Molecular and Cellular Mechanisms of Adaptive Vascular Growth
Chapter 3

Natural Killer Cells and CD4+ T-Cells Modulate Collateral Artery Development


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Abstract

Objective: The immune system is thought to play a crucial role in regulating collateral circulation (arteriogenesis), a vital compensatory mechanism in patients with arterial obstructive disease. Here, we studied the role of lymphocytes in a murine model of hind limb ischemia.

Methods and Results: Lymphocytes, detected with markers for NK1.1, CD3 and CD4, invaded the collateral vessel wall. Arteriogenesis was impaired in C57BL/6 mice depleted for Natural Killer (NK)-cells by anti-NK1.1 antibodies and in NK-cell-deficient transgenic mice. Arteriogenesis was, however, unaffected in Jα281-knockout mice that lack NK1.1+ Natural Killer T (NKT)-cells, indicating that NK-cells, rather than NKT-cells are involved in arteriogenesis. Furthermore, arteriogenesis was impaired in C57BL/6 mice depleted for CD4+ T-lymphocytes by anti-CD4 antibodies, and in major histocompatibility complex (MHC)-class-II-deficient mice that more selectively lack mature peripheral CD4+ T-lymphocytes. This impairment was even more profound in anti-NK1.1-treated MHC-class-II-deficient mice that lack both NK- and CD4+ T-lymphocytes. Finally, collateral growth was severely reduced in BALB/c as compared with C57BL/6 mice, two strains with different bias in immune responsiveness.

Conclusions: These data show that both NK-cells and CD4+ T-cells modulate arteriogenesis. Promoting lymphocyte activation may represent a promising method to treat ischemic disease.
Introduction

Collateral artery development (arteriogenesis) is crucial for prevention and recovery of tissue ischemia caused by arterial occlusive disease.\(^1\) Most patients with arterial obstructions in lower extremities have well developed collateral arteries resulting in mild or no ischemic symptoms at all. Furthermore, it is a well known clinical observation that exercise improves walking distance, possibly by stimulating collateral blood flow.\(^2\) Some patients, however, show impaired collateral formation, resulting in disabling claudication or even tissue loss ultimately requiring limb amputation. To date, it is unknown why individual differences in collateral formation occur. Since there is an extensive variability of the immune system within human populations\(^3\);\(^4\), and evidence is accumulating that immune responses play a crucial role in arteriogenesis, we here focus on cellular components of the immune system and their effects on collateral formation.

Several studies have implicated a role of the innate immune system by showing a function of monocytes in collateral formation.\(^5\)-\(^8\) In addition, it was recently shown that collateral formation is hampered in T-lymphocyte-deficient nude mice\(^9\) and CD4 knockout mice\(^10\), suggesting a role of the adaptive immune system in collateral formation, possibly involving CD4+ T-helper cells. CD4+ T-helper cells are active in secreting multiple cytokines and in modulating trafficking of other cellular components of the immune system, e.g. monocytes/macrophages.\(^11\) Several cytokines that are produced by lymphocytes, such as interleukin 10, 12 and 18, play a role in collateral formation.\(^12\)-\(^14\) In addition, expression of multiple inflammatory genes, including lymphocyte-related markers and cytokines, is elevated in murine hind limbs after femoral artery ligation.\(^15\) Together, these findings imply that the lymphocyte system contributes to collateral formation. However, which specific subsets of lymphocytes play a role in collateral formation is unknown.

Subsets of lymphocytes, such as T-cells, but also Natural Killer (NK) cells and Natural Killer T (NKT) cells, have been suggested to play a role in vascular remodeling, both in physiological conditions, e.g. remodeling of fetal blood supply during pregnancy\(^16\), and in pathological conditions, e.g. atherosclerotic plaque progression.\(^17\)-\(^19\) Recently, it was proposed that the inflammatory responses involved in plaque progression also contribute to collateral formation.\(^20\) However, to our knowledge, a role of either NK-cells or NKT-cells in collateral formation has not been previously reported.

NK-cells, a component of the innate immune system, mediate cellular cytotoxicity among other activities and produce chemokines and inflammatory cytokines such as interferon-\(\gamma\) and tumor necrosis factor.\(^21\) They are important in attacking pathogen-infected cells, especially during early phases of an infection.\(^21\);\(^22\) Furthermore, there
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are indications that NK-cells exert an immunoregulatory effect, for example via crosstalk with dendritic cells.\textsuperscript{23} NKT-cells are a population of NK1.1\textsuperscript{+} T-cells that share some characteristics with NK-cells. Key features of NKT-cells include heavily biased T-cell receptor gene usage, CD1d restriction, and high levels of cytokine production, particularly interleukin-4 and interferon-γ.\textsuperscript{24} The NKT system has been suggested to act as a bridge between innate and adaptive immunity.\textsuperscript{25} In the present study, the roles of CD4\textsuperscript{+} T-helper cells, NK-cells and type I NKT-cells in collateral formation were compared. We show for the first time that NK-cells play a role in collateral formation. In addition, we show that CD4\textsuperscript{+} T-helper cells are involved. There was no evidence for a role of type I NKT-cells in collateral formation.

Materials and methods

Mouse models

Experiments were approved by the committee on animal welfare of the Netherlands Organization for Applied Scientific Research (TNO). For all experiments male mice were used, aged 10-12 weeks. C57BL/6 mice (TNO) were used as standard. BALB/c mice, CB6F1 (C57BL/6 x BALB/c F1) mice (Harlan), Major Histocompatibility Complex Class II-deficient mice and C57BL/6 littermates (Taconic Farms) were purchased. Jα281-knockout mice (C57BL/6 background) were kindly provided by Dr Masaru Taniguchi (Chiba, Japan).\textsuperscript{26} NK-cell-deficient transgenic mice (C57BL/6 background) were kindly provided by Dr Wayne M. Yokoyama (St. Louis, USA). The latter mice were generated using a transgenic construct consisting of Ly49A cDNA under control of the mouse granzyme A gene, leading to defective natural killing activity and deficiency in NK1.1\textsuperscript{+} CD3\textsuperscript{-} cells, but not other lymphocytes, as described.\textsuperscript{27}

In vivo depletion studies

C57BL/6 mice were depleted of CD4\textsuperscript{+} cells by three consecutive intraperitoneal injections of 100 μg of the anti-CD4 antibody GK1.5 given every other day before surgery, and 7 days after surgery.\textsuperscript{28} Isotype-matched rat IgG (Sigma) was used as control. For depletion of NK1.1\textsuperscript{+} cells, C57BL/6 and MHC-class-II-deficient mice received intraperitoneal injections of 250 μg of anti-NK1.1 antibody (PK136) or isotype-matched mouse IgG2a (TNO) as control 5 days and 1 day before surgery, and twice a week after surgery.\textsuperscript{29} Depletion was confirmed in peripheral blood by fluorescence-activated cell sorting analysis using antibodies against CD3 and CD4 (Pharmingen BD) for CD4 depletion or antibodies against NK1.1 and NKG2A/C/E (Pharmingen BD) for NK1.1 depletion.
Surgical procedure

Mice were anesthetized with a combination of Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen) intraperitoneally before surgery. Ischemia of the left hind limb was induced by electro-coagulation of the left common femoral artery proximal to the bifurcation of superficial and deep femoral artery, as described. To analyze cytokine RNA expressions in muscles containing collateral arteries, adductor muscles were dissected from both upper limbs at 3 and 7 days after surgery in anaesthetized mice, and subsequently snap frozen in liquid nitrogen and stored at –70°C for further analysis. Subsequently, to analyze lymphocyte accumulation around collaterals in histological sections, mice were sacrificed followed by intracardial perfusion fixation, as described. A 5 mm-thick transversal slice of the whole upper limb was dissected at the level of femoral artery occlusion. This was done in a similar fashion for the contra-lateral control limb. After removal of the femur, tissues were dehydrated and embedded in paraffin. Five mm-thick cross-sections were serially cut for immunohistochemical analysis.

Angiography

To study collateral vessel development, post-mortem angiography of both hind limbs was performed using polyacrylamide-bismuth contrast (Sigma) at various time points after femoral artery occlusion, as described. Grading of collateral filling was performed in a single blinded fashion by two independent observers and was based on the Rentrop Score. Grading was as follows: 0=no filling of collaterals, 1=filling of collaterals only, 2=partial filling of distal femoral artery, 3=complete filling of distal femoral artery.

Perfusion Imaging

Blood flow of both paws was measured at baseline, immediately after surgery, and serially over 4 weeks, using Laser Doppler Perfusion Imaging (Moor Instruments), as previously reported. To control for temperature variability, animals were kept in a double-glassed vessel filled with water at constant temperature of 37°C for 5 minutes and during subsequent measurements. Perfusion was expressed as a ratio of left (ischemic) to right (non-ischemic) limb.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections using antibodies for CD3 (Serotec), CD4 (Abcam) and NK1.1 (PK136). The number of positively stained cells per microscopic field was scored in a single-blinded fashion (magnification x100). Scoring was as follows: 1=0-5 positive cells per field, 2=5-10 positive cells per field, 3= >10 positive cells per field. Mean arteriole diameter was quantified from randomly photographed sections stained for α-smooth muscle actin...
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(DAKO) using image analysis (Qwin, Leica). A minimum of 10 fields per section was analyzed.

For CD45, MAC3 and PCNA experiments, 5 μm-thick paraffin-embedded sections of muscle were re-hydrated and endogenous peroxidase activity was blocked for 20 minutes in methanol containing 0.3% hydrogen peroxide. Immunohistochemistry was performed using the avidin-biotin-horseradish peroxidase system (DakoCytomation). Heat-induced antigen retrieval was performed for 10 minutes using citrate buffer (pH 6.0) before incubation with antibodies. Sections were incubated overnight with primary antibodies against CD45 (rat-anti-mouse, BD Pharmingen), MAC3 (1:50, rat-anti-mouse, BD Pharmingen), or PCNA (1:100, mouse-anti-human, Calbiochem). After rinsing in PBS, detection was carried out with goat-anti-rat biotinylated antibody (1:300, Jackson) for CD45 and MAC3, and horse-anti-mouse biotinylated antibody (1:400, Vector Laboratories) for PCNA, for 60 minutes at room temperature. The signal was visualized using the NovaRED substrate kit (Vector Laboratories) and sections were counterstained by Mayer’s hematoxylin. Negative controls without primary antibody were run for each sample. Number of cells stained for the various markers was determined in regions with morphological signs of ischemia, such as atrophy of muscle and infiltrating cells. Single-blinded counting of positive cells was performed in 5 microscopic fields (magnification x400) per mouse in these regions.

RNA analysis

Total RNA was extracted from frozen adductor muscle tissue using the RNeasy fibrous tissue midi kit (QIAGen) according to the manufacturer’s protocol. To prevent contamination of genomic DNA in PCR, RNA samples were treated with DNase prior to cDNA synthesis using RNase-free DNase (QIAGen) according to the manufacturer’s protocol. One microgram of total RNA was reversed transcribed into cDNA in a final volume of 233 ml using Ready-To-Go You-Prime First-Strand Beads (Amersham) according to the manufacturer’s protocol. Samples were stored at –20°C until PCR analysis.

For TNFα, TLR-4, MCP-1, GM-CSF and the housekeeping gene HPRT, intron-spanning primer-probe sets were designed using Primer Express™ software (Perkin Elmer, Table 1). TaqMan gene expression assays for IL-1β, IL-4, IL-6 and IFNγ were purchased (Applied Biosystems). Samples were normalized to HPRT housekeeping gene expression. Real-time RT-PCR was performed using Q-PCR mastermix (Eurogentec) in a 25ml reaction volume. After 2 minutes of incubation at 50°C the enzyme was activated by incubation at 95°C for 10 minutes followed by 40 PCR cycles consisting of 15 seconds denaturation at 95°C and hybridization at 60°C for 1 minute.
Taqman Primers and Probes for real-time quantitative RT-PCR

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Table 1

Statistical analysis

Results are expressed as mean±SEM. Comparisons between means were performed using the Student T-test or one-way ANOVA as appropriate. Ordinal scores (angiography, immunohistochemistry) were compared using Pearson Chi-square test. A nonlinear mixed model was additionally used to analyze perfusion data. The model assumes an exponential approach to an asymptote: y = a (1 – e-ct). Log(c) instead of c itself was used to guarantee that it will be a positive number, and to compare rates on a relative scale. We used the library nlme, written for the Open Source system R to estimate the mixed models. To test whether groups were different in the rate of perfusion recovery, the fixed effects for log(c) were compared: the difference of the means was divided by the pooled standard error to derive a z-score. Mann-Whitney and Wilcoxon Signed Rank tests were used for CD45, MAC3, and PCNA experiments. A p-value <0.05 was considered statistically significant.

Results

Mouse strains with different bias in immune responsiveness show a different ability to develop collateral arteries

Lymphocyte-mediated immune responses differ between C57BL/6 and BALB/c mouse strain. We hypothesized that this could be associated with a different ability to grow collateral arteries. To test this, unilateral femoral artery occlusion was
performed in both C57BL/6 and BALB/c mice. Indeed, severe foot necrosis was observed after surgery in most BALB/c mice, whereas only sporadic necrosis of toes was observed in C57BL/6 mice. This was paralleled by angiographic findings; collateral formation was severely impaired in BALB/c mice, whereas C57BL/6 mice demonstrated rapid collateral formation with functional collateral arteries already present at 3 to 7 days after surgery (Figure 1A). Quantification of angiographic images showed a significant decrease of angiographic Rentrop score in BALB/c versus C57BL/6 mice at 7 and 14 days after surgery (Rentrop score 1.6±0.3 versus 2.4±0.2 (p=0.02, n=8) and 2.2±0.3 versus 3.0±0 (p=0.01, n=6), respectively) (Figure 1B). In addition, diameters of arterioles in adductor muscle were decreased in BALB/c mice (Figure 1C). Correspondingly, ischemic to non-ischemic laser-doppler paw perfusion ratios were markedly decreased in BALB/c as compared with C57BL/6 mice throughout the observation period after surgery (p<0.01 at all time points, n=5) (Figure 1D). In contrast, in C57BL/6 x BALB/c F1 mice perfusion recovery after femoral artery occlusion was as rapid as in the C57BL/6 parent strain, indicating a dominant effect of C57BL/6-genes on collateral formation (n=5) (Figure 1D). The 3 groups differed in rate of perfusion recovery according to a nonlinear mixed model (fixed effects: -1.900±0.143 (BL/6), -3.863±0.126 (BALB/c) and -1.205±0.231 (F1), p<0.001 (BL/6 vs BALB/c), p<0.001 (F1 vs BALB/c), p=0.005 (F1 vs BL/6)) (Figure 1D).

Local lymphocyte accumulation accompanies development of collateral arteries

To study whether subsets of lymphocytes accumulate differently between C57BL/6 and BALB/c strains in areas where collaterals are formed, immunohistochemistry for both CD3 and CD4 was performed in upper limbs after unilateral femoral artery occlusion (n=3). Immunohistochemistry for NK1.1 was only performed in C57BL/6 mice, since BALB/c mice do not express the NK1.1-marker. Cells positively staining for CD3, CD4 or NK1.1 were present in adventitias of collateral arteries and increased in number over time after femoral artery occlusion (Figure 2A-C). In non-operated contra-lateral limbs very few lymphocytes were detected. Moreover, C57BL/6 mice accumulated significantly more lymphocytes than BALB/c mice at various time points (Figure 2A and B). Gene expressions of several lymphocyte-related cytokines were studied in adductor muscle tissues by real-time RT-PCR (n=6) (Table 2); IFNγ represents a typical T-helper 1 cytokine, whereas IL-4 and IL-6 represent T-helper 2 cytokines, IL-1β, IL-6, IFNγ, TNFα, MCP-1 and GM-CSF play a role in NK-cell function and regulation, and TLR-4 represents a molecule higher up in the inflammatory cascade.
Figure 1 A Representative angiographic image of upper hind limb 7 days after femoral artery occlusion in C57BL/6 and BALB/c mouse. Collateral artery growth was severely hampered in BALB/c as compared with C57BL/6 mice. B Quantification of angiographic collateral arteries (Rentrop score) (n=4-9). *p<0.05. C Quantification of arteriole diameter in adductor muscle stained for α-smooth muscle cells (*P<0.05, **P<0.01, n=3). D Ischemic/non-ischemic laser-doppler paw perfusion ratios in C57BL/6 mice, BALB/c mice and C57BL/6 x BALB/c F1 hybrid mice. As compared with C57BL/6 mice, perfusion recovery was markedly decreased in BALB/c mice, however, similar rapid perfusion recovery was observed in C57BL/6 x BALB/c F1 mice (n=5). *p<0.05, **p<0.01 (black *=as compared to C57BL/6, gray *=as compared to F1), trend lines according to nonlinear mixed model.
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Figure 2 Representative photomicrographs of lymphocytes (arrows) located in adventitia of collateral arteries (*=lumen of collateral artery, x300) and quantification of the number of lymphocytes per microscopic field in upper limbs of C57BL/6 and BALB/c mice (score 1-3) (n=3). *=p<0.05, **=p<0.01 (for C57BL/6 as compared to BALB/c), O=p<0.05, OO=p<0.01 (as compared to pre-operative value). A-C Immuno-staining for CD3, CD4 and NK1.1, respectively.
Gene expressions in ischemic limbs were significantly increased for IL-1β, IL-4, INFγ and GM-CSF, whereas decreased for IL-6 in C57BL/6 as compared to BALB/c mice. This difference in cytokine expressions between strains reached significance only at 3 days after femoral artery occlusion, paralleled by an increased number of CD4-positive cells around collaterals in C57BL/6 as compared to BALB/c mice at that time. Gene expressions of TNFα, TLR-4 and MCP-1 were comparable between both strains, being significantly elevated in ischemic as compared to non-ischemic limbs at 3 days.

**Impaired collateral formation in the absence of CD4+ T-lymphocytes**

To more specifically determine which types of lymphocytes are involved in collateral formation, first, the role of CD4+ T-lymphocytes was assessed. Femoral artery occlusion was performed in C57BL/6 mice depleted for CD4+ cells by anti-CD4 antibodies. Percentage of CD4+CD3+ cells in peripheral blood was markedly reduced in CD4-depleted mice in comparison with control mice, as determined by flow cytometric analysis on the day of surgery (p<0.01, n=5) (Figure 3A). This indicated successful depletion of CD4+ cells. Collateral formation was impaired in CD4-depleted mice as compared with control mice 7 days after femoral artery occlusion, as shown by angiography (Rentrop score 2.2±0.4 versus 3.0±0.0, respectively, p=0.03) (Figure 3B, C). Furthermore, laser-doppler paw perfusion recovery was decreased 7 days after femoral artery occlusion in anti-CD4 antibody-treated mice (p=0.06) (Figure 3D). Rate of perfusion recovery was decreased in CD4-depleted mice according to a nonlinear mixed model (fixed effects: -1.766±0.361 (control) and -0.998±0.273 (anti-CD4), p=0.045) (Figure 3D). These data indicate involvement of CD4+ cells in collateral artery development.
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Figure 3 A Absence of CD4+ cells of gated CD3+ cells in peripheral blood of CD4-depleted C57BL/6 mice, but not in control mice, as indicated by flow cytometry at the day of surgery. B Representative angiographic image of upper hind limb 7 days after femoral artery occlusion in CD4-depleted or control mice. Collateral arteries (arrows) were less developed in CD4-depleted mice. C Quantification of angiographic collateral arteries at 7 days (Rentrop score) (n=5). *p=0.03. D Ischemic/non-ischemic paw perfusion ratios in CD4-depleted mice as compared with control mice. Perfusion recovery was decreased in CD4-depleted mice 7 days after femoral artery occlusion (n=5). †p=0.06, trend lines according to nonlinear mixed model.
Subsequently, femoral artery occlusion was performed in major histocompatibility complex (MHC) class-II-deficient mice, which are characterized by aberrant maturation of MHC-class-II-restricted CD4+ T-lymphocytes in the thymus and therefore selectively lack mature peripheral CD4+ T-helper cells. Laser-doppler paw perfusion recovery was significantly decreased throughout the observation period of 4 weeks following femoral artery occlusion in MHC-class-II-deficient mice as compared with their C57BL/6 littermates, indicating involvement of CD4+ T-helper cells in collateral formation (p<0.05 at all time points, n=10) (Figure 4A, B). Rate of perfusion recovery was decreased in MHC-class-II-deficient mice according to a nonlinear mixed model (fixed effects: -1.150±0.160 (BL/6WT) and -1.861±0.258 (MHC-class-II−/−), p=0.010) (Figure 4B).

To exclude the possibility that the lack of lymphocytes in our model compromised the clearance of debris from ischemic tissues and fostered inflammation, thereby indirectly influencing blood flow recovery, ischemic hind limb muscle was stained for CD45, MAC3 and PCNA, as a pan-leukocyte marker, macrophage marker and proliferation marker, respectively. Morphological signs of ischemia were similar in MHC-class-II-deficient mice (n=6) and control mice (n=4) 3 days after femoral artery occlusion. Furthermore, the number of positive cells stained for all markers was not significantly different between ischemic muscle regions of MHC-class-II-deficient mice and ischemic muscle of control mice (Figure 5). These data indicate that the lack of lymphocytes does not cause by itself inflammation of the ischemic tissues.

Figure 4 A Representative laser-doppler perfusion image of paws 7 days after left femoral artery occlusion (arrow indicates ischemic paw) in C57BL/6 wild-type mouse and MHC-class-II-deficient mouse. Perfusion recovery after femoral artery occlusion was decreased in MHC-class-II-deficient mice. B Ischemic/non-ischemic paw perfusion ratios (n=10). *p<0.05, **p<0.01, trend lines according to nonlinear mixed model.
Figure 5 Number of positive cells stained for CD45 (A), MAC3 (B), and PCNA (C) in ischemic muscle regions of MHC-class-II-deficient mice (n=6) and ischemic muscle of control littermates (n=4). *p<0.05.

Impaired collateral formation in the absence of NK1.1-positive cells

Since in C57BL/6 mice collateral arteries developed much faster than the anticipated time required to generate a CD4+ T-cell response, the role of Natural Killer (NK) cells in collateral formation was assessed, as NK-cells are known as rapidly reacting lymphocytes.21;22 For this, femoral artery occlusion was performed in C57BL/6 mice treated with the NK-cell-depleting antibody anti-NK1.1, or control antibody. The percentage of NK1.1+ cells in peripheral blood was markedly reduced in NK1.1-depleted mice as compared with control mice, as determined by flow cytometric analysis on the day of surgery (p<0.01, n=6) (Figure 6A). A sustained reduction in NK1.1+ cells was revealed by flow cytometric analysis 14 days after femoral artery occlusion (data not shown). The rate of laser-doppler paw perfusion recovery was decreased in NK1.1-depleted mice as compared with control mice according to a
nonlinear mixed model (fixed effects: -1.438±0.184 (control) and -2.125±0.178 (anti-NK1.1), p=0.004) (Figure 6C). Moreover, comparisons at each time point showed that perfusion recovery was significantly decreased in NK1.1-depleted mice at 3 and 7 days after femoral artery occlusion, but was comparable to controls at 14 days and thereafter (p=0.005, 0.009 and 0.44, respectively, n=6) (Figure 6B, C). Therefore, to visualize collateral formation, angiography was performed already 3 days after femoral artery occlusion. At this time point, collateral growth was indeed significantly impaired in NK1.1-depleted mice as compared with control mice (Angiographic score 0.7±0.3 versus 1.8±0.5, respectively, p=0.04, n=6) (Figure 6D). Together, these data reveal a role for NK1.1+ cells in collateral artery formation, which seemed to be most dominant in early phases of collateral growth.

Additional impairment of collateral formation in CD4+ T-lymphocyte-deficient mice after NK1.1 depletion

To study whether NK1.1+ cells and MHC-class-II-restricted CD4+ T-cells exert their effects independently, we wished to study the effect of the absence of NK1.1+ cells on collateral artery growth in mice lacking peripheral CD4+ T-helper cells. Therefore, femoral artery occlusion was performed in MHC-class-II-deficient mice treated with NK1.1 antibodies. Laser-doppler paw perfusion recovery was markedly decreased in NK1.1 antibody-treated MHC-class-II-deficient mice as compared with non-treated MHC-class-II-deficient mice at 3 and 7 days after femoral artery occlusion (p=0.07 and 0.01, respectively, n=4) (Figure 6E). Moreover, at 7 days after femoral artery occlusion, paw perfusion recovery was decreased by 55% in NK1.1 antibody-treated MHC-class-II-deficient mice as compared with their control C57BL/6 littermates, whereas MHC-class-II-deficient or NK1.1-depleted mice showed a decrease of 22% or 20% as compared with their respective controls. Overall, the 3 groups differed in rate of perfusion recovery according to a nonlinear mixed model (fixed effects: -1.246±0.125 (BL/6WT), -1.952±0.222 (MHCII-/-) and -2.354±0.161 (MHCII -/-+Anti-NK1.1), p=0.003 (BL/6WT vs MHCII-/-), p<0.001 (BL/6WT vs MHCII -/-+Anti-NK1.1), p=0.071 (MHCII -/- vs MHCII -/-+Anti-NK1.1)) (Figure 6E). These findings indicate independent roles for NK1.1+ cells and MHC-class-II-restricted CD4+ T-cells in collateral artery growth.

Impaired collateral formation in Natural Killer cell-deficient mice, but not in mice that lack Natural Killer T-cells

The studies described above clearly point to an independent role for CD4+ T-helper cells and NK1.1+ cells in collateral formation, but do not address the question whether NK1.1-positive NK-cells or NKT-cells are involved in collateral artery growth. The latter cells also express the NK1.1-marker24, and are present in mice deficient for MHC class II as they are restricted by CD1d-molecules.
Figure 6 A Absence of NK1.1+ cells in peripheral blood of NK1.1-depleted mice, but not in control mice, as indicated by flow cytometry for both NK1.1 (y-axis) and NKG2A/C/E (x-axis) at the day of surgery. B Representative laser-doppler perfusion images of paws 3 days after left femoral artery occlusion (arrow indicates ischemic paw). C Ischemic/non-ischemic paw perfusion ratios (n=6). **p<0.01. In NK1.1-depleted mice, perfusion recovery after femoral artery occlusion was significantly decreased in the first 7 days after femoral artery occlusion. Trend lines according to nonlinear mixed model. D Quantification of angiographic collateral arteries 3 days after femoral artery occlusion (Rentrop score) (n=6). *p=0.04. Collateral formation was markedly impaired in NK1.1-depleted mice. E Perfusion recovery after femoral artery occlusion was significantly decreased in NK1.1 antibody-treated MHC-class-II-deficient mice as compared with untreated MHC-class-II-deficient mice (n=4). *p<0.05, **p<0.01 (black*=as compared to MHC-class-II-deficient mice, gray*= as compared to C57BL/6 WT mice), trend lines according to nonlinear mixed model.

To further define the cell type involved in promoting collateral formation, we therefore wished to study collateral artery growth in mice that selectively lack either NKT-cells or NK-cells. For this, first, femoral artery occlusion was performed in Jq281-knockout...
mice, which have a selective loss of Vα14 NKT-cells, leaving other lymphocytes, including NK-cells, intact. Collateral arteries were well developed in Ja281-knockout mice 7 days after femoral artery occlusion (Figure 7A). Angiographic score was not significantly different as compared with control C57BL/6 wild-type mice (n=5) (Figure 7B). There was no significant difference in paw perfusion recovery in time after femoral artery occlusion between Ja281-knockout mice and control C57BL/6 mice (n=7, fixed effects according to non-linear mixed model: -1.520±0.158 (BL/6WT), -1.540±0.214 (Ja281−/−), p=0.470), indicating that type I NKT-cells are not crucial for collateral artery development (Figure 7C). Second, femoral artery occlusion was performed in transgenic mice with a profound and selective deficiency in NK-cells and defective natural killing. Perfusion recovery was impaired in NK-cell-deficient mice at most time points after femoral artery occlusion (n=8, fixed effects according to non-linear mixed model: -0.992±0.222 (BL/6WT), -1.535±0.178 (NKD), p=0.028) (Figure 7D). These findings identify the involvement of NK-cells, but not type I NKT cells in collateral formation. Together, these data indicate that the above-described effects of either CD4 or NK1.1 depletion on collateral artery growth were the results of depletion of T-cells or NK-cells, respectively, but not type I NKT-cells.

Discussion

In the present study, we provide evidence for a role of both NK-cells and MHC-class-II-restricted CD4+ T-cells in collateral formation in mice with acute hind limb ischemia. Several studies have recently indicated that the immune system plays a crucial role in collateral artery growth. These studies focused on a role for monocytes/macrophages. However, roles for inflammatory cell types other than monocytes in collateral formation have only scarcely been reported. Recently, it was shown that collateral formation is hampered in CD4 knockout mice. Although CD4 can also be expressed by monocytes, NKT-cells or dendritic cells, these data suggest a role for CD4+ T-helper lymphocytes on collateral growth. The purpose of the present study was to determine in more detail the role of specific subsets of lymphocytes in collateral artery development. For illustration of the hypothetical interaction of the lymphocyte subtypes studied with respect to the initiation of arteriogenesis see Figure 8. We combined two methods, namely laser-doppler perfusion imaging and post-mortem angiography, to assess collateral artery formation. The former technique provided us with the most quantitative data, and enabled us to follow perfusion recovery in time, whereas the latter technique, being an endpoint measurement, was used to confirm that perfusion recovery relates to visual growth of collateral arteries. We first studied the role of the immune system in collateral formation using C57BL/6 and BALB/c strains that have different genetic backgrounds and display different bias in lymphocyte-mediated immune responses.
Figure 7 A Representative angiographic image of upper hind limb 7 days after femoral artery occlusion in C57BL/6 wild-type mouse or Ja281-knockout mouse, selectively lacking type I NKT-cells. Collateral formation was unaffected in Ja281-knockout mice. B Quantification of angiographic collateral arteries 7 days after femoral artery occlusion (Rentrop score) (n=5). C Ischemic/non-ischemic paw perfusion ratios in Ja281-knockout mice as compared with C57BL/6 wild-type mice (n=7). Perfusion recovery was unaffected in Ja281-knockout mice. Trend lines according to nonlinear mixed model. D Perfusion recovery was impaired in transgenic mice that selectively lack NK-cells (n=8). *p<0.05, **p<0.01, †p<0.07, trend lines according to nonlinear mixed model.

For instance, it was shown that upon infection CD4+ T-cells of C57BL/6 and BALB/c mice show distinct cytokine patterns; C57BL/6 mice represent a prototypic T-helper 1 responder strain, whereas BALB/c mice are considered to be a T-helper 2 responder strain. Furthermore, apart from T-cells, NK-cells may also react differently in C57BL/6 as compared with BALB/c mice. For example, C57BL/6 mice are resistant, whereas BALB/c mice are susceptible to murine cytomegalovirus infection.
The polymorphic NK-cell receptor gene Ly-49H has been shown to account for this cytomegalovirus resistance effect.\textsuperscript{36} Finally, BALB/c mice have fewer thymic and peripheral NKT-cells than C57BL/6 mice.\textsuperscript{37} Our data clearly show that collateral formation is markedly reduced in BALB/c mice as compared with C57BL/6 mice. This was paralleled by decreased accumulation of lymphocytes and changed expression profile of innate cytokines around collaterals in BALB/c mice, suggesting a role of lymphocytes in collateral formation. Although expression of IL-6 was relatively increased in BALB/c mice, expression of IL-4 was decreased, indicating that the retarded collateral formation could not simply be explained by a preferential expression of Th2 cytokines. In
general, expressions of most innate cytokines were increased in C57BL/6 as compared to BALB/c mice, suggesting pro-arteriogenic actions of these cytokines. It should be noted, however, that a better developed pre-existing collateral network in C57BL/6 mice as compared to BALB/c mice may partly explain the rapid collateral formation in C57BL/6 mice. In line with this, we observed a 1.3-fold increase in arteriole diameter in adductor muscle before femoral artery occlusion and increased perfusion recovery immediately after femoral artery occlusion in C57BL/6 mice as compared to BALB/c mice. Nevertheless, we here provide evidence that other, immune-related factors play an additional role in the differences in collateral formation between both strains. Subsequently, we specified which subsets of lymphocytes are involved in collateral formation in C57BL/6 mice using antibody-depletion strategies and transgenic models. Our data clearly point to a role of MHC-class-II-restricted CD4+ T-cells involved in arteriogenesis. Although this study did not address the phenotype of this CD4+ T-cell, it is tempting to speculate that CD25+CD4+ regulating T-cells are participating. These CD4+CD25+ regulating T-cells recognize, in contrast to “conventional” CD4+ T-cells (i.e. Th1- and Th2 cells), self-antigens and are involved in maintenance of normal tissue homeostasis after insult. Moreover, they express an activated phenotype as also exemplified by expression of the activation marker CD25, indicating that they can respond swiftly to disruption of anatomical integrity. Furthermore, although collateral formation was not affected in Jα281-knockout mice, indicating that type I NKT-cells are not involved in this process, our data cannot exclude a role for type II NKT-cells. These T-cells are CD1d-restricted but Jα281-negative and thus still present in Jα281-knockout mice. More detailed analysis of this cell type could be of interest as they could be responsible, like CD4+CD25+ regulatory T-cells, for the influx of CD4+CD3+ cells around collaterals relatively rapid after femoral artery occlusion. Nonetheless, the observed effects on the time course of perfusion recovery after femoral artery occlusion in the absence of NK- and/or T-cells suggest that NK1.1-positive cells play a role in the initiation of collateral formation, whereas T-cells play a role throughout the whole process. The early action of NK-cells in collateral formation is in accordance with the fact that NK-cells are rapidly reacting inflammatory cells, providing the first line of defense against invading pathogens. The role of NK-cells in the context of innate immunity is well characterized in response to tumor cells, transplanted cells, and several classes of pathogens, including many viruses and intracellular bacteria. NK-cell activation is regulated by activating and inhibitory receptors. An important activating NK-receptor, NKG2D, is expressed by all NK-cells. Its ligands, e.g. RAE-1 molecules in mice or MHC-Class-I-related (MIC) molecules in man, are expressed upon cellular stress. Furthermore, MHC-class-I antigen expression is upregulated under ischemic conditions in animal models. Therefore, it is tempting to hypothesize that cellular stress, induced by ischemia or elevated shear-stress in collaterals upon arterial occlusion, upregulates RAE-1/MIC or MIC-like molecules. Subsequent triggering of NKG2D may lead to NK-cell
activation and thereby release of inflammatory factors that are involved in collateral artery development. Interestingly, poor collateral formation as observed in BALB/c mice was completely abolished in C57BL/6 x BALB/c F1 mice that carry alleles of both C57BL/6 and BALB/c strain, indicating a dominant genetic effect of the C57BL/6 genome. One possible explanation may be that a modulated NK-receptor-ligand interaction, as reported in BALB/c mice, leads to poor collateral growth by inadequate NK-cell activation. Receiving NK-receptor-ligand-related genes from the C57BL/6 background may reverse this deleterious effect. Another explanation may contribute to the effect of particularly the NK cells on arteriogenesis as well. Differences in pre-existing collateral networks may be genetically determined, which may explain why one patient forms an adequate collateral network or responds well to arteriogenic treatment, and the other patient does not. Interestingly, leucocytes were recently proposed to play a role in retinal vascular remodeling or pruning during development. A role for the immune system in embryonic development of a collateral network is yet to be determined, but it presents a challenging additional hypothesis for the contribution of NK cells in collateral formation.

We can conclude that both NK-lymphocytes and CD4+ T-lymphocytes are involved in collateral formation in mice, since the absence of these subpopulations of lymphocytes interferes with arteriogenesis. However, it should be taken into account that the evidence obtained in our mouse models, is mainly indirect evidence for the proposed therapeutic beneficial use of lymphocytes for inducing arteriogenesis. Future research will determine whether and how lymphocytes play a role in arteriogenesis in man. Stimulation of arteriogenesis by specific activation of defined lymphocyte subsets might be a promising treatment for patients with ischemic disease.

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