$^-$ cells in peripheral blood never exceeded 1%. Considering the low percentage of CD4$^+$CD3$^-$ cells found in peripheral blood it was surprising that more than 4% of MLN cells were CD4$^+$CD3$^-$ in 17.5-day-old embryos. Since MLN isolated from day 16–18 embryos cannot be dissected completely free of surrounding tissue, the estimated percentage of CD4$^+$ cells measured by fluorescence-activated cell sorting (FACS) could be low. Indeed, sections of MLN taken from day 16 embryos showed that approximately 30% of the cells expressed CD4, while CD3 was absent.

To test whether CD4$^+$CD3$^-$ cells could also be found

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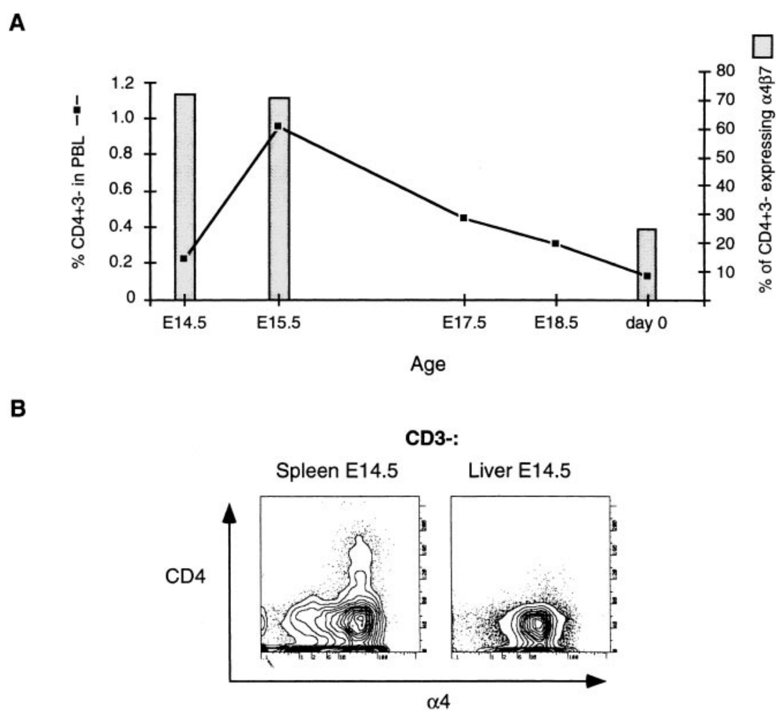

Figure 1. $CD4^+CD3^-$ Cells Can Be Found in Fetal Peripheral Blood and Spleen but Not in Fetal Liver

(A) Percentage of $CD4^+CD3^-$ cells in peripheral blood leukocyte preparations from 14-, 15-, 17-, and 18-day-old embryos and newborn mice. The percentage of α4β7-expressing $CD4^+CD3^-$ cells in 14- and 15-day-old embryos and in newborn mice was determined using MAb Datk-32, GK1.5, and 145–2C11, recognizing α4β7, CD4, and CD3 respectively.

(B) Analysis of fetal liver and fetal spleen from 14-day-old embryos for the presence of $CD4^+CD3^-$ cells in combination with expression of α4. $CD4^+CD3^-$ cells could be detected in fetal spleen, while no obvious population of $CD4^+CD3^-$ cells could be observed in fetal liver.

in other lymphoid organs, fetal spleen, liver, and thymus were analyzed. Analysis of fetal spleen showed that $CD4^+CD3^-$ cells could also be found in fetal spleen (E13.5–E17.5) with frequencies ranging from 0.2%–2.5% around E14.5 (Figure 1B and data not shown). From fetal days 11 to 15.5, the liver is the principal repository of multipotent hematopoietic stem cells (Morrison et al., 1995). FACS analysis of cells from E12.5 to E14.5 revealed that $CD4^+$ cells were infrequent and attempts to sort $CD4^+CD3^-$ cells from fetal livers were unsuccessful (Figure 1B and data not shown). Thus, while it is likely that $CD45^+CD4^+CD3^-$ cells are derived from fetal liver hematopoietic stem cells, if they are, this phenotype must be induced in secondary sites (such as the fetal spleen). We then tested whether the thymus, the other major lymphopoietic organ at 12–17 days postconception, harbored the cells. Analysis of the fetal thymus for $CD4^+CD3^-$ cells revealed that this population was undetectable. Thus $CD4^+CD3^-$ $α4β7^+$ cells are first found in early (13.5 days postconception) fetal spleens.

Newborn Lymph Node $CD4^+CD3^-$ Cells Are Lymphotoxin-$β^+$ and Express Lymphoid Cytokine Receptors

$CD4^+CD3^-$ cells belong to the hematolymphoid lineages since the leukocyte common antigen CD45 (Ly5) was uniformly expressed on $CD4^+CD3^-$ cells (Table 1). FACS analysis of newborn $CD4^+CD3^-$ cells derived from lymph nodes showed that the cells express ICAM-1, CD44, and IL-7Rα at high levels, while about 75% of the cells express the IL-2Rα chain (CD25) (Table 1). Staining for the common γ chain of the IL-2 receptor (IL-$2Rγ_c$) showed that all $CD4^+CD3^-$ cells express this subunit common to the IL-2, IL−4, IL−7, IL-9, and IL−15 receptor (Table 1).

One early cellular event, described here, is the selective entry of $CD4^+CD3^-$ cells into the developing lymph nodes. Recently, multiple mutant mouse strains that lack lymph nodes have been described. Null mutations of lymphotoxin-α (LTα), LTβ, RelB, and IL-2Rγ chain all result in lack of lymph nodes (Lo et al., 1992; Togni et al., 1994; Banks et al., 1995; Burkly et al., 1995; Cao et al., 1995; Weih et al., 1995; Eugster et al., 1996; Koni et al., 1997). In addition, injection of LTβ-R-Ig chimeras into pregnant mothers results in lack of development of PLN in newborn mice (Rennert et al., 1996). In addition, B cell follicle formation also seems to involve signaling through the tumor necrosis factor (TNF) and LTβ receptor (Pasparakis et al., 1996; Rennert et al., 1997). The cellular and molecular events that are involved in the formation of lymph nodes and follicles have not been defined.

Could early colonization of LN by $CD4^+CD3^-$ cells be involved in the formation of lymph nodes or follicles? Analysis of $CD4^+CD3^-$ cells for expression of LTβ, RelB, or IL-2Rγ chain was performed. FACS analysis of $CD4^+CD3^-$ cells, using an LTβ-R-Ig fusion protein, showed expression of LTβ on $CD4^+CD3^-$ in MLN at E18.5 (Figure 2A). Subsequently, $CD4^+CD3^-$ cells from MLN, taken within 6 hr after birth, were sorted into lysis buffer and reverse-transcribed to obtain cDNA. Analysis of cDNA with specific primers for *relB* and *LT*β showed both bands of the appropriate size, which could be amplified with one internal primer for each band (Figure 2B). In addition, we also observed expression of IL-2Rγ chain on $CD4^+CD3^-$ cells (Figure 2C).

$CD4^+CD3^-LTβ^+IL\text{-}2Rγ^+$ cells, derived from MLN at birth, are negative for the natural killer (NK)– specific antigens NK1.1 (Koo and Peppard, 1984; Yokoyama and Seaman, 1993) and 2B4 (Garni-Wagner et al., 1993) (Table 1). Staining in situ for the dendritic cell markers

Table 1. Phenotypic Characterization of $CD4^+$ $CD3^-$ Cells Isolated from MLN on Day 0

Surface Antigen	Expression
ICAM-1	+
CD44	+
α4	+
β7	+
α4β7	+
L-selectin	−
MHC class I	+
MHC class II	+(±50%)
TCR αβ	−
TCR γδ	−
CD3	−
CD5	−
sIgM	−
B220	−
Ly5	+
Thy1.1	+
Thy1.2	Low to medium
IL-2R	+(±75%)
IL-7R	+
IL-2Rγ_c	+
Sca-1	−
c-Kit	− to low
Mac-1	−
Gr-1	−
NLDC-145	−
CD11c	−
B7-1	−
B7-2	−
CD40	−
NK1.1	−
FcγRIII/II	Low to medium
CR1/CR2	−

NLDC-145 and N418, recognizing DEC-205 and CD11c (integrin p150α) respectively (Kraal et al., 1986; Metlay et al., 1990), showed that $CD4^+CD3^-$ cells lack expression of both markers. No accessory molecules, such as B7-1, B7-2, or CD40 could be observed at normal levels of expression at this stage of development. All $CD4^+$ $CD3^-$ cells lack expression of CD5. Staining with monoclonal antibody (MAb) 2.4G2, recognizing FcγRIII/II (CD16/32), showed expression of this receptor on $CD4^+CD3^-$ cells. No expression of CR1/CR2 could be detected.

To study whether $CD4^+CD3^-$ cells expressed markers of more primitive hematolymphoid progenitors, we tested for the presence of Sca-1 and c-Kit. These markers have been described to be present on hematopoietic stem cells and T cell progenitors (Spangrude et al., 1991; Akashi and Weissman, 1996; Randall et al., 1996). $CD4^+$ $CD3^-$ cells do not express Sca-1 and show low, if any, expression of c-Kit (Table 1).

$CD4^+CD3^-$ Cells Are Neither T Lymphocytes nor T Lymphocyte Precursors

$CD4^+CD3^-$ cells lack expression of CD3, while mature T lymphocytes normally express CD3 proteins as part of the T cell receptor complex. To test whether $CD4^+CD3^-$ cells are T lymphocytes, $CD4^+CD3^-$ cells from MLN of newborn mice were stained for T cell receptor (TCR) αβ, TCR γδ, and Thy1. Stainings for TCR showed no expression of TCR αβ or TCR γδ (Table 1). To study Thy1 expression, we analyzed both Thy1.1 and Thy1.2 in C57BL/KA-Thy1.1 and C57BL/KA-Thy1.2 mice, respectively. In C57BL/KA-Thy1.1 mice the expression of Thy1.1 on $CD4^+CD3^-$ cells was high, while the expression of Thy1.2 on these cells from Thy1.2 mice was low to medium (Table 1) (In these two congenic lines, the same relative differential expression of Thy1 is also found on adult bone marrow hematopoietic stem cells [Spangrude and Brooks, 1992]).

Subsequently, newborn nude mice were analyzed to test whether the dysplastic thymus could affect the number of $CD4^+CD3^-$ cells that could be found in lymph nodes. In MLN of control, heterozygous, and homozygous nude mice, we observed $CD4^+CD3^-$ cells in comparable numbers, suggesting that the existence of $CD4^+CD3^-$ cells is thymus independent.

To examine more exhaustively the possibility that $CD4^+CD3^-$ cells belong to the T lymphocyte lineage, the configuration of the TCR was analyzed. Since rearrangement of the β chain of the TCR precedes rearrangement of the α chain, we used primers to determine whether Dβ1.1 to Jβ1.7 or Dβ2.1 to Jβ2.7 were rearranged. $CD4^+CD3^-$ cells from newborn MLN were sorted into lysis buffer and genomic DNA was analyzed by polymerase chain reaction (PCR). Figure 3A shows that the TCR genes of $CD4^+CD3^-$ cells are in germline configuration when analyzed for rearrangement from Dβ1.1 to Jβ1.7 and Dβ2.1 to Jβ2.7, while control $CD8^+$ thymocytes show rearrangement of the TCR β chain (Figure 3A). In addition, analysis of cDNA derived from sorted $CD4^+CD3^-$ cells showed no expression of the pre-T cell receptor-α (pTα) when analyzed by PCR with seminested primers (data not shown).

To analyze the potential of $CD4^+CD3^-$ cells as precursors to T lymphocytes, $CD4^+CD3^-$ cells were placed in a fetal thymic organ culture (FTOC). After culturing the cells for 2 weeks in irradiated *Rag2*$^{-/-}$ thymic lobes, no maturation toward the T lymphocyte lineage could be observed (data not shown).

$CD4^+CD3^-$ Cells Are Neither B Cells nor Precursors to the B Lymphocyte Lineage

Our results suggest that $CD4^+CD3^-$ cells do not belong to the T lymphocyte lineage since the TCR genes are in germline configuration, no expression of pTα can be detected, and $CD4^+CD3^-$ cells do not develop toward the T lymphocyte lineage when placed in an FTOC. The question arises: do they belong to the class of lymphocytes that rearrange and express antigen receptors at all? $CD4^+CD3^-$ cells were analyzed for characteristics of B lymphocytes. Staining of $CD4^+CD3^-$ cells, derived from MLN taken from newborn mice within 24 hr after birth for B220 and sIgM, showed that all $CD4^+CD3^-$ cells are negative for both (Table 1). In addition, analysis of genomic DNA from $CD4^+CD3^-$ cells with primers to analyze IgH chain genes showed that genes for the IgH chain are in germline configuration, while B cells show rearranged bands (Figure 3B). As a negative control, T cells were analyzed: sorted T cells were resorted twice to ensure purity. In this population we observe the genes for IgH chain in germline arrangement and some rearranged bands, a phenomenon previously described for T cells (Kurosawa et al., 1981).

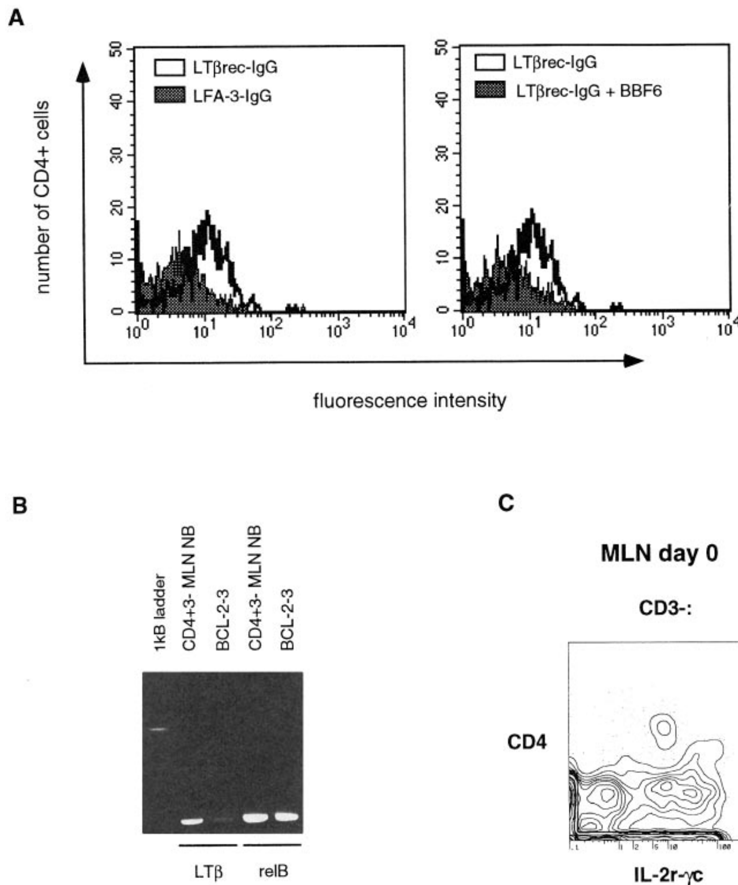

Figure 2. $CD4^+CD3^-$ Cells Express LTβ, RelB, and IL-$2R\gamma_c$

(A) Staining of $CD4^+CD3^-$ cells for surface expression of LTβ using murine LTβ-R-Ig. As a negative control, cells were incubated with LFA-3-Ig or LTβ-R-Ig in the presence of anti-LTβ MAb (BBF6). MLN were isolated from embryos at 18.5 days of gestation (E18.5). At this time point no $CD4^+CD3^+$ cells could be observed, and when double staining for CD4 and LTβ-R-Ig was performed, all $CD4^+$ cells showed low to medium staining with LTβ-R-Ig but no staining with LFA-3-Ig or LTβ-R-Ig in the presence of blocking anti-LTβ MAb.

(B) Analysis of cDNA from $CD4^+CD3^-$ cells isolated from MLN on day 0 for *LT*β and *relB* by RT-PCR showed that bands for both *LT*β as well as *relB* could be observed when seminested primers were used.

(C) Staining of $CD4^+CD3^-$ cells, taken from MLN on the day of birth, for IL-$2R\gamma_c$ showed that all $CD4^+CD3^-$ cells were positive.

To test ultimately whether $CD4^+CD3^-$ cells had no progenitor–progeny relation to B lymphocytes, we placed sorted $CD4^+CD3^-$ cells on S17 stromal cells in the presence of IL-7. Although the bone marrow cells gave B cell colonies at a frequency of 0.1%, no maturation toward B cell lineage could be observed for $CD4^+CD3^-$ cells.

To test whether $CD4^+CD3^-$ cells belong to the rearranging antigen receptor lymphocyte class, we analyzed the presence of mRNA for recombinase genes *Rag1* and *Rag2* by reverse transcription (RT)–PCR. $CD4^+CD3^-$ cells, derived from MLN of newborn mice, were sorted into lysis buffer and reverse transcribed for the production of cDNA. PCR reactions with primers specific for *Rag1* and *Rag2* showed that $CD4^+CD3^-$ cells do not have mRNA for these two recombinase genes (Figure 3C). Analysis of *Rag2*$^{-/-}$ and severe combined immunodeficient (SCID) mice revealed a diminished but distinguishable population of $CD4^+CD3^-$ cells (data not shown; see also Figure 6B, below). Therefore it is possible that lymphogenesis might affect the genesis and/or maintenance of $CD4^+CD3^-$ cells.

$CD4^+CD3^-$ Cells Can Be Cultured in IL-2 and Generate $NK1.1^+$ NK Cells

Since the majority of $CD4^+CD3^-$ cells express CD25 (IL-2Rα) and the IL-$2R\gamma_c$ chain, $CD4^+CD3^-$ cells could potentially proliferate and differentiate in response to IL-2. $CD4^+CD3^-$ cells from MLN, taken from newborn mice within 24 hr after birth, were sorted and cultured in the presence of IL-2. At the population level, the cells showed morphological characteristics of activation and proliferated (Figure 4A). To assess the frequency of $CD4^+CD3^-$ cells that are responsive to IL-2, $CD4^+CD3^-$ cells were double sorted and single cells were deposited into separate wells of 96-well plates. The cells were cultured in IL-2, on AC-6.2.1 feeder layers, and were analyzed for clonal expansion. After approximately 10 days, outgrowth of cells was obvious and the frequency of IL-2–responding cells was determined. Approximately 10%–20% of the wells showed clonal expansion of cells, varying among experiments. Further analysis of the clones showed that ±80% of them expressed NK1.1, and all NK1.1-expressing clones were able to kill YAC-1 cells in the presence of concanavalin A (Figure 4B). Since NK1.1 is not expressed on $CD4^+CD3^-$ cells when analyzed directly after isolation from newborn lymph nodes, and freshly isolated $CD4^+CD3^-$ cells lack the ability to kill YAC-1 cells, differentiation of $CD4^+CD3^-$ progenitors into NK cells must have occurred (Figure 4C).

$CD4^+CD3^-$ Cells Contain APC Precursors

When $CD4^+CD3^-$ newborn MLN cells were stained for expression of I-A or I-E, approximately 50% of the cells showed expression of class II (Figure 5A). To test whether $CD4^+CD3^-$ cells are precursors for macrophages or dendritic cells, we plated a sorted population

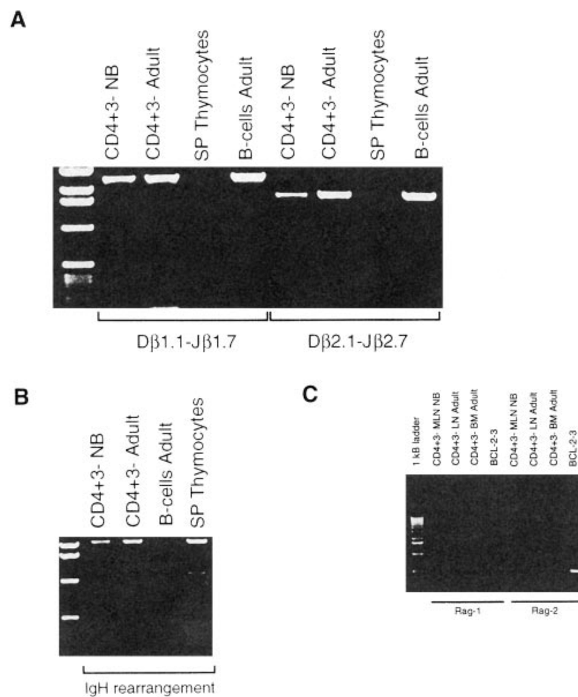

Figure 3. CD4^{+}CD3^{-} Cells Rearrange Neither the TCR β chain nor the IgH Chain, and They Do Not Contain mRNA Coding for the Recombinase Enzymes RAG1 and RAG2

(A) Characterization of CD4^{+}CD3^{-} cells as potential members of the T lymphocyte lineage. Analysis of the β chain of the TCR in genomic DNA isolated from triple-sorted CD4^{+}CD3^{-} cells showed no rearrangements but germline configuration of the gene. As a positive control, CD8^{+} cells were sorted from the thymus, and as a negative control, B lymphocytes were sorted from adult MLN.

(B) Characterization of CD4^{+}CD3^{-} cells as potential B lymphocyte lineage members. Genomic DNA isolated from triple-sorted CD4^{+}CD3^{-} cells showed no rearrangement of the IgH chain genes. As a positive control, B lymphocytes sorted from adult MLN were used and CD8^{+}CD3^{+}CD4^{-} cells sorted from the thymus were used as a control for germline configuration.

(C) Analysis of cDNA from CD4^{+}CD3^{-} cells isolated from MLN on day 0 and 8 weeks after birth and from bone marrow at 8 weeks after birth for the presence of mRNA coding for the recombinase enzymes RAG1 and RAG2. In all CD4^{+}CD3^{-} samples cDNA for *Rag1* and *Rag2* could not be found by RT-PCR. As a positive control, a B cell lymphoma, BCL2-3, was used (Chen et al., 1994).

of CD4^{+}CD3^{-} cells, derived from MLN, in methylcellulose in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), according to the method described by Inaba et al. (Inaba et al., 1993). As a positive control, we sorted major histocompatibility (MHC) class II–negative precursors from bone marrow that can give rise to macrophages and dendritic cells when grown in semisolid culture in the presence of GM-CSF (Inaba et al., 1993). After a culture period of 14 days, we observed multiple colonies in our positive bone marrow control, but no colonies in the cultures of CD4^{+}CD3^{-} cells in GM-CSF. Even after prolonged culture periods no colonies from CD4^{+}CD3^{-} cells could be observed, suggesting that the CD4^{+}CD3^{-} population does not contain clonal activity for macrophages or dendritic cells inducible by GM-CSF alone (data not shown).

Since approximately 50% of CD4^{+}CD3^{-} cells taken from MLN within 24 hr after birth express MHC class II molecules, we were interested to determine whether CD4^{+}CD3^{-} cells could present antigen. CD4^{+}CD3^{-} cells were sorted from MLN within 24 hr after birth and cultured overnight with the pigeon cytochrome C–specific T cell hybridoma 2B4 (Hedrick et al., 1982) in the presence and absence of cytochrome C. As a positive control for antigen presentation, a lipopolysaccharide-activated B cell line was used. After 18 hr of culture, the supernatants were analyzed for the presence of IL-2 as a measure of the activation of 2B4 cells by cytochrome C on class II–positive cells. Production of IL-2 by 2B4 cells could be induced by CD4^{+}CD3^{-} cells in the presence of cytochrome C. However, on a cell-to-cell basis the induction of IL-2 production by CD4^{+}CD3^{-} cells was much lower when compared to lipopolysaccharide-stimulated B cells (data not shown).

To test whether CD4^{+}CD3^{-} cells could further differentiate into antigen-presenting cells (APC), we cultured the cells overnight in the presence of IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994) and subsequently placed the cells as effector cells in an allogeneic mixed lymphocyte reaction (MLR). As a control, CD4^{+}CD3^{-} cells cultured overnight in medium alone and CD4^{-} cells cultured with and without IL-4 and GM-CSF were used. As a positive control, dendritic cells isolated from PLN were used. CD4^{+}CD3^{-} cells cultured overnight in IL-4 and GM-CSF were as potent in inducing allogeneic T lymphocytes to proliferate as were purified dendritic cells (Figure 5B). CD4^{+}CD3^{-} cells cultured in medium alone and CD4^{-} cells were not able to induce allogeneic T lymphocytes to proliferate (Figure 5B). Thus, CD4^{+}CD3^{-} cells contain potent progenitors of dendritic APC. During the course of our studies it was reported that thymic precursors, isolated from mature thymus, were induced to proliferate and differentiate to dendritic cells when grown in the presence of a different mixture of five to seven cytokines (Saunders et al., 1996). Since in these studies GM-CSF was not necessary for differentiation, we tested whether CD4^{+}CD3^{-} cells could also differentiate into dendritic APC in the absence of GM-CSF. We therefore cultured the cells in IL-1β, IL-3, IL-7, and TNFα. This mixture of cytokines induced proliferation of CD4^{+}CD3^{-} cells and cluster formation characteristic for dendritic cells as described (Saunders et al., 1996). However, after 5 days of culture, 10% of the cells were found to express low levels of MHC class II, while the remaining 90% were negative, in contrast with the CD4^{lo} precursors found in the thymus (Saunders et al., 1996). The class of cells that develop in this cytokine mixture needs to be determined.

CD4^{+}CD3^{-} Cells Can Be Found in B Cell Areas

To determine the location of CD4^{+}CD3^{-} cells within the lymph nodes, cryostat sections of newborn MLN and PLN were stained for CD3 and CD4. In lymph nodes taken within 24 hr after birth, CD4^{+}CD3^{-} cells could be found throughout the lymph nodes. At this time very few T cells are present in the lymph nodes and distinct B and T cell areas are absent (Friedberg and Weissman, 1974; R.E.M., unpublished data). Upon staining of the lymph node sections at 2, 3, and 4 days after birth with

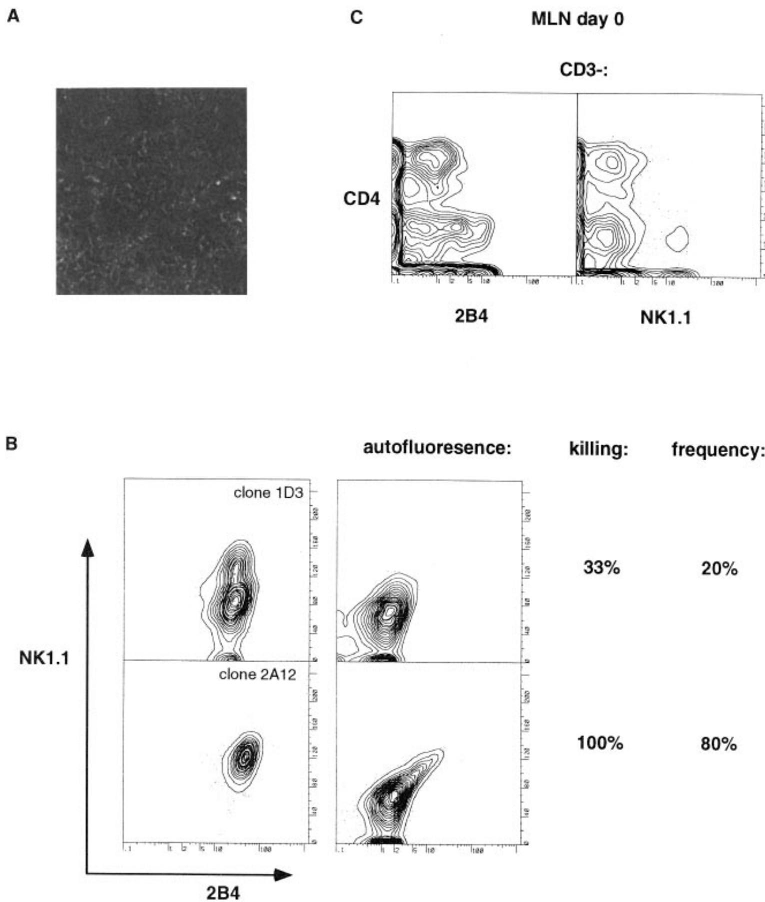

Figure 4. CD4$^+$CD3$^-$ Cells Can Proliferate in the Presence of IL-2 and Differentiate into NK Cells, While Freshly Isolated CD4$^+$CD3$^-$ Cells Do Not Express NK Cell–Specific Antigens

(A) Overnight culture of bulk-sorted CD4$^+$ CD3$^-$ cells in the presence of IL-2 (250 U/ml) showed that CD4$^+$CD3$^-$ cells responded well to this cytokine.

(B) Culture of CD4$^+$CD3$^-$ cells in IL-2 on AC-6.2.1 feeder layers. CD4$^+$CD3$^-$ cells were sorted twice and deposited at a single cell/ well in a 96-well plate containg AC-6.2.1 feeder layers and IL-2 (250 U/ml). Within 10 days growth could be observed in 10%–20% of the wells, varying among experiments. The colonies were analyzed for NK surface markers by staining with MAb NK1.1 and 2B4 and their ability to kill YAC-1 cells in the presence of concanavalin A. Cells derived from NK1.1$^+$ colonies could kill 100% of the target cells at a E:T ratio of 20, while lack of NK1.1 surface molecule coincided with less efficient killing of the target cells. Eighty percent of the colonies that grew out in the presence of IL-2 expressed NK1.1.

(C) Analysis of freshly isolated CD4$^+$CD3$^-$ cells for the expression of NK surface markers by staining with MAb NK1.1 and 2B4 show that CD4$^+$CD3$^-$ cells lack expression of NK surface markers when taken directly from MLN on day 0.

MAb 6B2, recognizing B220 (Coffman and Weissman, 1981), B cell follicles could easily be distinguished at 4 days after birth. At 2 days after birth the distinction between B and T cell areas became obvious. Lymph node sections of 2-day-old animals analyzed for CD4$^+$ CD3$^-$ cells revealed CD4$^+$CD3$^-$ cells in the cortical area, where B cell follicles were then starting to form (Figure 6A). When PLN from SCID mice were analyzed at 10 days after birth, CD4$^+$CD3$^-$ cells had accumulated in the cortical area of the lymph nodes (Figure 6B). That CD4$^+$CD3$^-$ cells specifically migrate to the B cell follicles could further be confirmed in transfer studies. Ly5.2–expressing CD4$^+$CD3$^-$ cells, isolated from lymph nodes of newborn mice, were transferred into C57BL6/Ly5.1 hosts on the day of birth. At 8 days after transfer, Ly5.2$^+$ donor cells were always found in B cell follicles, surrounded by FDC-M2$^+$ cells (Figures 6C and 6D).

Recently, the chemokine receptor BLR1 has been shown to play a role in B cell migration to the follicles (Forster et al., 1996), where we also observe relatively high numbers of CD4$^+$CD3$^-$ cells and where we find the cells after transfer. Therefore, cDNA derived from sorted CD4$^+$CD3$^-$ cells was analyzed for expression of *Blr1* by seminested PCR. As expected from our transfer studies, we observed *Blr1* expression in all lymph node CD4$^+$ CD3$^-$ cDNA samples tested (Figure 7).

Discussion

A cell population characterized by uniform expression of CD4 and lack of CD3 can be found at relative high numbers in neonatal lymph nodes (Kelly and Scollay, 1992; Mebius et al., 1996). Our results show that CD4$^+$CD3$^-$ cells demonstrate uniform expression of CD45, and thus belong to the hematopoietic system and form a newly described subset of hematolymphoid cells. CD4$^+$CD3$^-$ cells enter the lymph nodes during fetal life and 0–1 days after birth, using the Peyer's patch homing receptor, integrin $\alpha4\beta7$ on their surface, and its ligand MAdCAM-1 on the opposing HEV (Mebius et al., 1996). The population contains NK cell precursors and dendritic APC precursors, and most cells tend to localize in B cell follicles.

Further characterization of CD4$^+$CD3$^-$ cells shows that they rearrange neither the β chain of the TCR nor the IgH chain, that they do not express pTα, and that they lack mRNA for the recombinase enzymes RAG1 and RAG2, suggesting that CD4$^+$CD3$^-$ cells do not belong to the T or B lymphocyte–rearranging subset of hematolymphoid cells. That CD4$^+$CD3$^-$ cells do not belong to the B lymphocyte lineage is further supported by their inability to differentiate into B220-expressing B cells in IL-7–containing S17 cultures. That CD4$^+$CD3$^-$ cells do not belong to the T lymphocyte lineage is further substantiated by the presence of CD4$^+$CD3$^-$ cells in neonatal lymph nodes from nude, *Rag2*$^{-/-}$, and SCID mice and by their inability to differentiate into T lymphocytes when placed in FTOC, as shown for CD4$^+$CD3$^-$ cells isolated from human peripheral blood (Bruno et al., 1997). However, human CD4$^+$CD3$^-$ cells present in peripheral blood express pTα and low levels of CD3-ϵ and have initiated rearrangements of the TCR β chain—all

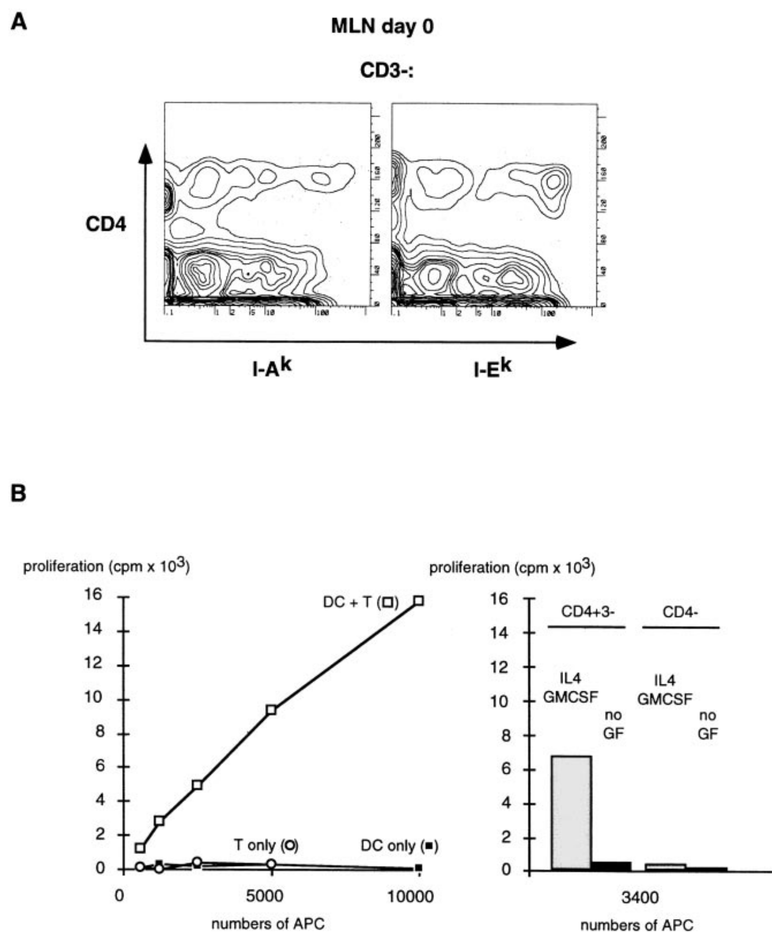

Figure 5. Fifty Percent of CD4$^+$CD3$^-$ Cells Express MHC Class II, and Overnight Culture in IL-4 and GM-CSF Induces CD4$^+$CD3$^-$ Cells to Become Potent APC

(A) Analysis of newborn CD4$^+$CD3$^-$ cells for the expression of MHC class II molecules showed that approximately 50% of the cells expressed I-A^k or I-E^k.

(B) CD4$^+$CD3$^-$ cells were cultured overnight in the presence of IL-4 and GM-CSF. As a control, CD4$^+$CD3$^-$ cells were cultured overnight with media alone (no growth factor). Also, all cells from MLN except CD4$^+$CD3$^-$ cells were exposed to the same culture conditions (with or without IL-4 and GM-CSF). The cells were subsequently mixed with 10^5 allogeneic T lymphocytes. As a positive control, purified dendritic cells, isolated from PLN and MLN, were mixed with the same number of T lymphocytes, and as a negative control, T cells or dendritic cells alone were placed in a well. After 4 days [^{3}H]thymidine (1 μCu/well) was added, and after 6 hr the incorporation of [^{3}H]thymidine was measured, showing that CD4$^+$CD3$^-$ cells cultured overnight in IL-4 and GM-CSF are as potent in inducing an allogeneic MLR as are dendritic cells isolated from lymph nodes.

features absent in the cells described here (Bruno et al., 1997).

CD4$^+$CD3$^-$ cells could not be found in the fetal liver or thymus between days E12.5 and E15.5. The population was present in the spleen, as early as the spleen could be isolated (E13.5), suggesting that the fetal spleen could be the site of their development. However, analysis of neonatal lymph nodes from spleenless Hox11$^{-/-}$ mice shows that CD4$^+$CD3$^-$ cells are present; thus, the spleen per se is not mandatory for their development (data not shown). Another place of origin could be the aorta-gonad-mesonephros region, which has recently been identified as a preliver intraembryonic site that harbors precursors of adult type hematopoietic stem cells (Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993; Müller et al., 1994).

The observed activities for NK cells and APC within one cell population are noteworthy, since a common human lymphoid precursor that gives rise to T and B lymphocytes, NK cells, and dendritic cells has been described (Galy et al., 1995), as has a mouse cell population with the same potentials (Wu et al., 1991; Wu et al., 1996; Kondo et al., unpublished data). From adult thymus, a precursor population, with low expression levels of CD4, that has the ability to generate dendritic, B, T lymphoid, and NK cells, can be isolated (Wu et al., 1991, 1996; Ardavin et al., 1993; Matsuzaki et al., 1993; Saunders et al., 1996). However, this activity cannot be seen when these cells are isolated from fetal thymus. The low expression level of CD4 on these precursors and the inability to find these cells in fetal and newborn animals (Antica et al., 1993) suggest that this precursor population resembles one unrelated to the CD4$^+$CD3$^-$ cells described here. Another population of early fetal thymocytes, which can give rise to T lymphocytes and NK cells, has been described (Rodewald et al., 1992). Although this population has similarities to the CD4$^+$CD3$^-$ cells described here, such as expression of FcγRIII/II, IL-2Rα, and Thy1 and maturation into NK cells in response to IL-2, expression of CD4 is absent on these cells (Rodewald et al., 1992).

Immunohistological stainings of fetal and neonatal lymph nodes show that CD4$^+$CD3$^-$ cells are scattered throughout the lymph nodes until 2 days after birth, when they tend to localize in the cortical area of the lymph node. This localization can be observed in the absence or presence of classic B and T lymphocytes, suggesting that the localization of CD4$^+$CD3$^-$ cells does not depend on the presence of rearranging lymphocytes. Expression of the putative chemokine receptor BLR1, directing B cell migration to splenic follicles, can be detected in CD4$^+$CD3$^-$ cells, suggesting a role for BLR1 in the positioning of CD4$^+$CD3$^-$ cells in the cortical area. This is further supported by our transfer studies, in which we always found the donor cells localized in the B cell follicles. This localization of CD4$^+$CD3$^-$ cells

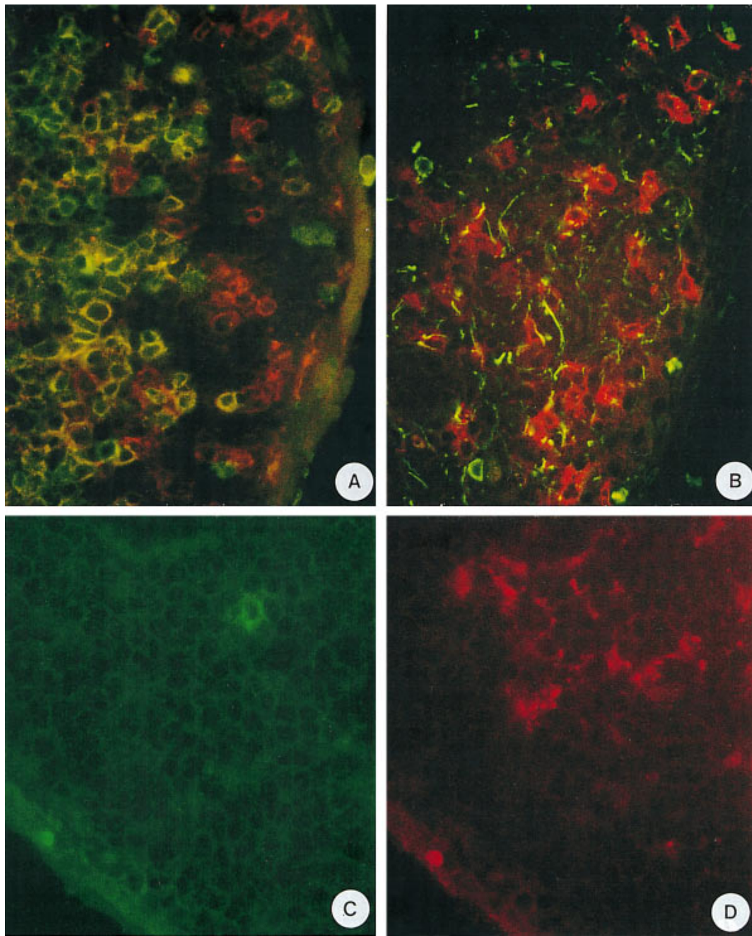

Figure 6. $CD4^+CD3^-$ Cells Can Be Found in the Cortical Area of Developing Lymph Nodes and Localize in B Cell Follicles upon In Vivo Transfer

(A) Double staining of PLN from 2-day-old mice for CD4 (rhodamine) and CD3 (fluorescein isothiocyanate). With the combination of red and green all $CD4^+CD3^-$ cells are red, while $CD4^+CD3^+$ cells are yellow. At this time point B and T cell areas in PLN can be distinguished. Most $CD4^+CD3^-$ cells can be found in the cortical area of the lymph nodes.

(B) Double staining of PLN from 10-day-old SCID mice for CD4 (rhodamine) and CD3 (fluorescein isothiocyanate). All the $CD4^+CD3^-$ cells line up in the cortical area.

(C and D) Double staining of day 8 lymph node derived from a C57BL6/Ly5.1 mouse that received 2000 sorted $CD4^+CD3^-$ cells (derived from C57BL/Ka (Ly5.2) mouse) intravenously on the day of birth for donor marker Ly5.2 (C) and in the same section for FDC-M2 (D). The Ly5.2 donor cell can be found among the FDC-M2–expressing cells in the primary follicles.

around the time that follicles are being formed, before follicular dendritic cells (FDC) can be detected, is suggestive of a third progenitor activity for $CD4^+CD3^-$ cells as FDC precursors. However, FDC are MAdCAM-1^+, and these precursors express the MAdCAM-1 ligand integrin $\alpha4\beta7$ (Berlin et al., 1993; Szabo et al., 1997; R. E. M., unpublished data). Previous experiments have indicated that in adult radiation chimeras, FDC are not bone marrow derived, which has led to the conventional wisdom that they are stromal elements independent of hematopoietic lineages (Humphrey et al., 1984). However, if FDC precursors are produced only from hematopoietic precursors in late fetal/early neonatal life, and if they use the integrin $\alpha4\beta7$/MAdCAM-1 adhesion pair to enter neonatal lymph nodes, adult radiation chimeras could not test whether they are derived from hematopoietic stem cells. Because FDC are $CD45^-$ ($Ly5^-$) (Schriever et al., 1991), we cannot use Ly5 congenic pairs to test the hypothesis that FDC are also derived from $CD4^+CD3^-$ cells. At this point we can only surmise that $CD4^+CD3^-$ cells are FDC precursors or cells that aid in the development of FDC.

Another cell population with dendritic morphology present in germinal centers has been described recently (Grouard et al., 1996). These cells are defined by expression of CD4 and CD11c and lack of CD3 expression (Grouard et al., 1996). $CD4^+CD3^-$ cells, described here, are similar to these $CD4^+CD11c^+CD3^-$ dendritic cells (GCDCs) in the expression of markers such as CD44, CD45, and ICAM-1, and lack of CD3, $TCR\alpha\beta$, $TCR\gamma\delta$, B7-1, and B7-2 (Grouard et al., 1996). The localization of GCDCs in germinal centers and $CD4^+CD3^-$ cells in B cell follicles suggests that these subsets could be related. However, $CD4^+CD3^-$ cells lack expression of CD11c, one of the three antigens used to identify GCDCs (Grouard et al., 1996). In addition, the ultrastructural characteristics of $CD4^+CD3^-$ cells are very similar to early precursors, while GCDCs have characteristics of dendritic cells (Grouard et al., 1996; data not shown). The observation that $CD4^+CD3^-$ cells become strong inducers of an allogeneic reaction after overnight culture in IL-4 and GM-CSF, while GCDCs can induce strong proliferation of allogeneic T cells without prior culture, supports the idea that the $CD4^+CD3^-$ cell population could contain precursors to GCDCs.

$CD4^+CD3^-$ cells show surface expression of $LT\beta$ and IL-$2R\gamma_c$, and at the mRNA level, using RT-PCR, contain message for *LT*β, *relB*, and *Blr1*. It is noteworthy that a cell population that is relatively abundant in the developing lymph nodes expresses four markers ($LT\beta$, RelB, γ_c receptor, and BLR1) involved in the formation of lymphoid tissue architecture. Blocking of $LT\beta$ by in utero injections of $LT\beta$-R-Ig, and null mutation of $LT\alpha$, $LT\beta$, IL-$2R\gamma_c$, and RelB all result in the absence of at least

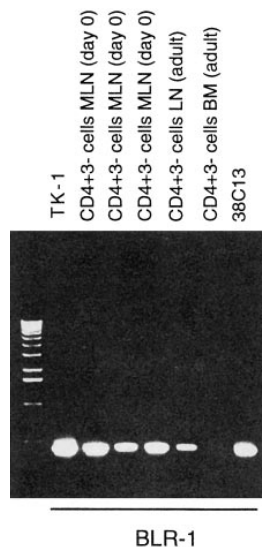

Figure 7. CD4$^+$CD3$^-$ Cells Contain mRNA for the Putative Chemokine Receptor BLR1

Analysis of cDNA from CD4$^+$CD3$^-$ cells isolated from MLN on day 0 and 8 weeks after birth and from bone marrow at 8 weeks after birth for the presence of mRNA coding for the putative chemokine receptor BLR1. In all CD4$^+$CD3$^-$ samples from lymph nodes, cDNA for *Blr1* could be found by RT-PCR. However, CD4$^+$CD3$^-$ cells sorted from bone marrow lacked mRNA for *Blr1*. As a positive control, two different lymphomas were used: 38C13 and TK-1 (Butcher et al., 1980; Gallatin et al., 1983).

PLN and a disorganized spleen that lacks B cell follicles (Lo et al., 1992; Togni et al., 1994; Banks et al., 1995; Burkly et al., 1995; Cao et al., 1995; Weih et al., 1995; Eugster et al., 1996; Koni et al., 1997). Blocking of BLR1 inhibits the maturation of B cell follicles (Forster et al., 1996). We propose that CD4$^+$CD3$^-$ cells play a role in the initiation of lymphoid structures during ontogeny. Of interest is the independent observation that in Peyer's patch development, expression of CD4 in the absence of CD3 can be observed, while simultaneously the expression of IL-7R is present in these early structures, suggesting that CD4$^+$CD3$^-$IL-7R$^+$ cells in the developing Peyer's patch and LN are similar (Spencer et al., 1986; Adachi et al., 1997). To test whether there indeed is a role for CD4$^+$CD3$^-$ cells in the formation of lymph nodes, CD4$^+$CD3$^-$ cells need to be specifically eliminated, perhaps genetically, by virtue of CD4 expression and lack of CD3 expression. We have yet not found a mutant mouse strain that lacks CD4$^+$CD3$^-$ cells in developing lymph nodes, even in the smallest lymph nodes that we have analyzed. Our hypothesis would be that CD4$^+$CD3$^-$ cells provide accessory cells in lymphoid structures, necessary to form lymph nodes or lymphoid tissue architecture. It will be important to see whether CD4$^+$CD3$^-$ cells are also involved in autoimmune and chronic inflammatory diseases in which lymph node–like structures are formed in extralymphatic tissues (Pozzilli et al., 1993; Kratz et al., 1996).

In summary, we have described a novel subset of the hematopoietic lineage. The population is characterized by expression of CD4 and LTβ and lack of CD3. The population demonstrates homogeneous expression or lack of expression of many surface markers, except IL-2R and MHC class II, which seem to divide the population. We observed the following activities within the CD4$^+$CD3$^-$ population: in response to IL-2, a fraction of CD4$^+$CD3$^-$ cells turn into NK cells; in reponse to IL-4 and GM-CSF, CD4$^+$CD3$^-$ cells differentiate into APC; and in response to IL-1β, IL-3, IL-7, and TNFα, CD4$^+$ CD3$^-$ cells proliferate. Our hypothesis that CD4$^+$ CD3$^-$ cells play a role in the initiation of lymph nodes is supported by several observations: expression of LTβ, RelB, IL-2Rγ_c, and BLR1, all involved in the formation of the lymph nodes; the early appearance of CD4$^+$CD3$^-$ cells in beginning lymph nodes; and the selective expression of MAdCAM-1 on HEV, allowing CD4$^+$CD3$^-$ cells to accumulate in the lymph nodes during late fetal and early neonatal life.

Experimental Procedures

Animals

C57BL/KA-Thy1.1, C57BL/KA-Thy1.2, BALB/c, and AKR mice were maintained in the laboratory animal colony at Stanford University School of Medicine.

Antibodies and Staining

The following antibodies and reagents were used for staining: GK1.5 (anti-CD4); 145-2C11 (anti-CD3); KT-31 (anti-CD3); 3A10 (anti-γδ-TCR); H57-597 (anti-αβ-TCR), 3E2 (anti-ICAM-1); IM781 (anti-CD44); R1-2 (anti-α4); M298 (anti-β7); Datk-32 (anti-α4β7); Mel-14 (anti-L-selectin); 53-7.3 (anti-CD5); AF6-88.5 (anti-H-2K^b); 30-H12 (anti-Thy1.2); 7D4 (anti-IL-2Rα, CD25); 2.4G2 (anti- FcγIII/IIR, CD16/32); NLDC-145 (anti-DEC205); 6B2 (anti-B220); M1/70 (anti-Mac-1); 8C5 (anti-Gr-1); AL1-4A2 (anti-Ly5.1); 2B8 (anti-c-Kit, CD117); 19XE5 (anti-Thy1.1); TUGm3 (anti-IL-2 receptor γ chain) (Kondo et al., 1993, 1994); A7R34 (anti-IL-7 receptor) (Sudo et al., 1993); E13 (anti-Sca-1); 11-5.2 (anti-I-A^k) (Pharmingen); 14-4-4S (anti-I-E^b) (Pharmingen); NK1.1 (anti-NK cell marker) (Pharmingen); N418 (anti-CD11c); 1G10 (anti-B7-1) (Pharmingen); GL1 (anti-B7-2) (Pharmingen); 1C10 (anti-CD40); 7G6 (anti-CR1/CR2) (Pharmingen); mouse LTRβ fused to human IgG1 (Browning et al., 1997). Stained cells were analyzed using a dual laser FACS (Becton Dickinson Immunocytometry Systems, Mountain View, CA), gating out dead cells and debris and sometimes blocking the CD16/32 FcR with MAb 2.4G2. Immunohistochemistry was carried out as described previously (Mebius et al., 1991).

Preparation of Genomic DNA and cDNA for PCR Analysis

Genomic DNA of sorted cell populations was prepared by sorting approximately 800 cells directly into 10 μl 1× PCR buffer containing 0.5% Tween 20 and 100 μg/ml proteinase K and then placing them for 40 min at 56°C followed by 10 min at 94°C to inactivate proteinase K.

cDNA was generated from cell populations sorted directly into RT reaction buffer as described (Chang et al., 1991). A maximum of 1000 cells were deposited in 20 μl of RT/lysis buffer and cDNA prepared.

PCR Analysis

Genomic DNA was analyzed for rearrangement of the β chain of the TCR or the IgH chain by amplifying 0.2 μl of the first PCR reaction with internal primers. Per reaction, 0.5 mM of each primer, 0.4 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 U of Taq polymerase (Boehringer Mannheim) were used. The first amplification cycle (30 s/2 min/3 min at 94°C/63°C/72°C, respectively) was repeated nine times, followed by 25 cycles (30 s/1 min/3 min at 94°C/63°C/72°C, respectively).

cDNA was analyzed for the presence of *Rag1*, *Rag2*, *LTβ*, *Blr1*, and *relB*. For *LTβ*, *Blr1*, and *relB*, 1 μl of the first PCR reaction was amplified with one internal 5′ primer in combination with the same 3′ primer to ensure specificity. For *relB*, *Blr1*, and *LTβ* primers,

the amplification cycles were identical to the ones used with the rearrangement primers (T_{anneal}: *relB*, 63°C; *LT*β, 57°C; and *Blr1*, 58°C). For *Rag1* and *Rag2* primers, the first amplification cycle (30 s/30 s/45 s at 94°C/62°C/72°C, respectively) was repeated four times and followed by 21 amplification cycles at a lower annealing temperature (30 s/30 s/45 s at 94°C/58°C/72°C, respectively).

The following oligonucleotides were used for PCR:

5′ PCR Primers

*D*β*1.1* external: 5′ GAGGAGCAGCTTATCTGGTG 3′
*D*β*1.1* internal: 5′ GGTAGACCTATGGGAGGGTC 3′
*D*β*2.1* external: 5′ TAGGCACCTGTGGGGAAGAAAC 3′
*D*β*2.1* internal: 5′ GTATCACGATGTAACATTGTG 3′
DQ52: 5′ GACTAAGCGGAGCACCACAG 3′
Rag1: 5′ TGCAGACATTCTAGCACTCTGG 3′
Rag2: 5′ CACATCCACAAGCAGGAAGTACAC 3′
relB 3F external: 5′ TGATCCACATGGAATCGAGAGC 3′
relB 1F internal: 5′ TGCTTCCATATCCCTTCCTGGGCTG 3′
*LT*β 3F external: 5′ TGGTGACCCTGTTGTTGGCAGT 3′
*LT*β 5F internal: 5′ TATCACTGTCCTGGCTGTGCTG 3′
*Blr1*F: 5′ TTCCTGTTCCACCTCGCAGTAG 3′

3′ PCR Primers

*J*β*1.7* external: 5′ AAGGGACGACTCTGTCTTAC 3′
*J*β*1.7* internal: 5′ ACCATGGTCATCCAACACAG 3′
*J*β*2.7* external: 5′ TGAGAGCTGTCTCCTACTATC 3′
*J*β*2.7* internal: 5′ GGAAGCGAGAGATGTGAATC 3′
JH4#2: 5′ CTCTCAGCCGGCTCCCTCAGGG 3′
Rag1: 5′ ACATCTGCCTTCACGTCGAT 3′
Rag2: 5′ TCCCTCGACTATACACCACGTCAA 3′
relB 2R external: 5′ CCCAGTGCCTCCTCTGTTACCTCAG 3′
*LT*β 4R external: 5′ GCTCAGGGTTGAGGTCAGTT 3′
Blr1-2R external: 5′ ACTAAAATGGCCACCCTGACC 3′
Blr1-3R external: 5′ CCATCCCATCACAAGCATCGG 3′

NK Cell Frequency and NK Cytotoxic Activity

$CD4^+CD3^-$ cells sorted at one cell/well in 96-well plates were grown in the presence of IL-2 on AC-6.2.1 feeder layers. After 7–10 days the colonies grown were tested by FACS for an NK-like phenotype by staining with NK-specific MAb 2B4 and NK1.1 (Pharmingen). NK cytotoxic activity of the different colonies was measured using the microcytotoxicity assay developed and described by Aguila and Weissman (1996). The percentage killing was calculated relative to the control, which contained YAC-1 cells in the absence of any effector cells.

MLR

T lymphocytes were isolated from PLN and MLN (C57BL/6 mice) using MACS beads. Dendritic APC were isolated by layering lymph node suspensions; enriched by negative selection of Mac-1$^+$, B220$^+$, and Thy1$^+$ cells using Dynabeads (Dynal AS, Oslo, Norway) onto metrizamide, as described before; and subsequently irradiated (3000 rad) (Knight et al., 1983). $CD4^+CD3^-$ cells were sorted and cultured overnight in the presence or absence of IL-4 (800 U/ml) and GM-CSF (2000 U/ml) before placement in a MLR assay. Both $CD4^+CD3^-$ and dendritic APC were derived from BALB/c mice. Different concentrations of $CD4^+CD3^-$ cells or dendritic APC were mixed with 10^5 T lymphocytes in a U-bottom 96 well plate. After 4 days of incubation [^{3}H]thymidine was added to the different wells (1 μCi/well), and after 6 hr the incorporation of [^{3}H]thymidine was measured. The incorporation of [^{3}H]thymidine was used as a measure of the proliferation of T lymphocytes in response to the allogeneic APC to which they were exposed.

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