NK/NKT Cells and Atherosclerosis

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16.1 Introduction

The immune response is operationally divided into the two arms of innate and adaptive immunity. The innate immune response is characterized by the involvement of a large variety of cells and mechanisms that have three important characteristics: (1) they are based on the recognition of defined patterns present in molecules of microbial origin, (2) they utilize receptors that have been selected by co-evolution of host and pathogen and not those which undergo gene rearrangements and selection during the developmental phases of the organism, and (3) they are ready to become activated and thus offer immediate reaction to invading pathogens, representing important first lines of defense. The innate immune system has been commonly considered to lack the capacity for immunological memory. Mechanisms of adaptive immune response, on the other hand, rely on use of gene rearrangement of receptors that recognize unique epitopes on individual molecules and are positively selected during development as well as during the response by the best fit with recognized antigen. Immunological memory

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is a hallmark of adaptive immunity. Both innate and adaptive immunity play important roles also in recognition of self-antigens and represent key players in the pathogenesis of chronic inflammatory and autoimmune diseases.

In addition to innate and adaptive immunity, the immune system has evolved a series of cells which share some features of both types of immunity. These are T cells expressing T cell receptors (TCR) capable of recognizing self antigens. These T cells are not deleted during thymic development, are continuously activated in a sub-optimal manner in the periphery and become fully activated in exceptional conditions, including infection, cellular stress, tumor transformation and chronic inflammation. In humans these cells may recognize self-lipid antigens presented by CD1 antigen-presenting molecules, or still-unknown antigens presented by MR1 or self phosphorylated non-peptidic metabolites such as TCR γδ cells.

Atherosclerosis, being a chronic inflammatory disease, is characterized by involvement of both innate and adaptive immune responses. The past years have revealed a large number of mechanisms that participate in initiation and chronic development of lesions and also in their aggravation, finally resulting in plaque rupture. The contributions of adaptive and innate immunity in atherosclerosis have already been extensively reviewed [1–3], and thus we review only those mechanisms in which Natural Killer (NK) and Natural Killer T (NKT) cells are involved. The extensive, but clearly not all-inclusive, spectrum of investigations on the participation of NK and NKT cells in atherosclerosis is summarized in Table 16.1.

16.2 Natural Killer Cells in Atherosclerosis

NK cells belong to the innate immune system and develop within the bone marrow [4]. Although secondary lymphoid tissues are suspected to be the principal sites from which NK cells evolve [5] NK cells receive signals from both inhibitory and activatory receptors [6] that regulate the development and function [7] of this circulating lymphocyte population. The killer immunoglobulin-like receptors (KIR) and the CD94/NKG2A heterodimer, a lectin-like receptor, are among the best characterized inhibitory receptors. Both types of receptors recognize epitopes on human class I leukocyte antigen (HLA) molecules, thus representing a second type of immune recognition of these molecules in addition to that of TCR [8]. According to in vitro experimental data inhibitory signals usually overrule activation signals. Nevertheless, many activatory receptors are also expressed by NK cells. They include CD2, CD16, NKG2D, killer activatory receptors (KAR) and Toll-like receptors (TLR) [9]. Most of these activatory receptors do not recognize HLA molecules, thus focusing NK cell activation on a variety of target molecules. Once a cell loses expression of major histocompatibility complex (MHC) class I molecules (e.g. due to a viral infection), activatory receptors efficiently trigger NK cells, leading to killing of target cells. Activated NK cells may also secrete cytokines and growth factors including interferon-γ (IFN-γ), tumor

Table 16.1 Participation of NK and iNKT cells in atherosclerosis

Cells	Marker or model	Outcome, function, or mechanism	Effects on atherosclerosis progression	k/o model	References
NK	Beige	NK functionally defective	Up	LDLr(−/−)	[16]
NK	Ly49A	NK functionally defective	Down		[17]
NK	Ly49A	NK functionally defective	Down	ApoE(−/−)	[17]
NK	CAD patients	CAD, 7β-hydroxycholesterol	Down		[21]
Lipid reactive T cells	LXR and PPAR-γ (in F1B hamster)	Metabolism, CD1a/CD1d upregulation			[48–50]
γδ-T	Statins	LDL lowering, gamma delta T stimulation	Up/down		[54, 56, 58]
NKT		NKT absence	Down	CD1d + ApoE(−/−)	[60, 63]
iNKT	α-GalCer	iNKT activation	Up	ApoE(−/−)	[60]
iNKT	α-GalCer	iNKT activation	Unchanged	CD1d + ApoE(−/−)	[63]
iNKT	RT–PCR	Vα14-Jα18 transcripts		ApoE(−/−)	[61]
NKT	WTD		Early down	CD1d + LDLr(−/−)	[64]
iNKT	WTD	Crossing, adoptive transfer	Down	Jα18 + LDLr(−/−)	[65]
iNKT	α-GalCer	Atheroprotective	Down	LDLr(−/−)	[67]
iNKT	α-GalCer		Unchanged	ApoE(−/−)	[67]
NK/NKT	CD137	Human artery, reduced NK/NKT cells		CD137(−/−), ApoE(−/−)	[72–74]
NK/iNKT	CXCL12:CXCR4/ VLA-4	Shear stress induced, reciprocal usage by NK/iNKT			[77, 78]
NKT	MCP-1	Cell adhesion, released by type 2 NKT	Down	MCP-1 + LDLr(−/−), MCP-1r + ApoE(−/−)	[63, 85, 87, 88]
iNKT	CXCL16:CXCR6	iNKT homing, homeostasis/pro-inflammatory function	Down:up		[90–92, 95–97]
iNKT	CXCR3	High on iNKT, adiponection represses ligands	Up	ApoE + adiponectin(−/−)	[41, 95, 98, 99]

(continued)

Table 16.1 (continued)

Cells	Marker or model	Outcome, function, or mechanism	Effects on atherosclerosis progression	k/o model	References
NK/NKT	CXCR1 and CXCR2	Il-8 involvement in adhesion, high affinity CXCR2 solely on iNKT	Down	LDLr + IL-8r(−/−)	[101, 104]
iNKT	Human patients	Il-8 involvement in neovascularization	Up		[36]
iNKT	LIGHT	Hyperlipidemia via reduction of hepatic lipase	Up		[115]
	Endothelial lipase	In plaques			[117]
	Angiopoietin-like 3	Hypolipidemia in men and mice			[118, 119]
		Lower cholesterol and triglycerides		Wt, ApoE(−/−), LDLr(−/−)	[120–122]
		Elevated endothelial lipase		Angiopoietin-like 3(−/−)	[123–125]
	CD40/CD40L and OX40/OX40L		Up		[129, 130]
	Transgenic OX40L		Up		[130]
		Antagonizing anti-OX40L antibody can lower plaque burden, important for iNKT activation			[131–134]
NK/iNKT	TRAIL	Associated with overall increased cardiovascular mortality, found in plaques, high expression on NK/iNKT			[137]
iNKT	CYLD	Up by shear stress, iNKT survival factor, ICOS induction	Linked, down		[140–143]
		TGF-β reduction and impaired generation of regulatory T cells	Up	ICOS(−/−)	[145]

necrosis factor-α (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [6].

Immunoglobulin-like and lectin-like receptor gene families are encoded in the leukocyte receptor complex (LRC) and natural killer complex (NKC) loci, respectively. Due to strong selection pressure on the immune system by evolution, receptors and their respective ligands acquired high diversity as exemplified by the highly polymorphic KIR and MHC class I molecules. Surprisingly, mouse NK cell receptors and their MHC class I binding partners dramatically diverge from human equivalents. From this one can reason the difficulties experienced in finding appropriate mouse models for human diseases involving NK cells. Non-human primate models hold great promise but are still poorly genetically defined [7].

NK cells are generally thought not to be sufficient to cause atherosclerotic lesions although they are found among plaque infiltrating cells [10, 11]. More specifically, the shoulder region of atherosclerotic plaques is the prevalent location for NK cells, which have been detected immunohistologically in all stages of atherosclerosis [12]. Lesion fate might also be influenced by the responsiveness of NK cells to cytokines such as IFN-α/β, interleukin (IL)-12, IL-15 and IL-18 which are also found in the plaque cytokine milieu [1]. However, there is no direct evidence of this involvement.

Experimental atherosclerosis models almost exclusively rely upon two genetically engineered atherosclerosis-prone mice, namely apolipoprotein E (ApoE) knockout (k/o or (−/−)) mice (ApoE(−/−)) developing spontaneous hyperlipidemia and atherosclerosis [13] and low-density lipoprotein (LDL) receptor-deficient mice (LDLr(−/−)). The latter develop severe hypercholesterolemia and atherogenesis when fed a Western-type diet (WTD) but only modest disease on a normal diet [14, 15]. In the LDLr(−/−) model, and unlike in humans, NK cells were experimentally verified during early-stages of lesion formation only [16, 17], thus suggesting a possible involvement in the initiation of lesions. In these initial lesions, NK cells accounted for 0.1–0.5% of total infiltrating lymphocytes. This number may seem too small for a population involved in lesion initiation. However, considering that NK cells have activatory receptors which recognize common ligands and not TCR receptors specific for unique antigens, their low number in lesions is compatible with an important active role.

The participation of NK and NKT cells in atherosclerosis has been studied in mouse models of atherosclerosis [18]. One major drawback to NK cell research is the lack of a proper k/o mouse model that is completely deficient in NK cells [19]. However, two models in which NK cells are functionally deficient exist and have been investigated in the context of atherosclerosis. The beige mouse shows decreased, but incomplete loss of NK cell activity, and an otherwise very complex phenotype. WTD feeding failed to induce atherosclerosis in beige mice but increased plaque size in beige mice bred onto the LDLr(−/−) background [16], suggesting that NK cells might reduce rather than facilitate development of atherosclerotic lesions. A second model is represented by the granzyme A promoter-controlled Ly49A transgenic mouse. The NK cell inhibitory receptor Ly49A is specific for the MHC class I molecules H-2D(d) and D(k) and its transgenic

expression leads to absence of functional NK cells. Bone marrow transfer from granzyme A promoter controlled Ly49A transgenic mice into irradiated LDLr(−/−) mice resulted in 70% reduction of plaque burden [17]. Crossing the Ly49A transgenic with LDLr(−/−) or ApoE(−/−) mice reduces formation of early-stage atherosclerotic lesions significantly [18]. Taken together, and in opposition to findings in the beige mouse model, these data suggest that NK cells might be involved in aggravation of atherosclerosis. However, while the Ly49A transgenic model does provide important information on the role of NK cells, the evidence is inconclusive. Other Ly49A-expressing populations like NKT and a part of CD8 T cells [20] might also be affected and their contribution to lesion progression cannot be excluded. Strong evidence for a modification of NK cells comes also from a study of patients with coronary artery disease (CAD) in whom a reduction in NK cell numbers and a concomitant reduction in cytotoxic activity of the remaining population was reported [21]. Whether this is associated with increased NK cell susceptibility to 7β-hydroxycholesterol [22] and/or to margination of most active NK cells remains to be elucidated.

16.3 A Short Introduction on Natural Killer T Cells

CD1d is recognized by two sets of NKT cells that are termed type 1 and type 2 NKT cells. A semi-invariant TCR Vα24-Jα18 chain in humans and Vα14-Jα18 chain in mice defines type 1 NKT cells which are also named invariant NKT (iNKT) cells. The invariant TCR α-chain of iNKT cells mostly pairs with TCR Vβ11 chain in humans and with TCR Vβ 8.2, 7 or 2 chains in mice. The finding of multiple TCR β-chains in mice [23] and TCR β-chain junctional diversity in humans led to the term semi-invariant for the TCR of iNKT cells. Type 2 NKT cells express a less limited TCR repertoire without apparent biases in the Vα and Vβ chains [24]. Two important features have greatly facilitated the study of iNKT cells, namely the semi-invariant TCR αβ-chain and the pan-reactivity to α-galactosylceramide (α-GalCer), a glycosphingolipid isolated from the marine sponge *Agelas mauritianus* [25].

NKT cell numbers vary between mouse strains and between human individuals. In humans, they range from less than 0.001% to over 1% of total blood lymphocytes. They express surface markers often present on NK cells and not MHC-restricted T cells. However, they may release a large variety of soluble factors and exert effector functions similar to those of MHC-restricted T cells. Since iNKT cells are constantly activated in a sub-optimal manner, when they are fully activated they immediately respond. This functional characteristic makes the behavior of this cell population similar to that of cells of innate immunity. For this reason, they are commonly considered a bridge between innate and adaptive immune responses.

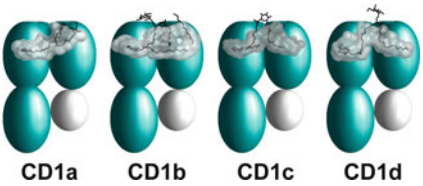

Fig. 16.1 Schematic view of CD1 antigen-presenting molecules loaded with representative lipid ligands. Each CD1 molecule contains three domains (depicted in *green*) and has evolved a unique network of hydrophobic pockets (depicted in *grey*) that allow binding of lipid antigens with different structures. In the figure each CD1 molecule is filled with a different lipid (depicted in *black*). The number and volumes of hydrophobic pockets differ significantly. CD1b pockets have a volume of 2,200 $Å^3$. CD1c and CD1d pockets have intermediate volumes of 1,780 $Å^3$ and 1,650 $Å^3$, respectively. CD1a has the smallest pocket volume of 1,280 $Å^3$. Each CD1 molecule is non-covalently complexed with β2-microglobulin (depicted as a *white sphere*). Lipid-specific TCR's dock on top of the complexes formed by antigen and CD1 molecules

16.4 CD1 the Antigen-Presenting Molecule for Lipids

The CD1 antigen-presenting molecules (APM) for lipids are structurally related to the MHC class I family but have evolved to bind and present lipidic antigens to T cells instead of peptides [26]. Their existence has been known for more than two decades [27] but it is only during the last decade that the functions of the highly conserved mammalian CD1 proteins are being appreciated [28]. Mice express only the CD1d protein whereas humans express four additional CD1 family members (CD1a, CD1b, CD1c and CD1e) that bind lipid antigens [29, 30]. CD1a, CD1b and CD1c are referred to as group 1 CD1 molecules according to sequence homologies. CD1d is the sole member of group 2, whereas CD1e is not expressed on the cell surface and participates in intracellular lipid antigen processing and loading onto other CD1 molecules [31] (Fig. 16.1).

Presentation of lipid antigens is controlled by tissue- and cell-specific distribution of each CD1 protein. CD1a, CD1b and CD1c proteins are found primarily on antigen presenting cells (APC) [32], whereas CD1d has a much broader distribution among lymphoid and myeloid lineage cells [33]. All CD1 molecules are expressed in atherosclerotic lesions [34], suggesting their involvement in local lipid antigen presentation. CD1d is distributed also outside the hematopoietic system and its presence on parenchymal and endothelial cells (EC) contrasts with group 1 CD1 molecules [32]. Extraordinarily high levels of CD1d were found also on vascular smooth muscle cells (SMC) in all tissues [35]. This unique distribution enables CD1d to present lipid antigens within plaques. Since also non-professional APC express CD1d, it is also possible that the response of CD1d-restricted T cells, including iNKT cells, might have different outcomes according to which APC is engaged. We have recently reported that CD1d is preferentially expressed in advanced, neovascularized lesions [36]. This might be the result of increased macrophage infiltration within plaques and/or to a local altered transcription activity.

Another important issue is that each CD1 molecule has evolved unique hydrophobic pockets where lipid antigens are tightly bound [37–39]. For example, CD1b

has four pockets connected with each other and may bind very long lipids up to 80 Carbons long. CD1a and CD1c have two pockets, called A′ and F′, which are capable of binding lipid antigens with one or two alkyl chains. In both CD1a and CD1c the F′ pocket is made by a deep groove shape open to the solvent, thus facilitating lipid antigen binding and exchange. CD1d is instead made of two pockets, A′ and F′, that are both closed at the bottom. This feature allows binding of antigens with one or two alkyl chains whose length is fixed. Since CD1 molecules are not polymorphic, they have evolved unique structural features that allow optimal binding of a large variety of lipid shapes.

16.5 The Antigenic Potential of Lipids Accumulating in Plaques and Role of Peroxisome Proliferator-Activated Receptors

Lipid accumulation in lipid-laden "foam cells" has been mostly interpreted as an effect of atherosclerotic disease progression. However, lipids might also become active participants in stimulating innate and adaptive immunity. Even before the emergence of "foam cells" lipoproteins accumulate in intimal regions of the artery due to their ability to bind to components of the extracellular matrix (ECM) [40]. ECM localization effectively sequesters the lipoproteins from plasma antioxidants and favors oxidative modifications. Whether LDL oxidation occurs primarily within serum before sequestration or upon intima accumulation remains debated. Importantly, oxidized LDL is thought to cause inflammatory responses, thus directly facilitating atherosclerosis progression [3, 41].

Lipid accumulation has been related to early atherosclerosis. The source of the lipids can be diverse. Phenotypic changes of the cells present in the plaque can induce accumulation of large amounts of lipids produced by endogenous metabolic pathways [42]. This accumulation may result either from defective catabolism or from accelerated de novo synthesis following prolonged cell signaling. Furthermore, changes in the blood lipid transport system may also influence the amounts and type of accumulated lipids. Variation of the proteins present within lipoprotein particles may alter the lipid species present during disease. Even the lipid storage organ, adipose tissue, can secrete modulators of inflammation [43], leading to a vicious circle of activatory lipids causing inflammation which in turn induces accumulation of more lipids and continuous pro-inflammatory responses from the same adipose tissue.

A key role in regulation of lipid metabolism is exerted by peroxisome proliferator-activated receptors (PPAR) and liver X receptor (LXR) that were initially characterized in metabolic diseases [44] and in myocardial energy metabolism [45]. These ligand-activated nuclear receptors mediate their functions by co- and trans-repression or transactivation of various target genes [46]. Recent studies revealed an important influence of these nuclear receptors also on inflammatory pathways in atherosclerosis [47] and cardiovascular diseases [46]. PPAR-α has attracted particular attention since its selective targeting by agonist ligands induced a better anti-atherosclerotic response in comparison to agonists of LXR and PPAR-γ in

WTD-fed F1B hamster [48]. PPAR-γ has been attributed involvement in immune regulation related to CD1 genes since it prevents expression of CD1a [49] and induces upregulation of CD1d via retinoic acid synthesis [50] with consequential facilitated activation of NKT cells [51].

A field that has not yet received appropriate attention is that linking nutrition, lipid metabolism, lipid immunogenicity and inflammatory immune response. Consumption of flavonoid-containing nutrition (e.g. fruits, vegetables and tea) decreases the risk of inflammation-mediated diseases through stimulation of PPAR transcriptional activity by flavonoids such as apigenin, chrysin, and kaempferol [52]. This strongly hints of the crucial importance of healthy nutrition and the detrimental eating behavior of Western societies. A better understanding of how PPAR and LXR stimulation [53] contributes to lipid-mediated immunity would help to define therapeutic nutrition or treatments for metabolic diseases associated with chronic inflammation [44].

Another lipid pathway associated with immune responses is that of mevalonate, which is important for many cellular activities, including cholesterol synthesis and protein prenylation. Statins are used in therapies to lower blood LDL levels and for treatment of atherosclerosis [54]. The benefit of statin treatment to cardiovascular disease has been controversial. One explanation for the varying outcomes might be statin-induced impairment of CD1d-mediated antigen presentation due to inhibition of prenylation via blockade of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme of the mevalonate pathway and the main cholesterol producer in the liver [55]. Concerning blockade of the mevalonate pathway by statins, one should also consider the T cell population which expresses the TCR γδ (γδ-T cells) and whose antigen isopentenylpyrophosphate is blocked after statin treatment [56]. γδ-T cells have been found in lesions [57]. It is also noteworthy that bacterial infections may upregulate the mevalonate pathway and lead to activation of γδ-T cells [58].

A final deliberation on lipids accumulating in plaques is whether they are immunogenic for human CD1-restricted T cells. There is no published study showing that plaques contain lipids capable of stimulating CD1 group 1- or CD1d-restricted T cells. However, this is a highly possible event since the immune system does not ignore the abundance and diversity of lipids, and lipid-specific T cells may distinguish minimal structural modifications in lipid antigens [38, 59]. Intra-plaque accumulation of altered self-lipids or lipids derived from invading bacterial cells may represent ideal antigens for a local immune response. Future studies should address this issue in order to identify the function and potential role of CD1-restricted T cells in plaque inflammation.

16.6 iNKT Cells in Animal Models of Atherosclerosis

Some considerations must be taken into account when evaluating animal models for atherosclerosis. Firstly, the existing murine knockout models of atherosclerosis do not closely mimic human atherosclerosis, but rather represent models for human

familial hypercholesteromia, i.e. purely genetically determined variants of the disease. Secondly, lesions formed in atherosclerosis mice models are unlikely to progress to a rupture-prone stage as typically occurs in human atherosclerosis [1]. Thirdly, murine lipid metabolism, lipoproteins and important components of the immune system (e.g. mice express only CD1d) differ significantly from their human counterparts.

Three studies have reported that iNKT cells are important in the ApoE(−/−) model of atherosclerosis and under a high cholesterol diet the presence of iNKT cells was inferred [60–62]. One investigation used molecular approaches (i.e. RT-PCR and spectratyping) to show that mRNA of TCR V$\alpha14^+$ oligoclonal populations is detectable within aortic fatty streaks [62]. This TCR chain is prototypic of iNKT cells, and although the study did not provide sequence information it is remarkable that this Vα chain was monotypic and detected in almost all investigated samples.

Assessment of iNKT cell contribution to disease is achievable by experiments with CD1d(−/−) or TCR Jα18(−/−) mice targeting NKT or iNKT cells, respectively. In CD1d/ApoE-double-deficient mice, and compared to ApoE(−/−) mice, a reduction in atherosclerosis [60] and a 25% decrease in lesion size [63] was reported. Furthermore, administration of α-GalCer to ApoE(−/−) mice exacerbated atherosclerosis [60], whereas this treatment did not affect lesion size in CD1d/ApoE-double-deficient mice [63]. CD1d(−/−) mice on an atherogenic diet had significantly smaller lesions than wild-type controls [61]. Noteworthy in these studies is that the type of diet differently affected CD1d-dependent reduction in atherosclerotic plaque size, ranging from 25% for a chow diet to 70% for an atherogenic diet. Atherosclerosis development in wild-type or ApoE(−/−) mice on an atherogenic diet showed association with Vα14-Jα18 transcripts in the atherosclerotic arterial walls. This could indicate recruitment of iNKT cells to lesional sites [61].

Along the same line, LDLr(−/−) mice reconstituted with CD1d(−/−) bone marrow after lethal irradiation developed significantly less atherosclerosis compared to transfer of wild-type bone marrow [61], suggesting that NKT cells are sufficient to induce early-stage atherosclerotic lesions. Crossing the atherosclerosis-susceptible LDLr(−/−) mice to CD1d(−/−) mice led to drastically decreased lesion size after several weeks on WTD but no difference in Th1 and Th2 cytokine mRNA [64]. However, this difference was lost after prolonged periods (i.e. after 8–12 weeks).

Data first mentioned in a review [18] and subsequently reported [65] inferred the participation of iNKT cells in atherosclerosis by crossing LDLr(−/−) mice with Jα18(−/−) mice, which uniquely lack iNKT cells; in F1 mice a slower lesion formation compared to LDLr(−/−) mice was observed. Furthermore, iNKT cells proved pro-atherogenic also in an adoptive transfer model of atherosclerosis, whereby splenocytes from Vα14/Jα18 TCR trangenic mice after several weeks on WTD were transferred to immunodeficient RAG1/LDLr-double-deficient mice [66]. Interestingly, it was reported that serum from hypercholesterolemic mice may induce CD1d-dependent activation of an iNKT cell hybridoma when dendritic cells are used as APC [66]. This underscores the importance of endogenous lipids in

the activation of iNKT cells during disease progression. A recent study showed that delivery of exogenous antigen (α-GalCer) could be atheroprotective in combination with WTD in LDLr(−/−) but not in ApoE(−/−) mice [67]. This finding is in contradiction with most published studies and deserves further comment. In that study atherosclerosis was induced by placing perivascular collars around the carotid arteries, whereas previous studies measured atherosclerotic lesions in the aortic sinus. The authors attribute their discrepant findings to a potentially defective lipid-presentation on CD1d molecules due to the lack of ApoE and thereby defective lipid delivery. However, discrepancies might also derive from use of a distinct model of atherosclerosis and the anatomic site under observation. Future investigations should address the relevance of anatomical location when lesion induction and/or progression are investigated.

Even more important is the need to understand the role and contribution of self lipids to iNKT cell activation. Most studies in which iNKT cells were stimulated used the pan-activatory capacity of α-GalCer, and the role of endogenous lipids has been widely neglected. However, since a pro-atherogenic role of iNKT cells in the absence of exogenous stimulation has been demonstrated, it is important to identify which endogenous lipids are involved. The lipid antigens might be generated elsewhere in the body and carried by lipoproteins accumulating within fatty streaks, or they might be produced within the atherosclerotic plaque upon local alterations of lipid metabolism.

An additional level of complexity is provided by the presence of iNKT cells with different functional properties. Abundance of iNKT cells with pro-inflammatory capacity might positively influence atherosclerotic lesion progression, whereas a prevalence of anti-inflammatory iNKT cells might have opposite effects. Interestingly, both bacterial infections and the presence of commensal bacteria may directly affect iNKT cells [68]. Therefore, an indirect effect of infections might be expansion of the pro-inflammatory pool of iNKT cells with worsening effects on atherosclerosis. In the same line is a report that only the $CD4^+$ iNKT subpopulation is pro-atherogenic in an adoptive transfer model. This cell population is characterized by reduced expression of the Ly49 inhibitory receptors and therefore they show a low activation threshold [69]. Among the transferred subpopulations, the $CD4^+$ iNKT cells were able to secrete more pro-inflammatory cytokines including IFN-γ, IL-2 and TNF-α when stimulated with α-GalCer [70].

One interesting marker is CD137, a member of the TNFR family originally called “induced by lymphocyte activation” (ILA). CD137 may co-stimulate iNKT cells during inflammation [71]. In addition, CD137 is expressed in arterial tissue specimens of human atherosclerosis patients and its activation in ApoE(−/−) mice induces plaque inflammation [72]. CD137(−/−) mice show reduced numbers and altered function of iNKT cells [73]. Thus, CD137 might be involved in activation of pro-atherogenic iNKT cells. Additionally, CD137(−/−) mice also present lower NK cells levels. Recent findings introduced a combination of anti-DR5, anti-CD137, and anti-CD1d as effective immunotherapy for various established tumors in mice [74]. A similar therapeutic strategy remains to be tested in atherosclerosis mouse models.

In a recent pioneering study in humans the presence of iNKT cells in atherosclerotic plaques was proven [36]. iNKT cell lines isolated from plaques were found to have an extremely low activation threshold. This observation could be of crucial importance since it might reflect a selective accumulation of iNKT cells with high affinity TCR inside plaques, or a lack of NK inhibitory receptors on lesional iNKT cells which could thereby be triggered by minimal doses of antigen. Future studies should analyze expression of inhibitory receptors on iNKT cells isolated from plaques and peripheral blood of the same donors.

16.7 Role of iNKT Cells Within Atherosclerotic Lesions

Relatively little is known about the direct role of iNKT cells within atherosclerotic plaques. Here, we have gathered the fragmented information relevant to plaque recruitment of iNKT cells, local activation and possible effector functions. In many cases there is experimental evidence that some mechanisms are important for both plaque generation and iNKT cell biology. We describe this literature and attempt to correlate the most important issues, although there is still no experimental evidence that iNKT cell involvement in plaque evolution depends on these mechanisms. According to a sequential mechanistic order of events iNKT cells first adhere to EC, then migrate within atherosclerotic tissue, and then become activated within plaques where they exert different effector functions. We follow this order in commenting upon the literature reports.

16.7.1 Adhesion of iNKT Cells to Endothelial Cells

Accumulation of leukocytes in tissues is essential for effective host defence, and for this purpose the cells must interact with and penetrate the vessel wall and migrate in the tissue [75, 76]. Leukocyte interactions with vascular endothelium are highly orchestrated processes that include the capture of free-flowing leukocytes from the blood with subsequent leukocyte rolling, arrest, firm adhesion, and ensuing diapedesis [75, 76]. Cell adhesion molecules play a crucial role in orchestrating these processes, and a variety of families of adhesion molecules that mediate the interaction of circulating leukocytes and vascular EC have been identified [75, 76]. Adherence of NK and NKT cells to EC occurs similarly to other T cells with respect to P- and E-selectins and the LFA-1:ICAM-1 pair in response to CXCL12 [77]. An exception is represented by the independent, differential usage of VLA-4 and CXCR4 in the bone marrow vasculature under shear flow [77]. The migratory response to CXCL12 of NK cells is directly proportional to the CXCR4 expression levels whereas the affinity of VLA-4 is inversely correlated (e.g. low CXCR4 and high affinity VLA-4 co-exist and VLA-4 can be rapidly activated by CXCL12). The CXCL12:CXCR4 couple is essential for NK development in adult mice [78]. Reciprocally, iNKT cells express high CXCR4 levels and low-affinity VLA-4. Both cell types were able to adhere, but only NKT showed trans-endothelial

migration. Thus a weak signal permits arrest and adhesion of the cells, whereas only high levels of CXCR4 induce diapedesis.

Vascular cell adhesion molecule (VCAM) 1 is the receptor for VLA-4 and appears to be critical for the development of atherosclerotic lesions. Its expression in EC overlying foam cells in the lesion is increased in rabbits fed an atherogenic diet [79]. Even more importantly, VCAM-1 expression precedes the appearance of macrophages and foam cell development in the intima [80]. The expression of VCAM-1 is controlled by the combined action of TNF-α and IL-4 [81], with IL-4 reducing the threshold level of TNF-α needed for induction of the VCAM-1 gene [82].

16.7.2 The Network of Chemokines and Cytokines Regulating iNKT Cells

A large orchestra of cytokines [80] and chemokines [83] is involved in atherosclerotic disease progression. Since the ones affecting NK cells have recently been reviewed [84] we focus on those possibly relevant to iNKT cells. Most of the major players are shared between cells of the innate and the acquired branches of the immune system, although some important differences among immune cell populations are present.

An important chemokine in atherosclerosis is the monocyte chemotactic protein-1 (MCP-1). MCP-1/LDLr-double-deficient mice on a high-cholesterol diet have less lipid deposits in the aorta [85]. ApoE(−/−) mice lacking the receptor for MCP-1 similarly showed low development of aortic plaques [86]. These results are explained by the fact that in MCP-1(−/−) mice, fewer macrophages are attracted to the aortic wall. Importantly, also human monocytes show increased adhesion to EC in the presence of MCP-1 [87]. Following α-GalCer treatment, an early burst of cytokines, which include MCP-1, occurs [63]. It is unclear whether MCP-1 is released by iNKT cells since human iNKT cell clones expanded and activated in vitro are negative for this chemokine (our unpublished data). Interestingly, type 2 NKT cells may release this cytokine [88]. However, due to lack of surface markers for these cells, it remains difficult to assess their function in vivo and their role in atherosclerosis development.

An intriguing protein pair is the chemokine CXCL16 and its receptor CXCR6 [89]. CXCL16 is atheroprotective [90, 91] whereas its receptor promotes atherosclerosis [92]. These opposing effects could reflect different modes of action of the conformationally distinct soluble and membrane-bound forms of CXCL16 [93]. The transmembrane form is upregulated by atherogenic lipids and in turn increases high-density lipoprotein (HDL) uptake and facilitates reverse cholesterol transport in macrophages [94]. Nevertheless, soluble CXCL16 could compete for the binding site on CXCR6 by stabilizing a distinct CXCR6 conformation, thus blocking adhesion of cells via CXCR6 [93]. Whether membrane-bound CXCR6 also binds to another pro-inflammatory soluble factor, different from CXCL16, is an additional possible mechanism, which at present cannot be formally excluded. This pair

is also important for iNKT cell homing [95], homeostatic regulation in the periphery [96] and pro-inflammatory function [97].

Unlike conventional T cells, human NKT cells express varying levels of CXCR6, CCR5 and CCR2 [98] but high levels of CXCR3 [99]. Most murine NKT cells showed similar CXCR3 expression and migrated in response to its ligands [95]. Monokine induced by interferon-γ (MIG)/CXCL9, a CXCR3 ligand, was a potent inducer of iNKT and NK cell chemotaxis in all tissue locations. A logical physiological consequence would be the avoidance of CXCR3 ligand secretion by plaque tissue cells in order to restrict NKT cell access to lesions. Indeed, lysophosphatidylcholine, a major component of oxidized LDL and thus abundant in atherosclerotic lesions, inhibits MIG, interferon-γ-induced protein 10 kDa (IP-10, CXCL10) and interferon-inducible T-cell α chemoattractant (I-Tac, CXCL11) at a posttranscriptional level in EC [100]. Likewise, adiponectin represses production of CXCR3 ligands as demonstrated in ApoE/adiponectin–double-deficient mice which have higher levels of, for example, IP-10 [41]. Additionally, the lesions in these mice are exacerbated and present more infiltrating $CD4^+$ T lymphocytes, pointing to mitigative effects of adiponectin in atherosclerosis by means of attenuated CXCR3 ligand expression and thus less recruitment of T lymphocytes to evolving plaques.

16.7.3 IL-8 Secretion by iNKT Cells and Its Role in Atherosclerosis

Another important chemokine is CXCL-8 (IL-8) which interacts with the chemokine receptors CXCR1 and CXCR2. Monocytes produce IL-8 and express both chemokine receptors. These molecules contribute to atherogenesis as shown by studies in IL-8k/o mice. Less monocytes and macrophages accumulate in atherosclerotic lesions of LDLr/IL-8 receptor double-deficient mice [101]. Furthermore, IL-8 stimulates firm adhesion of monocytes to vascular endothelium [102] and attracts NK cells [103]. Importantly, murine iNKT cells are the sole T cell population expressing CXCR2, a high-affinity murine IL-8 receptor, and migrate in response to MIP-2, the murine IL-8 homologue, in chemotaxis assays [104]. These findings strengthen the role of IL-8 as a key chemoattractant during atherosclerotic lesion development.

Il-8 may also play an important role in intraplaque neovascularisation. This is supported by different lines of evidence obtained in our laboratories [36]. We found that $CD1d^+$ cells preferentially associate near areas of neovascularisation in established plaques and that both CD1d-expressing cells and iNKT cells may accumulate within the same areas. When iNKT cells were activated with lipid antigens, they showed proangiogenic effects supported by enhanced microvascular sprout formation in an in vitro angiogenesis assay. This effect was associated with EC migration and was dependent on soluble factors release by activated iNKT cells. Unexpectedly, iNKT cells isolated from plaques showed strong release of IL-8 and blocking anti-IL-8 monoclonal antibodies completely inhibited the promigratory effect. Collectively these data strongly suggest that iNKT cells may influence the

neovascularisation within intima of atherosclerotic tissue, thus directly contributing to the establishment of vulnerable plaques.

The importance of IL-8 is supported by a series of investigations with human cells in vitro and in animal studies [105]. IL-8 has been detected in atheromatous tissue [106–108] and is released by monocytes stimulated with oxidized LDL and cholesterol [108, 109]. IL-8 has pleiotropic effects which include attraction of neutrophils [105], accumulation of macrophages in the intima [102], adhesion of monocytes to EC [110], induction of SMC proliferation and chemotaxis [111] and recruitment of $CD8^+$ effector T cells within tissue [112]. IL-8 has already been proposed as an important mediator of angiogenesis in cardiovascular lesions contributing to plaque growth [107]. Our findings [36] add iNKT lymphocytes to the group of IL-8-producer cells and suggest a role of local T cell activation also on neovascularisation. Local activation of iNKT cells by lipid antigens may well have pathogenic consequences on both plaque chronic inflammation and neovascularisation, which together may contribute to plaque destabilization. These functions might also be exerted by other cell types such as monocytes and T cells recognizing non-lipid antigens in plaques. The identification of plaque-derived lipid antigens will provide new hints on the mechanisms inducing local adaptive immune response and perhaps disclose novel immunotherapeutic approaches.

16.8 Contribution of iNKT Cells to Inflammatory Response and Regulation of Lipid Metabolism

TNF-α is involved in acute phase reactions and systemic inflammation. The importance of inflammatory processes from the onset of atherosclerosis to its final complications has emerged over the past decades, and some anti-inflammatory treatments have reached practical applications [41, 113]. The atherosclerotic plaque has been recognized as not inert, but a very dynamic zone within which there is an enormous amount of ongoing biological processes including a variety of common inflammatory mechanisms [80].

One potential pro-atherogenic mechanism of iNKT cells relates to release of pro-inflammatory cytokines. Human plaque-derived iNKT cells release a large variety of pro-inflammatory cytokines upon antigen stimulation [36]. An important finding is that these plaque-derived T cells show a very low activation threshold, since they release large amounts of cytokines when stimulated with extremely low antigen doses. iNKT cells isolated from circulating blood instead release similar cytokine levels only when stimulated with 5–50 times higher antigen doses. The molecular mechanism underlying the high reactivity of plaque resident iNKT cells remains to be investigated, and it might represent a selection within the tissue, which facilitates the retention of more reactive cells. However, since the plaque antigen(s) has not yet been identified, it is unknown whether a high reactivity response might apply also to other lipid antigens.

The stimulation of mouse iNKT cells through injection of α-GalCer, the archetypical iNKT cell agonist, leads to induction of pro-inflammatory effects.

Indeed, in a mouse model of atherosclerosis α-GalCer injection increases the number and size of plaques and elicits a potent cytokine storm with massive release of Th1 and Th2 cytokines, together with elevated plasma levels of IL-6 and MCP-1 [63, 114]. An important comment on these studies is that human disease is not associated with a cytokine storm, but rather with a continuous local activation of inflammatory responses. Therefore, the massive response to α-GalCer injection does not resemble the pathological mechanism of human disease.

A second pro-atherogenic mechanism relates to regulation of lipid metabolism. Transgenic mice expressing the LIGHT (tumor necrosis factor superfamily, member 14) molecule exclusively on T cells showed hyperlipidemia with high levels of cholesterol and triglycerides [115]. When these T cells were transferred into wild type mice, cholesterolemia developed and this was mediated by iNKT cells. Overexpression of LIGHT on iNKT cells leads to a marked reduction of the hepatic lipase enzyme, which is one of the main regulators of lipidemia. The reason why iNKT cells and not other T cell populations are responsible for these important metabolic changes is unknown. The physiological preferential activation of iNKT cells in mouse liver might explain the local interaction of LIGHT overexpressed on iNKT cells with lymphotoxin-beta receptor (the LIGHT natural receptor), which is physiologically expressed by many cell types including liver hepatocytes. Whether this mechanism applies also to human iNKT cells remains unclear. However, the low number of iNKT cells in the human liver together with their moderate expression of LIGHT under physiological conditions requires cautious evaluation and demands additional investigations.

That activated T cells may directly influence lipid metabolism is an important concept highlighted for the first time by the foregoing mouse studies. Conceivably this metabolic regulation may occur also in other anatomical microenvironments, including plaque tissue. Indeed, a close relative of hepatic lipase is the endothelial lipase that is mainly synthesized by vascular EC [116]. SMC and macrophages are also able to express endothelial lipase, albeit at lower levels. It is possible that chronic intraplaque lymphocyte activation leads to high expression of LIGHT on the surface of T cells, which in turn downregulates endothelial lipase in surrounding cells. This might cause a local alteration of lipid metabolism, with lipid accumulation and perhaps generation of lipid antigens capable of triggering CD1-restricted T cells.

Endothelial lipase is found within human atherosclerotic lesions [117]. One major inhibitory factor controlling endothelial lipase is angiopoietin-like protein 3, a secreted protein involved in human familial combined hypolipidemia [118]. The mouse gene has been shown as causal in a hypolipidemic mouse strain [119] and its inactivation lowered cholesterol and triglyceride levels in wild-type [120], ApoE(−/−) and LDLr(−/−) mice [121, 122]. In angiopoietin-like 3(−/−) mice the activity of endothelial lipase is elevated because no suppression can take place [123–125]. What is unclear is the connection between angiopoietin-like 3 deficiency and lower LDL cholesterol. This is especially important since neither lipoprotein nor hepatic lipase are associated with LDL cholesterol [126]. The tissue source for angiopoietin-like 3 is almost exclusively liver but the producing cell population remains unknown.

Data accumulated over the past years points to a close cross-talk between the immune system and lipid homeostasis and might offer some explanation for the frequently linked occurrence of hyperlipidemia and systemic inflammation [127]. In addition to the angiopoietin family of secreted molecules, also the TNF/TNFR superfamily, comprising more than 40 members, has a decisive impact on metabolism and inflammation. The eponymous family member, TNF-α, is overexpressed in adipose tissue of patients suffering from the metabolic syndrome which predisposes to atherosclerosis [127]. Among the TNF/TNFR superfamily, CD40/CD40L and OX40/OX40L [128] participate in plaque inflammation and atherosclerosis progression as shown in mice [129, 130] and by genetic single-nucleotide polymorphism analysis in humans [130]. Indeed, polymorphic OX40L variation increases the possibility of a heart attack. Furthermore, transgenic OX40L overexpression exacerbates atherosclerosis whereas deficiency is beneficial [130]. Even treatment with antagonizing anti-OX40L antibody can lower plaque burden in ATH-susceptible mice [131]. The OX40/OX40L pathway is highly important for iNKT cells since it is involved in iNKT induction of allergic airway inflammation [132] as well as iNKT-mediated anti-tumour effects [133, 134]. Whether local OX40/OX40L interaction between activated iNKT cells and CD1d-expressing cells contributes to plaque development and lesion progression has not been investigated.

Another TNF family member, TNF-related apoptosis-inducing ligand (TRAIL) [135], has been linked to atherosclerosis since it was found in atherosclerotic tissue. Several functions have been attributed to TRAIL including promotion of EC and SMC migration and proliferation as well as regulation of the vascular tone [136]. TRAIL can also be found as a soluble protein and in the elderly, low plasma levels of soluble TRAIL is associated with overall increased cardiovascular mortality [137]. NK and iNKT cells express high levels of TRAIL, which is directly involved in a concanavalin A-induced hepatitis model [138]. These effects are mediated by the interaction of TRAIL with its receptor DR5, a molecule that is upregulated on hepatocytes by steatosis and free fatty acids and is expressed also by EC [139]. Interestingly, statin treatment reduces TRAIL expression on T cells and also reduces their capacity to kill EC in vitro [139]. Similar therapeutic effects of statins might occur in plaques infiltrated by NK and iNKT cells, which are the circulating cells expressing the highest levels of TRAIL.

An unexpected link between atherosclerotic lesion generation and iNKT cell survival is provided by the de-ubiquitinating enzyme cylindromatosis (CYLD). This enzyme attenuates NF-κB activity and reduces neointima build-up [140]. It is synthesized primarily by SMC in both non-diseased and plaque tissue and is upregulated in response to mechanical injuries including shear stress [141]. CYLD also facilitates IL-7-mediated iNKT cell survival [142] and enhances the expression of inducible costimulatory molecule (ICOS), an important co-stimulatory molecule for iNKT cells [143] and required for their function and homeostatsis [144]. Accordingly, during arterial stress CYLD is upregulated and might exert a double-edged sword effect by both reducing local inflammation and facilitating iNKT survival. Importantly, ICOS deficiency exacerbates atherosclerosis because it leads

to reduced levels of transforming growth factor β (TGF-β) and impaired generation of regulatory T cells [145].

16.9 Lipid-Specific Immunity and the Future of Atherosclerosis Diagnosis and Prevention

The identification of lipids as important antigens driving the immune response has revealed novel pathogenic mechanisms and may offer new approaches to immunotherapy and diagnosis of atherosclerosis. The culprit antigenic lipids in atherosclerosis remain unknown and future studies will doubtless reveal their nature. Their identification will provide important clues regarding the metabolic alterations leading to their generation and accumulation. This will divulge how the immune response recognizes cells within plaques, providing local contribution to chronic inflammation. A new series of studies will be necessary to investigate whether important associations exist between the generation and accumulation of these lipids and the genetic predisposition, diet and environmental circumstances of patients. This knowledge will increase our understanding of the pathogenesis of atherosclerosis and also may offer new clues as to why animal models with features close to human disease are difficult to establish.

The antigenic lipids will likely become important therapeutic targets too. New drugs capable of blocking their synthesis or facilitating their catabolism will be useful to reduce intra-plaque activation of CD1-restricted T cells, with immediate effects on local inflammation. However, it will be crucial to carefully evaluate whether drug-induced alterations of lipid metabolism will have other beneficial or detrimental effects within plaque lesions.

Identification of lipid antigens will also broaden the scope of molecules to be used in a new generation of vaccines. So far oxidized lipoproteins have been proposed and in some models they have shown beneficial effects [146]. The option of inducing antigen-specific T cells with anti-inflammatory functions is a possibility that can be achieved by modern biotechnology. The instruction of T cells towards an anti-inflammatory T-helper 2 or a T-regulatory functional phenotype has been achieved in some vaccination models [147, 148]. These approaches can be attempted also in atherosclerosis, although it will be difficult to demonstrate immediate beneficial effects since human disease has a very long course. This temporal issue might represent a significant caveat preventing the interest of pharmaceutical companies and delaying approval by institutional authorities.

An important new analytical application will be the determination of the type and level of antigenic lipids in patients with atherosclerosis. If these lipids are present also in serum, their routine measurement may become feasible. Large patient cohorts will have to be investigated in order to evaluate whether altered levels of antigenic lipids correlate with disease stage and eventually with its progression. Modern lipid analysis is performed by mass-spectrometry, which provides structure identification and good semi-quantitative measurement. However, this type of analysis requires expensive, technically demanding apparatus and

data evaluation by appropriately trained personnel. These aspects present important limitations, which however might be solved by introduction of novel smaller apparatus that are less costly and simpler to operate.

Finally, lipid antigen identification may create a platform for new hypothesis-driven pathogenic mechanisms, which can be supported by genome-wide association studies in humans [118] and meta-analyses of such studies [126]. The large amount of data provided by these studies often reveals suspected genes whose association with disease is not yet comprehended. It is probable that understanding the role of a new class of lipids will provide useful links to a cohort of these genes and facilitate the integrated evaluation of genetic, immunological and biochemical findings. The ultimate goal is the detailed study and understanding of the many faces of atherosclerotic disease, with a systematic view to the interactome of each component.

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