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Involvement of CD8+ T cell subsets in early response to vascular injury in patients with peripheral artery disease *in vivo*

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Abstract

Aims—Adaptive immunity is critical in vascular remodelling following arterial injury. We hypothesized that acute changes in T cells at a percutaneous transluminal angioplasty (PTA) site could serve as an index of their potential interaction with the injured vascular wall.

Methods and Results—T cell subsets were characterised in 45 patients with Rutherford 3–4 peripheral artery disease (PAD) undergoing PTA. Direct angioplasty catheter blood sampling was

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Conflict of Interests

The authors declare no existing conflicts of interest regarding the publication of this paper.

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performed before and immediately after the procedure. PTA was associated with an acute reduction of α/β -TcR CD8⁺ T cells. Further characterisation revealed significant reduction in pro-atherosclerotic CD28nullCD57⁺ cells, effector (CD45RA+CCR7⁻) and effector memory (CD45RA-CCR7⁻) cells, in addition to cells bearing activation (CD69, CD38) and tissue homing/adhesion markers (CD38, CCR5).

Conclusions—The acute reduction observed here is likely due to the adhesion of cells to the injured vascular wall, suggesting that immunosenescent, activated effector CD8⁺ cells have a role in the early vascular injury immune response following PTA in PAD patients.

Introduction

Atherosclerosis is an immune and inflammatory disease [1]. Recent positive results from immune targeted intervention outcome studies such as the CANTOS trial [2] emphasise a need for better understanding and focused targeting of this process. Immune responses to modified lipoproteins (such as minimally oxidized / B phenotype / small dense LDLs) have been identified as key initiators of atherosclerotic plaque development [3]. This is linked to immune cell recruitment into the vessel wall, which is facilitated by endothelial dysfunction and increased expression of adhesion molecules and immune cell recruitment [4, 5]. T cells are one of the cells recruited early to the dysfunctional vasculature and may precede and orchestrate recruitment of key cells including pro-atherosclerotic macrophages, neutrophils or B cells. While CD4⁺ T cells have been implicated in the initiation of atherosclerosis, recent data indicate the role of CD8⁺ T cell activation and recruitment at early stages of the disease.

Recruitment of monocytes and lymphocytes from the peripheral blood to the intima of the vessel wall represents the primary event in atherogenesis [6] but is also essential in regulation of plaque stability and long term vascular remodelling. T cell-derived cytokines including interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), and interleukin 17 (IL-17) have all been implicated. The expansion of immunosenescent CD28null (either CD4⁺ or CD8⁺) T cells is characteristic for unstable plaques. These are terminally differentiated T cells that have lost CD28 expression [7] and secrete TNF- α and IFN- γ , contributing to plaque destabilisation and vascular remodelling [7]. Most studies, however, were performed in animal models, with human data relating primarily to coronary artery disease, while peripheral arterial disease has been far less studied. Several inflammatory biomarkers, including high sensitivity C-reactive protein (CRP) [8], soluble adhesion molecules (such as sICAM [9]), and TNF α [10], are associated with both the incidence and severity of PAD [11], and greater systemic inflammation is associated with increased cardiovascular morbidity and mortality in PAD [12]. Interestingly, decreased CD4⁺ T cells are linked to cardio- and cerebrovascular events and cardiovascular mortality in long term follow up [13].

Endovascular treatment of PAD allows the control of symptomatic disease in many cases. It is minimally invasive and still a very efficient way to combat arterial stenosis and occlusion, although its effects are possibly hindered by restenosis and vascular remodelling resulting from vascular damage. Inhibition of chemotaxis, in particular CCR5-dependent mechanisms,

prevents vascular remodelling leading to restenosis in mice [14]. In particular, this prevents Th1 and Th17 responses [15]. While balloon or wire injury models have been well characterised in mice [15], our understanding of restenosis in humans is based primarily on vein grafts, which represent advanced disease [16]. It is very difficult to address early events in the setting of vascular injury and restenosis in humans. Therefore, we postulated that acute changes in T cell subsets [17] following percutaneous transluminal angioplasty (PTA) could serve as an index of their potential interaction with the vascular wall (Figure 1). Such a model would allow us to address this issue in patients *in vivo*, while so far only attempts with *in vitro* arterial culture models have been made [18]. This may allow for identification of immune cells potentially interacting with the vascular wall following endovascular procedures and therefore, provide valuable targets for future preventive and therapeutic interventions.

Materials and methods

Patients

A total of 45 patients with stage 3 or 4 PAD using Rutherford's classification, who required PTA for severe claudication or rest pain, were recruited from the Malopolska Endovascular Prospective Registry [19]. Significant stenosis (but not totally occluded) was diagnosed in all patients, localised in the iliac (5[11%]), femoral (33[73%]) and popliteal (7[16%]) arteries. None of the patients had calcified lesions. Two substudies (Table 1) characterising immune cell subpopulations in blood flowing through vascular segments undergoing PTA were performed. Patients with clinical or biochemical features of an inflammatory process that would modify immune readouts such as ischaemic and trophic ulcerations, chronic kidney disease, neoplastic disease, or elevated CRP were excluded. In the first substudy, PTA or sham procedure was performed (Cohort 1; n=12) in a random, cross-over fashion to determine the acute effects of PTA on major T cell subpopulations. Immune cell subpopulations identified by the first substudy were then studied in a larger validation cohort (Cohort 2; n=33). Symptoms of peripheral ischaemia (including baseline Rutherford's scale classification and ankle-brachial index (ABI)) were recorded in all subjects. Pre-procedural imaging studies (ultrasound colour Doppler or CT angiography) were performed to plan the endovascular treatment. PTA was performed in iliac, femoral, or popliteal vascular segments, depending on clinical indications. The study was approved by the Jagiellonian University Ethics Committee (KBET/282/B/2013). All patients have given their written informed consent.

Procedure and blood sampling

Vascular access was achieved by common femoral artery puncture and 6F arterial introducer sheath. Following diagnostic angiography of the target vessel, *sample before PTA* (Figure 1) was collected in ethylenediaminetetra acetic acid (EDTA)-containing vials directly from the balloon catheter sized to the reference vessel diameter (40-60mm in length and 5-7mm in diameter). None of these used drug eluting balloons. Immediately following this, balloon angioplasty was performed in the narrowed, non-calcified artery. *Sample after PTA* was collected from the balloon catheter at the level of balloon deflation immediately after balloon deflation (within 15 seconds). The time between two samplings was on average

2.5±0.5 minutes. The time gap between sampling was minimised to be as short as possible, but not to interfere with the proper standardised endovascular treatment procedure. Additionally, in the selected group of initial n=12 patients, a sham sampling procedure was performed to investigate possible individual variability and sampling related effects. Blood was sampled from the arterial introducer twice within 2.5±0.5 minutes. No vascular intervention was performed between samplings. All of the above blood samples (10ml) were immediately placed on ice and transferred to the laboratory within 45 minutes and analysed.

In a separate, additional group of patients (n=6) that did not differ clinically from the main study population, a longer time course with repeated sampling after 2min+/-0.5min And 7 min+/-0.5 min was performed.

Flow cytometric analysis of cell population and antigen expression on lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by standard gradient centrifugation using Pancoll human (PAN Biotech GmbH, Aidenbach, Germany). PBMCs were further suspended in phosphate-buffered saline (PBS) containing 1% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies, USA) and were used immediately after isolation. A total of 5×10^5 PBMCs were stained for 20 minutes with fluorochrome-conjugated monoclonal antibodies. The following monoclonal antibodies were used: anti-CD3-PerCP (clone SK7), anti-CD4-APC (clone SK3), anti-CD4-PE-Cy7 (clone SK3), anti-CD8-APC-H7 (clone SK1), anti-CD45RA-FITC (clone L48), anti-CD197-PE-Cy7 (clone 3D12), anti-CD19 APC-H7 (clone SJ25C1), anti-CD16-PE (clone 3G8), anti-CD56-PE-Cy7 (clone B159), anti-TCRg/d-PE (clone 11F2), anti-TCRa/b-FITC (clone T10B9.1A-31), anti-CD28-APC (clone CD28.2), anti-CD57-FITC (clone NK-1), anti-CD25-PE (clone M-A251), anti-CD69-FITC (clone FN50), anti-CD38-APC (clone HIT2), anti-CD195-PE-Cy7 (clone 2D7/CCR5) (BD, Pharmingen, CA, USA). After staining, cells were washed twice with PBS containing 1% FBS. Cells were processed in the FACSVerse flow cytometer (Becton Dickinson, CA, USA) and analysed using FlowJo software (TreeStar, USA). Lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC) signals from PBMC and T cells were gated according to CD3 expression. Percentages of CD4, CD8, and Double Negative T cells (DN-T) were assessed. Each of the subpopulations was next analysed for presence of naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-) and, CD45RA+ effector (CD45RA+CCR7-) cells. B cells were identified from lymphocytes with confirmation of CD19 expression and NK cells were gated with confirmation of co-expression of CD16 and CD56 antigens. In T cells and their subsets the expressions of surface activation markers were then assessed. Finally, absolute number of leukocytes and their subsets per mm^3 were calculated based on quantitative white cell differential count which was performed for every sample independently. Fluorescence Minus One (FMO) controls were used to determine the positivity of evaluated antigens.

Statistics

Data was described using mean with standard deviations or median with the first and third quartiles for continuous variables and as counts and percentages for nominal variables. Continuous variables were compared using Students t-test with or without correction for

unequal variances (verified using Levene test) or Mann-Whitney U test for non-normally distributed data (verified using Shapiro-Wilk test). Data from before and after the intervention were compared using paired version of tests above. Relative and absolute changes were also analysed. Nominal variables were compared using Pearson χ^2 test or Fisher Exact test when appropriate. Data management, statistical analysis, and statistical discovery were performed using JMP®, Version 12. SAS Institute Inc., Cary, NC, 1989-2012 and the software environment for statistical computing R. Two-sided p values <0.05 were considered significant.

Results

Patient characteristics

The clinical characteristics of patients reflected a typical profile of patients with a high burden of cardiovascular risk (Table 1). There were no significant differences in general patient characteristics between the initially characterised population and the validation population. Ischaemia was evaluated using Rutherford's scale, ankle-brachial index (ABI), and examination of arteries using ultrasound with colour Doppler or CT angiography. Ischaemic symptoms as evaluated by Rutherford's scale equalled 3 in 50 subjects and 4 in 11 others. Only patients with *de novo* non-calcified lesions and no previous target vessel intervention were included. No signs of local or systemic inflammation were present in any of the cases at the time of PTA. As expected for such a patient population, subjects were chronically treated with aspirin (75mg) as well as atorvastatin (20-40mg) and clopidogrel (75mg).

Utility of the novel model for assessment of acute changes in leukocyte subpopulations in blood flowing out of the culprit lesion

Sequential measurement of leukocyte content and their subpopulations in the blood flowing through the lesion before and immediately after PTA allows for a consistent detection of content reduction in several leukocyte subpopulations, which is likely associated with the adhesion of these cells to the injury site. These shifts were not observed in response to the sham procedure (Figure 2). We expected to detect an increase in several plaque-specific immune cells, however, this was not observed using the proposed approach (Figure 2).

Reduction in the number of leukocytes in blood outflowing from the vascular injury site

The comparison of main subsets of peripheral blood lymphocytes in the initial study revealed that the absolute number of T cells per mm^3 of blood outflowing from the treated lesion was significantly decreased after PTA, while no change was observed in patients undergoing a sham procedure (Figure 2). The same effect was observed in the group of 33 patients in the validation cohort undergoing PTA (Figure 3A). Extensive phenotyping using multicolour flow cytometry identified key T cell subpopulations which showed reduction following PTA, indicative of their possible adhesion to the balloon injury site. The analysis of individual T cell subsets revealed that the number of CD8+ cells was decreased predominantly in the samples after angioplasty in comparison to before reperfusion (Figure 2 and Figure 3C). The numbers of CD4+ and CD3+CD4-CD8- (DN)-T cell subpopulations remained unchanged (Figure 3B, D). Similarly, absolute numbers of B-lymphocytes and NK

cells remained unchanged (Figure 1A, B supplement), although preliminary studies showed a decrease in the number of NK cells (Figure 2). To gain additional insight into the diminished T cell population, the numbers of TCR $\alpha\beta$ and $\gamma\delta$ were assessed. The analysis of blood samples outflowing from the angioplasty site revealed a decreased number of TCR $\alpha\beta$ in comparison to before PTA and reperfusion. No significant changes were observed in T cells expressing TCR $\gamma\delta$ (Figure 3D).

Effector CD8+ T cells and response to angioplasty

To assess how angioplasty affects naïve and memory T cell subsets in blood flowing through the vascular lesion, we determined the percentage of cells expressing CD45RA and/or CD197 (CCR7) antigens. Analysis of CD8+ T cells revealed that angioplasty led to a reduction in the absolute number of both effector memory (CD45RA-CCR7-, Figure 2, 4C, 4E) and CD45RA+CCR7- effector cells in the blood (Figure 2, 4D, 4E). There were no significant changes in the absolute number of naïve (CD45RA+CCR7+, Figure 2, 4A) nor in central memory cells (CD45RA-CCR7+, Figure 2, 4B) upon contact with the angioplasty site. No differences were observed within CD4 T cell subsets (Figure 2supplement).

Activated and CCR5+ CD8+ T cells are recruited to vascular injury sites

To further characterise the changes in the prevalence of activated T cells, the expression of early activation marker (CD69) and late activation marker (CD25) was assessed. Flow cytometry analysis revealed a decreased number of CD69+ T cells following vascular injury in the outflowing blood in comparison to the sample collected before balloon deflation (Table 2). This effect was particularly observed in CD8+ lymphocytes. There were no statistically significant changes in the number of T cells bearing the late activation marker CD25 (Table 2). Interestingly, the numbers of both CCR5+ and CD38+ T cells were decreased after vascular injury, but this effect reached statistical significance only in the group of 33 patients (Table 2). Decreased expression of CCR5 and CD38 were particularly pronounced in CD8+ T lymphocytes (Table 2).

Immunosenescent T cells are recruited to vascular injury sites

We found a decreased number of CD28null T cells after angioplasty (Table 2, Figure 5A). This was also accompanied by a decreased number of T cells expressing CD57 antigen (Table 2, Figure 5B). Analysis of individual T cell subsets revealed that a decreased number of CD28null as well as CD57 positive cells was seen in CD8+, but not CD4+ lymphocytes (Table 2, Figure 2).

Time-course of T cell recruitment to vascular injury sites

Additional experiments included sampling within 2 and 7 minutes after PTA to investigate how time affects leukocyte counts. The reduction in CD8 T cell subsets (CD28null and CD57+, Figure 3 A and B supplement, n=6) as well as CD45RA effector CD8+ T cells (Figure 3 D supplement) was observed in all time points and did not return to pre-treatment values within the studied timeframe. This was also true for other CD8+ T cells however the effect was moderate and did not reach statistical significance at 7-minute time-point (Figure 3 C supplement).

Discussion

PTA is a therapeutic procedure alleviating the symptoms in a number of atherosclerotic conditions [20]. Its effectiveness in both coronary artery disease and peripheral arterial disease is, however, limited by the process of remodelling leading to intimal proliferation and recurrence of vascular occlusion [21–23]. The role of immune and inflammatory factors have been identified in mouse models with an important role for T cells in early remodelling with subsequent recruitment and local proliferation of macrophages [24–26]. Reconstitution of immunodeficient mice with human peripheral blood mononuclear cells (PBMC) promotes neointima formation after carotid wire injury while reducing neointima formation after femoral cuff injury [27]. While this process has been extensively studied in animal models, insight into early events in humans is limited. In order to identify immune cells potentially recruited very early in the process of vascular injury, we hypothesised that immune cell recruitment/adhesion to the site of vascular injury/PTA would be associated with acute reduction of their content in blood flowing out of the PTA site immediately upon balloon deflation. PTA exposes the subendothelial space in atherosclerotic vessels [28]. This may result in a release of immune cells from atherosclerotic plaques but may also enhance blood cell adhesion as an early response to injury [27].

Indeed, we report here that effector and immunosenescent CD8⁺ T cells bearing chemokine receptor CCR5 may preferentially adhere to the injured vessel wall after balloon deflation. This effect was also observed in longer time points suggesting that vascular injury still persists. These cells exhibit effector memory (CD45RA-CCR7⁻) and CD45RA+CCR7⁻ effector characteristics. Further analysis indicated that CD8⁺ cells demonstrating features of pro-atherosclerotic dysregulation and immunosenescence (CD28null/CD57⁺) [29], as well as cells bearing early activation marker (CD69) were significantly locally recruited following PTA. These changes were absent in sham sampling procedures and were not observed in other cell populations. Identification of possible CD8 T cell interactions with injured vascular wall, while novel in humans, is in line with studies showing the importance of CD8⁺T cells in the vascular injury response in mice [30, 31]. Interestingly, CD8 knockout increased neointimal formation and TNF levels in injured arteries and was also associated with delayed re-endothelialization [31]. The effect of CD8⁺ T cells on reduced neointima formation after arterial injury was attributed in part to increased function of the CD28high phenotype. These T cells have cytolytic activity against syngenic smooth muscle cells (SMC) which express the B7-1 molecule [30]. In humans, however, we have observed recruitment of CD28null cells rather than CD28high CD8⁺ cells, which are immunosenescent [32], secrete proinflammatory cytokines, and exert greater cytotoxicity [33]. Higher frequencies of CD8⁺CD28nullCD57⁺ cells are associated with an increased prevalence of carotid artery lesions in patients infected with human immunodeficiency virus (HIV) [34]. In the present study we have identified that apart from CD28nullCD57⁺ characteristics, recruited CD8⁺ cells can be identified by CD38. This is important because CD38 is involved in lymphocyte homing and it can regulate lymphocyte adhesion to endothelial cells by interaction with CD31 [35]. CD38 is regulated upon T cell activation and differentiation, and is characteristic for memory cells. T cells expressing a high level of CD38 have reduced proliferation but display an increased production of IL-2 and INF- γ

[36]. Thus, while initial studies suggested protective effects of CD28^{high}CD8⁺ T cells in vascular injury, activated immunosenescent cells may have an opposite effect. Indeed, depletion of CD8⁺ T cells by antibodies led to reduced atherosclerosis, apoptosis, and necrosis in the plaque of Apoe^{-/-} mice [37]. CD8 T cells promote atherosclerosis through the expression of TNF- α and cytotoxic molecules such as Granzyme B and Perforin. Granzyme or Perforin-deficient CD8⁺ T cells failed to promote atherosclerosis when transferred into T cell-deficient mice [37]. In human atherosclerotic plaques, CD8⁺ T cells represented up to 50% of the lymphocytes in advanced lesions [38], but the role in vascular injury response to interventional procedures remains unknown.

Identification of the cells participating as a potential first line response to vascular injury may be valuable from the point of view of focusing future mechanistic model studies of vascular injury on these cells. Arterial wall remodelling is common both in primary atherosclerotic lesions and after transcatheter interventions and is a major determinant of lumen size and therefore clinical outcomes. During balloon angioplasty in humans, the rigid atherosclerotic plaque is separated from the more compliant vessel wall components (plaque-free intima, media, and adventitia). Tears usually extend deeply into the vessel wall [39]. This may lead to a rapid release of plaque specific cells, which we had hoped to detect as well. However, no major increases in any of the studied subsets of T cells were observed. This may indicate that only minimal numbers of such cells are released to circulation following PTA injury. The other option is that their release is very rapid and occurs only in the initial seconds, while adhesion/recruitment to the site of injury is more long lasting.

Our study also had some inherent limitations, which was observational and aimed at identifying, for the first time, interesting potential future targets for mechanistic investigations. These results will initiate further *ex vivo* cultures and stimulation studies, cell-based imaging, cytokine evaluation and genomic methods that will enhance interpretation and our understanding of the role of CD8⁺ T cell recruitment in this early phase of vascular injury response. Finally, while local recruitment is one of the explanations for the decrease in CD8 T cells following PTA, it should be considered that these could be recruited elsewhere, which would likely affect our measurements, even if they were obtained locally from the culprit site. This possibility, however, should not diminish the potential importance of pin-pointing an early response to PTA even if it was more systemic, rather than localized. We have focused on T cells in relation to their particular importance in the early stages of atherosclerosis and restenosis. Other cells may play an important role as well. However, in a recent study, we did not observe a rapid change of monocyte content using a similar approach, although in that study a sham procedure was not performed [40].

In summary, we have measured a rapid reduction of T cell subsets likely resulting from the adhesion and homing to the injured vascular wall to identify immunosenescent and activated effector CD8⁺ cells as part of an early vascular injury immune response in human PAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Ketelhuth DF, Hansson GK. Adaptive Response of T and B Cells in Atherosclerosis. *Circulation research*. 2016; 118:668–678. [PubMed: 26892965]
- [2]. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *The New England journal of medicine*. 2017; 377:1119–1131. [PubMed: 28845751]
- [3]. Li Y, Lu Z, Huang Y, Lopes-Virella MF, Virella G. F(ab')₂ fragments of anti-oxidized LDL IgG attenuate vascular inflammation and atherogenesis in diabetic LDL receptor-deficient mice. *Clinical immunology (Orlando, Fla.)*. 2016; 173:50–56.
- [4]. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *The Journal of clinical investigation*. 1997; 100:2153–2157. [PubMed: 9410891]
- [5]. Urbanski K, Ludew D, Filip G, Filip M, Sagan A, Szczepaniak P, Grudzien G, Sadowski J, Jasiewicz-Honkisz B, Sliwa T, Kapelak B, et al. CD14+CD16++ "nonclassical" monocytes are associated with endothelial dysfunction in patients with coronary artery disease. *thrombosis and haemostasis*. 2017; 117:971–980.
- [6]. Marino F, Guasti L, Tozzi M, Schembri L, Castiglioni L, Molteni E, Piffaretti G, Castelli P, Cosentino M. Gene expression of adhesion molecules in endothelial cells from patients with peripheral arterial disease is reduced after surgical revascularization and pharmacological treatment. *International journal of vascular medicine*. 2013; 2013 412761.
- [7]. Duftner C, Goldberger C, Falkenbach A, Wurzner R, Falkensammer B, Pfeiffer KP, Maerker-Hermann E, Schirmer M. Prevalence, clinical relevance and characterization of circulating cytotoxic CD4+CD28- T cells in ankylosing spondylitis. *Arthritis research & therapy*. 2003; 5:R292–300. [PubMed: 12932293]
- [8]. Ridker PM, Stampfer MJ, Rifai N. Novel risk factors for systemic atherosclerosis: a comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. *Jama*. 2001; 285:2481–2485. [PubMed: 11368701]
- [9]. Pradhan AD, Rifai N, Ridker PM. Soluble intercellular adhesion molecule-1, soluble vascular adhesion molecule-1, and the development of symptomatic peripheral arterial disease in men. *Circulation*. 2002; 106:820–825. [PubMed: 12176954]
- [10]. Pradhan AD, Shrivastava S, Cook NR, Rifai N, Creager MA, Ridker PM. Symptomatic peripheral arterial disease in women: nontraditional biomarkers of elevated risk. *Circulation*. 2008; 117:823–831. [PubMed: 18227386]
- [11]. Pande RL, Brown J, Buck S, Redline W, Doyle J, Plutzky J, Creager MA. Association of monocyte tumor necrosis factor alpha expression and serum inflammatory biomarkers with walking impairment in peripheral artery disease. *Journal of vascular surgery*. 2015; 61:155–161. [PubMed: 25095746]
- [12]. Vidula H, Tian L, Liu K, Criqui MH, Ferrucci L, Pearce WH, Greenland P, Green D, Tan J, Garside DB, Guralnik J, et al. Biomarkers of inflammation and thrombosis as predictors of near-term mortality in patients with peripheral arterial disease: a cohort study. *Annals of internal medicine*. 2008; 148:85–93. [PubMed: 18195333]
- [13]. Szomjak E, Der H, Kerekes G, Veres K, Csiba L, Toth J, Peter M, Soltesz P, Szodoray P. Immunological parameters, including CXCL8 (IL-8) characterize cerebro- and cardiovascular events in patients with peripheral artery diseases. *Scandinavian journal of immunology*. 2010; 71:283–291. [PubMed: 20384872]
- [14]. Zernecke A, Liehn EA, Gao JL, Kuziel WA, Murphy PM, Weber C. Deficiency in CCR5 but not CCR1 protects against neointima formation in atherosclerosis-prone mice: involvement of IL-10. *Blood*. 2006; 107:4240–4243. [PubMed: 16467202]

- [15]. Smirnova NF, Gayral S, Pedros C, Loirand G, Vaillant N, Malet N, Kassem S, Calise D, Goudouneche D, Wymann MP, Hirsch E, et al. Targeting PI3Kgamma activity decreases vascular trauma-induced intimal hyperplasia through modulation of the Th1 response. *The Journal of experimental medicine*. 2014; 211:1779–1792. [PubMed: 25073791]
- [16]. Mitra AK, Gangahar DM, Agrawal DK. Cellular, molecular and immunological mechanisms in the pathophysiology of vein graft intimal hyperplasia. *Immunology and cell biology*. 2006; 84:115–124. [PubMed: 16519729]
- [17]. Brandt D, Sergon M, Abraham S, Mabert K, Hedrich CM. TCR(+)CD3(+)CD4(-)CD8(-) effector T cells in psoriasis. *Clinical immunology (Orlando, Fla.)*. 2017; 181:51–59.
- [18]. Voisard R, Fischer R, Osswald M, Voglic S, Baur R, Susa M, Koenig W, Hombach V. Aspirin (5 mmol/L) inhibits leukocyte attack and triggered reactive cell proliferation in a 3D human coronary in vitro model. *Circulation*. 2001; 103:1688–1694. [PubMed: 11273998]
- [19]. Krzanowski M, Drelicharz L, Belowski A, Partyka L, Sumek-Brandys B, Ramakrishnan PK, Nizankowski R. Costs of Real-Life Endovascular Treatment of Critical Limb Ischemia: Report from Poland-A European Union Country with a Low-Budget Health Care System. *Annals of vascular surgery*. 2016; 31:111–123. [PubMed: 26616505]
- [20]. Hsiao ST, Spencer T, Boldock L, Prosseda SD, Xanthis I, Tovar-Lopez FJ, Van Beusekom HM, Khamis RY, Foin N, Bowden N, Hussain A, et al. Endothelial repair in stented arteries is accelerated by inhibition of Rho-associated protein kinase. *Cardiovascular research*. 2016; 112:689–701. [PubMed: 27671802]
- [21]. Schillinger M, Minar E. Percutaneous treatment of peripheral artery disease: novel techniques. *Circulation*. 2012; 126:2433–2440. [PubMed: 23147770]
- [22]. Ryan TJ, Bauman WB, Kennedy JW, Kereiakes DJ, King SB 3rd, McCallister BD, Smith SC Jr, Ulliot DJ. Guidelines for percutaneous transluminal coronary angioplasty. A report of the American Heart Association/American College of Cardiology Task Force on Assessment of Diagnostic and Therapeutic Cardiovascular Procedures (Committee on Percutaneous Transluminal Coronary Angioplasty). *Circulation*. 1993; 88:2987–3007. [PubMed: 8252713]
- [23]. Wu JH, Li Y, Zhou YF, Haslam J, Elvis ON, Mao L, Xia YP, Hu B. Semaphorin-3E attenuates neointimal formation via suppressing VSMCs migration and proliferation. *Cardiovascular research*. 2017; 113:1763–1775. [PubMed: 29016743]
- [24]. Kovacic JC, Gupta R, Lee AC, Ma M, Fang F, Tolbert CN, Walts AD, Beltran LE, San H, Chen G, St Hilaire C, et al. Stat3-dependent acute Rantes production in vascular smooth muscle cells modulates inflammation following arterial injury in mice. *The Journal of clinical investigation*. 2010; 120:303–314. [PubMed: 20038813]
- [25]. Fan Y, Zhang J, Chen CY, Xiao YB, Asico LD, Jose PA, Xu JC, Qian GS, Zeng CY. Macrophage migration inhibitory factor triggers vascular smooth muscle cell dedifferentiation by a p68-serum response factor axis. *Cardiovascular research*. 2017; 113:519–530. [PubMed: 28165114]
- [26]. Barhoumi T, Fraulob-Aquino JC, Mian MOR, Ouerd S, Idris-Khodja N, Huo KG, Rehman A, Caillon A, Dancose-Giambattisto B, Ebrahimian T, Lehoux S, et al. Matrix metalloproteinase-2 knockout prevents angiotensin II-induced vascular injury. *Cardiovascular research*. 2017; 113:1753–1762. [PubMed: 29016715]
- [27]. Moser J, van Ark J, van Dijk MC, Greiner DL, Shultz LD, van Goor H, Hillebrands JL. Distinct Differences on Neointima Formation in Immunodeficient and Humanized Mice after Carotid or Femoral Arterial Injury. *Scientific reports*. 2016; 6 35387.
- [28]. Pasternak RC, Baughman KL, Fallon JT, Block PC. Scanning electron microscopy after coronary transluminal angioplasty of normal canine coronary arteries. *The American journal of cardiology*. 1980; 45:591–598. [PubMed: 7355756]
- [29]. Youn JC, Yu HT, Lim BJ, Koh MJ, Lee J, Chang DY, Choi YS, Lee SH, Kang SM, Jang Y, Yoo OJ, et al. Immunosenescent CD8+ T cells and C-X-C chemokine receptor type 3 chemokines are increased in human hypertension. *Hypertension (Dallas, Tex. : 1979)*. 2013; 62:126–133.
- [30]. Dimayuga PC, Chyu KY, Lio WM, Zhao X, Yano J, Zhou J, Honjo T, Shah PK, Cercek B. Reduced neointima formation after arterial injury in CD4-/- mice is mediated by CD8+CD28hi T cells. *Journal of the American Heart Association*. 2013; 2:e000155. [PubMed: 23702879]

- [31]. Zhang JM, Wang Y, Miao YJ, Zhang Y, Wu YN, Jia LX, Qi YF, Du J. Knockout of CD8 delays reendothelialization and accelerates neointima formation in injured arteries of mouse via TNF- α inhibiting the endothelial cells migration. *PloS one*. 2013; 8:e62001. [PubMed: 23658704]
- [32]. Weng NP, Akbar AN, Goronzy J. CD28(-) T cells: their role in the age-associated decline of immune function. *Trends in immunology*. 2009; 30:306–312. [PubMed: 19540809]
- [33]. Dumitriu IE, Araguas ET, Baboonian C, Kaski JC. CD4+ CD28 null T cells in coronary artery disease: when helpers become killers. *Cardiovascular research*. 2009; 81:11–19. [PubMed: 18818214]
- [34]. Kaplan RC, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, Xue X, Hunt P, Karim R, Kern DM, Hodis HN, et al. T cell activation and senescence predict subclinical carotid artery disease in HIV-infected women. *The Journal of infectious diseases*. 2011; 203:452–463. [PubMed: 21220772]
- [35]. Dengler TJ, Pober JS. Human vascular endothelial cells stimulate memory but not naive CD8+ T cells to differentiate into CTL retaining an early activation phenotype. *Journal of immunology* (Baltimore, Md. : 1950). 2000; 164:5146–5155.
- [36]. Sandoval-Montes C, Santos-Argumedo L. CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. *Journal of leukocyte biology*. 2005; 77:513–521. [PubMed: 15618297]
- [37]. Kyaw T, Winship A, Tay C, Kanellakis P, Hosseini H, Cao A, Li P, Tipping P, Bobik A, Toh BH. Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation*. 2013; 127:1028–1039. [PubMed: 23395974]
- [38]. Gewaltig J, Kummer M, Koella C, Cathomas G, Biedermann BC. Requirements for CD8 T-cell migration into the human arterial wall. *Human pathology*. 2008; 39:1756–1762. [PubMed: 18706675]
- [39]. Nobuyoshi M, Kimura T, Ohishi H, Horiuchi H, Nosaka H, Hamasaki N, Yokoi H, Kim K. Restenosis after percutaneous transluminal coronary angioplasty: pathologic observations in 20 patients. *Journal of the American College of Cardiology*. 1991; 17:433–439. [PubMed: 1991900]
- [40]. Maga P, Mikolajczyk TP, Partyka L, Krzanowski M, Malinowski KP, Nizankowski R. Percutaneous Transluminal Angioplasty in Patients with Peripheral Arterial Disease Does Not Affect Circulating Monocyte Subpopulations. *BioMed research international*. 2016; 2016 2708957.

Figure 1. Sampling process.

Blood was obtained before (A) and immediately during/after (C) PTA balloon deflation (B) to study immune cells potentially released or adhering to the injury site.

Figure 2. Heatmap presenting 22 cell types with ratio of post- and pre-procedure level significantly different between patients who underwent either Sham procedure or PTA. Colour of each square corresponds to a fold change (FC) of cell level that was calculated as a ratio of post- and pre-procedure level of each analysed cell type. Ratios were compared between 2 studied groups (i.e. Sham (n=12) and PTA (n=12); each patient is depicted in a single column) using t test. Out of 57, 22 cell types, which are depicted on the heatmap, demonstrated FC significantly different between 2 studied groups after Bonferroni correction for multiple testing. Heatmap was generated using R (ver. 3.2.2) software and pheatmap package (ver. 1.0.8).

Figure 3. T cells and their subsets before and after angioplasty.

Absolute numbers per mm³ and ratios of T cells (Panel A) and their subsets CD4+ (Panel B), CD8+ (Panel C) and TCR g/d+ (Panel D) were assessed in peripheral blood collected before and after percutaneous transluminal angioplasty (PTA). Boxes represent the 25th and 75th percentiles and horizontal lines the median.

**p<0.01 vs Before PTA.

Figure 4. CD8+ T cell subsets before and after angioplasty.

Absolute numbers per mm³ and ratios of naïve (CD45RA+CCR7+, Panel A), central memory (CD45RA-CCR7+, Panel B), effector memory (CD45RA-CCR7-, Panel C), CD45RA+ effector (CD45RA+CCR7-, Panel D) cells were assessed in peripheral blood collected before and after percutaneous transluminal angioplasty (PTA). Panel E shows flow cytometric examples of CD8 subpopulations depending on expressions of CD45RA and CCR7 markers. Boxes represent the 25th and 75th percentiles and horizontal lines the median.

**p<0.01 vs Before PTA.

Figure 5. Markers of immunosenescence in CD8+ T cells and their subsets before and after angioplasty.

Absolute numbers per mm³ and ratios of CD28null T cells (Panel A) and CD57+ (Panel B) CD8+ T cells were assessed in peripheral blood collected before and after percutaneous transluminal angioplasty (PTA). Representative dot plots of CD28 and CD57 expressions in CD8+ T cells are shown (Panel A and Panel B, respectively).

Boxes represent the 25th and 75th percentiles and horizontal lines the median.

**p<0.01 vs Before PTCA.

Table 1
Demographic and clinical data of patients undergoing lymphocyte population sampling and evaluation.

	Initial Sham/PTA population	Validation PTA population	p
N	12	33	-
Sex (M:F)	10:2	28:5	-
Age (mean±SD)	66±11	69±9	0.32
Risk factors:			
Smoking (n; %)	10 (83.3%)	22 (66.7%)	0.25
Hypertension (n; %)	10 (83.3%)	22 (66.7%)	0.25
Diabetes (n; %)	4 (33.3%)	11 (33.3%)	1
Hypercholesterolaemia (n; %)	7 (58.3%)	19 (57.6%)	0.97
CAD (n; %)	9 (75%)	19 (57.6%)	0.28
Medication in use:			
Beta-blockers (n; %)	9 (75%)	16 (48.5%)	0.1
ACE-I (n; %)	9 (75%)	21 (63.6%)	0.47
Ca ²⁺ channel blockers (n; %)	1 (8.33%)	6 (18.2%)	0.36
Acetylsalicylic Acid (n; %)	12 (100%)	33 (100%)	1
Insulin (n; %)	4 (33.3%)	11 (33.3%)	1
Diuretics (n; %)	9 (75%)	21 (63.6%)	0.47
Statins (n; %)	12 (100%)	33 (100%)	1
Clopidogrel (n; %)	12 (100%)	33 (100%)	1

CAD — coronary artery disease; ACE-I - angiotensin-converting-enzyme inhibitors

Table 2
Acute changes of activated and immunosenescent T cells and their subsets before and after angioplasty

	PTA	T cells (cells/mm ³)	CD4+ cells (cells/mm ³)	CD8+ cells (cells/mm ³)	DN-T cells (cells/mm ³)
CD25	Before	409 (305;531)	339 (224;411)	46 (22;114)	3 (2;5)
	After	372 (292;489)	297 (194;383)	43 (24;102)	2 (1;4)
CD69	Before	31 (20;37)	8 (6;12)	14 (10;20)	2 (1;5)
	After	20 ** (16;31)	8 (5;12)	10 * (7;12)	2 (1;3)
CD38	Before	248 (153;342)	196 (113;295)	28 (18;46)	5 (4;8)
	After	218 ** (139;304)	184 (115;247)	22 ** (13;31)	5 (3;6)
CCR5	Before	213 (164;379)	82 (57;122)	98 (62;179)	18 (10;33)
	After	156 ** (110;266)	62 (48;104)	72 ** (41;99)	14 (9;21)
CD28null	Before	220 (127;332)	15 (3;46)	158 (81;231)	11 (6;16)
	After	131 ** (73;223)	11 (2;27)	80 ** (54;127)	8 (3;13)
CD57	Before	292 (211;436)	81 (53;103)	164 (95;255)	11 (8;25)
	After	193 ** (155;264)	59 ** (41;91)	97 ** (76;134)	9 (5;18)

Absolute numbers of CD25, CD69, CD38, CCR5, CD28null and CD57 positive cells per mm³ were measured in peripheral blood collected before and after percutaneous transluminal angioplasty (PTA). The medians and the (25th and 75th) percentiles are indicated.

*
p<0.05 vs Before PTA

**
p<0.01 vs Before PTA.