Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation

Seghers, L.

6

C57BL/6 NK gene complex involved in vascular remodeling in general

L. Seghers[1,2,5] Msc; M.R. de Vries[1,5] Bsc; J. van Bergen[3] PhD; R.E.M. Toes[4] PhD; V.W.M. van Hinsbergh[2] PhD; P.H.A. Quax[1,5] PhD

1 Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
2 Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands
3 Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands
4 Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
5 Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

Submitted for publication

Abstract

Objective: The immune system plays an important role in vascular remodeling. Monocytes, CD4+ and CD8+ T cells were already proven to be modulators of vascular remodeling. NK cells were indicated to contribute to vascular remodeling in atherosclerotic lesion development and recently in revascularisation. Genetic differences between C57BL/6 and BALB/c mice, particularly in the Natural Killer gene Complex (NKC) where linked to differences in revascularization. Here we expand the role of NK cells, and in particular of the C57BL/6 NKC, in vascular remodeling in general.

Methods and Results: C57BL/6, BALB/c and BALB.B6-CMV1^{r} (CMV1^{r}) mice, of which the latter is congenic for the C57BL/6 NKC, were used in two different models of vascular remodeling, induction of intimal hyperplasia by femoral artery cuff placement and vein graft remodeling by placement of a venous interposition in the mouse carotid artery.
NK cell depleted C57BL/6 mice demonstrated significant reduced intimal hyperplasia after cuff placement compared to controls. Subsequently, cuff placement and vein grafting in C57BL/6, BALB/c mice and in BALB/c mice congenic for the C57BL/6 NKC, BALB.B6-CMV1^{r} (CMV1^{r}), resulted in profound intimal hyperplasia in C57BL/6 mice, but also in CMV1^{r} mice, whereas this was significantly reduced in BALB/c mice. CD45 staining for leukocytes, showed significant larger leukocyte infiltration in both C57BL/6 and CMV1^{r} vein graft segments, compared to BALB/c vein graft segments.

Conclusions: These data demonstrate a role for NK cells in vascular remodeling in general.

Introduction

Vascular remodeling is a multi factorial process that induces structural alteration of the vessel wall composition [1] and applies to several vascular diseases, such as atherosclerosis [2], vein graft disease, and also contributes to outward growth of collateral arteries [3, 4]. The immune system plays an important role in vascular remodeling. Leukocyte subsets such as monocytes, CD4+ and CD8+ T lymphocytes were already proven to be modulators of vascular remodeling [2, 5]. Recently, NK cells were demonstrated to contribute to vascular remodeling processes in atherosclerosis [6] and revascularization [7]. In another recent study, genetic differences in the Natural Killer gene Complex (NKC) between C57BL/6 and BALB/c mice were demonstrated to explain differences in collateral artery growth (Seghers et al, unpublished data; chapter 5 of this thesis). It is not clear whether these genetic NKC differences could also explain the strain dependent differences in other vascular remodeling processes [8].

Shear stress alterations or direct injury induces endothelial cell activation, which is often the initial step in vascular remodeling. As a response vessel wall permeability increases, and activated endothelial cells secrete chemo attractants such as monocyte chemo-attractant protein-1 (MCP-1) and express increased levels of cellular adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1). In this initial phase, MCP-1 attracted monocytes adhere to and invade in the vessel wall and then contribute to the local inflammatory cascade by production of pro-inflammatory cytokines and growth factors [2]. MCP-1 is also a chemo-attractant for NK cells [9] that are found present in early atherosclerotic lesions and in the vessel wall of remodeling collateral arteries [7]. In a somewhat later phase Natural Killer T cells and T lymphocytes arrive at the site of remodeling [2].

Vascular remodeling is different between patients, as for example observed in atherosclerotic plaque composition [10], vein graft thickening and in collateral artery growth [11]. It is not entirely clear why this variation exists, but is suggested that this is due to differences in immune response. This is supported by the observation of strain dependent differences in vascular remodeling between mouse strains. We and others observed that C57BL/6 mice display better collateral artery growth when compared to BALB/c mice [7, 12, 13]. This can in part be explained by a different immune bias between these two strains, existing of a difference in T-helper response [14] and genetic differences in the Natural Killer gene complex (NKC) [15], a gene locus on chromosome 6 encoding multiple activating and inhibitory NK cell receptors such as NKG2D, Nkrp1c (NK1.1), CD94/NGK2, and the highly polymorphic Ly49 receptor family [16, 17]. Compared to C57BL/6, BALB/c mice lack a 200kb region containing encoding members of the Ly49 receptor family. As a consequence the BALB/c strain has only 7 Ly49 genes, whereas the C57BL/6 NKC possesses 12 Ly49 genes.

In a recent study of our group, it was shown that the NKC differences in part explain the strain dependent variation in collateral artery growth between C57BL/6 and BALB/c (Seghers et al, unpublished data; chapter 5 of this thesis). Remarkably, the presence of the C57BL/6 NKC in these congenic BALB/c mice, led to a substantial improvement of their naturally poor collateral artery growth towards the rapid collateral artery growth of C57BL/6 mice. This was shown in a BALB/c mouse strain, which is congenic for the entire C57BL/6 NKC.
Originally, this congenic BALB.B6-CMV1^{r} mouse strain, was generated by Scalzo and colleagues to map genes involved in murine cytomegalovirus (mCMV) resistance [18]. By the expression of the entire C57BL/6 NK cell receptor repertoire on their NK cells, these congenic mice, exhibit a level of resistance towards mCMV comparable to that of C57BL/6 mice [19].
In this study we want to further expand our work on the role of NK cells in processes of vascular remodeling in a non-atherosclerotic setting. In particular, we want to study the effect of strain dependent differences in the Natural Killer gene complex on general vascular remodeling. Therefore, we hypothesize that differences in Natural Killer gene complex between C57BL/6 and BALB/c mice result in strain dependent differences in general vascular remodeling. To study this we use the congenic BALB.B6-CMV1^{r} mice and C57BL/6 and BALB/c mice.

Materials and methods

Mice

Animal experiments were approved by the LUMC-animal welfare committee. For all experiments male mice were used, aged 12-16 weeks. C57BL/6 mice (Charles River, L'Arbresle Cedex, France) and BALB/c mice (Charles River, L'Arbrescle Cedex, France) were purchased. Jα281-knockout mice (C57BL/6 background) were kindly provided by Dr Masaru Taniguchi (Chiba, Japan) [20]. BALBc.B6-CMV1^{r} breeding couples were kindly provided by Dr. W. M. Yokoyama (St. Louis, USA). All animals were fed regular chow diet (Sniff Spezialdiäten GMBH, Soest, Germany) ad libitum. For surgery, mice were anesthetized by using Midazolam (5 mg/kg; Roche, Woerden, The Netherlands), Medetomide (0.5 mg/kg; Orion, Espoo, Finland), and Fentanyl (0.05 mg/kg; Janssen Pharmaceutics, Beerse, Belgium).

Cuff induced intimal hyperplasia mouse model

Both left and right femoral arteries were dissected from their surroundings. A non-constrictive polyethylene cuff (Portex, 0.40 mm inner diameter, 0.80 mm outer diameter,

and 3.0 mm length) was placed loosely around the femoral artery bilaterally and kept in situ for 21 days. Mice were sacrificed after 21 days and cuff segments were harvested after perfusion fixation with 4% formaldehyde. Tissue handling and analysis of intimal hyperplasia was performed as described [21]. Briefly, for quantification of intimal thickening, elastic laminae were visualized with Weigert's elastin staining. Six sequential representative sections per vessel segment were used to quantify the amount of intimal hyperplasia, using image analysis software (Qwin, Leica, Rijswijk, The Netherlands).

Vein graft mouse model

A venous interposition was placed in the mouse carotid artery as described previously [22, 23]. Caval veins of donor mice were placed as vein grafts in the left carotid artery of recipient mice. Mice were sacrificed 28 days after surgery for histological analysis. Vein graft segments were harvested after perfusion fixation with 4% formaldehyde, fixated overnight and paraffin-embedded using an automated tissue processor (Leica). Cross-sections were made throughout the embedded vein grafts. Six representative sections per vessel segment were stained with Haematoxylin-Phloxine-Saffron (HPS) for histological and morphometric analysis (Leica). Vein graft thickening was defined as the area between lumen and adventitia and determined by subtracting the luminal area from the total vessel wall area.

Immunohistochemistry

Staining for CD45 was performed on 5 μm-thick paraffin-embedded sections of vein grafts that were were incubated overnight with a primary antibody against CD45 (1:100 rat-anti-mouse, BD Pharmingen, United States of America), as described [7]. Negative controls without primary antibody were run for each sample.

Quantification of CD45 immunostained sections

Intensity of CD45 stained vein graft segments were performed single blinded, scoring the intensity in a range from 1 to 3 in 6 consecutive sections per mouse. 1: < 25 CD45 positive cells / section of vessel wall, 2: 25-50 CD45 positive cells / section of vessel wall, 3: >50 CD45 positive cells. Scores from each individual mouse were averaged.

NK cell depletion

For depletion of NK1.1+ cells, CMV1^{r} mice received intraperitoneal injections of 200μg of anti-NK1.1 antibody (PK136) or of an isotype-matched mouse IgG2a control antibody 5 days, 3 days and 1 day before surgery, and twice a week after surgery. Depletion was confirmed in peripheral blood by fluorescence-activated cell sorting (FACS) -analysis using antibodies against NK1.1 and CD3 (BD Biosciences, San Jose, USA) just before surgery and 14 days after surgery.

NK cell activation assay

1 x 10^7 splenocytes were cultured in 24-wells plates coated with either anti-NKp46 antibody (R&D Systems Europe, Oxon, United Kingdom), isotype-matched control (goat IgG, R&D Systems Europe, Oxon, United Kingdom) antibody (both at 5μg / well), or in medium containing 0.1 μg/ ml Phorbol 12-Myristate 13-Acetate (PMA) and 0.5 μg/ ml ionomycin (Sigma-Aldrich, St. Louis, United States of America). Intracellular staining for interferon-gamma (IFN-γ, BD Biosciences, San Jose, USA) was performed after a 4-h incubation at 37ºC in the presence of Brefeldin A. Percentages of IFN-γ producing NK cells were obtained by gating on CD3ε-, NK1.1+ or CD3ε-, DX5+ cells during FACS analysis on BD FACS LSR II using BD FACS Diva software v6.0 (BD Biosciences, San Jose, USA). For each mouse, the percentage of NK cells producing IFN-γ in response to NKp46 incubation was normalized for the response to PMA/ionomycin as follows: (NKp46 – control antibody)/(PMA/iono – control antibody) x 100%.

Statistical analysis

Results are expressed as mean±SEM. Comparisons between medians were performed using the Mann Whitney test as appropriate. A p-value <0.05 was considered statistically significant.

Results

NK cells, but not NK-T cells, contribute to intimal hyperplasia

Several leukocyte subsets such as monocytes, T lymphocytes, NK-T cells, and NK cells were demonstrated to play a role in atherosclerotic lesion formation. In this study, we wanted to expand the role of NK cells in vascular remodeling, by demonstrating a role for NK cells in the induction of intimal hyperplasia in a non-atherosclerotic setting. Therefore, NK cells were depleted in C57BL/6 mice that subsequently received a non-constrictive cuff around the femoral artery.

Flowcytometric (FACS) analysis of peripheral blood samples on the day of surgery confirmed that NK-cell depletion had been successful (Figure 1A), and the depletion was sustained as revealed by FACS analysis 14 days after surgery (data not shown).

21 days after cuff placement NK1.1 depleted C57BL/6 mice displayed significant reduced intimal hyperplasia compared to controls (2265±324 μm^2 vs. 5251±627 μm^2; Figure 1B-D). As a consequence lumen stenosis was significantly reduced by approximately 50% in NK1.1 depleted mice when compared to controls. The intima/media (I/M) ratio was also significantly reduced in NK1.1 depleted C57BL/6 mice as compared to controls.

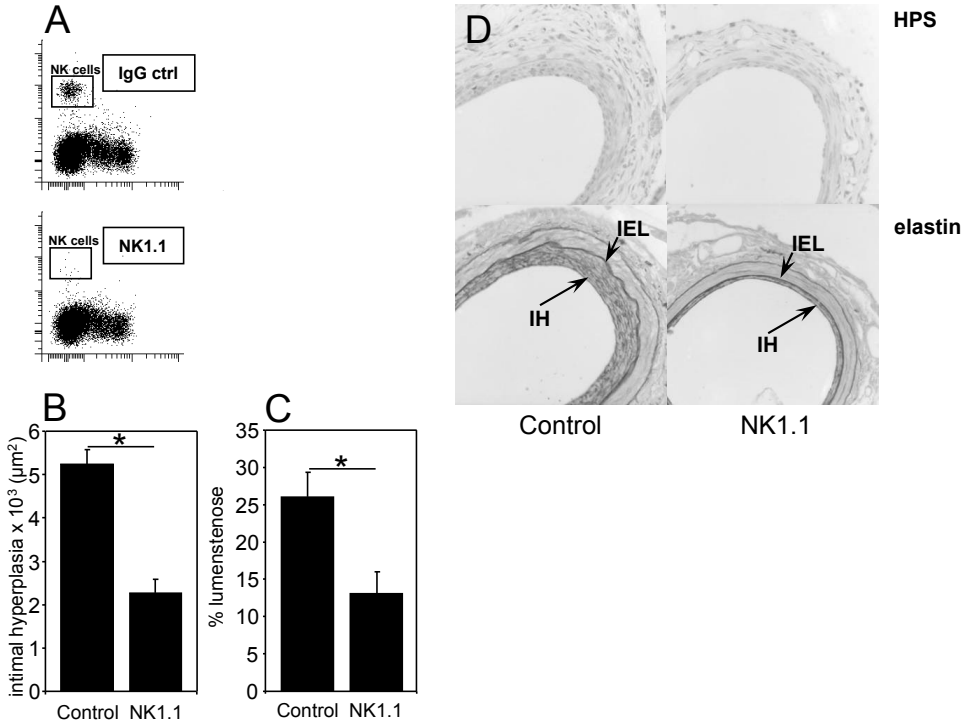

Figure 1. A. Representative of flow cytometric analysis for NK cells in peripheral blood from NK1.1 depleted C57BL/6 mice and IgG treated controls at the moment of cuff placement; average NK cell percentages of lymphocyte population: NK1.1 0.16±0.02% and IgG control 2.90±0.18%. Gated for CD3ε- NK1.1+ cells. **B.** Intimal hyperplasia in cuffed femoral artery segments of NK1.1 depleted C57BL/6 mice (n=10) and controls (n=10) after 21 days. NK1.1 depleted C57BL/6 mice show a significant reduction of intimal hyperplasia. **C.** Percentage of lumen stenosis by intimal hyperplasia in NK1.1 depleted C57BL/6 mice and controls after 21 days. **D.** Representative microscopic photographs (magnification 40x) of HPS and elastin stained cuffed femoral artery segments from NK1.1 depleted C57BL/6 mice and controls 21 days after cuff placement; arrows indicate intimal hyperplasia (IH) or internal elastic lamina (IEL). * p-value <0.01. *See color figure on page 226.*

Since NK1.1 mediated depletion also depletes NK-T cells, and NK-T cells were previously indicated to play a role in atherosclerotic lesion formation, we studied cuff induced formation of intimal hyperplasia in NK-T deficient Jα281-knockout mice (Jα281$^{-/-}$) on a C57BL/6 background and littermate controls. As depicted in figures 2A-C, no differences in cuff induced intimal hyperplasia were observed in Jα281$^{-/-}$ mice when compared to controls.

The significant reduction in intimal hyperplasia and subsequent reduced lumen stenosis in NK cell depleted mice, but not in NK-T cell deficient mice, indicates a role for NK cells, but not NK-T cells, in the formation of intimal hyperplasia in a non-atherosclerotic setting. With this indication we can relevantly study the impact of the strain dependent NKC differences in this process of vascular remodeling.

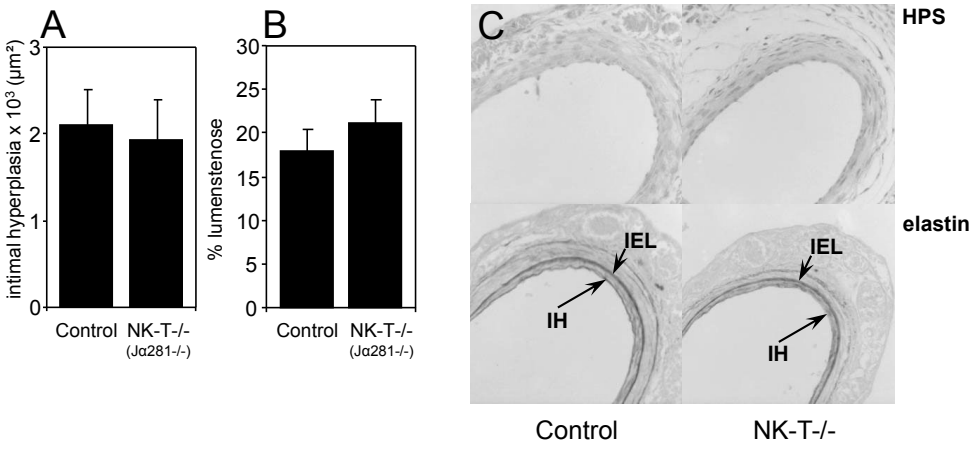

Figure 2. A. Average intimal hyperplasia in cuffed femoral artery segments of NK-T deficient ($J\alpha281^{-/-}$) mice (n=8) and controls (n=8), showing no significant differences between the two groups after 21 days. **B.** Percentage of lumen stenosis by intimal hyperplasia in $J\alpha281^{-/-}$ mice and controls. **C.** Representative microscopic photographs (magnification 40x) of HPS and elastin stained cuffed femoral artery segments from $J\alpha281^{-/-}$ mice and controls 21 days after cuff placement; arrows indicate intimal hyperplasia (IH) or internal elastic lamina (IEL). *See color figure on page 227.*

The C57BL/6 NK gene complex increases intimal hyperplasia in BALB/c mice

Our group previously demonstrated that genetic differences in NK cell gene complex (NKC) between C57BL/6 and BALB/c mice results in different arteriogenesis, another concept of vascular remodeling. By presence of the C57BL/6 NKC in congenic BALB/c, their naturally poor arteriogenic response was recovered towards the rapid arteriogenic response of C57BL/6. Since it is demonstrated that NK cells contribute to the formation of intimal hyperplasia, we subsequently wanted to study the effect of these strain dependent NKC differences in different processes of vascular remodeling, such as cuff induced intimal hyperplasia and vein grafting.

To study the role of the differences in NKC between C57BL/6 and BALB/c in vascular remodeling, non-constrictive cuffs were placed around the femoral arteries of C57BL/6, BALB/c and of the congenic BALB/c mouse strain congenic for the C57BL/6 NKC, BALB.B6-CMV1^{r} (CMV1^{r}), and we compared formation of cuff induced intimal hyperplasia between these mouse strains in the cuffed femoral artery segments that were harvested after 21 days.

Our analysis of the vessel wall revealed that CMV1^{r} mice displayed an approximate 3 fold significant increase in intimal hyperplasia, as compared to BALB/c (Figure 3A). The extent of intimal hyperplasia in CMV1^{r} mice was equal to that of C57BL/6 mice. Measurement of the media, demonstrated that this was significantly thicker in CMV1^{r} mice as compared to their background BALB/c strain, which suggests more smooth

muscle cell proliferation in the congenic strain. Lumen stenosis in CMV1^r mice was also significantly increased by approximately 2.5 fold (Figures 3B-E).
Together, these data indicate that a factor within the C57BL/6 NK gene complex induces significant formation of intimal hyperplasia. Furthermore, the increase of medial thickness in CMV1^r mice, suggests that more smooth muscle cell proliferation has occurred than in BALB/c mice.

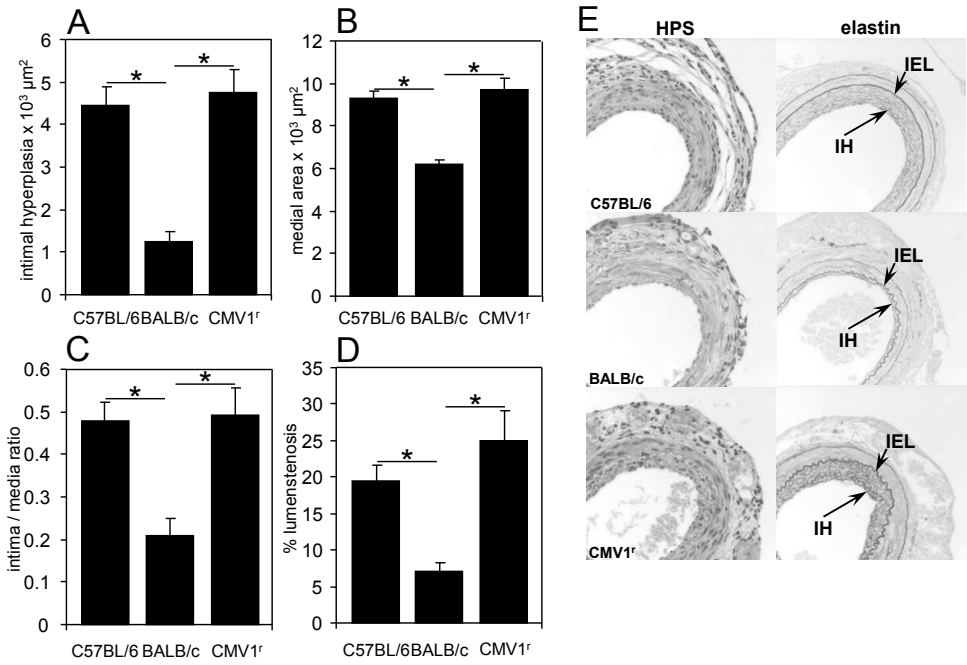

Figure 3. Morphometric quantification of cuffed femoral artery segments from C57BL/6 (n=9), BALB/c (n=5) and CMV1^r mice (n=9) 21 days after cuff placement.
A. Average area of intimal hyperplasia (μm^2). **B.** Average medial area (μm^2). **C.** Average intima / media ratio. **D.** Percentage of lumenstenosis. **E.** Representative microscopic photographs (magnification 40x) of HPS and elastin stained cuffed femoral artery segments 21 days after cuff placement; arrows indicate intimal hyperplasia (IH) or internal elastic lamina (IEL). * p-value <0.01.
See color figure on page 227.

The C57BL/6 NK gene complex contributes to profound vein graft remodeling

To further establish a role for the C57BL/6 NKC in general vascular remodeling, we assessed the role of the NKC in our second model for vascular remodeling; the mouse model for vein graft disease. In C57BL/6, BALB/c and CMV1^r mice a venous (caval vein) interposition was placed in their left carotid artery which was left in situ for 28 days.
After 28 days the vein graft segments were analyzed, and it was revealed that CMV1^r mice displayed a strong increase in vein graft thickening by 104% when compared to

BALB/c mice (Figure 4A and D). Vein graft thickening in CMV1^r mice was slightly, but not significant, bigger than in C57BL/6. The total vessel area in CMV1^r mice was also significantly larger than that of BALB/c mice, and equaled that of C57BL/6 (Figure 4B). From these data, compared to standard caval vein diameters of all tested strains, it can be concluded that both CMV1^r and C57BL/6 mice demonstrated outward remodeling of the vein graft, whereas BALB/c mice showed reduced outward remodeling (Figure 4C). Presence of the C57BL/6 NKC in the congenic CMV1^r mice here contributed to increased vein graft remodeling and converted the reduced BALB/c response towards that of C57BL/6.

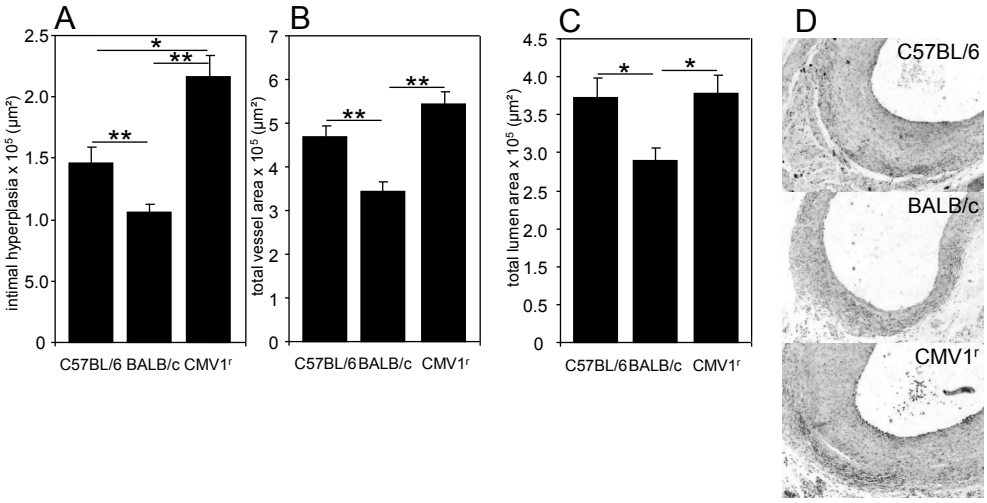

Figure 4. Morphometric quantification of vein graft segments from C57BL/6 (n=10), BALB/c (n=9), and CMV1^r mice (n=9) 21 days after engraftment.
A. Average area of intimal hyperplasia (μm^2). **B.** Average total vessel area (μm^2). **C.** Average total lumen area (μm^2). **D.** Representative microscopic photographs (magnification 20x) of HPS stained vein graft segments 21 days after engraftment from C57BL/6, BALB/c and CMV1^r mice. * p-value <0.05; ** p-value <0.01 *See color figure on page 228.*

Increased inflammatory cell influx in CMV1^r vein grafts

In order to study potential differences in the inflammatory response induced by NK activation, we analyzed the presence of leukocytes in vein graft segments by an immunohistochemical staining for CD45, a pan-leukocyte marker. As depicted in figure 5A, a profoundly stronger inflammatory reaction in C57BL/6 and CMV1^r mice was observed, which was reflected by larger inflammatory cell infiltrates when compared to BALB/c mice. Comparison of the CD45 staining between the three mouse strains, performed single blinded, revealed that the infiltrate of CD45 positive cells was significantly larger in both C57BL/6 and CMV1^r when compared to BALB/c mice (Figure 5B).

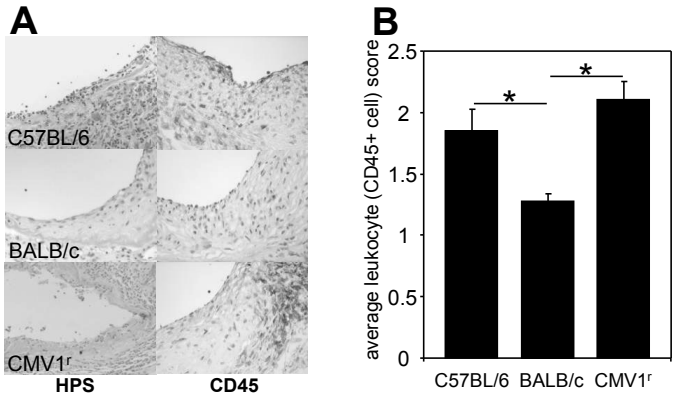

Figure 5. A. Representative microscopic photographs of CD45 stained vein graft segments, demonstrating differential influx of CD45 positive cells in to the vessel wall of C57BL/6, BALB/c, and CMV1^r mice. **B.** Quantification of CD45 positive cells that are present in the vessel wall expressed as average leukocyte score. * p-value <0.01. *See color figure on page 228.*

NK cell responsiveness is improved in BALB.B6-CMV1r compared to BALB/c

The presence of the C57BL/6 NKC in CMV1^r mice results in the development of NK cells expressing a different receptor repertoire compared to BALB/c. In recent years, it has become clear that the number and strength of inhibitory interactions between Ly49 receptors and MHC class I determine NK cell responsiveness [24, 25].

Based on the strain-dependent NKC differences, we hypothesized that the differences in responsiveness between CMV1^r and BALB/c NK cells might correlate with the differences in leukocyte accumulation in vein grafts, and the subsequent different phenotypes of vascular remodeling in these mice.

To investigate this, we employed a commonly used assay to measure NK cell responsiveness, measuring intracellular IFN-γ in NK cells upon crosslinking of activating receptors [24, 25]. To this end, splenocytes of C57BL/6 (n=6), BALB/c (n=6) and CMV1^r mice (n=6) were stimulated *in vitro* with plate-bound antibodies to NKp46, an activating NK receptor present in equal densities on all splenic NK cells in the three mouse strains. An isotype-matched control antibody and Phorbol-12-Myristate-13-Acetate (PMA) and ionomycin served as negative and positive control stimuli, respectively. NK cell responses to the different stimuli were analyzed by FACS, measuring the frequencies of IFN-γ positive cells within the NK cell population after 5 hours of stimulation.

The response of CMV1^r NK cells to NKp46 stimulation was quite similar to C57BL/6 NK cells, reaching an average 24.2±3.1% and 31.1±10.7% of the maximum response respectively (Figure 6). This IFN-γ response was significantly different from BALB/c NK cells ($p<0.01$) that responded poorly reaching only 2.7±0.9% of the maximum IFN-γ response after NKp46 stimulation. NK cell IFN-γ response to PMA / ionomycin stimulation was not different between strains.

These data demonstrate that CMV1^r NK cells have a significantly improved responsiveness when compared to BALB/c NK cells in reaction to a NK receptor specific activating stimulus, and that this difference is likely explained by NK cell receptors encoded by the C57BL/6 NKC.

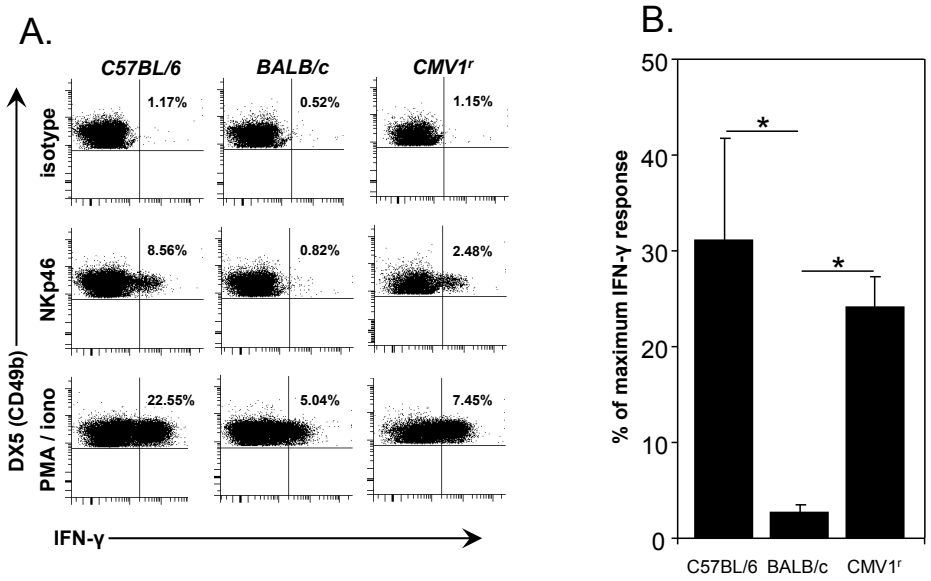

Figure 6. A. NK cells expressing DX5 (CD49b) and intracellular IFN-γ (previously gated on CD3ε-). Flow cytometry of IFN-γ expression in NK cells from C57BL/6, BALB/c and CMV1^r mice after stimulation with plate bound NKp46, goat IgG isotype control or with PMA / ionomycin. Representative of two independent experiments (n=3 mice per strain per experiment). **B.** NK cell responsiveness to specific NK receptor mediated activation by NKp46 in C57BL/6, BALB/c and CMV1^r NK cells expressed as average percentage of the maximum IFN-γ response that was achieved by non-specific PMA/ionomycin stimulation (data pooled from two independent experiments n=3 mice per strain per experiment).
Responsiveness to PMA/ionomycin non-specific stimulation was not different between the tested strains. Induction of NK cell IFN-γ production by NKp46 was significant when compared to 'background' IFN-γ production of NK cells incubated with isotype matched control (goat IgG) in all tested mousestrains (data not shown).
* p-value <0.01.

Discussion

In the present study we further expand the role of NK cells in different processes of vascular remodeling in a non-hypercholesterolemic setting. Furthermore, we demonstrated a contributory role for the C57BL/6 Natural Killer gene Complex in general vascular remodeling, since the presence of the C57BL/6 NKC in a congenic BALB/c mouse strain

induced profound vascular remodeling in two mouse models for vascular remodeling.
In the past two decades studies have focused on the role of different inflammatory cell subsets on vascular remodeling in an atherosclerotic setting [2, 5]. Here, we for the first time demonstrate a role for NK cells in vascular remodeling in a non-atherosclerotic setting. NK cell depletion resulted in significant reduction of intimal hyperplasia in our C57BL/6 mice. This is paralleled by previous studies that described significant reduction of atherosclerotic lesion development in LDL-$R^{-/-}$ mice without functional NK cells [6]. A role for NK-T cells was previously indicated in the induction of atherosclerotic lesion formation [26]. However, in our model the formation of intimal hyperplasia was not affected in NK-T cell deficient Jα281$^{-/-}$ when compared to controls. This indicates that type I NK-T cells are not implicated in the formation of intimal hyperplasia in a non-atherosclerotic setting. It should be taken into account that our data cannot exclude a role for type II NK-T cells, which are CD1d restricted but Jα281-negative and thus still present in Jα281$^{-/-}$ mice.
Vascular remodeling is an inflammatory process, which is initiated after endothelial cell damage and activation. Increased endothelial production of chemo-attractants, such as Monocyte Chemo-attractant Protein-1 (MCP-1) [9], and increased presentation of cellular adhesion molecules, such as Vascular Cellular Adhesion Molecule-1 (VCAM-1), facilitates recruitment, adhesion, and finally peri-vascular invasion of a number of inflammatory cell subsets, such as monocyte derived macrophages, but also, as it appears now, NK cells for which MCP-1 is also a chemo-attractant [27].
The second important finding in this study is that strain dependent differences in vascular remodeling in both cuff induced intimal hyperplasia and vein graft remodeling between C57BL/6 and BALB/c are explained by differences in the Natural Killer gene complex. Introduction of the C57BL/6 NKC in BALB/c congenic mice, BALB.B6-CMV1^{r} (CMV1^{r}), resulted in complete abolishment of the vascular remodeling phenotype of BALB/c towards the profound phenotype of C57BL/6 mice in two different mouse models for vascular remodeling. In both these mouse models BALB/c mice showed significant reduced vascular remodeling, whereas C57BL/6 mice and the congenic CMV1^{r} mice showed abundant remodeling.
These data are completely in line with our previous observation in arteriogenesis, another concept of vascular remodeling, where congenic CMV1^{r} mice demonstrated a substantial improvement of their naturally poor arteriogenic response towards the rapid arteriogenic response of C57BL/6.
Since CMV1^{r} mice express the C57BL/6 NKC, our data suggest that vascular remodeling is modulated by a factor that is present within the C57BL/6 NKC, but absent from the BALB/c NKC. Compared to the C57BL/6 NKC, the BALB/c NKC contains fewer functional genes encoding activating and inhibitory NK cell receptors [28].

These differences were shown to have consequences for NK cell responsiveness, since BALB/c NK cells exhibited significant hypo-responsiveness towards activating stimuli *in vitro*, and this was completely abolished in NK cells from the congenic BALB/c mouse strain (CMV1^r) towards the normal and adequate responsiveness of C57BL/6 derived NK cells.

We speculate that the presence of the additional NK cell receptors explain the greater NK cell responsiveness in CMV1^r mice, and it could well be that these differences in NK cell responsiveness between C57BL/6 and BALB/c contribute to the induction of a different inflammatory response. This might have resulted in the differences in leukocyte infiltration as observed in the vein graft segments of the three mouse strains used in our study. The inflammatory cell influx in vein graft segments was markedly increased in both C57BL/6 and CMV1^r, but not in BALB/c. This suggests that CMV1^r mice have a similar inflammatory response as C57BL/6 mice.

BALB/c mice have a T helper-2 (Th-2) response, associated with reduced vascular remodeling. C57BL/6 mice generally have a Th-1 response, that is associated with profound vascular remodeling [14]. This is also confirmed by the observed differences in vascular remodeling between C57BL/6 and BALB/c in our experiments. Both CMV1^r and C57BL/6 mice demonstrated more intimal hyperplasia and inward remodeling, which is seen often in Th-1 mediated responses [29]. In BALB/c mice remodeling is less pronounced. The increased inflammatory cell influx in vein graft segments in C57BL/6 and CMV1^r, but not in BALB/c mice, suggests that the hypo-responsive NK cell phenotype might indirectly contribute to the induction of a different inflammatory response and thereby reduced vascular remodeling.

In conclusion, the current study expands the role of NK cells in general vascular remodeling. Furthermore, we demonstrate that the genetic differences in the Natural Killer gene complex explain strain dependent differences in vascular remodeling. Presence of the C57BL/6 NKC results in a different phenotype of vascular remodeling, which might contribute to a different inflammatory cascade. These data suggest that a factor for profound vascular remodeling resides within the C57BL/6 NK cell gene complex.

Sources of funding

This study was sponsored by the EU European Vascular Genomics Network (LSHM-CT-2003-503254), the Dutch Program for Tissue Engineering (DPTE) grant VGT6747, and by the Netherlands Initiative for Regenerative Medicine. JvB was supported by the Landsteiner Foundation for Blood transfusion Research (grant 0515).

Disclosures

Conflict of Interest: none declared.

References

1. Gibbons GH, Dzau VJ. The emerging concept of vascular remodeling. *N Engl J Med* 1994 May 19;330(20):1431-8.
2. Hansson GK, Robertson AK, Soderberg-Naucler C. Inflammation and atherosclerosis. *Annu Rev Pathol* 2006;1:297-329.
3. Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res* 2004 September 3;95(5):449-58.
4. Heil M, Schaper W. Pathophysiology of collateral development. *Coron Artery Dis* 2004 November;15(7):373-8.
5. Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* 2009;27:165-97.
6. Whitman SC, Rateri DL, Szilvassy SJ, Yokoyama W, Daugherty A. Depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice. *Arterioscler Thromb Vasc Biol* 2004 June;24(6):1049-54.
7. van Weel V, Toes RE, Seghers L, Deckers MM, de Vries MR, Eilers PH, Sipkens J, Schepers A, Eefting D, van Hinsbergh VWM, van Bockel JH, Quax PHA. Natural killer cells and CD4+ T-cells modulate collateral artery development. *Arterioscler Thromb Vasc Biol* 2007 November;27(11):2310-8.
8. Harmon KJ, Couper LL, Lindner V. Strain-dependent vascular remodeling phenotypes in inbred mice. *Am J Pathol* 2000 May;156(5):1741-8.
9. Schober A, Zernecke A. Chemokines in vascular remodeling. *Thromb Haemost* 2007 May;97(5):730-7.
10. Feuchtner GM, Cury RC, Jodocy D, Friedrich GJ, Blumenthal RS, Budoff MJ, Nasir K. Differences in coronary plaque composition by noninvasive computed tomography angiography in individuals with and without obstructive coronary artery disease. *Atherosclerosis* 2011 March;215(1):90-5.
11. Hansen JF. Coronary collateral circulation: clinical significance and influence on survival in patients with coronary artery occlusion. *Am Heart J* 1989 February;117(2):290-5.
12. Chalothorn D, Clayton JA, Zhang H, Pomp D, Faber JE. Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains. *Physiol Genomics* 2007 July 18;30(2):179-91.
13. Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, Ziegelhoeffer T, Brandt U, Pearlman JD, Swartz HM, Schaper W. Impact of mouse strain differences in innate hindlimb collateral vasculature. *Arterioscler Thromb Vasc Biol* 2006 March;26(3):520-6.
14. Schulte S, Sukhova GK, Libby P. Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis. *Am J Pathol* 2008 June;172(6):1500-8.
15. Anderson SK, Dewar K, Goulet ML, Leveque G, Makrigiannis AP. Complete elucidation of a minimal class I MHC natural killer cell receptor haplotype. *Genes Immun* 2005 September;6(6):481-92.
16. Ryan JC, Naper C, Hayashi S, Daws MR. Physiologic functions of activating natural killer (NK) complex-encoded receptors on NK cells. *Immunol Rev* 2001 June;181:126-37.
17. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol* 2003 April;3(4):304-16.

18. Scalzo AA, Lyons PA, Fitzgerald NA, Forbes CA, Shellam GR. The BALB.B6-Cmv1r mouse: a strain congenic for Cmv1 and the NK gene complex. *Immunogenetics* 1995;41(2-3):148-51.

19. Cheng TP, French AR, Plougastel BF, Pingel JT, Orihuela MM, Buller ML, Yokoyama WM. Ly49h is necessary for genetic resistance to murine cytomegalovirus. *Immunogenetics* 2008 October;60(10):565-73.

20. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 1997 November 28;278(5343):1623-6.

21. Eefting D, Schepers A, de Vries MR, Pires NM, Grimbergen JM, Lagerweij T, Nagelkerken LM, Monraats PS, Jukema JW, van Bockel JH, Quax PH. The effect of interleukin-10 knock-out and overexpression on neointima formation in hypercholesterolemic APOE*3-Leiden mice. *Atherosclerosis* 2007 August;193(2):335-42.

22. Schepers A, Eefting D, Bonta PI, Grimbergen JM, de Vries MR, van W, V, de Vries CJ, Egashira K, van Bockel JH, Quax PH. Anti-MCP-1 gene therapy inhibits vascular smooth muscle cells proliferation and attenuates vein graft thickening both in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2006 September;26(9):2063-9.

23. Zou Y, Dietrich H, Hu Y, Metzler B, Wick G, Xu Q. Mouse model of venous bypass graft arteriosclerosis. *Am J Pathol* 1998 October;153(4):1301-10.

24. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 2005 June 1;105(11):4416-23.

25. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, French AR, Sunwoo JB, Lemieux S, Hansen TH, Yokoyama WM. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 2005 August 4;436(7051):709-13.

26. van Puijvelde GH, van Wanrooij EJ, Hauer AD, de VP, van Berkel TJ, Kuiper J. Effect of natural killer T cell activation on the initiation of atherosclerosis. *Thromb Haemost* 2009 August;102(2):223-30.

27. Shimizu K, Mitchell RN. The role of chemokines in transplant graft arterial disease. *Arterioscler Thromb Vasc Biol* 2008 November;28(11):1937-49.

28. Proteau MF, Rousselle E, Makrigiannis AP. Mapping of the BALB/c Ly49 cluster defines a minimal natural killer cell receptor gene repertoire. *Genomics* 2004 October;84(4):669-77.

29. Mitchell RN, Libby P. Vascular remodeling in transplant vasculopathy. *Circ Res* 2007 April 13;100(7):967-78.