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The macrophage-TCRαβ is a cholesterol-responsive combinatorial immune receptor and implicated in atherosclerosis

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ABSTRACT

Recent evidence indicates constitutive expression of a recombinatorial TCRαβ immune receptor in mammalian monocytes and macrophages. Here, we demonstrate *in vitro* that macrophage-TCRβ repertoires are modulated by atherogenic low density cholesterol (LDL) and high-density cholesterol (HDL). *In vivo*, analysis of freshly obtained artery specimens from patients with severe carotid atherosclerosis reveals massive abundance of TCRαβ^{+} macrophages within the atherosclerotic lesions. Experimental atherosclerosis in mouse carotids induces accumulation of TCR bearing macrophages in the vascular wall and TCR deficient rag$^{-/-}$ mice have an altered macrophage-dependent inflammatory response. We find that the majority of TCRαβ bearing macrophages are localized in the hot spot regions of the atherosclerotic lesions. Advanced carotid artery lesions express highly restricted TCRαβ repertoires that are characterized by a striking usage of the Vβ22 and Vβ16 chains. This together with a significant degree of interindividual lesion repertoire sharing suggests the existence of atherosclerosis-associated TCRαβ signatures. Our results implicate the macrophage-TCRαβ combinatorial immunoreceptor in atherosclerosis and thus identify an as yet unknown adaptive component in the innate response-to-injury process that underlies this macrophage-driven disease.

1. Introduction

Macrophages are key effector cells in chronic inflammatory diseases such as atherosclerosis and tuberculosis [1,2]. Based on their myeloid origin and their phagocytic activity they serve fundamental functions in innate immunity and are thought to rely solely on invariant germline encoded immune receptors [3].

Recent evidence reveals the existence of T cell receptor (TCR)-based recombinatorial immune receptors in myeloid phagocytes and thus challenges the long-held view that variable host defense is limited to lymphocytes. This is based on the demonstration that subsets of human and murine neutrophils constitutively express variable TCRαβ immunoreceptors [4,5]. Further evidence indicates life-long expression of the neutrophil TCRαβ [6] and its implication in autoimmune disease [7] and chronic periodontitis [8]. The existence of TCR-based immune receptors in the granulocyte lineage is further supported by the identification of a functional TCRγδ in eosinophils [9]. Separate from the granulocyte lineage, most recent studies revealed constitutive expression of combinatorial TCRαβ and TCRγδ immunoreceptors in macrophages [10,11], the second major phagocyte population. Together, these findings predict adaptive immune mechanisms outside T cells that rely on

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myeloid TCR [12,13]. Along with this, evidence has accumulated throughout the past few years indicative of adaptive properties and mechanisms of innate memory in macrophages [14–17].

The presence of TCRαβ combinatorial immunoreceptors in macrophages suggests their potential involvement in macrophage-dependent pathologies. Indeed, TCRαβ expressing macrophages have been implicated in granuloma formation in tuberculosis [10]. Given this and the fact that macrophages play a central role in the pathogenesis and progression of atherosclerosis [18–20], we tested whether the novel macrophage-TCRαβ is implicated in this inflammatory disease, the leading cause of death in Western societies.

2. Materials and methods

2.1. Collection of tissues and CD14$^+$ monocytes

Carotid endarterectomy specimens were obtained from randomly selected patients ($n = 10$) who had undergone elective vascular surgery at the Department of Thoracic and Vascular Surgery, Medical University of Göttingen. Written informed consent from all patients had been obtained. The study was approved by the Ethics Committee of the Medical University of Göttingen, Germany (Permit Number: 27/611). Each specimen was trisected for RT-PCR, frozen section and routine histopathology immediately after surgery. CD14$^+$ human monocytes (>99% purity) were isolated from healthy donors as previously reported [10,11] and aliquots were cultivated for 5 days in X-VIVO 10 serum-free medium (Cambrex, 5×10^5 cells/ml) in the presence of M-CSF to induce differentiation into macrophages.

2.2. Immunostaining and immunoblotting

Five micrometer tissue sections were immunostained as detailed in the Supplements.

Immunoblot analyses were performed with standard techniques as previously reported [6,10] using the mouse anti-human TCRαF1 and TCRβF1 antibodies (Thermo Scientific).

2.3. RT-PCR, TCRαβ CDR3 length spectratyping and CDR3$_\beta$ clonotype analysis

RT-PCR expression profiling of components of the TCR machinery and size spectratyping of the TCR Vα and the antigen-binding Vβ CDR3 regions were performed on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter) as previously reported [4,10]. A global repertoire analysis of all Vβ chains ($n = 25$) was established by electronic superimposition of individual Vβ$_i$ repertoires. The sequences of the PCR primers used in this study can be requested from the authors. Authenticity of all relevant PCR products was confirmed by sequencing. To determine the detailed CDR3 clonotypes for Vβ16, specific RT-PCR amplification products were cloned using standard methodology (TOPO TA Cloning Kit, Invitrogen) and the cDNA sequences encoding the Vβ16 CDR3 regions identified from at least 10 randomly picked clones.

2.4. Laser capture microdissection and ex situ TCR Vβ16 clonotyping

Approximately 2–16 CD68$^+$ cells from the shoulder regions of arterectomy specimens were microdissected using a laser microdissection system (P.A.L.M. Microlaser Technologies) and a Zeiss Axiovert fluorescence microscope (Carl Zeiss Microimaging) as previously described [11]. RT-PCR profiling for GAPDH, the TCRβ constant chain, Vβ16, and the macrophage marker CD163 was performed and the Vβ16 repertoire diversity was assessed by CDR3 spectratyping.

2.5. Induction of carotid atherosclerosis in mice

Experimental carotid atherosclerosis was induced in rag1 deficient mice and wildtype littermates as indicated in the Supplements [21,22].

2.6. Cholesterol import/export assay

Cholesterol foam cell development was induced in freshly obtained human monocytes as previously described [23,24]. For this, aliquots of 10^8 macrophages were incubated in the presence of enzymatically degraded, non-oxidized LDL (eLDL) [25] (40 μg/ml) for 24 h. Subsequently, macrophages were incubated with HDL$_3$ (100 μg/ml) for an additional 24 h to induce cholesterol removal from the cell. Controls were either incubated in the absence of cholesterol or only in the presence of eLDL (control in HDL$_3$ deloading experiments). Only LPS-free preparations were used as assessed by limulus endotoxin assay.

3. Results

3.1. Excessive accumulation of TCRαβ$^+$ macrophages in human lesions of atherosclerosis

Recent evidence revealed the massive presence of TCRαβ bearing macrophages in tuberculous granulomas and demonstrated their implication in TNF-dependent granuloma formation [10]. Similarly as in tuberculosis, macrophages play a pivotal role in the pathogenesis of atherosclerosis [1,18,19]. Therefore, we examined whether the novel macrophage immunoreceptor is involved in this important inflammatory disease. First, we looked for evidence of macrophage-TCR expression in human carotid artery atherosclerosis. Carotid endarterectomy (CEA) specimens were obtained from randomly selected patients with severe carotid artery stenosis who had undergone elective vascular surgery ($n = 10$; 5 males, 5 females) (Table S1). Each specimen was trisected for RT-PCR, frozen section and routine histopathology, respectively. The majority of the individuals had a history of hypertension and displayed a heterogenous spectrum of additional risk factors. Histological analysis of randomly selected CEA sections revealed the excessive presence of macrophages/foam cells which stained positively for TCRαβ in 9 out of 10 patients (Figs. 1A, S1 and S2). The near-complete absence of CD2 cells in adjacent sections showed that these cells were not T lymphocytes or NK cells (Fig. S3). Consistent with this, TCRαβ/CD68 doublestaining demonstrated that the majority of the CD68$^+$ lesion macrophages express the TCRαβ (Fig. 1B). Large quantities (85–95%) of TCRαβ$^+$/CD68$^+$ cells were typically clustered in the shoulder regions of the plaques and areas bordering on the lipid cores. Consistent with immunostaining, RT-PCR profiling and immunoblotting demonstrated abundant expression of the TCR α- and β-chains in the CEA specimens (Fig. 1C and D).

TCRβ repertoire analyses of 9 CEA specimens by CDR3 spectratyping demonstrated that only a minor fraction of the human TCR Vβ chains (Vβ1–Vβ25) was expressed in the atherosclerotic lesions (Figs. 2A and S4). These included Vβ7, Vβ22 and Vβ16, respectively, with the latter showing the highest expression frequency (5 out of 8 CEA). Detailed TCR Vβ16 clonotype analysis by sequencing of the CDR3 regions in two randomly selected CEA specimens (patient 1, patient 4) confirmed expression of diverse yet highly restricted Vβ16 repertoires (Fig. 2B). We noted that one Vβ16 variant

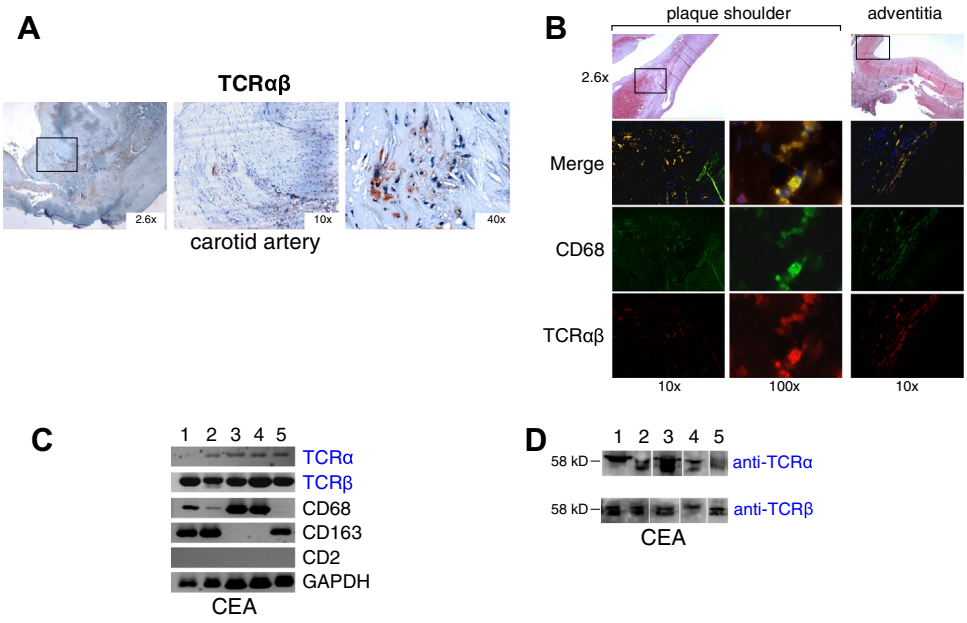

Fig. 1. Massive accumulation of TCRαβ$^+$ macrophages in human carotid artery atherosclerosis. (A) Light microscopy immunostaining of TCRαβ in a human carotid endarterectomy specimen (CEA 1) with severe atherosclerosis. Note the large abundance of TCRαβ expressing macrophages (brown) at various magnifications. The results are representative of 9 randomly selected individuals with clinically apparent carotid artery atherosclerosis. The carotid artery specimen was obtained during vascular surgery and immunostaining was performed on immediately PFA-fixed (TCRαβ, CD2) and frozen sections (TCRαβ/CD68 doublestaining), respectively. Frame indicates the magnified area. (B) Accumulation of TCRαβ$^+$/CD68$^+$ cells in the shoulder region of a representative atherosclerotic lesion assessed by fluorescence double immunostaining (frozen sections). (C) RT-PCR shows expression of the TCR α and β constant chain genes in the CEA specimens from five representative patients (1–5). Expression of macrophage marker genes (CD68 and CD163) and the absence of detectable RT-PCR signals for CD2 indicates that no marked lymphocyte or NK cell infiltration had occurred in these lesions. (D) Immunoblot demonstrates expression of the TCRαβ chains in the same carotid artery specimens as in (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(HRQRFTDTQY) was expressed in the carotid artery lesions from both patients.

Next, we tested whether macrophages express TCR Vβ16 variants in carotid lesions *in situ*. For this, small clusters of randomly selected immunostained CD68$^+$ macrophages (~ 4–16 cells) were laser-microdissected from CEA material and subjected to CDR3 Vβ16 spectratyping. This *ex situ* RT-PCR TCRβ repertoire profiling revealed a single Vβ16 peak in five out of six laser-microdissected specimens indicative of monoclonal Vβ16 gene expression (Figs. 2C and S5). Consistent with this, cloning and sequencing demonstrated that only a single Vβ16 variant (HRQRFTDTQY) was expressed by the microdissected lesion macrophage cluster in the CEA from patient 2. Intriguingly, this Vβ16 variant was identical with the Vβ16 clonotype that was expressed in the atherosclerotic lesions from patients 1 and 4, respectively. Also, we found evidence for monoclonal Vα14 expression in the microdissected lesion macrophages from three out of six specimens (Figs. 2C and S5). *Ex situ* clonotyping thus reveals that clustered lesion macrophages express unique macrophage-TCRαβ variants. Moreover, this demonstrates that common TCRβ clonotypes are expressed by lesion macrophages from distinct patients as exemplified for TCR Vβ16.

3.2. Experimental atherosclerosis induces accumulation of TCRαβ$^+$ macrophages in the vascular wall

Our observation that lesion macrophages in carotid plaques express distinct TCRβ repertoires suggested an implication of the novel immunoreceptor in the pathogenesis of carotid atherosclerosis. To test this hypothesis, we used a model of murine atherosclerosis which was established in our laboratory. In this approach, macrophage-rich atherosclerotic lesions of the carotid and neointima formation are induced by ferric chloride injury followed by 3 weeks of high fat diet [22]. To specifically address the role of the macrophage-TCR in this vascular model, development of carotid atherosclerosis was compared between wildtype ($n = 6$) and recombinase rag1 deficient mice ($n = 7$) that lack the functional TCR. Three weeks after injury all mice developed a pronounced inflammatory response under the fat-rich diet that was dominated by macrophages and displayed near absence of lymphocytes (data not shown). Immunostaining for the mouse TCRαβ routinely revealed numerous TCR positive cells in the media of wildtype mice with typical macrophage appearance (Fig. 3A) These cells colocalized with the F4/80$^+$ cells consistent with the presence of TCRαβ bearing macrophages (Fig. 3B).

In contrast, no TCRαβ expression was seen in recombination defective rag1$^{-/-}$ mice (not shown). Morphometric analysis demonstrated that ablation of the macrophage-TCR resulted in a significantly reduced medial cross-sectional area relative to wildtype mice (15.7 μm^2 vs. 24.1 μm^2, $p = 0.023$) (Fig. 3C and D). These results demonstrate that experimental atherosclerosis triggers accumulation of TCRαβ$^+$ macrophages in the carotid wall. Moreover, they strongly suggest that the media thickness of carotid lesions depends on the presence of macrophage combinatorial immune receptors.

3.3. Macrophage-TCR Vβ repertoire expression is modulated by cholesterol

Finally, we determined whether an interrelationship exists between macrophage cholesterol trafficking and TCRαβ expression. For this, an *in vitro* cholesterol import and export model using enzymatically modified LDL (eLDL) and HDL_3 was utilized [23,24]. Using this approach, we investigated whether the expression of the TCRαβ constant chains and genes for essential constituents of the TCR signalling complex (CD3ζ, ZAP70, LAT and Fyn) are regulated by cholesterol import/export in macrophages. Uptake of eLDL for

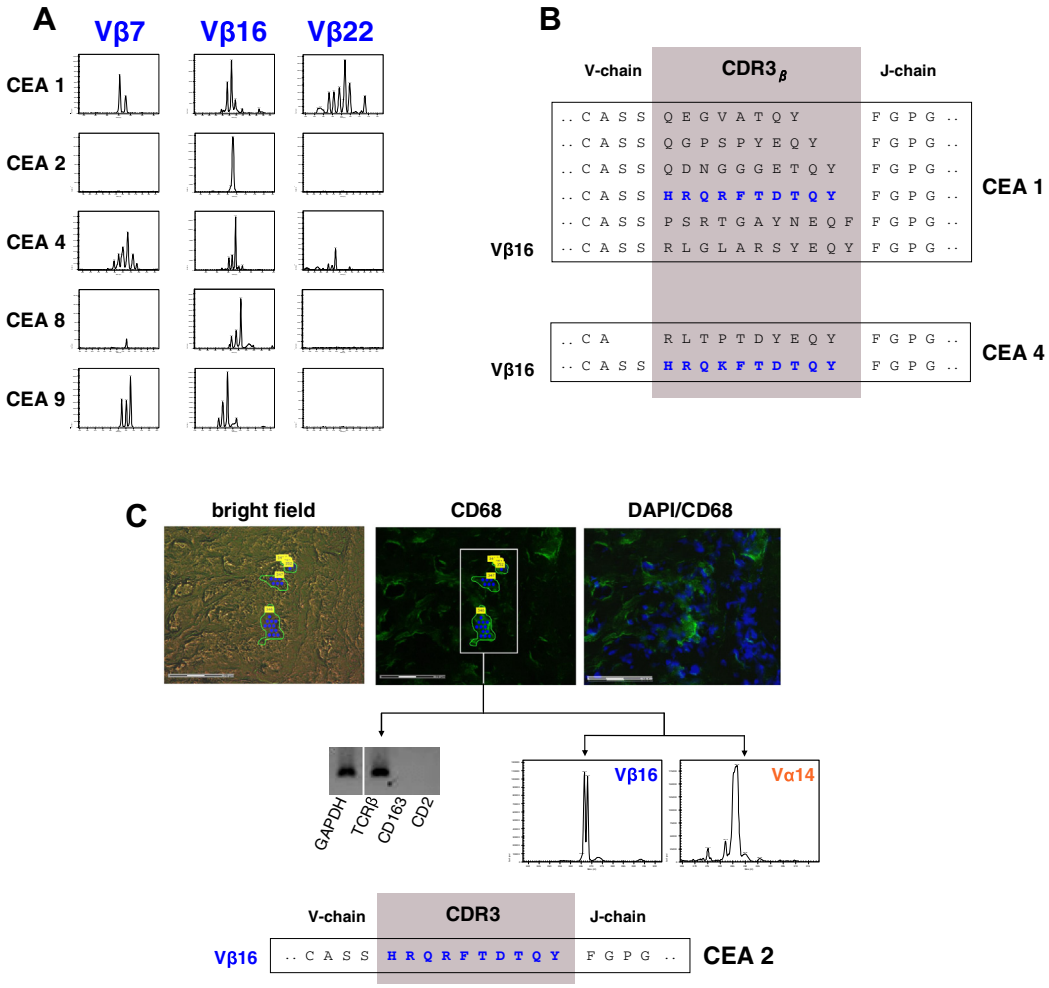

Fig. 2. TCR Vβ repertoires expressed in advanced carotid atherosclerosis are highly restricted. (A) CDR3 spectratyping shows the repertoire diversities of the most frequently expressed TCRβ chains (Vβ16, Vβ22 and Vβ7), representatively shown for five CEA specimens. (B) TCR Vβ16 repertoires shown on the detailed clonotype level. Amino acid (aa) sequences of expressed TCR Vβ16 CDR3 variants are representatively shown for patients 1 and 4 (CEA 1, CEA 4). Identified $CDR3_{\beta}$ segments are shaded. The sequences of the flanking V- and J-chain segments are indicated. Note that the Vβ16 clonotype HRQRFTDTQY (blue) is expressed in the atherosclerotic lesions of both patients. (C) *Ex situ* TCR clonotype analysis demonstrates monoclonal TCR Vβ16 restriction and expression of a dominant Vα14 chain in $CD68^{+}/CD2^{-}/TCR\alpha\beta^{+}$ lesion macrophages. The amino acid sequence of the identified Vβ16 CDR3 variant is indicated (bottom). Cell clusters of $CD68^{+}$ immunostained macrophages (6–8 cells, encircled) were isolated by laser capture microdissection and the expressed Vαβ repertoires were analyzed. RT-PCR documents expression of the TCRβ constant chain, the macrophage marker CD163 and the lymphoid lineage marker gene CD2. Scale bar, 75 μm. The results were obtained for CEA 2. For results of additional patients see Supplementary Fig. S5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

24 h had no significant effect on the regulation of these genes. In contrast, HDL_3 mediated cholesterol efflux induced downregulation of TCRβ and most signaling components within 24 h (Fig. 4A). CDR3 spectratyping revealed that eLDL ingestion by macrophages (72 h) induced significant repertoire changes of the most frequently used TCRβ chains in human carotid artery lesions (Vβ16, Vβ22 and Vβ7) relative to untreated controls (Fig. 4B). Moreover, global analysis of all TCR Vβ chain repertoires (Vβ1–25) demonstrated that cholesterol uptake generally increased repertoire diversity of the expressed TCR Vβ chains. Clonotype sequencing of Vβ16, the predominant TCRβ chain in lesion macrophages, confirmed on the detailed amino acid level that the expressed Vβ16 variants had changed during cholesterol uptake (Fig. S6A). Consistent with the observed negative effect of HDL_3 on expression of the TCR complex, we noted that HDL_3 mediated cholesterol export (24 h) generally induced TCRβ repertoire narrowing in cholesterol enriched macrophages (Figs. 4C and S6B). Together, these *in vitro* results identify cholesterol import/export as a potent *in vitro* modulator of macrophage-TCRβ repertoire expression.

4. Discussion

Macrophages are the most abundant immune cells in the atherosclerotic lesion and play a key pathogenic role in atherosclerosis [18,20]. Here, we demonstrate that the novel combinatorial TCRαβ macrophage immune receptor is implicated in this important disease.

We find that >80% of the lesion macrophages in advanced human carotid artery atherosclerosis from 9 out of 10 randomly selected patients express recombinatorial TCRαβ immune receptors. This reveals that massive accumulation of $TCR\alpha\beta^{+}$ macrophages is a general feature of atherosclerosis and indicates the involvement of macrophage-based specific host defense mechanisms in plaque formation.

The highest frequency of TCRαβ bearing macrophages was typically observed in the shoulder regions of the plaques and the areas bordering on the lipid cores. Since these sites are known for high inflammatory activity or represent the macrophage–cholesterol contact zone [1,19,26], our findings localize macrophage-TCRαβ

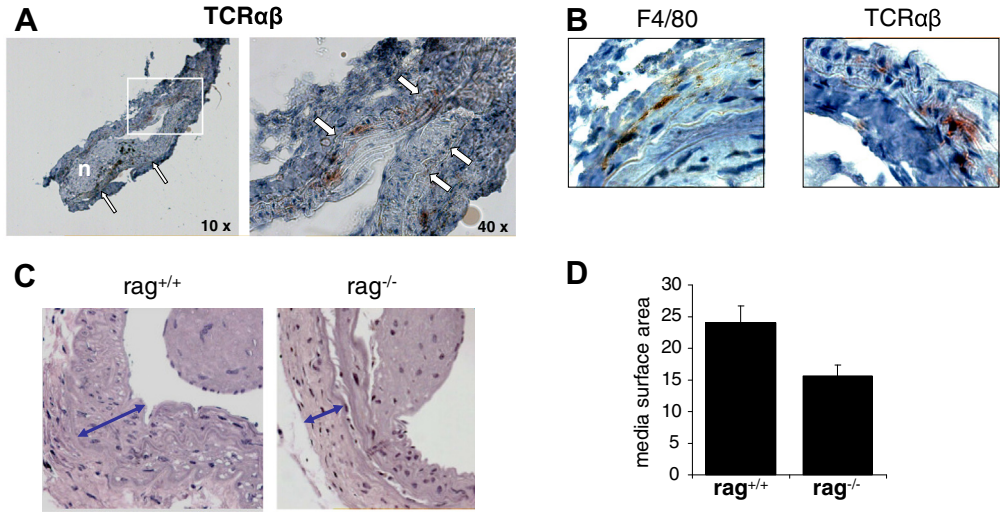

Fig. 3. Implication of the macrophage-TCR in experimental atherosclerosis in mouse carotid arteries. (A) Experimental atherosclerosis in the mouse carotid artery induces accumulation of TCRαβ^{+} macrophages (brown) in the neointima, media and adventitia, respectively. Shown is a representative stenotic carotid artery 3 weeks post vascular injury from a recombination competent rag1$^{+/+}$ mouse (wildtype). Arrows highlight the outer elastic laminae of the media. n, neointima. The frame indicates the magnified area (40×, right). Reactive atherosclerosis of the carotid artery was induced by ferric chloride and maintenance of the mice on a fat-rich diet for a total of 4 weeks as previously reported [21,22]. (B) Infiltrating macrophages (F4/80^{+}) and TCRαβ^{+} cells colocalize in the media of the reactive carotid stenosis. Immunocytochemistry was performed on two adjacent frozen sections with antibodies to the murine macrophage marker F4/80 and the mouse TCRαβ, respectively. Immunostaining is shown for a representative wildtype mouse. (C) Reduced media surface areas in stenotic carotid arteries of TCR recombination defective rag1$^{-/-}$ mice. The H&E sections shown are representative of six wildtype (rag1$^{+/+}$) and an equal number of rag1$^{-/-}$ mice. Arrows represent the media diameter. (D) Quantitative analysis of the media cross-sectional areas. Surface areas are indicated in μm^2. The data represent the means ± SD of 6 mice in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

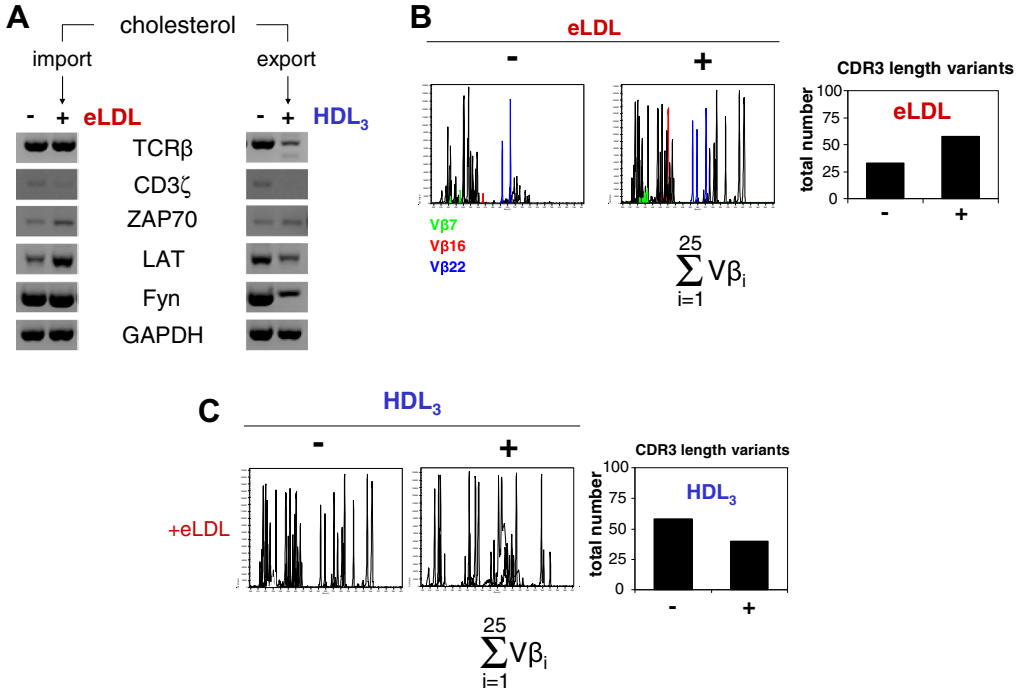

Fig. 4. TCR Vβ repertoire expression in macrophages is modulated by cholesterol uptake and release. (A) RT-PCR expression profiling in macrophages during cholesterol import and export. Note that high-density lipoprotein (HDL_3) mediated cholesterol export inhibits the expression of the TCRβ gene and multiple components of the TCR signaling complex. Peripheral blood monocytes were differentiated into macrophages for 5 days and loaded with atherogenic enzymatically modified LDL cholesterol (eLDL, 40 μg/ml) for an additional day. Subsequently, macrophages were incubated with HDL_3 (100 μg/ml) for 1 day to induce cholesterol removal from the cells. (B) Global analyses of all TCR Vβ repertoires (Vβ1–25) in macrophages in response to eLDL uptake for 72 h. The repertoires for the Vβ7, Vβ16 and Vβ22 chains, respectively, which are most frequently expressed in human carotid artery atherosclerotic lesions, are highlighted by color coding for orientation. (Box) Quantitative analysis of all expressed Vβ CDR3 length variants after 72 h of cholesterol loading. (C) Synopsis of the global repertoire changes (Vβ1–25) and quantification of the expressed Vβ CDR3 length variants in response to HDL_3 mediated cholesterol export. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dependent immune responses to the "hot-spot" regions of atherosclerotic lesions.

Classical T-lymphocyte TCR-mediated antigen-specific mechanisms have previously been implicated in the pathogenesis

of traditional atherosclerosis [19,27,28]. The abundant presence of combinatorial TCRαβ in lesion macrophages, we report here, identifies a second system of TCR dependent host-defense in the atherosclerotic plaque. Given that macrophages typically considerably outnumber the quantities of T cells in mature plaques, it is possible that the macrophage-TCRαβ may be more critical to the pathogenesis of advanced lesions than the canonical TCRαβ.

Consistent with roles of the macrophage-TCRαβ in atherogenesis, we find that experimental induction of carotid atherosclerosis in mice triggers accumulation of TCRαβ$^+$ cells in the artery wall. In fact, the finding that ablation of the TCR (rag$^{-/-}$ mice) in this model leads to significantly reduced media thickness strongly supports the view that the macrophage-TCRαβ contributes to the pathogenesis of atherosclerosis.

This is also supported by our observation that macrophage-TCRβ repertoire expression is modulated *in vitro* by cholesterol uptake and release. These results establish a direct link between macrophage cholesterol trafficking and the novel macrophage combinatorial immune receptor. Excessive uptake of cholesterol by macrophages in the arterial wall and formation of lipid-laden foam cells play a pivotal role during the early phase of the atherosclerotic lesion development [18]. Thus, our finding that ingestion of atherogenic eLDL cholesterol by macrophages induces TCRβ repertoire expression *in vitro* suggests that the macrophage-TCR may be induced early during atherogenesis. Consistent with this, we noted that repertoire diversity of those Vβ chains that were predominantly expressed in mature lesions (Vβ7, Vβ22 and Vβ16) increased after ingestion of atherogenic eLDL cholesterol. It is thus conceivable that phagocytic uptake of atherogenic cholesterol may function as an inducer of TCR expression in atherogenesis *in vivo*.

Carotid artery lesions routinely expressed individual-specific, highly restricted Vβ repertoires that are characterized by the predominant usage of the Vβ7, Vβ22 and Vβ16 genes, respectively. In particular, TCR Vβ16 was expressed in >60% of the CEA specimens studied. It is currently unclear whether this is a peculiarity of carotid artery atherosclerosis or a more general feature of advanced atherosclerotic lesions. Our findings are consistent with a previous study by others demonstrating expression of restricted Vβ repertoires and overrepresentation of TCR Vβ16 in aortic valve lesions from apoE null mice [28]. It should be noted that in this work the authors solely attributed their findings to T cells although no effort was made to identify the cellular source of TCR expression.

The atherosclerotic lesions included in this study were largely free of significant quantities of T cells as assessed by immunohistochemistry and RT-PCR expression profiling of lineage markers. This indicated that the observed highly restricted expression of only few Vβ chains (Vβ7, Vβ22 and Vβ16) indeed originated from macrophages. Consistent with this, laser-microdissection-based *ex situ* clonotyping revealed that randomly selected clusters of CD68$^+$ lesion macrophages share expression of a monoclonal TCR Vβ16 chain. The majority (60%) of these macrophages displayed monoclonal TCR Vα14 coexpression suggesting that carotid lesion macrophages produce TCR Vα14/β16 variants. Together, these results strongly suggest that lesion macrophages engage in targeted immune responses which may lead to expression of atherosclerosis-associated TCRαβ signatures.

The exact molecular mechanisms through which the macrophage-TCRαβ combinatorial immunoreceptor is implicated in atherogenesis still remain to be defined. In this context, it will be particularly challenging to identify the antigenic structures that are targeted by the TCR bearing macrophages in atherosclerotic lesions. It is likely that the Vβ variants expressed by lesion macrophages are directed against lipid antigens and/or their degradation products. It is also conceivable that altered structures of the arterial wall, which accumulate during progression of atherosclerosis, are targeted by TCRαβ bearing macrophages. Given that the TCRαβ facilitates phagocytosis, we speculate that the TCRαβ$^+$ rich regions represent microenvironments of enhanced phagocytic activity.

Collectively, this study identifies an unrecognized macrophage-dependent flexible host-defense mechanism in atherosclerosis. Moreover, it suggests the existence of atherosclerosis-related macrophage-TCRαβ signatures that may represent novel molecular targets for the diagnosis and treatment of this important inflammatory disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.034.

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