Neonatally Induced Inactivation of the Vascular Cell Adhesion Molecule 1 Gene Impairs B Cell Localization and T Cell–dependent Humoral Immune Response

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Abstract

Vascular cellular adhesion molecule (VCAM)-1 is a membrane-bound cellular adhesion molecule that mediates adhesive interactions between hematopoietic progenitor cells and stromal cells in the bone marrow (BM) and between leukocytes and endothelial as well as dendritic cells. Since VCAM-1–deficient mice die embryonically, conditional VCAM-1 mutant mice were generated to analyze the in vivo function of this adhesion molecule. Here we show that interferon-induced Cre-loxP–mediated deletion of the VCAM-1 gene after birth efficiently ablates expression of VCAM-1 in most tissues like, for example, BM, lymphoid organs, and lung, but not in brain. Induced VCAM-1 deficiency leads to a reduction of immature B cells in the BM and to an increase of these cells in peripheral blood but not in lymphoid organs. Mature recirculating B cells are reduced in the BM. In a migration assay, the number of mature B cells that appears in the BM after intravenous injection is decreased. In addition, the humoral immune response to a T cell–dependent antigen is impaired. VCAM-1 serves an important role for B cell localization and the T cell–dependent humoral immune response.

Key words: conditional VCAM-1 mutant mice • B cell development • lymphocyte migration • cre/loxP • bone marrow

Introduction

The vascular cell adhesion molecule (VCAM)-1 (CD106) was originally described as a cytokine-induced glycoprotein (INCAM-110) expressed on human vascular endothelial cells (1). In mice, two isoforms of VCAM-1 are produced by alternative splicing. One form contains seven Ig-like domains, a transmembrane region, and a short COOH-terminal cytoplasmic tail (2–4), while the second, cytokine and LPS-inducible form, contains the first three domains only and is attached to the cell membrane by a glycosylphosphatidylinositol anchor (5). VCAM-1 directs the recruitment of leukocytes to sites of inflammation by binding predominantly to α4β1 integrin (very late antigen [VLA]-4; CD49d/CD29) and with lower affinity to α4β7 integrin (6–8).

Binding of the seven domain form of VCAM-1 to its ligands is mediated by the first and fourth Ig-like domain (9, 10).

A contribution of the VCAM-1/VLA-4 adhesion pathway for leukocyte migration in rheumatoid arthritis, lupus nephritis, inflammatory bowel disease, allograft rejection, atherosclerosis, contact hypersensitivity, and experimental autoimmune encephalomyelitis has been suggested (11–18). Blocking VCAM-1/VLA-4–mediated cellular adhesion by mAbs often results in a less severe course of these diseases in animal models.

Differentiation and proliferation of hematopoietic progenitor cells occur in intimate contact with the bone marrow (BM) microenvironment which is composed of stromal cells and extracellular matrix proteins. Since stromal cells secrete numerous factors that are necessary for the growth and differentiation of hematopoietic precursor cells, it may be critical for these cells to stay in the BM in order to be exposed to a cytokine milieu and to receive all signals necessary for maturation. VCAM-1 is constitutively expressed on BM stromal cells (19). Several reports demonstrate that VCAM-1 plays a crucial role in hematopoietic progenitor cell trafficking and lodgement of transplanted murine he-
matopoietic progenitor cells to BM (19–22). Other authors have shown the significance of cytokines for hematopoietic progenitor cell (HPC) mobilization in vivo by the treatment of mice with fms-like tyrosine kinase 3 (Flt3) ligand (23). This suggests that cytokines may alter the expression or the activation state of adhesion molecules. VCAM-1 is also constitutively expressed on follicular dendritic cells (24, 25), which serve a critical role for antigen presentation. The VLA-4–VCAM-1 interaction is involved in the adhesion of human B cells to follicular dendritic cells of germinal centers in vitro, which is hypothesized to affect the immune response maturation in the germinal center (24–27). Furthermore, a link between VCAM-1 function and rescue of germinal center B cells (26–28) or thymocytes (29) from apoptosis has been reported. Finally, VCAM-1 may also function in maturation and costimulation of T cells (30–32).

Since mice homozygous for the VCAM-1 deletion die early during embryogenesis (33–35), most experiments regarding the function of VCAM-1 have so far been carried out either in vitro or using mAbs blocking the function of VCAM-1. A very small number of VCAM-1–deficient mice survive the detrimental effects of the VCAM-1 mutation on embryogenesis (34). These mice exhibit elevated numbers of circulating blood mononuclear leukocytes (34); however, no phenotype with regard to hematopoiesis has been reported (36). It remains questionable whether these mice represent a suitable tool for evaluation of postnatal VCAM-1 function. To circumvent the limitations of the above mentioned approaches for the evaluation of the in vivo function of VCAM-1 for postnatal life, we used inducible gene targeting (37). This approach leads to the absence of VCAM-1 protein in most organs of mice in which the VCAM-1 gene was deleted by IFN-induced Cre-loxP–mediated recombination. Hereby, the critical function of VCAM-1 for retention of B cells during maturation in the BM and for localization of mature B cells in the BM could be established. Moreover, VCAM-1 plays a critical role in the humoral immune response to a T cell–dependent antigen.

**Materials and Methods**

**Mice and Conditional Gene Inactivation.** Mice homozygous for the loxP flanked (floxed) VCAM-1 gene (35) and Mx-cre transgenic mice (37) were bred and crossed to generate homozygous VCAM-1 floxed mice carrying the Mx-cre transgene. In each litter, mice with and without the Mx-cre transgene were generated. Neonatally, at days 1, 4, and 7 the mice were injected with 10^6 U of IFN-α/102 intraperitoneally to inactivate the VCAM-1 gene in mice bearing the Mx-cre transgene and to use the Mx-cre nontransgenic mice as controls. At the age of 5 wk, mice were typed for the Mx-cre transgene by Cre–specific PCR and Southern blot analysis. The different genotypes, Mx-cre;VCAM-fl/wt mice (conditional VCAM-1 mutant mice) and VCAM-fl/fl mice (control mice), were identified at the expected Mendelian ratio. The mice were kept in a conventional animal facility. Sentinel animals were checked regularly according to the FELASA health monitoring report (38) and mice were used for studies at the age of 6–8 wk to exclude any influence of IFN-α treatment on the B and T cell pool (39).

**Southern Blot Analysis.** Tissue of different organs from conditional VCAM-1 mutant mice was washed with PBS and digested at 56°C with 100 μg/ml proteinase K (Boehringer) in lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS) overnight. 1 vol of isopropanol was added to the lysate and the precipitate was recovered. Depending on the size of the precipitate the DNA was dispersed in 100–400 μl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5. The prepared DNA was digested with BamHI (New England Biolabs, Inc.) and probed with a mixture of two nonradioactive labeled with the Gene Images™ labeling kit (Amerham Pharmacia Biotech). In an agarose gel the deleted VCAM-1 gene migrated at 4.5 kb, whereas the floxed gene gave two signals of 6 and 3 kb in size.

To test mice for the presence of the Mx-cre transgene tail, DNA was digested with BamHI and probed with a 750-bp BamHI-XbaI (New England Biolabs, Inc.) fragment of the Cre-coding region, resulting in two bands at 5 and 3.5 kb.

**Quantification of the Deletion.** To assess the degree of VCAM-1 gene deletion in various tissues, DNA from organs of three different conditional VCAM-1 mutant mice was prepared and analyzed by at least two independent Southern blot each. The amount of deletion was calculated by scanning the signals of the 3-kb floxed and the 4.5-kb deleted VCAM-1 gene fragment generated during different exposure times on X-OMAT film (Eastman Kodak Co.) in a FluorS Multilimage (Bio-Rad Laboratories).

**PCR.** To test for the presence of the Mx-cre transgene, a 1-kb fragment of the coding region was amplified by standard PCR procedure using the two primers cre– (5′-CAA TCT AAC CTA GAC CGG CTT ACA C-3′) and cre+ (5′-CAT GCC CAT TTT CCA GCA-3′). Tissue from tail biopsies was lysed overnight at 56°C with 100 μg/ml proteinase K (Boehringer) in lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS), precipitated with isopropanol, and resuspended in 150 μl Tris-HCl, 0.1 mM EDTA, pH 7.5. 1 μl of this DNA solution was used for PCR analysis in 50 nl vol overlayed with mineral oil. 200 mM dNTPs (Boehringer), 20 pmol of each primer, and 5 U Taq polymerase (produced in our own laboratory) were added, and the PCR reaction was performed on a thermal cycler (TRIO-Thermoblock; Biometra) in 10 mM Tris-HCl, pH 8.3, at 25°C, 50 mM KCl, and 3 mM MgCl2 (35 cycles: 40 s at 94°C, 1 min at 60°C, and 1.5 min at 72°C). After the last cycle, samples were incubated for another 10 min at 72°C and subsequently analyzed by gel electrophoresis on a 1% agarose gel.

**Immunoprecipitation.** Mice were killed and the prepared organs were washed several times in PBS. Tissue was then homogenized and cells were disrupted with glass beads (Braun-Melsungen) in TBS (0.5% SDS, 1% Triton, 0.025 mM EDTA, 0.1 M Tris, pH 8.0) containing protease inhibitors as described (40). After adding 0.5% NP-40, membrane proteins were extracted at 4°C overnight. Cell debris was removed by high speed centrifugation and 0.4 ml of the supernatant was subjected to immunoprecipitation. After incubation with biotinylated goat anti–mouse IgG1 Ab (Dianova) overnight at 4°C and precipitation of unspecific bound proteins through adding streptavidin–coupled agarose (Sigma-Aldrich) for another night at 4°C, preclearing was finished by centrifugation preceding precipitation of VCAM-1 protein from the supernatant. For that purpose the supernatant was incubated again overnight at 4°C with biotinylated rat anti–mouse VCAM-1 Ab (Clone 429; BD Pharmingen) after another 24 h at 4°C in the presence of streptavidin–coupled agarose and precipitation by centrifugation. The pellet was washed two times in PBS and separated on a 10% acrylamide gel. Proteins were
blotted on a PVDF membrane (Immobilon-P; Millipore) and VCAM-1 protein was detected with polyclonal goat anti-VCAM-1 Ab (sc-1504; Santa Cruz Biotechnology, Inc.) and anti–goat IgG-horseradish peroxidase (Dako) using an ECL detection kit (Amer sham Pharmacia Biotech).

Immunohistochemistry. Frozen sections of spleen were fixed in acetone, incubated with 0.1% phenylhydrazine (Sigma-Aldrich) in PBS, and blocked with PBS containing 4% FCS. For immunostaining, biotinylated rat anti–mouse VCAM-1 mAb (clone 429; BD PharMingen), rat anti–mouse VCAM-1 mAb (MK-2, 9DB3, V.7H1, V.4B12.1, V.6C3; provided by Dr. D. Vestweber, University of Muenster, Muenster, Germany), or polyclonal goat anti–VCAM-1 Ab (sc-1504; Santa Cruz Biotechnology, Inc.) was used. Primary Abs were detected with peroxidase-conjugated streptavidin (Dianova) or peroxidase-conjugated anti–rat IgG (Jackson ImmunonoResearch Laboratories) or anti–goat IgG (Santa Cruz Biotechnology, Inc.). The detection was performed using 3,3′-diaminobenzidine (DAB) substrate kit (Amersham Pharmacia Biotech).

Results

Conditional Gene Targeting of VCAM-1. To circumvent embryonic lethality in VCAM-1−/− deficient mice (33, 34) we set out to delete the VCAM-1 gene in mice in a conditional manner using the Mx−/cre system (37). In this approach the target gene is flanked by loxP sites and Cre is expressed after IFN injection. This leads to a Cre−/loxp−mediated deletion of the target gene. We have crossed mice in which the proximal promoter sequences and the first two exons of the VCAM-1 gene are flanked by loxP sites (VCAM-1lox/lox mice; reference 35), with Mx−cre transgenic mice (37; Fig. 1 A). On days 1, 4, and 7 after birth, mice homozygous for the VCAM-1 mutation and transgenic for Mx−cre (Mx−cre;VCAM1lox/lox mice) were treated with 106 U IFN-α1/α2 (IFN-α) (54) to induce Cre−mediated recombination. Mice were analyzed for deletion of the VCAM-1 gene at the age of 6–8 wk.

VCAM-1lox/lox mice (control mice) and Mx−cre; VCAM-1lox/lox mice (conditional VCAM-1 mutant mice) did not exhibit any detectable deletion of the VCAM-1

Affinity maturation of serum IgG1 Ab was assessed in sera of NP-CG-immunized mice by measuring the ratio of binding to NP14-BSA/NP20-BSA (50). Germinal centers were stained in splenic sections with biotinylated peanut agglutinin (Vector Laboratories), detected with streptavidin–horseradish peroxidase (Dianova), developed with diaminobenzidine, and counterstained with hematoxylin (Sigma-Aldrich) by standard procedure (51).

Quantification of Plasma Cells. IgG-secreting plasma cells in 10-mo-old mice were stained with TRITC (rhodamine)-conjugated goat anti–mouse IgG mAb (Southern Biotechnology Associates, Inc.) in cytopsins of spleen and BM (52). Each cytopsin contained 2 × 103 cells. At least 14 cytopsins of two control and two conditional VCAM-1 mutant mice were counted.

Bromodeoxyuridine Treatment. Mice (two control and two conditional VCAM-1 mutant mice) were fed with bromodeoxyuridine (BrdU) at a concentration of 1 mg/ml with 1 M sucrose in drinking water for 3 d. During the labeling period the drinking water was protected from light. Mice were then killed and surface staining of single cell suspensions was performed as described previously (53). 5–10 × 106 cells were first surface stained with FITC-conjugated Abs against B220 (mAb RA33.A1.2C5) or IgM (mAb R33.23-24-12). After fixation in methanol and denaturation of the DNA, cells were stained with a biotinylated anti–murine IgD (BD PharMingen), rat anti–mouse VCAM-1 mAb (MK-2, 9DB3, V.7H1, V.4B12.1, V.6C3; provided by Dr. D. Vestweber, University of Muenster, Muenster, Germany), or polyclonal goat anti–VCAM-1 Ab (sc-1504; Santa Cruz Biotechnology, Inc.) was used. Primary Abs were detected with peroxidase-conjugated streptavidin (Dianova) or peroxidase-conjugated anti–rat IgG (Jackson ImmunonoResearch Laboratories) or anti–goat IgG (Santa Cruz Biotechnology, Inc.). The detection was performed using 3,3′-diaminobenzidine (DAB) substrate kit (Amersham Pharmacia Biotech). The detection was performed using 3,3′-diaminobenzidine (DAB) substrate kit (Amersham Pharmacia Biotech). The detection was performed using 3,3′-diaminobenzidine (DAB) substrate kit (Amersham Pharmacia Biotech).
Gene targeting of VCAM-1 in spleen without IFN-α treatment. After IFN-α application, VCAM-1 deletion was complete in the spleens of conditional VCAM-1 mutant mice, while there was no deletion detectable in control mice (Fig. 1 B). This suggests that conditional targeting of the VCAM-1 gene using the Mx-cre system is efficient and does not exhibit any obvious leakiness either in the absence of IFN-α application or in the absence of the transgene. After induced deletion, conditional VCAM-1 mutant mice did not exhibit any overt disease and could be kept for at least 12 mo. When different organs were assessed by Southern blotting for deletion of the VCAM-1 gene in conditional VCAM-1 mutant mice after IFN-α treatment, it became evident that the extent of the deletion varied. While the deletion was complete in, for example, BM, liver, lymph nodes, spleen, and thymus, only partial deletion was observed in heart (45%), intestine (85%), kidney (84%), and lung (69%), and only minimal deletion could be detected in brain (16%; Fig. 1 C).

To confirm that conditional deletion of the VCAM-1 gene led to a lack of protein expression, Western blot analysis for VCAM-1 protein and immunohistochemical detection using mAbs against VCAM-1 were performed. By Western blot analysis, there was no VCAM-1 protein detectable in BM, liver, lymph nodes, spleen, and thymus of conditional VCAM-1 mutant mice after IFN-α treatment, while a minor protein band was recognizable in the lung and heart (Fig. 1 D). By contrast, abundant VCAM-1 protein with a molecular mass of ~110 kD was present in the brain of these mice, as expected from the results of the Southern blot analysis (Fig. 1 C).

The immunohistochemical analysis confirmed the absence of VCAM-1 expression after conditional targeting of the VCAM-1 gene. A set of anti-VCAM-1 mAbs binding to different epitopes was used for indirect immunohistochemical detection. Frozen tissue sections from spleens of control mice after IFN-α treatment readily exhibited staining for VCAM-1 (Fig. 1 E). By contrast, no VCAM-1 expression was detectable in spleens of conditional VCAM-1 mutant mice after IFN-α application (Fig. 1 E).

Thus, the level of genomic deletion as assessed by Southern blot analysis corresponds well with the lack of protein expression of VCAM-1 as measured by Western blot analysis or immunohistochemistry.

Figure 1. Efficient conditional ablation of VCAM-1 in vivo. (A) Southern blotting strategy for detection of IFN-α-induced VCAM-1 gene deletion. (B) Southern blot analysis of splenic DNA of mice without (lanes 1 and 2) and with (lanes 3 and 4) IFN-α injection (probed for VCAM-1). Without either the cre transgene (lanes 1 and 3) or IFN-α induction (lane 2), deletion of the VCAM-1 gene did not occur, as indicated by the 3- and 6-kb bands. By contrast, deletion is almost complete after IFN-α induction in the presence of the cre transgene (lane 4), resulting in a 4.5-kb signal. (C) Southern blotting of various tissues from IFN-α–treated, conditional VCAM-1 mutant mice revealed a high degree of deletion in most organs, but almost no deletion in brain (LI, liver; LN, lymph node; LU, lung; SP, spleen; TH, thymus; BR, brain; HE, heart; IN, intestine; KI, kidney). (D) Western blot analysis of VCAM-1 (110 kD) in conditional VCAM-1 mutant and control mice. After IFN-α-induced Cre expression, VCAM-1 could not be detected in BM, liver, lymph node, spleen, and thymus of conditional VCAM-1 mutant mice (lanes 4, 6, 8, 10, and 11), while minor traces of VCAM-1 are detectable in heart, intestine, and lung (lanes 2, 5, and 7) and brain contains VCAM-1 levels similar to control mice (lane 1). (E) Immunohistochemical analysis of spleen reveals VCAM-1 expression in control mice (left) and by contrast, no VCAM-1+ cells in spleen of conditional VCAM-1 mutant mice (right).
VCAM-1 Retains Immature B Cells in the BM. Spleen, lymph nodes, thymus, and BM from IFN-treated conditional VCAM-1 mutant mice and from IFN-treated control mice were analyzed for cellularity and lymphocyte subsets by flow cytometry. While there was no major difference in overall cell number and expression of B220, IgM, IgD, CD3, CD4, and CD8 in spleens, lymph nodes, and thymus, a marked alteration of B cell subset distribution was detected in BM. There was a consistent, but statistically not significant, small reduction of nucleated cells in the BM of conditional VCAM-1 mutant mice (1.5 ± 0.64 × 10⁷ nucleated cells per femur for control mice, 1.2 ± 0.41 × 10⁷ nucleated cells per femur in conditional VCAM-1 mutant mice, P = 0.27). However, the percentages of cells within the lymphocyte gate were similar (32.5 ± 5.6% in control mice and 33.4 ± 6.3% in conditional VCAM-1 mutant mice), whereas the fraction of B220+ cells within these gates is slightly reduced in VCAM-1-deficient animals (14.7 ± 3.7% in control mice versus 10.6 ± 2.9% in conditional VCAM-1 mutant mice, P < 0.05). Taken together, this results in a reduced absolute number of B220+ cells per femur in BM of conditional VCAM-1 mutant mice (1.4 ± 0.6 × 10⁶ B220+ cells per femur) compared with control mice (2 ± 0.5 × 10⁶ B220+ cells per femur, P < 0.05). Hardy et al. have developed a nomenclature to distinguish the different stages of B cell development, with the most immature pro-B cells present in fraction A while mature B cells establish fraction F (42). In conditional VCAM-1 mutant mice fraction E, which comprises the immature B cells expressing IgM but no IgD, is reduced by a mean of 40% compared with fraction E in control mice (Fig. 2 A). This reduction of fraction E is even more pronounced when absolute cell numbers are calculated since the overall cell number and the number of B220+ cells are reduced in conditional VCAM mutant mice (see above; Fig. 2 B).

Fraction E cells comprise immature B cells that will soon leave the BM to enter the blood. Therefore, we wondered whether this B cell subset, which was reduced in the BM of conditional VCAM-1 mutant mice, would be detectable outside of the BM. In peripheral blood from conditional VCAM-1 mutant mice an increased number of white blood cells (16.3 ± 3.9 × 10⁹/mm³ blood versus 7.2 ± 1 × 10⁹/mm³ blood in control mice) was detected compared with control mice. The ratio between lymphocytes, neutrophils, and monocytes, as well as the fraction of B220+ cells within the lymphocyte gate, was not significantly altered (Table I). These findings are in accordance with experiments of anti–VLA-4 treatment in primates leading to granulocytosis as well as lymphocytosis (20, 21), whereas blocking of VLA-4 in rats caused a strong increase of mononuclear leukocytes and only a minor increase of polymorphonuclear leukocytes (55). However, in conditional VCAM-1 mutant mice there was a dramatic increase of IgM+IgD− cells (Fig. 3), a fraction of cells that could possibly represent the fraction E cells found to be diminished in the BM (Fig. 2, A and B). 30.5 ± 5.1% of all B220+ cells in conditional VCAM-1 mutant mice compared with 14.9 ± 1.8% of all B220+ cells in control mice belonged to the fraction of immature IgM+IgD− cells in peripheral blood (Fig. 3). This translates into a 3.5-fold increase of this population with regard to absolute cell numbers (1.72 ± 0.41 × 10⁹/mm³ blood in conditional VCAM-1 mutant mice versus 0.49 ± 0.04 × 10⁹/mm³ blood in control mice). In addition, about threefold elevated levels of B220+IgM−IgD− B cells, which may comprise B cells earlier during develop-

**Figure 2.** Altered B cell subsets in BM of conditional VCAM-1 mutant mice. (A) Flow cytometric analysis of BM cells within the lymphocyte gate (reference 45). B lineage cells are displayed by their CD45R/B220 and IgM expression. The letters in the rectangles indicate the different developmental stages according to Hardy et al. (reference 42). Conditional VCAM-1 mutant mice exhibit reduced numbers of B cells in fraction E and fraction F. 20,000 events were collected for both of the dot plots. More than 10 mice for both genotypes were analyzed, of which one representative experiment is depicted. (B) Comparison of absolute B cell numbers/femur (± SEM) between conditional VCAM-1 mutant mice (gray bars) and control mice (white bars). Values are calculated from four independent experiments including 8 control and 11 conditional VCAM-1 mutant mice. *Significantly different from value in control mice, P < 0.05.
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ment than fraction E, were detectable in peripheral blood of conditional VCAM-1 mutant mice (0.32 ± 0.13 × 10^9/mm³ blood in mutant mice versus 0.11 ± 0.04 × 10^9/mm³ blood in control mice).

Taken together, there is a sizeable shift in the peripheral blood B cell pool of conditional VCAM-1 mutant mice towards the more immature cells. This shift goes along with the loss of fraction E cells in the BM, which led us to hypothesize that VCAM-1 may be important for retention of B lineage cells during development in BM until they reach a certain developmental stage.

In contrast, the distribution of T cells was not significantly altered in conditional VCAM-1 mutant mice. T cell subsets (CD3, CD4, CD8) in thymus, spleen, peripheral blood, and lymph nodes were similar in conditional VCAM-1 mutant mice and control mice (data not shown).

B Cells Accumulating in the Blood of Conditional VCAM-1 Mutant Mice Are Immature Emigrants from the BM. Recently a novel mAb called 493 has been described which binds to a surface protein designated pB130-140 (48). This Ab discriminates between long-lived recirculating 493^+ B cells and various stages of B cell development, including fraction E cells which are either inside the BM or have just left this site and still express pB130-140. Since the reduction of B cells in the BM is accompanied by an increase of a similar B cell fraction in blood, we examined the origin of these B cells in blood according to their pB130-140 expression. As predicted, the fraction of 493^+ cells within the lymphocyte gate in peripheral blood of conditional VCAM-1 mutant mice was almost doubled compared with control mice (Fig. 4 A). A strong increase of the absolute number of 493^+IgM^+IgD^− B cells and a minute increase of the 493^+IgM^+IgD^− B cells were noted (Fig. 4 B). We evaluated whether immature B cells would also be detectable at higher numbers in spleens of conditional

Table I. Peripheral Blood Counts

| Blood cell types     | Control    | Conditional VCAM-1 mutant | Fold increment | P value |
|----------------------|------------|---------------------------|----------------|---------|
| RBCs (10^6/mm³)     | 8.7 ± 0.1* | 8.0 ± 0.6                 | 0.9            | 0.13‡   |
| Hemoglobin (g/dl)    | 14.2 ± 0.4 | 14.1 ± 0.7                | 1.0            | 0.92    |
| WBCs (10^3/mm³)     | 7.2 ± 1.0  | 16.3 ± 3.9                | 2.3            | 0.008   |
| L (% of WBCs)       | 71.9 ± 13.4| 70.1 ± 11.3               |                |         |
| B cells (% of L)    | 54.5 ± 0.5 | 51.2 ± 2.2                |                |         |
| T cells (% of L)    | 37.3 ± 6.0 | 32.2 ± 4.1                |                |         |
| N (% of WBCs)       | 19.9 ± 3.2 | 20.6 ± 4.8                |                |         |
| M (% of WBCs)       | 3.5 ± 0.8  | 3.4 ± 0.4                 |                |         |
| E (% of WBCs)       | 1.6 ± 1.2  | 2.4 ± 1.2                 |                |         |
| Ratio L/N/M/E       | 45:12:2:1  | 44:13:2:1.5               |                |         |

Hemoglobin levels as well as numbers of erythrocytes and leukocytes were evaluated in three control and seven mutant mice. E, eosinophils; L, lymphocytes; M, monocytes; N, neutrophils; WBC, white blood cell.

*SEM.
‡Student’s t test.

Figure 3. Increased number of immature B cells in peripheral blood of conditional VCAM-1 mutant mice. Flow cytometric analysis of peripheral blood B cells. In each experiment 20,000 events were acquired and subsequently gated for B cells (CD45R/B220^+ cells). The dot blots represent the CD45R/B220^+ cells within the lymphocyte gate in a control (top) and a conditional VCAM-1 mutant mouse (bottom) displayed by their IgM and IgD surface expression. In addition, the frequencies of mature (IgM^+IgD^+), immature (IgM^+IgD^−), and B220 only (IgM^+IgD^−) B cells are depicted as percentages of all B220^+ cells. More than 15 mice for both genotypes were analyzed, of which one representative experiment is shown.
VCAM-1 mutant mice. 10–20% of the immature B cells are supposed to reach the spleen as 493+ cells (48); however, the subsets of 493+ immature B cells have similar sizes in spleens of conditional VCAM-1 mutant mice and control mice. Compared with blood there is only a modest increase of 493+IgM+ cells detectable in spleens of VCAM-1 deficient mice (Fig. 4 C). There are several possible explanations: 493+ cells in peripheral blood of conditional VCAM-1 mutant mice have a reduced half life, 493 expression on these cells is rapidly downregulated, or, alternatively, these cells get trapped in sites other than the spleen.

To determine the transit time of IgM+ cells from BM to blood and spleen, we performed BrdU labeling of dividing cells. After a labeling period of 3 d, we recovered 21 ±

Figure 4. Expression of 493, a marker for immature B cells, in peripheral blood B cells. (A) Increased number of 493+IgM+ B cells in peripheral blood of conditional VCAM-1 mutant mice was detected. In each experiment, 50,000 events were acquired. The upper dot blots represent peripheral blood cells in a control (left) and a conditional VCAM-1 mutant mouse (right) to indicate gating for lymphocytes. The bottom displays the cells within the lymphocyte gate by their IgM and 493 surface expression. The frequencies of IgM-493-, IgM+493+, and IgM+493- cells are depicted as percentages of all lymphocytes. Out of three experiments, one representative flow cytometric study including three control and seven conditional VCAM-1 mutant mice is shown. (B) The graph illustrates the increase of 493+ B cells in peripheral blood of conditional VCAM-1 mutant mice compared with control mice. The mean absolute cell counts for leukocytes and lymphocytes determined in a separate experiment were used to calculate absolute numbers for the B cell subsets. White bars represent control mice; gray bars represent conditional VCAM-1 mutant mice. (C) Number of 493+ cells in spleen of two control mice (white bars) and two conditional VCAM-1 mutant mice (gray bars) are shown as a percentage of all lymphocytes. Immature IgD+ B cells are identified by their IgM surface expression, and mature B cells according to their IgD- staining. Values are depicted with the SEM from the flow cytometric analysis. *Significantly different from value in control mice, P < 0.05.
7.9% IgM+BrDU+ cells in BM, 20.8 ± 1.2% in blood, and 26.8 ± 1.2% in spleen of control mice (as percentage of all B220+ cells). In contrast, conditional VCAM-1 mutant mice showed lower numbers of IgM+BrDU+ cells in BM (15.8 ± 4.2%) and increased amounts in blood (28.6 ± 2.1%) and spleen (33.3 ± 3.4%). If absolute cell numbers are considered, the increase of newly generated IgM+ cells in the blood of conditional VCAM-1 mutant mice is even higher. Taken together, these results suggest that the IgM+IgD- B cells in peripheral blood of conditional VCAM-1 mutant mice are recent emigrants from the BM.

**Apoptosis of Immature B Cells in the Periphery.** Although the immature B cell compartment in blood is enlarged by premature transit of these cells from the BM (Fig. 4, A and B), no significant increase in immature B cells was observed in spleen (Fig. 4 C) or lymph nodes of conditional VCAM-1 mutant mice. Since increased apoptosis of B cells in blood could account for this observation, we quantified the number of apoptotic cells in the peripheral blood. In both control mice and conditional VCAM-1 mutant mice, 12% of the IgM+ cells were stained with annexin V (data not shown). Therefore, the frequency of apoptotic B cells does not seem to differ in blood from mutant and control animals.

**Recirculation of Mature IgM+IgD+ B Cells into the BM Is Influenced by VCAM-1 Expression.** In addition to the premature loss of immature B cells from the BM into the blood, we also observed a phenotype of the VCAM-1 deficiency for mature recirculating B cells. Fraction F cells in the BM were reduced by ~63% compared with control mice. These cells are mature B cells expressing IgM and IgD, which are thought to recirculate from the periphery into the BM (56). The number of fraction F cells significantly decreased from 2.4% (0.31 ± 0.06 × 10^6 cells per femur) in control mice to 0.9% (0.12 ± 0.1 × 10^6 cells per femur) of all nucleated cells in conditional VCAM-1 mutant mice (Fig. 2, A and B). We hypothesized that VCAM-1 may play a role in mediating migration of mature B cells into the BM. To address this we performed a short-term migration assay of mature B cells. Splenocytes of control mice were isolated and the immature CD43+ cells were depleted by MACS®. Enriched CD43− splenocytes, consisting mainly of IgM+IgD+ B cells, were labeled with PKH-26 and injected into the tail veins of conditional VCAM-1 mutant mice and control mice. First, in control mice we were readily able to detect PKH-26-labeled mature B cells in the BM after 20 h (rectangle F^D in Fig. 5, left dot plot), which supports the idea that mature B cells enter the BM. More important, about half of the PKH-26-labeled cells, which were detectable in BM of control mice, were found in BM of conditional VCAM-1 mutant mice (Fig. 5). The reduced number of PKH-26-labeled mature B cells that were detected in the BM of conditional VCAM-1 mutant mice after cell transfer in size parallels the observed reduction of fraction F (which is depicted by the rectangle F in Fig. 5, right dot plot) in these animals. This could be explained by an impaired recirculation of mature B cells into the BM caused by VCAM-1 deficiency. However, we cannot rule out other possible explanations for this phenotype: for example, an increasing loss of mature IgD+ B cells from the BM or a rapid death of these cells in BM as a result of the VCAM-1 deficiency. In addition, the short-term migration assay in the mutant animals might be influenced by the leukocytosis present in these mice; competition of the PKH-26-labeled cells with leukocytes present in the blood stream might affect the efficiency of PKH-26-labeled cells to enter the BM. The latter explanation is unlikely, as short-term migration assays performed with total splenocytes did not show any differences with regard to lymphocyte homing to spleen and peripheral lymph nodes in mutant and control mice, while the impaired recirculation to BM was confirmed (preliminary data not shown).

**Conditional VCAM-1 Mutant Mice Exhibit an Impaired T Cell–dependent Humoral Immune Response.** To investigate whether the function of B cells is affected by the VCAM-1 deficiency, mice were immunized with NP-CG. The specific immune response in conditional VCAM-1-deficient mice and control mice was monitored by measuring the NP-specific Ig levels at different time points. Before immunization, slightly but not significantly reduced levels of IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 were detected in sera of conditional VCAM-1 mutant mice (data not shown). Upon NP-CG immunization, conditional VCAM-1 mutant mice exhibited impaired primary and greater than fivefold reduced secondary IgG1 responses compared with control mice (Fig. 6). Affinity maturation of the Ab response against NP and generation of germinal centers in conditional VCAM-1 mutant mice did not seem to be affected (data not shown; see Materials and Methods). In addition, the numbers of IgG secreting plasma cells in BM and spleen of control and VCAM-1-deficient mice did not show any significant difference. 10-mo-old control mice contained 230 ± 85 IgG-secreting plasma cells/2 × 10^5 cells in BM and 77 ± 17 IgG-secreting plasma cells/2 × 10^5 cells in spleen, versus 247 ± 90 in BM and 75 ± 21 in

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**Figure 5.** Short-term migration of mature B cells into the BM. Flow cytometric analysis of CD45R/B220+ gated lymphocytes in BM of control mice and conditional VCAM-1 mutant mice 20 h after injection of mature splenic PKH-26–labeled B cells. The BM B cells are displayed by their IgD surface and PKH-26 expression. Rectangle F^D contains the donor-derived, PKH-26–labeled, recirculating mature B cells; rectangle F represents the fraction F of mature B cells present in the host. 0.06 ± 0.011% of all acquired events were recirculating B220+PKH-26+IgD+ B cells in control mice, while only 0.03 ± 0.01% of such cells were recovered from conditional VCAM-1 mutant BM. In total, 200,000 events were acquired for each dot plot; four control mice and four conditional VCAM-1 mutant mice were analyzed in two independent assays, of which one representative pair of mice is shown.
spleen of conditional VCAM-1 knockout mice. Although the generation of an efficient humoral immune response to a T cell–dependent antigen is dependent on VCAM-1, we could not distinguish whether this effect of the VCAM-1 deficiency is caused by an intrinsic B cell defect or is due to a defect in T and B cell interaction. Interestingly, when conditional VCAM-1 mutant mice were immunized with NP-Ficoll, which serves as a T cell–independent antigen, the immune responses were similar in mutant and control mice (anti-NP IgG1 preimmune levels less than 0.08 μg/ml in mutant and control mice; 14 d after immunization 47 ± 6.2 μg/ml in control and 46.1 ± 22.8 μg/ml in conditional VCAM-1 mutant mice).

**Discussion**

To study the in vivo function of the VCAM-1 gene, conditional VCAM-1 mutant mice were generated, since conventional gene targeting of VCAM-1 had resulted in embryonic lethality. VCAM-1lox/lox mice (here control conventional gene targeting of conditional VCAM-1 mutant mice were generated, since Fig. 1 C). In accordance with the marginal IFN-induced efficiency of deletion in heart, intestine, kidney, and lung spleen, and thymus. Southern blot analysis revealed almost alleles were efficiently deleted in BM, liver, lymph nodes, conditional VCAM-1 mutant mice). 6.2 mutant and control mice; 14 d after immunization 47

![Figure 6](image)

**Figure 6.** T cell–dependent immune response. Anti–NP-IgG1 levels in sera from control mice (white bars) and from conditional VCAM-1 mutant mice (gray bars) are depicted over 35 d after immunization. Two independent experiments were performed. The values shown were calculated from one experiment including three control mice and eight conditional VCAM-1 mutant mice. *Significantly different from value in control mice, P < 0.05.
mice, the development from pre-B cells to mature IgD
BM and peripheral blood in conditional VCAM-1 mutant
cells in the BM.

Apart from the altered distribution of B cells between
BM and peripheral blood in conditional VCAM-1 mutant
mice, the development from pre-B cells to mature IgD+ B
cells does not seem to be affected, given that we detected
similar numbers of mature B cells in lymph nodes and
spleen of conditional VCAM-1 mutant and control mice.
Therefore, it may be that only a limited number of immu-
Nate B cells, which are increased in blood of conditional
VCAM-1 mutant mice, undergoes transition to mature B
cells. We cannot rule out that these immature B cells are
functionally not equivalent to the immature B cells in
blood of control mice. Whether immature B cells in condi-
tional VCAM-1 mutant mice possess the same ability to re-
new the mature B cell pool like immature B cells in control
mice, which have spent a longer time in the BM, and
whether they exhibit the same half-life and homing prop-
erties are still unknown. It has recently been suggested that
the size of the peripheral mature B cell pool is autono-
mously regulated with the priority to maintain normal IgM
serum levels and is not a direct function of the number of
available B cell precursors (64). This mechanism may keep
the mature B cell pool constant in the conditional VCAM-1
mutant mice as well, as these mice exhibit normal Ig levels.
The immature B cells in blood, which are not used to re-
plenish the mature B cell pool of conditional VCAM-1
mutant mice, may leave the blood and may either be trapped or die in other compartments. However, immature
B cells do not accumulate in significant numbers in spleen
or lymph nodes of mutant mice. In addition, we could not
show that B cells undergo apoptosis in blood at significantly
higher rates than in control animals. In general, similar
statements apply also for T cells, e.g., enlarged amounts of
T cells in peripheral blood, but no increased amounts of
apoptotic T cells in blood or higher T cell numbers in sec-
dary lymphoid tissues of young mice.

During fetal life b1 integrins are critical for hematopoie-
sis (65) and B and T cell development postnatally depends on
a4 integrin (63, 66, 67). a4 integrin mediates efficient
attachment and transmigration of pre-B cells beneath the
BM stroma, which seems to be important for B cell devel-
opment. In the absence of a4 integrin, B and T cell devel-
opment is still possible, albeit at a very inefficient rate (67);
B cell development in a4 integrin-deficient chimeric mice
is impaired before the pro-B cell stage (66). By contrast,
our data suggest that B and T cells can develop in the ab-
sence of the a4b1 integrin ligand, VCAM-1. We therefore
hypothesize that, while VCAM-1 mediates retention of
immature B cells in the BM, other ligands of a4 integrin
serve additional functions during lymphopoiesis. The role
of the a4 integrin ligand fibronectin for localization of
HPCs is still controversial. In one study, treatment with re-
agents blocking binding to fibronectin (connecting sequence-
1 [CS-1] inhibitor, mAbs against a5b1) did not lead either to an increase in the number of HPCs in periph-
eral blood or to a reduction of HPCs in BM (22), while a
different study revealed increasing numbers of HPCs by in-
travenous injection of blocking peptides for all three pri-
mary fibronectin-binding sites (68). The expression of fms-
like tyrosine kinase 3 (Flt3) ligand and IL-3 receptor on
early B lineage cells and the in vitro finding that through
these receptors binding of VLA-4 and VLA-5 to fibronec-
tin is augmented (69, 70) support the idea that particularly
early developmental stages of B lineage cells may depend
on interaction with BM stromal cells via fibronectin.

An additional important observation in the BM of con-
tditional VCAM-1 mutant mice is the reduction of the ma-
ture B cells (fraction F) in this compartment (Fig. 2, A and
B). Short-term migration assays confirmed the hypothesis
that in mice mature B cells recirculate from the peripheral
blood to the BM, and showed that in the absence of
VCAM-1 the lodgement of mature B cells to the BM is
impaired (Fig. 5). Therefore, VCAM-1 not only plays a
role in recruitment of HPCs to the BM, as recently shown
(71), but is probably also involved in the recirculation of
mature B cells from peripheral blood to BM. This is sup-
ported by recent findings of Koni et al., who have shown
that migration of mature B cells into the BM is also im-
paired in TIE2Cre;VCAM-1flox/flox mice (72). The func-
tional significance of this recirculation of mature B cells to
the BM is still unclear.

Taken together, our results show that in addition to the
already described function of VCAM-1 for hematopoietic
progenitor localization VCAM-1 plays a crucial role for the
ordered trafficking of immature B cells and mature B cells
between BM and periphery. Since, apart from the leukocy-
tosis in peripheral blood (Table I), the observed conse-
quences of the conditional VCAM-1 mutation exclusively
affect B cells but not T cells (see Results), one can speculate
that during lymphoid development VCAM-1 expression in
BM becomes important for lymphocyte retention subse-
quent to the stage of a common lymphoid progenitor cell
and after the division into T and B cell lineage commitment.

mAbs against either intercellular adhesion molecule
(ICAM)-1, CD54, or VCAM-1 inhibit binding of human
germinal center B cells to follicular dendritic cells in vitro
(25). Moreover, ligation of the B cell receptor augments the
adhesion of B cells to VCAM-1 and fibronectin in germinal
centers by c-Met induction (73). The expression of VCAM-1
on follicular dendritic cells and the possible role of VCAM-1
for costimulation of T cells raised the question of whether
deletion of VCAM-1 would have an impact on the immune
response to a T cell–dependent antigen in vivo. The struc-
turally related adhesion molecule ICAM-1, which in vitro
had also been shown to participate in germinal center inter-
actions and to function as a costimulatory molecule for T
cells (25, 74), does not seem to participate in the humoral
immune response. ICAM-1–deficient mice generate normal
Ab responses to immunization with OVA (75). By contrast,
VCAM-1 is necessary for an efficient immune response against a T cell–dependent antigen, as shown after immunization of conditional VCAM-1 mutant mice (Fig. 6).

Blocking the VLA-4/VCAM-1 adhesion pathway has been demonstrated to increase apoptosis in human germinal center B cells (27). Therefore, VCAM-1 deficiency could also have affected affinity selection of B cells. However, we were unable to detect a difference in affinity maturation between conditional VCAM-1 mutant mice and control mice (data not shown). In addition, B cells in mutant animals were capable of mounting a robust T cell–independent humoral immune response (see Results). The impaired T cell–dependent humoral immune response could be explained by several mechanisms: B cell migration within the germinal center might be affected, and attachment of B cells to follicular dendritic cells might be impaired, leading to inefficient B cell proliferation. Alternatively, T cell help for mounting a humoral immune response might be impaired due to a lack of VCAM-1–mediated costimulation. Finally, recirculation of antigen–specific B cells to secondary lymphoid organs leading to a sustained immune response might depend on VCAM-1. We favor the idea that the reduced humoral immune response may be because of the absence of VCAM-1 expression on dendritic cells. At least IgG-secreting plasma cells are present in similar numbers in spleen and BM of control and mutant mice. Therefore, it is unlikely that an impaired migration of plasma cells to BM could explain the reduced humoral immune response in these mice.

In conclusion, the Mx-cre/loxP system can be used to inducibly delete genes which are expressed in endothelial cells. This approach was used to elucidate the in vivo function of VCAM-1 for B cell localization and humoral immune responses. We have detected a novel role for VCAM-1 in addition to its already known function for HPC migration, namely, its involvement in B cell homeostasis in BM and peripheral blood. VCAM-1 mediates retention of immature B cells in the BM and is probably involved in the recirculation of mature B cells from blood to the BM. Finally, VCAM-1 is critical for the T cell–dependent humoral immune response.

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