Novel immune cell-based therapies for atherosclerosis

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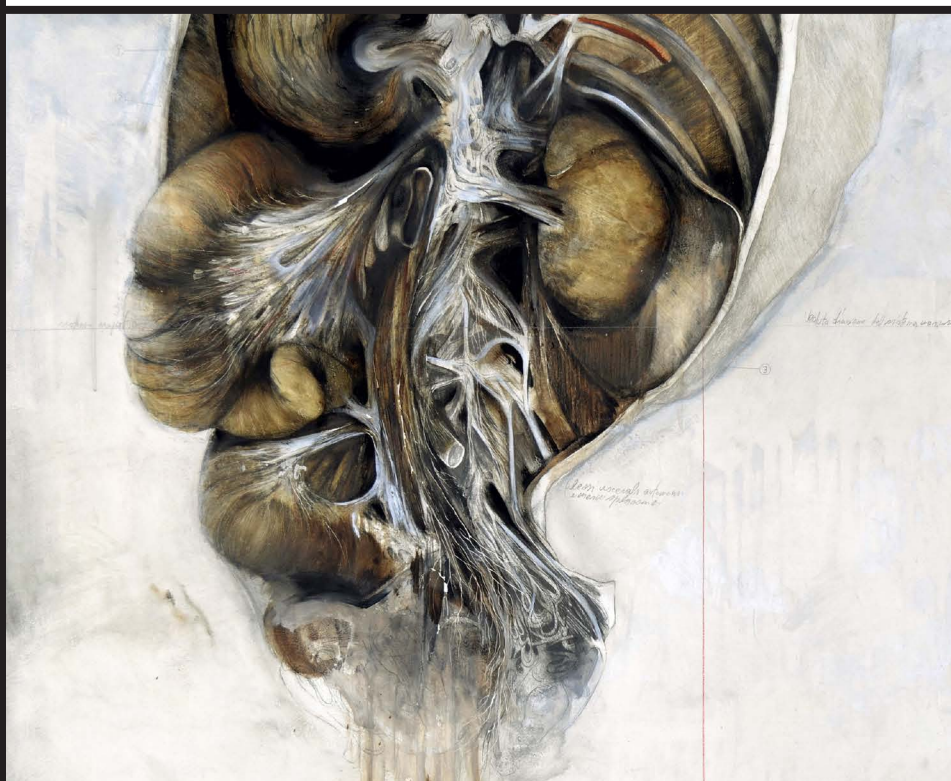
Modulation of Macrophages in Atherosclerosis by Heat-Killed *S. aureus*

Identification of a Novel Anti-Atherogenic Function for TLR2/PI3K-Signaling

Submitted



Abstract

Objective *Staphylococcus aureus* cell wall components can induce IL-10 responses by immune cells, which may result in atheroprotection. Here we thus investigated whether heat-killed *Staphylococcus aureus* (HK-SA) could reduce atherosclerosis by modulation of macrophage function.

Methods and Results We administered HK-SA twice weekly *intraperitoneally* to LDL receptor-deficient mice, which were subsequently put on a Western-type diet for six weeks. HK-SA administration resulted in an immediate significant 1.6-fold increase in IL-10 production by peritoneal cells and splenocytes, and a 12-fold increase in serum IL-10 levels. Moreover, aortic plaque ICAM-1, VCAM-1 and CCL2 expression were significantly downregulated by 40%. HK-SA-treated mice had reduced numbers of inflammatory Ly-6C^{hi} monocytes as well as Th1 and Th17 cells in circulation and spleen, respectively. Subsequently, attenuated leukocyte recruitment resulted in a significant inhibition of macrophage and T cell infiltration in atherosclerotic plaques, culminating in a significant 34% reduction of aortic root lesion sizes.

To determine the effects of *intraperitoneal* HK-SA treatment, we stimulated macrophages with HK-SA in vitro. This resulted in a significant TLR2-dependent increase in IL-10, arginase-1, iNOS, TNF-α, PD-L1, CCL22, and IDO expression. PI3K was found to crucially determine the balance of pro- and anti-inflammatory gene expression. The HK-SA-induced macrophage phenotype resembles M2b-like immunoregulatory macrophages.

Conclusion In contrast to the currently accepted pro-atherogenic role of TLR2 and PI3K, our data suggest that combined TLR2/PI3K-dependent signaling induced by HK-SA in macrophages is atheroprotective.

Introduction

Macrophages are found in all stages of atherosclerotic lesion development and outnumber any other cell type in the lesion[1,2]. They play a crucial role in clearing debris in the arterial wall, but are also involved in shaping immune responses. Therefore modulation of macrophages towards an anti-inflammatory phenotype is an attractive therapeutic goal.

The macrophage phenotype is determined by its local microenvironment, e.g. by cytokines and Toll-like receptor (TLR) agonists. IFN-γ and LPS induce classically activated M1 macrophages, characterized by e.g. iNOS and IL-12p70 expression[3,4]; while e.g. IL-4 can induce alternatively activated M2 macrophages, characterized by an upregulation of arginase-1 and IL-10 production[3,4]. The specific role of each macrophage subset in atherosclerosis is still under investigation. However, due to the production of anti-inflammatory mediators, such as IL-10, M2 macrophages are currently considered to be anti-atherogenic[5]. Indeed, we and others have previously confirmed that IL-10 can significantly protect against atherosclerosis[6,7]. This is due to the wide-ranging beneficial effects of IL-10, such as a reduction of CCL2 levels[8], reduced lesional macrophages[9], decreased lesional IFN-γ-producing T cells[6] and inhibition of T cell activation and proliferation[10].

Macrophages, as well as DCs, recognize pathogen- and danger-associated molecules in their microenvironment via their TLRs. This results in the maturation and activation of antigen-presenting cells (APCs) with subsequent production of pro-inflammatory cytokines. While mature DCs are vital for the activation of naïve T cells, both APCs can shape adaptive T cell responses and ensure long-lasting and specific responses.

TLRs recognize various highly conserved molecules and TLR2 and TLR4 signaling in the atherosclerotic plaque has been mainly associated with pro-inflammatory cytokine production by lesional macrophages[11,12]. TLR2 and TLR4 both recruit intracellular myeloid differentiation primary-response protein 88 (MyD88), and the MyD88 adaptor-like protein (MAL), which result in downstream activation of nuclear factor (NF)-κB and activating protein-1 (AP-1). TLR4 additionally results in TIR domain-containing adaptor protein inducing IFNβ (TRIF)/ TRIF-related adaptor molecule (TRAM)-dependent induction of NF-κB and AP-1, resulting in pro-inflammatory responses[13]. Another adaptor molecule, B-cell adaptor for PI3K (BCAP), was found to link TLRs to PI3K and was shown to reduce pro-inflammatory signaling in response to TLRs, e.g. in B cells and macrophages[13].

After pathogen elimination, it is vital to terminate the immune response to ensure a return to immune homeostasis without inducing damage to the affected tissue. LPS/TLR4 stimulation of monocytes results in a late anti-inflammatory IL-10 response to downregulate the primary induction of pro-inflammatory cytokines[14]. Similarly, it has been found that TLR2 can induce anti-inflammatory IL-10 responses[15]. Animal studies have established that certain bacteria, such as *Porphyromonas gingivalis* and *Yersinia* bacteria, induce a TLR2-dependent IL-10 production to evade the host's immune system, which can inhibit IFN-γ production by T cells[16,17]. Moreover,

administration of heat-killed *Staphylococcus epidermis* was found to potently induce IL-10 responses in APCs, which effectively inhibited massive T cell activation and toxic shock syndrome in mice[18]. This was attributed to peptidoglycan-embedded molecules in the *Staphylococcal* cell wall[18]. Indeed, peptidoglycan induced an IL-10 response in human macrophages via TLR2/PI3K, which resulted in the inhibition of T cell proliferation. However, stimulation of DCs with peptidoglycan resulted in an induction of T helper (Th) 1 and Th17 responses, indicating that not only the ligand and its kinetics, but also the cell type involved determines the type of immune response[19].

In this study we established that exposure of macrophages to heat-killed *Staphylococcus aureus* (HK-SA), which contains cell wall components such as peptidoglycan that are recognized by TLR2, induced anti-inflammatory IL-10-producing macrophages that protected LDL receptor-deficient ($LDLr^{-/-}$) mice from atherosclerosis development.

Material and Methods

Animals

C57BL/6, $LDLr^{-/-}$ and $TLR2^{-/-}$ mice were originally obtained from the Jacksons Laboratory, kept under standard laboratory conditions, and administered food and water *ad libitum*. All animal work was approved by the Ethics Committee for Animal Experiments of Leiden University and conforms to Dutch government guidelines.

Macrophage and peritoneal macrophage stimulations

Bone marrow cells were isolated from the tibias and femurs of C57BL/6 mice. The cells were cultured for seven days in RPMI supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, 0.1mM nonessential amino acids, 1% pyruvate (all obtained from PAA), 2mM L-glutamine (Thermo Fisher Scientific) in the presence of 10 ng/mL macrophage colony-stimulating factor (Peprotech) to obtain macrophages. Macrophage purity was assessed by CD11c, CD11b and F4/80 expression (flow cytometry) and routinely found to be above 90%. Peritoneal cells were isolated by peritoneal lavage, washed with PBS and left to adhere on plates for 2 hours at 37°C and 5% CO_2. Non-adherent cells were removed by aspiration and replaced with fresh RPMI, supplemented with 10% FCS, 100 U/mL penicillin/streptomycin and 2mM L-glutamine.

For in vitro stimulations, 1 x 10^5 cells per well were plated in 96-well flat-bottom plates (Greiner Bio-One) for analysis of cytokine production by ELISA or 5 x 10^5 per well in 24-well plates (Greiner Bio-One) for mRNA isolation. Cells were stimulated with indicated amounts of heat-killed *Staphylococcus aureus* (tlrl-hksa, Invivogen) for 24 hours.

Atherosclerosis

Atherosclerosis was induced in 10-12 weeks old female $LDLr^{-/-}$ mice by feeding a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter; Special Diet Services) for six weeks. Mice were treated twice weekly *intraperitoneally* with 10^8 colony-forming units of HK-SA (tlr-hksa, Invivogen) or PBS as a control. Treatment

was started one week prior to WTD and continued during the entire experiment.

Flow Cytometry

At sacrifice, blood and spleen were harvested. Single cell suspensions of spleens from $LDLr^{-/-}$ mice were obtained by using a 70 µm cell strainer (VWR International). Red blood cells were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently, 3 x 10^5 cells per sample were stained with the appropriate antibodies. The following antibodies were used: CD11b-eFluor450 (clone M1/70), CD11c-FITC (clone clone N418), CD4-PerCP (clone RM4-5; BD Biosciences), CD25-FITC (clone eBio3C7), FoxP3-APC (clone FJK-16s), Gata-3-PE (clone TWAJ), Ly-6C-PerCP (clone HK1.4), Ly-6G-FITC (clone 1A8; BD Biosciences), RORγt-PE (clone AFKJS-9), and T-bet-APC (clone eBio4B10). All antibodies were purchased from eBioscience, unless stated otherwise. For intracellular staining, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

Histological analysis

To determine plaque size, 10 µm cryosections of the aortic root were stained with Oil-Red-O and haematoxylin (Sigma Aldrich). Corresponding sections were stained for collagen using Sirius Red (Sigma Aldrich) or immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, 1:1000, Serotec Ltd.). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrate. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision anti-rabbit-HRP was used as secondary antibody (Immunologic). The section with the largest lesion and four flanking sections were analyzed for lesion size and collagen content, two flanking sections were analyzed for macrophage and T cell content. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area.

Real-time PCR

mRNA was isolated from macrophages and the aortic arch using the guanidium isothiocyanate method and reverse transcribed (RevertAid Moloney murine leukemia virus reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems) using SYBR Green technology. The expression was determined relative to the average expression of three household genes: Succinate dehydrogenase complex, subunit A, flavoprotein (Sdha), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). For used primer pairs refer to Table 1.

Gene	Forward	Reverse
Arg1	TGGCAGAGGTCCAGAAGAATGG	GTGAGCATCCACCCAAATGACAC
CCL22	TCTTGCTGTGGCAATTCAGA	GAGGGTGACGGATGTAGTCC
CCL2	CTGAAGCCAGCTCTCTCTTCCTC	GGTGAATGAGTAGCAGCAGGTGA
CD3	TCTGCTACACACCAGCCTCAAA	ATGACCATCAGCAAGCCCAGA
CD4	CAAAGTTCTCTCCATGTCCAACCTA	CACTCTTATAGGCCGTGATAGCTG
CD68	TGCCTGACAAGGGACACTTCGGG	GCGGGTGATGCAGAAGGCGATG
CD163	CAGTGCCCCTCGTCACCTTG	GATCTCCACACGTCCAGAACAGTC
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
ICAM-1	GTCCGCTTCCGCTACCATCAC	GGTCCTTGCCTACTTGCTGCC
IDO	CACTGCACGACATAGCTACCAGTC	TCCAGCCAGACAGATATATGCGGA
IL-10	GGGTGAGAAGCTGAAGACCCTC	TGGCCTTGTAGACACCTTGGTC
iNOS	CCTGGTACGGGCATTGCT	GCTCATGCGGCCTCCTTT
p35	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
Sdha	TATATGGTGCAGAAGCTCGGAAGG	CCTGGATGGGCTTGGAGTAATCA
TGF-β	AGGGCTACCATGCCAACTTCT	GCAAGGACCTTGCTGTACTGTGT
TNF-α	GCCTCTTCTCATTCCTGCTTGTG	ATGATCTGAGTGTGAGGGTCTGG
VCAM-1	AGACTGAAGTTGGCTCACAATTAAGAAG	AGTAGAGTGCAAGGAGTTCGGG

Table 1. Primer Pairs used for qPCR analysis. The relative expression of genes was determined relative to the average expression of the three household genes: succinate dehydrogenase complex, subunit A, flavoprotein (Sdha), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). Abbreviations: Arg1, arginase-1; IDO, indole 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor α; VCAM-1; Vascular cell adhesion protein 1.

Cytokine and analysis

IL-12p40 (eBiosciences), IL-10, IL-12p70, TNF-α, and CCL2 (all BD Biosciences) were determined by ELISA, according to manufacturer's protocol.

Serum cholesterol levels

Serum concentrations of total cholesterol were determined by enzymatic colorimetric assays (Roche Diagnostics). Absorbance was read at 490 nm. Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 µl of serum of each mouse using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia). Total cholesterol content of the effluent was determined as described above.

Statistical analysis

Values are expressed as mean±SEM. Data of two groups were analyzed by Student's

T-test, data of three groups were analyzed by one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, followed by Bonferroni post-testing. Statistical analysis was performed using Prism (GraphPad). Probability values of $P<0.05$ were considered significant.

Results

HK-SA potently induces IL-10 responses upon intraperitoneal administration

Peptidoglycan-embedded lipopeptides and glycopolymers in the *Staphylococcus aureus* cell wall have been shown to induce IL-10 responses[18,19]. We therefore determined whether *intraperitoneal* injections with 10^8 colony-forming units HK-SA could induce IL-10 responses in LDLr$^{-/-}$ mice. Indeed, two days after the injection, serum IL-10 levels were significantly increased by 12-fold in mice treated with HK-SA compared to controls, while IL-12 was not affected (Figure 1A).

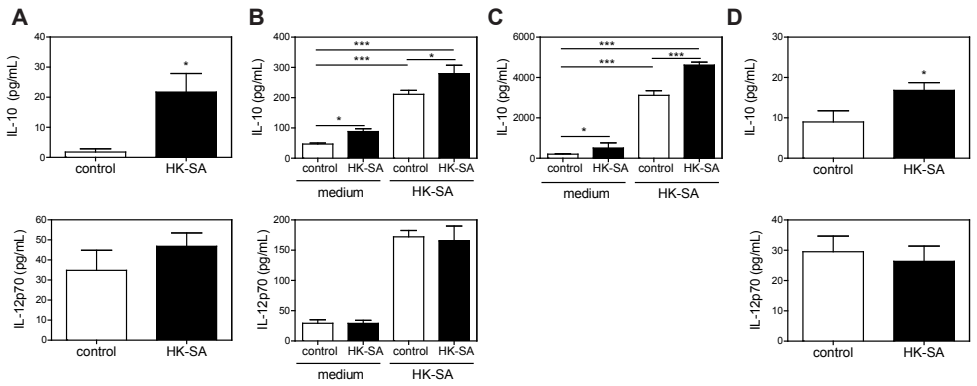


Figure 1. *Intraperitoneal* injection of HK-SA increases IL-10 responses in LDLr$^{-/-}$ mice. A. Two days after the injection of HK-SA cytokine levels in the plasma were measured. **B.** Splenocytes and **C.** peritoneal cells were isolated two days after injection of HK-SA and cultured in the presence of medium or HK-SA. **D.** After six weeks Western-type diet, cytokine levels in the plasma were measured. All cytokine responses were determined by ELISA. All values are expressed as mean±SEM and are representative of at least five mice.

We additionally isolated splenocytes and peritoneal macrophages two days after the HK-SA injection and cultured them ex vivo. We observed a significant 1.8-fold higher basal IL-10 production in cultured splenocytes in the HK-SA group (Figure 1B) and a 2.5-fold higher IL-10 production in cultured peritoneal macrophages from HK-SA-treated mice (Figure 1C). Upon re-stimulation with HK-SA, IL-10 production was significantly increased in splenocytes and peritoneal macrophages isolated from both groups. However, IL-10 responses in the HK-SA treated group were 1.6-fold higher than in the control group (Figure 1B and C). IL-12p70 responses were unaffected in splenocytes, while undetectable in peritoneal macrophages (Figure 1B and data not shown). Overall, these results suggest a significant in vivo IL-10 response upon HK-SA treatment.

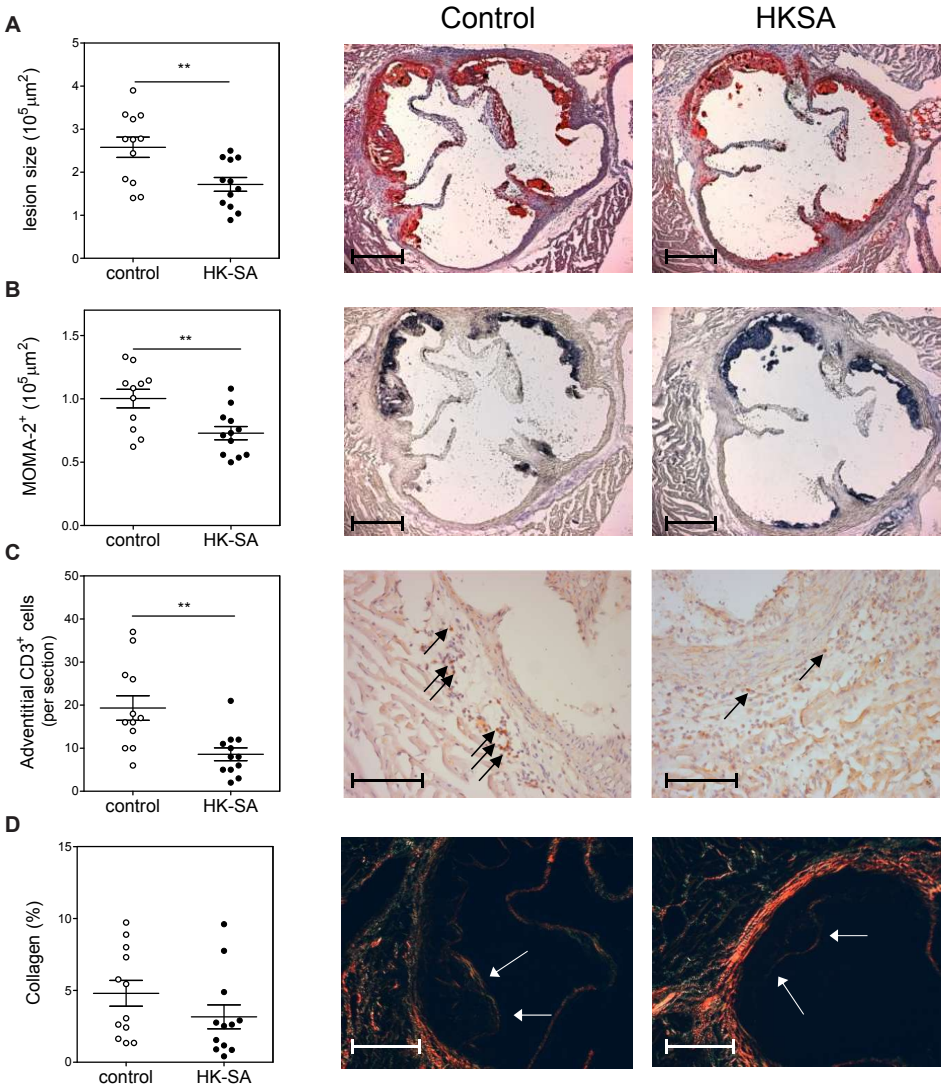


Figure 2. ***Intraperitoneal*** **injections of HK-SA reduce atherosclerotic lesion development. A.** After six weeks Western-type diet, lesion size in the three valve area of the aortic root was determined; representative cross-sections stained with Oil-Red-O and hematoxylin are shown. Scale bar, 300 µm. **B.** Macrophage positive area was determined by MOMA-2 staining. Scale bar, 300 µm. **C.** $CD3^+$ T cells were determined by αCD3 staining. Arrows indicate T cells. Scale bar, 100 µm. **D.** Collagen positive area was determined by Sirius Red staining under polarized light. Arrows indicate collagen-positive area. Scale bar, 200 µm. All values are expressed as mean±SEM and are representative of six mice. * P<0.05, ** P<0.01.

HK-SA treatment reduces atherosclerotic lesion development

We further determined whether *intraperitoneal* injections of HK-SA would be able to prevent atherosclerotic lesion development. We thus injected female $LDLr^{-/-}$ mice with 10^8 CFU of HK-SA twice weekly and after the first two injections we fed the mice a Western-type diet (WTD) for six weeks to induce atherosclerotic lesion development.

After six weeks, we still found a significant 2-fold increase of serum IL-10 levels in HK-SA-treated mice, suggesting increased IL-10 levels throughout our experiment, while IL-12 levels were not affected (Figure 1D).

Strikingly, HK-SA treatment resulted in a significant 34% reduction of aortic root lesion sizes compared to control mice (control: 2.6 x 10^5 ± 0.2 x 10^5 µm versus HK-SA: 1.7 x 10^5 ± 0.2 x 10^5 µm; Figure 2A). Additionally we observed a significant 27% reduction of macrophage positive area in the aortic root (control: 1.0 x 10^5 ± 0.1 x 10^5 µm versus HK-SA: 0.7 x 10^5 ± 0.1 x 10^5 µm; Figure 2B). Similarly, a 56% decrease in adventitial $CD3^+$ T cells in HK-SA-treated mice compared to control mice was observed (control: 19.3 ± 2.8 versus HK-SA: 8.6 ± 1.5 T cells per section; Figure 2C). No significant differences in collagen content between the two groups were observed (control: 4.7 ± 0.9% vs HK-SA: 3.2 ± 0.8%; Figure 2D). The observed effects were not due to effects on cholesterol or weight, as these did not differ between the groups (Figure 3).

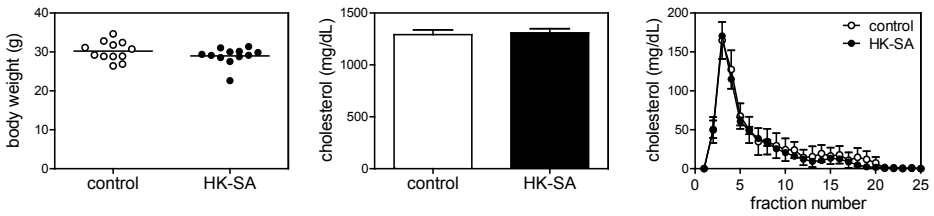


Figure 3. *Intraperitoneal* injections of HK-SA do not affect weight and cholesterol. After six weeks, mice were weighed and cholesterol levels were detected in the serum of mice. All values are expressed as mean±SEM and are representative of twelve mice. For FPLC analysis 3 mice per group were pooled.

HK-SA treatment reduces monocyte and T cell recruitment

To elucidate what caused the reduced macrophage and T cell content of lesions, we assessed whether vascular adhesion molecules were affected by the treatment after six weeks WTD. Indeed we observed a significant 3.7-fold reduction in ICAM-1 and a 1.8-fold reduction in VCAM-1 expression in the aortic arch upon HK-SA treatment (Figure 4A). Furthermore CCL2 expression was significantly reduced by 2.3-fold upon HK-SA treatment, while only a modest non-significant 19% reduction in serum CCL2 levels was observed (Figure 4B).

Because CCL2 plays a crucial role in the recruitment of monocytes to atherosclerotic lesions, we assessed monocyte responses. Overall monocyte numbers were not affected, but the number of inflammatory Ly-$6C^{hi}$ monocytes, which predominantly give rise to lesional macrophages[20], was significantly reduced by 31% (Figure 3C). Indeed, not only the amount of aortic root macrophages, but also CD68 expression in the aortic arch was significantly reduced by 81% in HK-SA-treated mice compared to controls, indicating a reduction of macrophages (Figure 4D).

ICAM-1 and VCAM-1 are also involved in diapedesis of T cells, possibly explaining low T cell numbers found in aortic root lesions. Similarly, we found that the expression of CD3 and CD4 were significantly decreased by 67% in the aortic arch (Figure 4E).

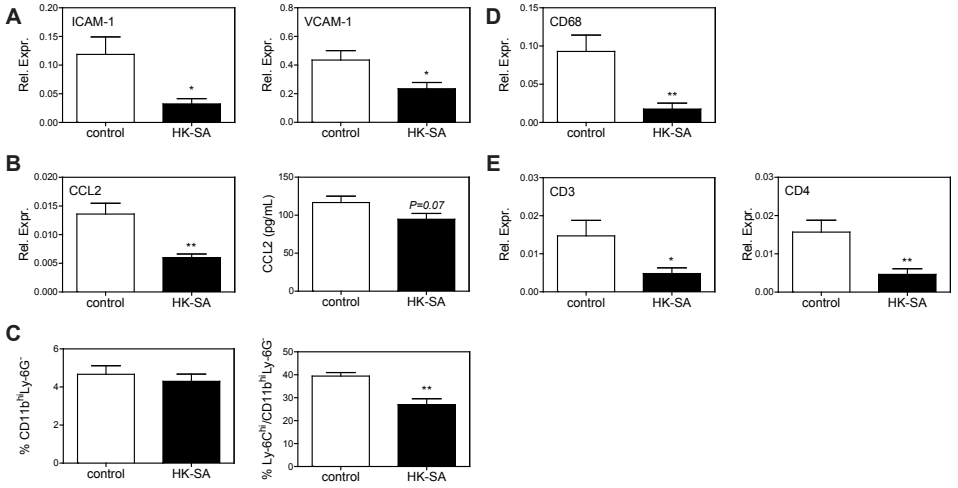


Figure 4. ***Intraperitoneal*** **injections of HK-SA decrease monocyte and T cell recruitment in LDLr mice. A.** Relative ICAM-1 and VCAM-1 mRNA expression in the aortic arch was determined by qPCR. **B.** CCL2 expression in the aortic arch was determined by qPCR and serum CCL2 levels were determined by ELISA. **C.** Circulating monocytes were determined as $CD11b^{hi}Ly\text{-}6G^{-}$ by flow cytometry. Inflammatory $Ly\text{-}6C^{hi}$ monocytes were determined as the percentage of total monocytes. **D.** Relative mRNA expression of CD68 and **E.** CD3 and CD4 in the aortic arch was determined by qPCR. Relative expression was determined compared to housekeeping genes (Sdha, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of at least six mice.* P<0.05, ** P<0.01.

HK-SA treatment reduces Th1, Th2 and Th17 responses in vivo

Lesional T cell responses are determined by induced T cell subsets and their proliferation. We thus monitored T cell responses in the circulation during the entire experiment and found no effects on Tregs or Th1 cells. However, Th2 numbers after three weeks WTD were decreased by 38% and Th17 cells were decreased by 37% after six weeks WTD (Figure 5A). Six weeks after induction of atherosclerosis, splenic $CD4^+$ T cell responses showed a significant 48% reduction in Th1 and a 31% reduction in Th17 responses, while no effects on Th2 and Treg responses were observed (Figure 5B). Next, we isolated splenocytes from control and HK-SA-treated mice after six weeks WTD and stimulated them ex vivo to assess their proliferative capacity. Upon treatment with HK-SA proliferation of splenocytes was decreased by 81% in HK-SA-treated mice, while upon stimulation with oxLDL and αCD3/αCD28 proliferation was decreased by 73% and 64%, respectively (Figure 5C). This indicates an overall reduced proliferative capacity of T cells in mice treated with HK-SA.

HK-SA induces an IL-10-producing immunoregulatory M2b-like phenotype in macrophages

To further delineate the effects of HK-SA on macrophages we cultured bone marrow-derived macrophages and treated these with increasing amounts of HK-SA (up to 10^8 colony-forming units). Indeed, we found a significant dose-dependent IL-10 production in response to increasing amount of HK-SA (Figure 6A). As atherosclerosis

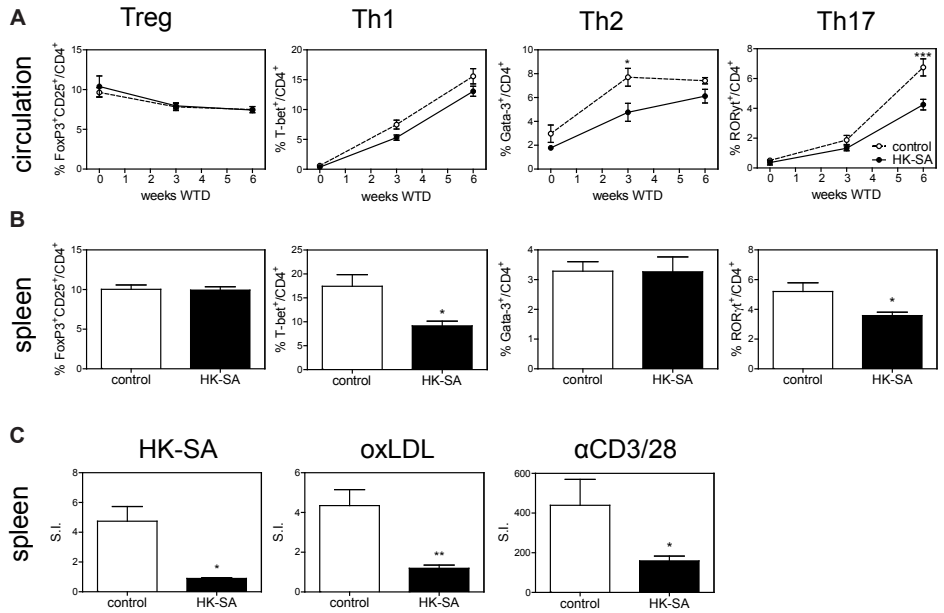


Figure 5. Intraperitoneal injections of HK-SA decrease inflammatory T cell responses. A. Treg ($FoxP3^+CD25^+$), Th1 ($T\text{-}bet^+$), Th2 ($Gata\text{-}3^+$), and Th17 ($ROR\gamma t^+$) cells within $CD4^+$ T cells were measured in the circulation throughout the entire experiment and **B.** in the spleen after six weeks WTD by flow cytometry. **C.** After six weeks WTD, splenocytes were isolated and cultured in the presence of 10^8 CFU of HK-SA, 5 µg/mL oxLDL, or 1 µg/mL αCD3/αCD28 for 72 hours. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation during the last 16 hours of culture. Proliferation is normalized for proliferation of controls (splenocytes without stimulation) and expressed as the stimulation index (S.I.). All values are expressed as mean±SEM and are representative of six mice.* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

is a predominantly Th1-driven disease, we determined whether HK-SA induced IL-12 production and thereby could exacerbate Th1 responses. We found that macrophages did produce large amounts of IL-12p40, which can heterodimerize to form IL-12 and IL-23, or form homodimers to block IL-12 function (Figure 6A). However, no IL-12 or IL-23 production could be observed (data not shown). This clearly indicates that treatment of macrophages with HK-SA could indeed be responsible for the strong IL-10 responses and the lack of IL-12 responses observed in vivo.

As recognition of pathogens is associated with macrophage activation and phenotype changes, we determined whether HK-SA exposure induced a specific macrophage subset. Exposure to HK-SA induced some markers of inflammatory M1 macrophages: a significant upregulation of iNOS (220-fold) and TNF-α (5-fold) was observed (Figure 6B). TNF-α and iNOS have also been described to be produced, in parallel with IL-10, by immunoregulatory M2b macrophages[21,22] and indeed we saw an 18-fold upregulation of IL-10 and a 45-fold upregulation of arginase-1, an M2 marker[23,24], upon treatment with 10^8 colony-forming units HK-SA (Figure 6C). Additionally we observed a 6-fold upregulation of CCL22, a 30-fold upregulation of IDO and a 6-fold upregulation of PD-L1; all anti-inflammatory markers[25,26] (Figure 6D). Other markers of M2 subsets, such as CD163 (M2a,c) and TGF-β (M2c)[23,24] were

significantly downregulated or not affected, respectively (Figure 6E). This expression profile indicates that macrophages are skewed towards immunoregulatory M2b macrophages with an overall anti-atherogenic phenotype upon HK-SA exposure.

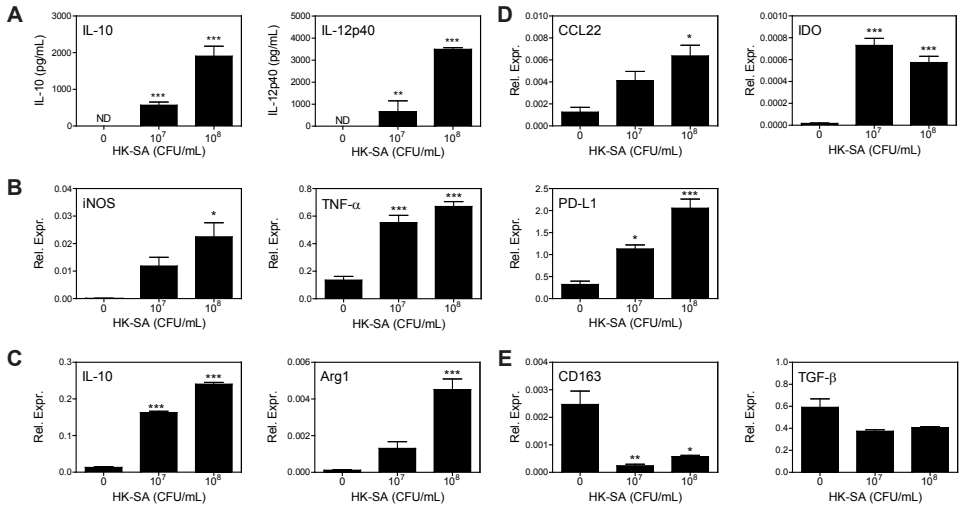


Figure 6. HK-SA induces a distinct macrophage phenotype. Bone marrow-derived macrophages were stimulated with indicated colony-forming units (CFU) of HK-SA. **A.** IL-10 and IL-12p40 responses were determined by ELISA. **B.** Markers for M1 macrophages, **C.** M2(b) macrophages, **D.** anti-inflammatory markers and **E.** M2a and M2c macrophages were determined by qPCR, relative to housekeeping genes (Sdha, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of three experiments. * P<0.05, ** P<0.01, *** P<0.001.

IL-10 responses to HK-SA are TLR2/PI3K-dependent

The *Staphylococcus aureus* cell wall contains TLR2 ligands and we therefore tested whether the observed anti-inflammatory IL-10 response was dependent on TLR2 ligation. Indeed, upon stimulation with 10^8 colony-forming units of HK-SA, macrophages of TLR2$^{-/-}$ mice produced 74% less IL-10 than wild-type controls. Moreover, deficiency of TLR2 also reduced TNF-α responses by 81% (Figure 7A), confirming that TLR2 recognition is needed for cytokine responses by macrophages to HK-SA. Other TLR2 ligands, including peptidoglycan, Pam3CSK4 and FSL-1 also showed a TLR2-dependent IL-10 induction (Figure 7B).

TLR2-induced IL-10 responses have been linked to PI3K activation upon receptor ligation[13]. We therefore treated macrophages with 10μM of the PI3K inhibitor wortmannin. We observed that PI3K inhibition dramatically reduced IL-10 responses by 80% in bone marrow-derived macrophages. On the other hand, PI3K inhibition significantly increased TNF-α and iNOS expression, and interestingly also induced a significant expression of IL-12. In line with IL-10 responses, also arginase-1 was significantly reduced. Surprisingly, IDO was however upregulated by PI3K inhibition (Figure 7C). Therefore, it seems that anti-inflammatory IL-10 responses are clearly PI3K-dependent and correlate with arginase-1 expression of macrophages. Interestingly, M1 markers and the anti-inflammatory enzyme IDO are however under

negative feedback control of PI3K, suggesting a central role for PI3K in determining the macrophage phenotype upon HK-SA treatment.

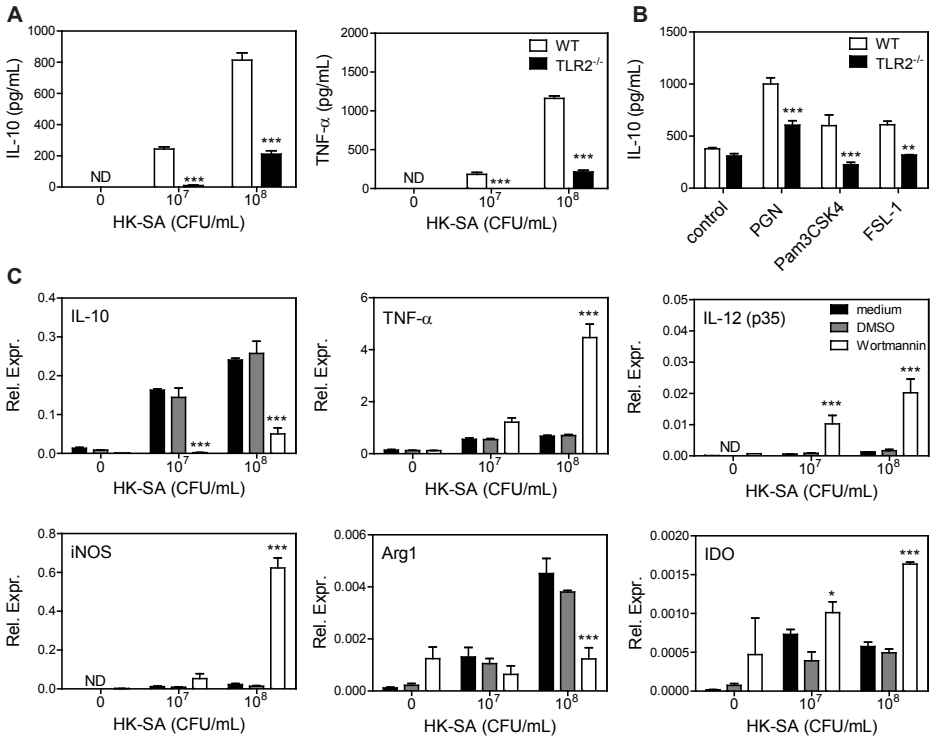


Figure 7. HK-SA-induced IL-10 responses are TLR2/PI3K-dependent. A. Bone marrow-derived macrophages from wild type (WT) or TLR2$^{-/-}$ mice were stimulated with indicated colony-forming units (CFU) of HK-SA or **B.** 10μg/mL of other TLR2 ligands. Cytokine responses are measured by ELISA. **C.** Bone marrow-derived macrophages were stimulated in the presence of the PI3K inhibitor Wortmannin (10μM) or vehicle control (DMSO). Relative expression was determined by qPCR, relative to housekeeping genes (Sdha, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of at least three experiments. * P<0.05, ** P<0.01, *** P<0.001. ND defines not determined.

Discussion

In this study we exploited the finding that exposure to HK-SA induces a strong anti-inflammatory IL-10 response in macrophages to treat atherosclerosis. We show that HK-SA treatment is a potent therapy to reduce both inflammation and atherosclerotic lesion development.

We found that in vitro treatment of murine macrophages with HK-SA induced a strong IL-10 response, while IL-12 and IL-23 responses were lacking, suggesting that exposure of macrophages to HK-SA in an inflammatory environment such as the atherosclerotic lesion may reduce inflammation. To determine whether HK-SA induced a distinct macrophage phenotype, we looked at the gene expression signature of macrophages upon HK-SA stimulation. As expected, recognition of HK-SA induced

the expression of some pro-inflammatory genes such as TNF-α and iNOS, considered as M1 markers. Interestingly, while nitric oxide together with IL-12 has been found to promote Th1[27], it can also induce Tregs in the absence of IL-12[28]. Moreover, nitric oxide expression by macrophages suppresses T cell proliferation[29,30]. Similarly, TNF-α can also have anti-inflammatory effects by promoting the survival and suppressive function of Tregs and by reducing effector T cell activation upon chronic exposure[31]. As such, both high levels of TNF-α and iNOS expression in combination with high IL-10 levels and absence of IL-12p70, as observed after HK-SA-exposure of macrophages, have been described as a hallmark of M2b macrophages[21,22]. M2b macrophages have been shown to be potent immunoregulators and can even protect mice from LPS-induced septic shock[22]. Additionally, the M2 marker arginase-1 is induced by HK-SA treatment. After stimulation with HK-SA, we find that macrophages also express anti-inflammatory IDO, PD-L1 and CCL22. IDO expression is known to suppress $CD4^+$ T cell responses and induce Tregs[25]. PD-L1 also plays a crucial role in inducing and maintaining Tregs[26], while CCL22 plays a role in Treg recruitment. We therefore conclude that HK-SA is capable of inducing a specific anti-inflammatory M2b-like macrophage signature.

The observed anti-inflammatory IL-10 response was largely dependent on TLR2. However, as IL-10 responses are not completely inhibited in $TLR2^{-/-}$ macrophages, other receptors (including scavenger receptors, NOD-like receptors, or other TLRs) recognizing HK-SA components likely contribute to induce IL-10 responses. For example, NOD-like receptor signaling is induced by peptidoglycan and triggers RIP2-dependent activation of NF-κB. The purified gram positive *Streptococcus pneumonia*, which mostly consists of peptidoglycan, was found to induce TLR2-dependent IL-10 responses, which were also largely dependent on NOD2-signaling[32]. However, *intraperitoneal* administration of only muramyl dipeptide, a NOD2-ligand and a component of peptidoglycan, increased atherosclerotic lesion development[33], indicating that combined signals might be needed.

Interestingly, we find that TLR2-induced IL-10 responses upon HK-SA exposure are largely dependent on PI3K. Indeed, previous studies found that TLR2 activation results in IL-10 responses that are partially PI3K-dependent[19,34,35]. Furthermore, TLR3, TLR7 and TLR9 have also been shown to recruit PI3K[13,36], indicating that other TLRs could possibly recognize components of HK-SA, e.g. single-stranded CpG-DNA, and contribute to IL-10 responses to HK-SA. Moreover, we confirm previous observations that PI3K exhibits a negative feedback on the induction of pro-inflammatory cytokine responses by TLRs[19,37–39]. We show that PI3K inhibition by wortmannin significantly increases TNF-α, IL-12p70 and iNOS responses of macrophages in response to HK-SA. Surprisingly PI3K also inhibits anti-inflammatory IDO expression by macrophages, suggesting that it plays a crucial role in balancing several pro- and anti-inflammatory responses to HK-SA in macrophages. Interestingly, PI3K has been suggested to be involved in determining the macrophage phenotype and increased PI3K signaling has been found to skew macrophages to an M2 phenotype[40]. In the context of atherosclerosis, only class Ib PI3K deficiency has been investigated and no effect on macrophage polarization was found, while atherosclerosis was significantly

attenuated[41,42]. Our study suggests that it will be interesting to determine the effect of targeted activation of (class Ia/Ib) PI3K in lesional macrophages.

In general TLR2 is known for the induction of pro-inflammatory responses. For example blocking TLR2 promotes graft acceptance upon renal transplantation[43] and reduces myocardial infarct sizes[44], due to reduced inflammation and tissue necrosis. In atherosclerosis, studies have shown that administration of Pam3Csk4 (TLR2/TLR1 agonist) and MALP2 (TLR2/TLR6 agonist) result in increased atherosclerotic lesion formation[45–47]. Deficiency of either TLR1 or TLR6 was found to have no effect on atherosclerosis in LDLr$^{-/-}$ mice[47]. Interestingly, the pro-atherogenic effect of TLR2 in mice was found to be associated to TLR2 signaling in non-bone marrow-derived cells[46,48].

In line with our findings, preclinical studies have shown that TLR2 signaling can also be exploited to reduce inflammation. *Intraperitoneal* injections of peptidoglycan and Pam3Csk4 were found to reduce ischaemia/reperfusion injury in a PI3K-dependent way[49]. Moreover, *intranasal* Pam3Csk4 administration suppressed asthma development[50], while *intraperitoneal* administration reduced type 1 diabetes[51], both via induction of IL-10 responses and Tregs. IL-10$^{-/-}$ mice have been found to have enhanced Th1 and Th17-like responses[52,53], thereby conversely significant amounts of IL-10 could result in a reduction of Th1 and Th17 responses. Indeed, we observe a significantly decreased induction of splenic Th1 cells and splenic Th17 cells, as well as circulating Th17 cells. The capacity of TLR2-dependent IL-10 production to inhibit IFN-γ-producing T cells has previously been described[16]. Moreover, IL-10 is known to indirectly prevent antigen-specific T cell activation by modulating APCs and directly by inhibiting T cell expansion[10]. In fact, we also observed a reduced proliferative capacity of splenic T cells.

In agreement with earlier observations that IL-10 reduces monocyte adhesion and recruitment[54], we found a significantly reduced expression of adhesion molecules, ICAM-1 and VCAM-1, as well as CCL2 in the aortic arch, resulting in reduced leukocyte recruitment to the lesions. Both macrophage and T cell presence was dramatically reduced in the aortic arch and the aortic root. A decrease of circulating inflammatory Ly-6C^{hi} monocytes, which is in line with reduced lesional macrophages, likely resulted from a decrease in CCL2 and a reduced recruitment of monocytes from bone marrow.

In summary, we demonstrate that HK-SA treatment significantly reduces atherosclerotic lesion development by 34%, likely through inducing IL-10-producing immunoregulatory M2b-like macrophages upon *intraperitoneal* treatment. Our results indicate that TLR2/PI3K activation in macrophages via HK-SA could be beneficial for atherosclerosis. Therefore, it will be important to determine which specific components of HK-SA are responsible for the observed protective effect. Eventually, it will be interesting to specifically target lesional macrophages, ideally with purified *Staphylococcus aureus* components. We believe our study will help to reassess the role of the TLR2/PI3K pathway in atherosclerosis and will support the discovery and further development of specific molecules to modulate macrophages via TLR2/PI3K pathways for atherosclerosis therapy.

References

1. Watanabe, T., Hirata, M., Yoshikawa, Y., Nagafuchi, Y. & Toyoshima, H. Role of macrophages in atherosclerosis. Sequential observations of cholesterol-induced rabbit aortic lesion by the immunoperoxidase technique using monoclonal antimacrophage antibody. *Lab. Invest.* 53, 80–90 (1985).
2. Gown, A. M., Tsukada, T. & Ross, R. Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am. J. Pathol.* 125, 191–207 (1986).
3. Murray, P. J. *et al.* Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* 41, 14–20 (2014).
4. Taghavie-Moghadam, P. L., Butcher, M. J. & Galkina, E. V. The dynamic lives of macrophage and dendritic cell subsets in atherosclerosis. *Ann. N. Y. Acad. Sci.* 1319, 19–37 (2014).
5. Chinetti-Gbaguidi, G. *et al.* Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARγ and LXRα pathways. *Circ. Res.* 108, 985–95 (2011).
6. Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* 85, e17–24 (1999).
7. Von Der Thüsen, J. H. *et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J.* 15, 2730–2 (2001).
8. Yoshioka, T. *et al.* Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice. *Gene Ther.* 11, 1772–1779 (2004).
9. Liu, Y. *et al.* Inhibition of atherogenesis in LDLR knockout mice by systemic delivery of adeno-associated virus type 2-hIL-10. *Atherosclerosis* 188, 19–27 (2006).
10. De Vries, J. E. Immunosuppressive and anti-inflammatory properties of interleukin 10. *Ann. Med.* 27, 537–41 (1995).
11. Miller, Y. I. *et al.* Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. *J. Biol. Chem.* 278, 1561–8 (2003).
12. Xu, X. H. *et al.* Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 104, 3103–8 (2001).
13. O'Neill, L. A. J., Golenbock, D. & Bowie, A. G. The history of Toll-like receptors - redefining innate immunity. *Nat. Rev. Immunol.* 13, 453–60 (2013).
14. De Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. & de Vries, J. E. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174, 1209–20 (1991).
15. Netea, M. G., Van der Meer, J. W. M. & Kullberg, B.-J. Toll-like receptors as an escape mechanism from the host defense. *Trends Microbiol.* 12, 484–8 (2004).
16. Gaddis, D. E., Maynard, C. L., Weaver, C. T., Michalek, S. M. & Katz, J. Role of TLR2-dependent IL-10 production in the inhibition of the initial IFN-γ T cell response to Porphyromonas gingivalis. *J. Leukoc. Biol.* 93, 21–31 (2013).
17. Sing, A. *et al.* Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med.* 196, 1017–24 (2002).
18. Chau, T. A. *et al.* Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nat. Med.* 15, 641–8 (2009).
19. Frodermann, V. *et al.* A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus. *J. Infect. Dis.* 204, 253–62 (2011).
20. Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J. Clin. Invest.* 117, 195–205 (2007).
21. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6, 13 (2014).
22. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25, 677–86 (2004).
23. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–69 (2008).
24. Wolfs, I. M. J., Donners, M. M. P. C. & de Winther, M. P. J. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. *Thromb. Haemost.* 106, 763–71 (2011).
25. Puccetti, P. & Grohmann, U. IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. *Nat. Rev. Immunol.* 7, 817–23 (2007).
26. Francisco, L. M. *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* 206, 3015–29 (2009).
27. Niedbala, W., Wei, X. Q., Piedrafita, D., Xu, D. & Liew, F. Y. Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol.* 29, 2498–505 (1999).

28. Niedbala, W. *et al.* Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25 T cells via p53, IL-2, and OX40. *Proc. Natl. Acad. Sci. U. S. A.* 104, 15478–83 (2007).
29. Bingisser, R. M., Tilbrook, P. A., Holt, P. G. & Kees, U. R. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J. Immunol.* 160, 5729–34 (1998).
30. Albina, J. E., Abate, J. A. & Henry, W. L. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* 147, 144–8 (1991).
31. Chen, X. & Oppenheim, J. J. Contrasting effects of TNF and anti-TNF on the activation of effector T cells and regulatory T cells in autoimmunity. *FEBS Lett.* 585, 3611–8 (2011).
32. Moreira, L. O. *et al.* The TLR2-MyD88-NOD2-RIPK2 signalling axis regulates a balanced pro-inflammatory and IL-10-mediated anti-inflammatory cytokine response to Gram-positive cell walls. *Cell. Microbiol.* 10, 2067–77 (2008).
33. Johansson, M. E. *et al.* Innate immune receptor NOD2 promotes vascular inflammation and formation of lipid-rich necrotic cores in hypercholesterolemic mice. *Eur. J. Immunol.* 44, 3081–92 (2014).
34. Martin, M. *et al.* Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. *J. Immunol.* 171, 717–25 (2003).
35. Polumuri, S. K., Toshchakov, V. Y. & Vogel, S. N. Role of phosphatidylinositol-3 kinase in transcriptional regulation of TLR-induced IL-12 and IL-10 by Fc gamma receptor ligation in murine macrophages. *J. Immunol.* 179, 236–46 (2007).
36. Chamberlain, N. D. et al. Ligation of TLR7 by rheumatoid arthritis synovial fluid single strand RNA induces transcription of TNFα in monocytes. *Ann. Rheum. Dis.* 72, 418–26 (2013).
37. Fukao, T. *et al*. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol.* 3, 875–81 (2002).
38. Guha, M. & Mackman, N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.* 277, 32124–32 (2002).
39. Fukao, T. & Koyasu, S. PI3K and negative regulation of TLR signaling. *Trends Immunol.* 24, 358–363 (2003).
40. Briken, V. & Mosser, D. M. Editorial: switching on arginase in M2 macrophages. *J. Leukoc. Biol.* 90, 839–41 (2011).
41. Chang, J. D. *et al.* Deletion of the phosphoinositide 3-kinase p110gamma gene attenuates murine atherosclerosis. *Proc. Natl. Acad. Sci. U. S. A.* 104, 8077–82 (2007).
42. Zotes, T. M. *et al.* PI3K p110γ deletion attenuates murine atherosclerosis by reducing macrophage proliferation but not polarization or apoptosis in lesions. *PLoS One* 8, e72674 (2013).
43. Farrar, C. A. *et al.* Inhibition of TLR2 promotes graft function in a murine model of renal transplant ischemia-reperfusion injury. *FASEB J.* 26, 799–807 (2012).
44. Arslan, F. *et al.* Treatment with OPN-305, a humanized anti-Toll-Like receptor-2 antibody, reduces myocardial ischemia/reperfusion injury in pigs. *Circ. Cardiovasc. Interv.* 5, 279–87 (2012).
45. Schoneveld, A. H. *et al.* Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. *Cardiovasc. Res.* 66, 162–9 (2005).
46. Mullick, A. E., Tobias, P. S. & Curtiss, L. K. Modulation of atherosclerosis in mice by Toll-like receptor 2. *J. Clin. Invest.* 115, 3149–56 (2005).
47. Curtiss, L. K., Black, A. S., Bonnet, D. J. & Tobias, P. S. Atherosclerosis induced by endogenous and exogenous toll-like receptor (TLR)1 or TLR6 agonists. *J. Lipid Res.* 53, 2126–32 (2012).
48. Liu, X. *et al.* Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids. *Atherosclerosis* 196, 146–54 (2008).
49. Ha, T. *et al.* TLR2 ligands induce cardioprotection against ischaemia/reperfusion injury through a PI3K/Akt-dependent mechanism. *Cardiovasc. Res.* 87, 694–703 (2010).
50. Nawijn, M. C. *et al.* TLR-2 activation induces regulatory T cells and long-term suppression of asthma manifestations in mice. *PLoS One* 8, e55307 (2013).
51. Filippi, C. M. *et al.* TLR2 signaling improves immunoregulation to prevent type 1 diabetes. *Eur. J. Immunol.* 41, 1399–409 (2011).
52. Berg, D. J. *et al.* Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J. Clin. Invest.* 98, 1010–20 (1996).
53. Yen, D. *et al.* IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J. Clin. Invest. 116, 1310–6 (2006).
54. Pinderski Oslund, L. J. *et al.* Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler. Thromb. Vasc. Biol.* 19, 2847–53 (1999).