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Original article

C57BL/6 NK cell gene complex is crucially involved in vascular remodeling

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ABSTRACT

Objective: NK cells are known to be involved in cardiovascular disease processes. One of these processes, vascular remodeling, may strongly differ between individuals and mouse strains such as the C57BL/6 and BALB/c. Moreover, C57BL/6 and BALB/c mice vary in immune responses and in the composition of their Natural Killer gene Complex (NKC). Here we study the role of NK cells, and in particular the C57BL/6 NKC in vascular remodeling and intimal hyperplasia formation.

Methods and results: C57BL/6, BALB/c and CMV1^r mice, a BALB/c strain congenic for the C57BL/6 NKC, were used in an injury induced cuff model and a vein graft model. NK cell depleted C57BL/6 mice demonstrated a 43% reduction in intimal hyperplasia after femoral artery cuff placement compared to control C57BL/6 mice ($p < 0.05$). Cuff placement and vein grafting resulted in profound intimal hyperplasia in C57BL/6 mice, but also in CMV1^r mice, whereas this was significantly less in BALB/c mice. Significant more leukocyte infiltrations and IFN-γ staining were seen in both C57BL/6 and CMV1^r vein grafts compared to BALB/c vein grafts.

Conclusions: These data demonstrate an important role for NK cells in intimal hyperplasia and vascular remodeling. Furthermore, the C57BL/6 NKC in CMV1^r mice stimulates vascular remodeling most likely through the activation of (IFN-γ-secreting) NK-cells that modulate the outcome of vascular remodeling.

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1. Introduction

Vascular remodeling is a multi-factorial process that induces structural alteration of the vascular wall [1]. Remodeling of the vessel wall is a significant factor in the manifestation of atherosclerosis, vein graft disease, arteriogenesis [2] and restenosis [3,4] but also in pulmonary arterial hypertension (PAH) [5] and decidua formation [6,7].

NK cells, a small subset of leukocytes, are known for direct killing of cells depending on their expression of NK-cell receptors specific for MHC class I molecules. NK cells respond upon activation by secretion of lytic granules containing perforin and granzymes, but also by secretion of interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α) and chemokines such as CCL3, CCL4 and CCL5. NK cells can be activated by CCL2, IL12 and IL18. Furthermore, NK cells influence other immune cells such as T cells, macrophages and dendritic cells directly by cell to cell contact or indirectly via cytokines [8]. Chemokines and cytokines involved in NK cell function are extensively researched and are proven modulators of vascular remodeling [9]. It has been demonstrated that NK cells contribute to atherosclerosis [10], arteriogenesis [2], PAH [11] and decidua formation (uterine NK cells) [6] but their precise role in intimal hyperplasia formation and vascular remodeling is not clear.

Patients differ in vascular remodeling capacity, as observed in atherosclerotic plaques and collateral artery growth [12,13]. It is suggested that this is due to differences in immune response. This is supported by strain dependent differences in vascular remodeling between mouse strains[14]. Especially differences in vascular responses between BALB/c and C57BL/6 are extensively investigated [2,15,16]. The differences in response are associated by a different immune bias between these two strains; C57BL/6 mice express a pro-inflammatory, T helper 1 (Th1)-associated profile whereas BALB/c mice show a repair-associated or Th2-like immune response [17]. Immuno-genetic differences between these two strains are found in the Natural Killer gene Complex (NKC). The NKC is 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 [18,19]. Compared to C57BL/6, BALB/c mice lack a 200 kb region encoding members

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of the Ly49 receptor family. As a consequence the BALB/c strain has only eight Ly49 genes, whereas the C57BL/6 NKC possesses fifteen Ly49 genes [20,21].

Scalzo and colleagues generated a congenic BALB.B6-CMV1^r mouse (CMV1^r) [22]. By the expression of the entire C57BL/6 NK cell receptor repertoire, these BALB/c mice, exhibit a level of resistance towards murine cytomegalovirus (CMV) comparable to that of C57BL/6 mice [23], rendering these mice resistant to CMV infections, unlike the BALB/c mice. We used this strain in comparison to BALB/c and C57BL/6 mice to study the involvement of the NKC in vascular remodeling.

Here we show that NK cells are involved in vascular remodeling as demonstrated by decreases in intimal hyperplasia in NK deficient mice and after NK cell depletion in an injury-induced restenosis model. In particular we demonstrate that the NKC is involved in vascular remodeling since expression of the C57BL/6 NKC in BALB/c mice results in a vascular remodeling that is comparable with that observed in C57BL/6 mice, with a shift towards an immune response characterized by IFN-γ production.

2. Materials and methods

2.1. 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 and BALB/c mice (Charles River) were purchased. Jα281-knockout mice (C57BL/6 background) were kindly provided by Dr Masaru Taniguchi [24]. BALBc.B6-CMV1^r and NK-cell deficient (NK$^{-/-}$) breeding couples were kindly provided by Dr. W. M. Yokoyama [25]. All animals received chow diet and water ad libitum. For surgery, mice were anesthetized by using midazolam (5 mg/kg, Roche), medetomidine (0.5 mg/kg, Orion) and fentanyl (0.05 mg/kg, Janssen Pharmaceutica). After the procedures mice were antagonized with atipamezol (2.5 mg/kg, Orion) and fluminasenil (0.5 mg/kg Fresenius Kabi).

2.2. Cuff induced intimal hyperplasia mouse model

Both left and right femoral arteries were dissected from their surroundings. A non-constrictive polyethylene cuff (inner diameter 0.4 mm, Portex) was placed loosely around the femoral artery bilaterally and kept in situ for 21 days [26]. Tissue handling and analysis of intimal hyperplasia were performed as described [27]. 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).

2.3. Vein graft mouse model

Venous interpositions were placed in the mouse carotid artery as described previously [27]. Caval veins of donor mice were engrafted 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. Cross-sections were made throughout the embedded vein grafts. Six representative sections per vessel segment were stained with Haematoxylin–Phloxin–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.

2.4. Immunohistochemistry

NK cells were detected with an FITC labeled DX5 (CD49b) antibody (Ebioscience) and DAPI (Vector) on 5 μm-thick frozen section of native femoral artery ($n = 6$), native caval vein ($n = 6$), cuffed femoral arteries 21 days after surgery ($n = 6$) and vein grafts 28 after surgery ($n = 6$), all on a C57BL/6 background.

Immunohistochemical staining for CD45 and IFN-γ were performed on 5 μm-thick paraffin-embedded sections of vein grafts that were incubated overnight with a primary antibody against CD45 (BD Pharmingen) or IFN-γ (Bio-connect). Negative controls with isotype matched antibodies were run for each sample.

Vein graft segments were analyzed by scoring the quantity of cells in a range from 1 to 3 in 6 consecutive sections per mouse. 1: <25 positive cells/section, 2: 25–50 positive cells/section, 3: >50 CD45 positive cells. Scores from each individual mouse were averaged.

2.5. NK cell depletion

For depletion of NK cells, C57Bl6 mice received intraperitoneal injections of 200 μg of anti-NK1.1 antibody (PK136) or 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) just before surgery and 14 days after surgery.

2.6. NK cell activation assay

1×10^7 splenocytes were cultured in wells of a 24-wells plate coated with either anti-NKp46 antibody (R&D Systems) isotype-matched control (goat IgG, R&D Systems) antibody (both at 5 μg/well), or in medium containing 0.1 μg/ml Phorbol 12-Myristate 13-Acetate (PMA) and 0.5 μg/ml ionomycin (Sigma–Aldrich). Intracellular staining for IFN-γ (BD Biosciences) was performed after a 4 h incubation at 37 °C in the presence of Brefeldin A. Percentages of IFN-γ producing NK cells were obtained by gating CD3ε-, DX5$^+$ cells during FACS analysis on BD FACS LSR II using BD FACS Diva software v6.0 (BD Biosciences).

2.7. Statistical analysis

Results are expressed as mean ± SEM. For in vivo testing comparisons between medians were performed using the Kruskall–Wallis or Mann–Whitney test. For in vitro NK cell responsiveness a One-way Anova or two-tailed Student's *t*-test was used. *P*-values <0.05 were regarded statistically significant.

3. Results

3.1. Presence of NK cells in remodeled vascular tissues

To demonstrate NK cells in remodeled vessels, immunohistochemistry was performed with antibodies directed at the NK marker LY49H (DX5). NK cells could be demonstrated in tissue sections of cuffed femoral arteries (Fig. 1B), although in limited amounts. Most NK cells that were seen in these sections were located in the adventitial region. *Comparable numbers of NK cells were found in 3 days old cuffed femoral arteries in C57BL/6, BALB/c and CMV1^r mice* (Online Supplement Fig. 1). No NK cells were found in sections of uninjured femoral arteries (Fig. 1A) or in native caval veins (Fig. 1C, D). NK cells could be detected in the intimal hyperplasia of vein grafts 28 day after surgery as demonstrated in Fig. 1E and F.

3.2. NK cells, but not NKT cells, contribute to arterial intimal hyperplasia

To demonstrate a role for NK cells in vascular remodeling, NK cells were depleted using antibodies directed at NK1.1 in C57BL/6 mice that subsequently received a non-constrictive cuff around the femoral artery ($n = 10$ mice/group). Flow cytometric (FACS) analysis of peripheral blood samples on the day of surgery confirmed that NK-cell depletion had been successful: average NK cell percentages of lymphocyte

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