



# When a Friend Becomes Your Enemy: Natural Killer Cells in Atherosclerosis and Atherosclerosis-Associated Risk Factors

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Atherosclerosis (ATS), the change in structure and function of arteries with associated lesion formation and altered blood flow, is the leading cause of cardiovascular disease, the number one killer worldwide. Beyond dyslipidemia, chronic inflammation, together with aberrant phenotype and function of cells of both the innate and adaptive immune system, are now recognized as relevant contributors to atherosclerosis onset and progression. While the role of macrophages and T cells in atherosclerosis has been addressed in several studies, Natural Killer cells (NKs) represent a poorly explored immune cell type, that deserves attention, due to NKs' emerging contribution to vascular homeostasis. Furthermore, the possibility to re-polarize the immune system has emerged as a relevant tool to design new therapies, with some successful examples in the field of cancer immunotherapy. Thus, a deeper knowledge of NK cell pathophysiology in the context of atherosclerosis and atherosclerosis-associated risk factors could help developing new preventive and treatment strategies, and decipher the complex scenario/history from "the risk factors for atherosclerosis" Here, we review the current knowledge about NK cell phenotype and activities in atherosclerosis and selected atherosclerosis risk factors, namely type-2 diabetes and obesity, and discuss the related NK-cell oriented environmental signals.

**Keywords:** natural killer cells, atherosclerosis, atherosclerosis-related risk factors, type-2 diabetes, obesity

## INTRODUCTION

Cardiovascular diseases (CVDs), the first cause of death worldwide, are characterized by an inflammatory microenvironment (1–3). An altered immune response can govern and impact both pathological resolution and/or progression of CVDs, as a consequence of systemic and tissue-local environments (4–7). In this context, it is worth noticing that atherosclerosis (ATS), the leading

cause of CVDs (8), is characterized by lipids accumulation, cell apoptosis, endothelial cells (ECs) increased permeability, fibrosis, and chronic inflammatory burden (9). In particular, EC dysfunction in ATS leads to proinflammatory cytokines production and immune cells recruitment within the atherosclerotic plaque (9). This tight connection between inflammation and CVDs has been recently reinforced by the results of two clinical trials (Canakinumab Anti-inflammatory Thrombosis Outcomes Study-CANTOS, and Low Dose Colchicine for Secondary Prevention of Cardiovascular Disease-LoDoCo) that show the benefit of targeting inflammation to lower the risk of CV events (10, 11). CANTOS investigators found that canakinumab (an interleukin-1 $\beta$ , IL-1 $\beta$ , neutralizing antibody) exerts a protective effect on thrombosis, targeting the IL-1 $\beta$  innate immunity pathway. Interestingly, the study shows that compared to placebo, the treatment also correlates with lower cancer mortality. Furthermore, the LoDoCo trial showed a preventive role of colchicine in the occurrence of cardiovascular events with inhibition of neutrophil chemotaxis and activation within a proinflammatory environment. In addition, another study showed that heart failure-associated inflammatory markers, including C-reactive protein, at the same time had a clear predictive value of new-onset cancer, independently of cancer risk factors (12). These data suggest that cancers and CVDs share some common mechanisms centered on inflammation and the immune response that may represent valuable novel targets for therapies. Moreover, ATS and cancer share risk factors such as obesity, diabetes mellitus, and hypertension (13), and pathophysiological pathways, such as chronic inflammation, oxidative stress, and alterations in immune cells phenotype and

functions. Of note, immune cells, both of innate and adaptive immunity, are characterized by extraordinary plasticity, thus they can adapt their phenotype and response (referred as immune cell polarization) to the hosting pathophysiological micro-(tissue/local) and macro-(peripheral blood/systemic) environment (14–19). These adaptation capabilities result in the ability of immune cells to acquire contrasting activities, as related to their “original commitment”, which is the defense of the host organism. These peculiar features have been observed in diverse chronic inflammatory-based disorders, ranging from CVD (6, 20, 21), to autoimmune diseases (22–24), to cancers (25–27).

The most investigated players in ATS are monocytes/macrophages, which, upon activation, support the subsequent specific T and B cells response (28, 29). However, recent preclinical and clinical evidence point to a role in ATS also for NK cells (30, 31), which are large granular lymphocytes of innate immunity, primarily involved in the immunosurveillance against virus-infected and malignant-transformed cells. NK cells can infiltrate the vessel wall, promoting atherosclerotic lesion development and producing perforin and granzyme B, thus leading to more vulnerable atherosclerotic lesions for atherothrombosis (32, 33).

Here, we review the current knowledge of NK cell phenotype and activities, discussing the environmental cues that can instruct NK cell behavior in ATS pathological context.

## NK Cells

NK cells originate in the bone marrow from CD34<sup>+</sup> hematopoietic stem cell (HSC) precursors, which generate common lymphoid progenitors (CLP), further committed to the NK cell progenitor (NKP), following the acquisition of IL-2/IL-15R $\beta$  subunit (CD122) that makes NK cells responsive to IL-15. Mature peripheral blood NK cells express CD16, CD57, and NKG2D, and display the ability to release perforin, granzyme, and IFN $\gamma$  (34–36).

The Neural Cell Adhesion Molecule/NCAM (CD56) and the FC gamma receptor III molecule (CD16) are the two major surface antigens used to discriminate NK cell subsets. CD56<sup>+</sup>CD16<sup>+</sup> NK cells (90–95% of total circulating NKs) are endowed with cytolytic functions, *via* antibody-dependent cellular cytotoxicity (ADCC), and release of perforin and granzymes, thus mediating the immunological synapsis between target and effector cell complex (34, 37). CD56<sup>bright</sup>CD16<sup>-</sup> (5–10% of circulating NKs) (34, 37) act by releasing pro-inflammatory and anti-tumor cytokines, such as IFN $\gamma$ , TNF $\alpha$ . Several studies, in particular within the recent single-cell era, demonstrated that NK cell subset classification may extend beyond this classical dichotomy of CD56<sup>dim</sup>CD16<sup>+</sup> and CD56<sup>bright</sup>CD16<sup>-</sup> NKs (38–41).

The local microenvironment, with its unique cellular interactions, provide relevant signals to shape NK cell phenotype and functions, both under physiological and pathological conditions (18, 42–45). During fetal development, NK cells acquire a peculiar phenotype, described as CD56<sup>superbright</sup>CD16<sup>-</sup>, termed decidual NKs (dNKs), endowed with increased ability to produce pro-angiogenic factors, such as vascular endothelial growth factor (VEGF),

**Abbreviations:** AAV, adeno associated virus; ADCC, antibody dependent cellular cytotoxicity; AOM, azoxymethane; ATS, atherosclerosis; BiP, binding immunoglobulin protein; BMI, body mass index; CAR, chimeric antigen receptor; CRC, colorectal cancer; CSF1R, colony-stimulating factor 1 receptor; CXCL, chemokine ligand (C-X-C motif); CVDs, cardiovascular diseases; DC, dendritic cell; dNK cells, decidual Natural Killer cells; ECs, endothelial cells; EOMES, eomesodermin; ER, endoplasmic reticulum; FOXP3, forkhead box P3; GM-1, ganglioside monosialic type 1; HbA1c, glycosylated hemoglobin; HFD, high fat diet; HIF-1, hypoxia inducible factor 1; IAP, integrin associated protein; IFN $\gamma$ , interferon  $\gamma$ ; IL-, interleukin-; ILCs, innate lymphoid cells; IRE1, inositol-requiring enzyme 1; LADA, latent autoimmune diabetes in adults; MCP-1/CCL2, monocyte chemoattractant protein-1; MHC, major histocompatibility complex; MICA, MHC class I chain-related protein A; MIP1- $\beta$ , macrophage inflammatory protein 1- $\beta$ ; MPs, matrix metalloproteinases; mTOR, mammalian target of rapamycin; NCAM, neural cell adhesion molecule; NCR, natural cytotoxicity receptor; NK, natural killer; NKG2A/D, Natural Killer receptor Group 2 A/D; NOD, non-obese diabetic; NLRP3, NOD-like Receptor Protein 3; oxLDL, Oxidized Low-Density Lipoprotein; OxPhos, Oxidative Phosphorylation; PB, Peripheral Blood; PDI, Protein Disulfide-Isomerase; PERK, Protein kinase R (PKR)-like Endoplasmic Reticulum Kinase; PIGF, Placental Growth Factor; RAE1, Retinoic Acid Early transcript 1; RAG, Recombinant Activating Gene; RIP, Rat Insulin Promoter; ROS, Reactive Oxygen Species; SREBP, Sterol regulatory Element Binding Protein; SMCs, Smooth Muscle Cells; sMICA, soluble MHC class I Chain-related Protein A; STAT, Signal Transducer and Activator of Transcription; sXBP1, spliced X-Box Binding Protein 1; TANKs, Tumor Associated Natural Killer cells; T-bet, T-box expressed in T cells; TCR, T Cell Receptor; TG, Transgenic; TINKs, Tumor Infiltrating NK cells; TNF $\alpha$ , TumorNecrosis Factor- $\alpha$ ; T2D, Type 2 Diabetes; UPR, Unfolded Protein Response; VEGF, Vascular Endothelial Growth Factor.

placental growth factor (PIGF), and CXCL8 (46, 47). Thus, in this particular scenario, dNK cells shift from killer to builder effector cells, being necessary for the appropriate formation of spiral arteries, which deliver oxygen and nutrients to the developing fetus (46, 47). Intriguingly, an expansion of pro-angiogenic, decidual-like NK cell subset has been found in solid cancers: these dNK-like, termed tumor-infiltrating (those present in tumor tissues-TINKs) and tumor-associated (NKs in the peripheral blood-TANKs) NK cells acquire a CD56<sup>bright</sup>CD16<sup>low</sup>CD9<sup>+</sup>CD49a<sup>+</sup> phenotype, can release pro-angiogenic factors and support EC proliferation, migration and ability to form capillary-like structures (18, 45, 48–51). Major mechanisms governing the alterations of NK cell activity in chronic inflammatory disorders include downregulation of the activation molecule NKG2D and the Natural Cytotoxicity Receptors (NCRs) NKp46, NKp44, and NKp30, together with decreased or increased capability to release IFN $\gamma$ , TNF $\alpha$ , perforin, and granzymes, according to the hosting environment (52–54).

## NK Cells in Atherosclerosis and Atherosclerosis-Related Risk Factors

ATS represents the most common pathophysiological alteration leading to ischemic heart disease and stroke (55). NK cells have been detected in atherosclerotic lesions in humans (56, 57) and mice (32, 33, 58, 59), mostly in advanced lesions, deep within plaques, and in shoulder regions of plaque (32). In addition, patients with advanced lesions show high levels of circulating NK cells (60). Here, we focus on NK in ATS and selected ATS-associated risk factors, i.e. type-2 diabetes, and obesity, analyzing the inflammatory environmental factors and signaling orchestrating different NK cells phenotype and functions, together with their interactions with other cells of the host organism.

## NK Cells in Atherosclerosis

There is contrasting evidence on the direct contribution and regulatory function of NK cells in ATS insurgence and progression, mainly as a consequence of the different murine models employed in various *in vivo* studies.

The first experiments investigating the role of NK cells in ATS have been conducted in beige mice, an animal model of NK cell functional deficient. In this murine model, Paigen et al. (61) reported no difference in ATS lesion size, suggesting no crucial role for NK cells in ATS. A second study using the NK cell functional deficient beige mice, crossed with LDLR deficient mice (beige, *LDLR*<sup>-/-</sup>), showed increased lesion size, as compared to control *LDLR*<sup>-/-</sup> mice, fed a high-fat diet (HFD). When reconstituted with the bone marrow (BM) of Ly49A transgenic mice (a murine model overexpressing the Ly49A receptor under the control of granzyme A promoter), *LDLR*<sup>-/-</sup> recipient animals exhibited smaller sizes of the lesions (62).

Further studies performed in *ApoE*<sup>-/-</sup> mice, in which NK cells were depleted by anti-asialo-GM-1 antibody, showed a significant reduction of the atherosclerotic lesion development (63). However, since several other cell types, such as myeloid cells, epithelial cells, and T-cell subsets express the glycolipid asialo-GM1 (64–67), the effects observed cannot be considered restricted to NK cells.

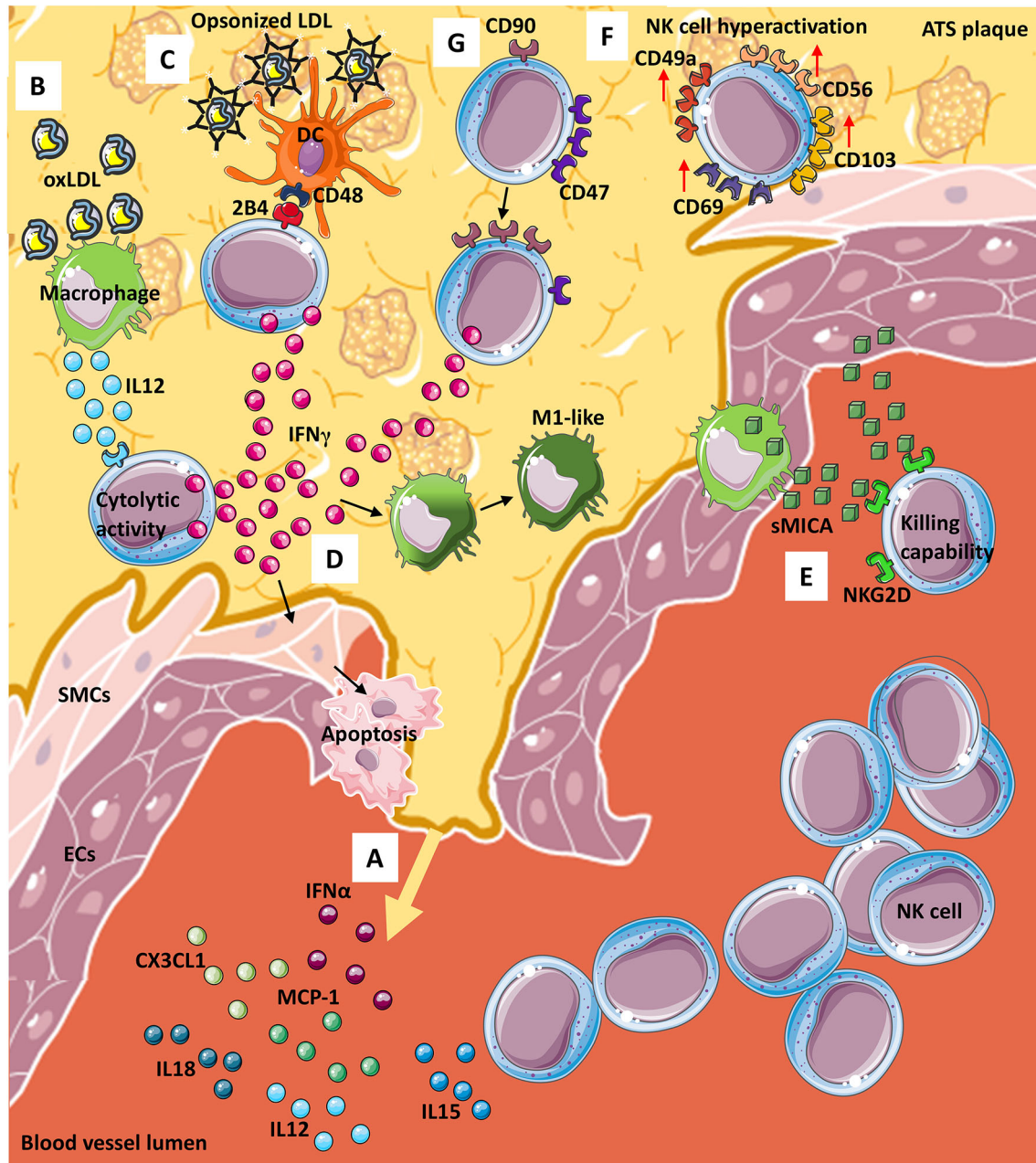
In another study, NK cells pre-activated with IL-2 were adoptively transferred into *ApoE*<sup>-/-</sup> *Rag2*<sup>-/-</sup> *IL2rg*<sup>-/-</sup> mice, resulting in increased ATS and necrotic core development, and IFN $\gamma$ , Perforin, and Granzyme B production, by transferred NK cells (32).

Various cytokines/chemokines are involved in NK cell recruitment, including monocyte chemoattractant protein-1 (MCP-1/CCL2), fractalkine (CX3CL1), IL-15, IL-12, IL-18, and IFN- $\alpha$  (68, 69). Moreover, IL-15, IL-12, and IL-18 drive NK cell pro-atherogenic features, by hyper-activating NK cells, either in a direct manner or *via* dendritic cells (DC) and monocyte/macrophages interaction (70–72) (**Figure 1A**). Atherosclerotic plaques are enriched in IL-12 producing macrophages, in response to oxLDL (73) (**Figure 1B**). IL-12 within the ATS plaque enhances NK cell cytolytic activity *via* IFN $\gamma$  production, resulting in plaque destabilization, through either the induction of smooth muscle cells (SMCs) apoptosis and/or secretion of matrix metalloproteinases (MMPs) (74). In addition, oxLDL supports the interaction between NK cells and DCs, in a CD48-2B4 contact-dependent manner (**Figure 1C**). Opsonized LDL favors the NK-DC crosstalk, *via* IL-12 and IFN $\gamma$ , resulting in altered DC editing/activation and/or selection of highly inflammatory M1-like macrophages (74) (**Figure 1D**). Both macrophages and endothelial cells within the ATS plaques have been reported to express NKG2D ligands that correlate with detectable serum levels of soluble Major Histocompatibility Complex (MHC) class I chain-related proteins A (sMICA), which in turn activated killing abilities in NK cells (75) (**Figure 1E**).

In human ATS, most of the current knowledge on NK cells comes from observational studies on lesion samples conducted by immunohistochemistry. As far as we know, phenotypic and functional features of NKs in ATS patients are still not completely known. In this context, a recent study from Bonaccorsi et al. characterized NK cells in atherosclerotic plaques of asymptomatic patients. They found that carotid plaques are enriched in CD56<sup>bright</sup>perforin<sup>low</sup> NK cells, which also express tissue-resident markers, such as CD103, CD69 and CD49a (76), (**Figure 1F**) with increased production of IFN $\gamma$  (76). These data suggest that hyperactivation of NK cells in carotid plaques may represent a relevant host-dependent mechanism determining plaque instability (**Figures 1F, G**).

In line with this hypothesis, using a murine model of adeno-associated virus (AAV)-induced hypercholesterolemia, Engelbertsen et al. (77) showed that loss of CD47 results in increased frequency of IFN $\gamma$  producing CD90<sup>+</sup> NK cells (77) (**Figure 1G**). Moreover, depletion of NK cells, using anti-NK1.1 monoclonal antibody, generates equalization of atherosclerotic burden, supporting that NK cell are involved in ATS progression in CD47-null mice (77). CD47 (an integrin-associated protein, IAP), is a transmembrane protein exerting multiple biological activities, ranging from regulation of efferocytosis to leukocyte trafficking (77, 78). There are contrasting reports, showing both pro-atherogenic and anti-atherogenic effects of CD47 and its ligands (77–79). Engelbertsen and colleagues (77) also found that CD90<sup>+</sup> NKs are expanded in the atherosclerotic





**FIGURE 1** | NK cells in atherosclerosis. From the site of atherosclerotic (ATS) plaque formation **(A)** many cytokines and chemokines are released into the blood, stimulating NK cells migration and entry within the plaque. Infiltrating NK cells can interact, by contact, with other immune cells such as **(B)** macrophages, that in presence of oxLDL, produce and release IL-12, thus potentiating cytolytic activity of NK *via* interferon  $\gamma$  ( $\text{IFN}\gamma$ ) release. OxLDL can also be present as opsonized particles **(C)** that support dendritic cells (DCs)-NK crosstalk which is also mediated by CD48-2B4 interaction and leads to **(D)**  $\text{IFN}\gamma$  release from NKs.  $\text{IFN}\gamma$  from NK cells exerts its effect on both **(D)** smooth muscle cells (SMCs) by inducing their apoptosis and macrophages by promoting M1-like phenotype switch. Furthermore, both **(E)** macrophages and endothelial cells (ECs) release soluble MICA (sMICA) that upon NKG2D binding on NK cells increase NK killing capability. Within ATS plaque, **(F)** a subset of NK cells display a hyperactive phenotype/behavior with increased expression of CD49a, CD56, CD69 and CD103 and NK cells **(G)** modify receptors expression downmodulated CD47 and increasing CD90 marker, leading to  $\text{IFN}\gamma$  release.

aorta and largely produce  $\text{IFN}\gamma$ , as compared to  $\text{CD90}^-$  NK cells (77).

Upregulation of cell cholesterol is a relevant hallmark of the ATS plaque, exacerbating the pro-inflammatory state that impact

on the plaque fate, by continuously damaging the ECs and SMCs, finally determining the plaque rupture. Of notice, upregulated cell cholesterol induce a signature of trained immunity (80). Trained immunity, a concept recently developed, refers to the

ability of innate immunity to develop a specific memory as for adaptive immunity (17). This innate immunological memory, leads to an augmented long-lasting proinflammatory immune response to a secondary stimulus, through metabolic and epigenetic rewiring of innate immune cells (17). Moreover, it has been demonstrated that, in addition to microbial stimuli, endogenous metabolites, such as oxLDL and lipoproteins, that characterize the plaque microenvironment, train the generation of pro-atherogenic monocytes and macrophages, by instructing these cells to produce ATS-supporting cytokines, such as GM-CSF, IFN $\gamma$ , IL-3 and MMPs. Mechanistically, this immune training is sustained by a metabolic switch from oxidative phosphorylation (OxPhos) to aerobic glycolysis and mevalonate synthesis, in monocytes, together with epigenetic modifications (17, 81).

In the case of ATS and all those pathologies where persistent chronic inflammation has a detrimental role, trained immunity can be considered a double edge sword. ECs, apart from their vascular function, are now considered as relevant immunoregulatory effectors in chronic inflammatory diseases (82, 83). Following cellular damage, ECs release large amount of IL-1 $\beta$  and IL-6 and increase the expression of adhesion molecules, like E-selectin (CD62E) and ICAM-1 (CD54), that further contribute to the exacerbate inflammation and trained immunity (80), including in ATS. In the case of ATS, the persistent activation of the inflammatory state, due to trained immunity, has been hypothesized as a relevant mechanisms linking non-resolving inflammation in ATS (84–86). As for macrophages, given the shared immune cell plasticity and capabilities to adapt to different pathophysiological environments, NK cells could be considered as key innate immune cells within the ATS plaque, both as a “soloists” or by interacting with atherogenic macrophages. In this context, the microenvironment of cholesterol-mediated exposure may also trigger trained immunity on NK cells, exacerbating their activity in the plaque, mimicking the same effect observed in macrophages.

Therefore, studies investigating the contribution of NK cells to ATS and their specific polarization state in ATS, still require more investigation.

ATS plaques are enriched in cholesterol that, through enzymatic reactions or by auto-oxidation with reactive oxygen species (ROS), is converted into oxysterol (87). Oxysterols can modulate NK cell metabolism and subsequently activity by inhibition of Sterol regulatory element binding protein (SREBP), which is required for proper cytokine-induced growth and effector function by NK cells (88). Indeed, both glycolysis and oxidative phosphorylation (OxPhos) are SREBP-dependent mechanisms and NK cells that cannot activate SREBP showed reduced glucose metabolism and impaired effector functions (88). Within ATS plaques, the higher amount of oxysterols can reduce NK cell functionality by blocking SREBP activity.

Related to the presence of cholesterol within ATS plaque, the NOD-like Receptor Protein 3 (NLRP3), an inflammasome component (89), is another player involved in both ATS onset and development and NK cell modulation. NLRP3

inflammasome (89) is a multimeric protein complex that, upon caspase-1 activation, leads to the release of inflammatory cytokines IL-1 $\beta$  and IL-18 (90).

Duewell and colleagues (90) have shown how cholesterol crystals are able to activate NLRP3 inflammasome, already in early stages of ATS, inducing inflammation and how this condition is impaired in mice deficient in components of the NLRP3 inflammasome, also following cholesterol crystal intraperitoneal injection (90). Moreover, it has been shown that *LDLr*<sup>-/-</sup> mice, transplanted with bone marrow (BM) from NLRP3-deficient mice, display an impaired development of early atherosclerosis (89), confirming the involvement of NLRP3 in ATS onset.

Interesting, IL-18 release, upon NLRP3 inflammasome activation, can impact not only NK cell recruitment (as mentioned before), but it has been shown that can affect NK cell cytotoxicity in murine model of cancer (91). Indeed, mice deficient in NLRP3 inflammasome components show increased growth of liver colorectal cancer (CRC) metastasis, in a mechanism dependent on the lower level of IL-18 and by the subsequent reduction of hepatic NK cell cytotoxicity (91). This data suggested that NLRP3 activation in ATS plaque could mediate NK cells recruitment and activation by IL-18 release.

Opposite evidence derives from a study on hepatocellular carcinoma (HCC) where NLRP3 has been reported to be involved in cancer development. In this study, HCC patients showed reduction and impairment in NK cells. Using *in vitro* co-culture system of NK cell line NK-92 and HCC cells, it has been shown that NLRP3 down-modulation in HCC cells induces lowered expression of metalloproteinase, subsequent to MICA upregulation which in turn increases NK-92 toxicity, through NKG2D binding (92). This result is further confirmed by exploiting a xenograft mouse model in which NLRP3 knock-out (KO) in HCC cell delays cancer development, reduces metastasis formation, and increases NK cell toxicity, through MICA-NKG2D interaction (92).

MICA is a surface protein that, upon proteinases cleavage, becomes soluble (sMICA) (93). MICA is overexpressed in both macrophages and endothelial cells within the ATS plaque and showed same function as sMICA in promoting NK cell cytotoxicity (94). Considering reported data, we can speculate that NLRP3 activation, by cholesterol crystals, affects the shedding of MICA from macrophages and endothelial cells by proteinases upregulation, and that sMICA increases NK cells recruitment within ATS plaque.

Given the complexity of ATS plaque environment, the molecular and cellular players that modulate its onset and development, and the continuous remodeling of the ATS plaque, it is not surprising that contrasting data suggest a dichotomous role of NK cells in ATS onset and development.

## Risk Factors for ATS: NK Cells in Type-2 Diabetes

Diabetes mellitus represents one of the major risk factors for CVDs, including ATS (95). Both type 1 and type 2 diabetes are accompanied by micro and macrovascular complications. Type 2

diabetes (T2D), a chronic complex disorder characterized by de-regulated inflammation and metabolic alterations, represents the most common form of the disease and affects about 95% of the diabetic population (96, 97). A major feature of T2D is represented by a peripheral resistance to the action of insulin and a failure of beta cells to compensate for this alteration, resulting in hyperglycemia. The biology of both cardiovascular and immune cells is altered by chronic or transient hyperglycemia and the consequent increased oxidative stress with ROS accumulation/production.

In diabetes, ROS production is driven by mitochondrial respiration, in response to glucose stimulation (98). When peripheral insulin resistance impaired glucose clearance, the continuous glycolytic flux increases ROS production (99) that exerts different effects, according to NK cells phenotype in the inflammatory microenvironment. In diabetes, ROS production is mainly induced by hypoxia (98) that, as ROS, can act as NK cell modulator.

Indeed, hypoxia modulates NK cell metabolism promoting glycolysis and reducing OxPhos that leads to enhanced cytotoxicity and increased IFN $\gamma$  production (99, 100). This hypoxia-related effect is also supported by MICA. Indeed, in inflammatory environment, such as in renal epithelial cells (101) and in cardiomyocytes (102), Hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) induces MICA upregulation that stimulates NK cytotoxicity and IFN $\gamma$  production (94). The chronic excess of glucose induces visceral adipose tissue (VAT) expansion and dysfunctions mediated by adipocytes and immune cells that contribute to hypoxia (103). Thus, in T2D VAT, we can speculate that immune cells, including NK cells, are involved in disease progression by modulating VAT microenvironment that in turn support inflammation and immune cell recruitment, by different mechanisms, including hypoxia.

Of note, even though high glucose concentration predisposes to ATS development, the use of lowering glucose drugs does not result in the reduction of the CV risk in diabetic subjects, a concept known as metabolic memory (104). In the case of immune cells, the persistent changes due to environmental alteration in nutrition and metabolism are referred to as “training”. Emerging data are supporting the idea that trained immunity can be associated with diabetes and its damaging effect on the CV system (105). Concerning innate immunity, several reports demonstrated the role of monocyte/macrophages in the process of ATS and increasing myelopoiesis in the diabetic bone marrow, but less is known on NK cells (106–108). Of note, in subjects with diabetes and diabetic atherosclerotic complications, the BM is dramatically remodeled, including deposition of pro-inflammatory adipocytes, decreased innervation, and vascularization with associated impaired hematopoiesis (109–113). The number of BM resident NK cells is increased in diabetic patients with or without ischemic complications, but no changes in the circulating cell pool were observed (109, 114).

A recently published meta-analysis, collecting results from 13 independent studies, showed that circulating NK cells number increase in T2D patients (n=491), compared to healthy subjects (n=1607) (115) (**Figure 2A**). However, other studies showed no

differences in the phenotype between NK cells in T2D and NK cells in healthy controls (116). Hyperglycemia, sedentary lifestyle, poor metabolic status, all represent peculiar features of diabetes and have been reported to reduce NK cell functionality (117). Functional alteration of NK cells, isolated from T2D patients, include reduced expression of NKP46 and NKG2D receptors, decreased cytotoxic activity *in vitro*, compared to healthy subjects (118) (**Figure 2B**). However, exposure to IL-15, a major cytokine involved in NK cell re-education and activation, restored NK cell functionality in T2D patients (117). Mechanistically, it has been demonstrated that endoplasmic reticulum (ER) stress, which is a crucial mediator of diabetes-associated complications, is induced by tunicamycin, a mediator for the unfolded protein response (UPR), with subsequent reduction of NKG2D and NKP46 expression (117). In addition, markers of UPR, such as BiP, PDI, and sXBP1, are increased in NK cells from T2D patients and ER stress is activated through PERK and IRE1 sensors, which are involved in UPR and that are causative of NKG2D down-modulation in NK cells from T2D patients (117) (**Figure 2C**).

Also, NKG2D expression in NK cells was found to be negatively correlated with glycated hemoglobin (HbA1c) level, suggesting that hyperglycemia could directly govern NK cell functional alterations (117). A correlation between hyperglycemia and NK cells activity has been also demonstrated by Kim et al. (119). In a recent study, Kim and colleagues (119) enrolled 49 participants, 21 with T2D, 15 with pre-diabetes, and 13 controls with normal glucose tolerance, to analyze NK cells activity modulation, as related to the diabetes stage. NK cells activity was measured by detecting circulating IFN $\gamma$  level together with HbA1c. They showed that HbA1c displayed an inverse linear correlation with NK cells activity (119), together with diabetes progression and they conclude that HbA1c is an independent predictor of NK cell activity in T2D patients (119).

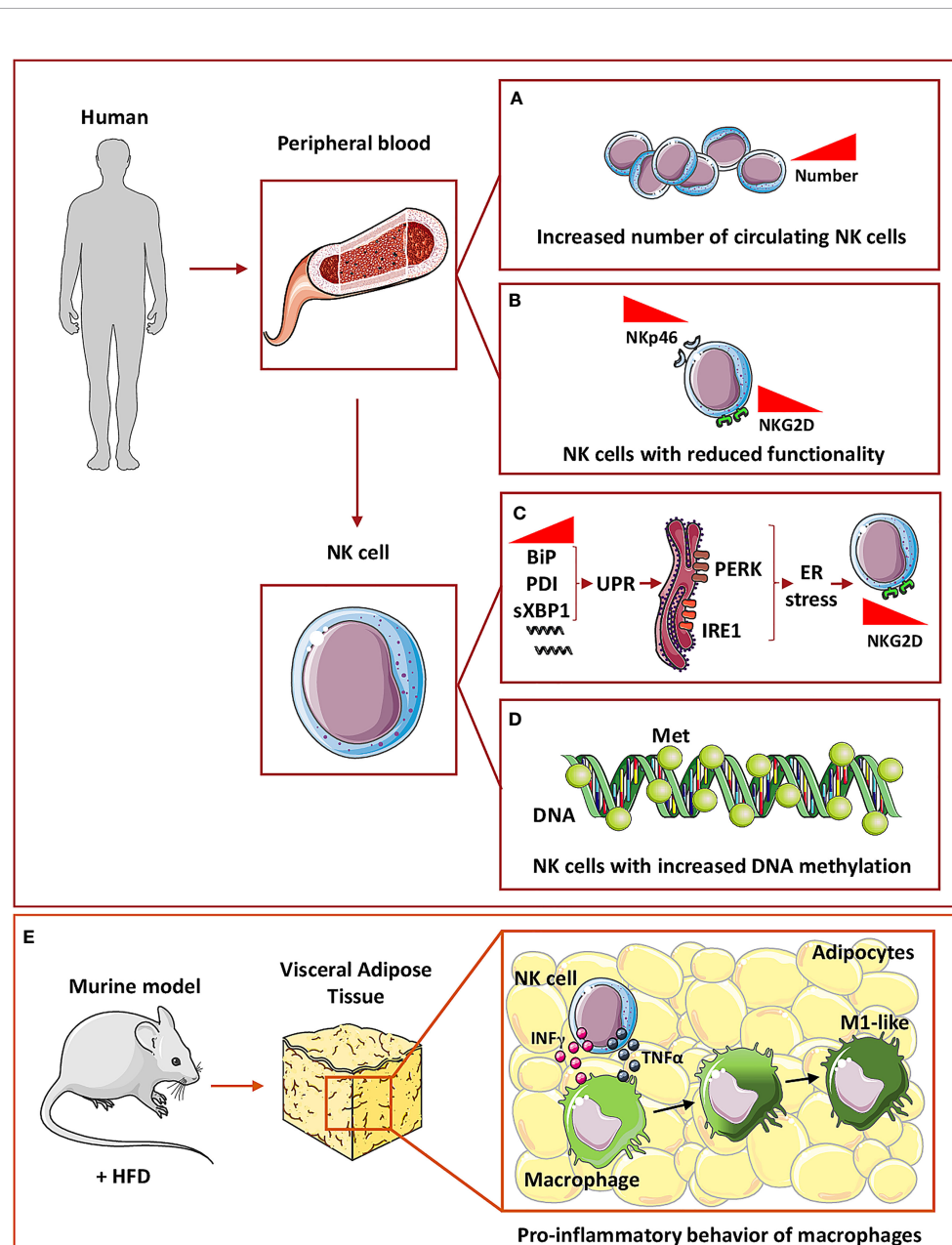
Moreover, epigenetic alterations functionally impact on immune cell effectors (120). While no global DNA methylation was observed in peripheral blood mononuclear cells, monocytes, lymphocytes, or T cells, NK cells from T2D patients exhibit increased methylation levels that positively correlate with insulin resistance, linking DNA methylation changes, immune cell function, and metabolic dysfunction (120) (**Figure 2D**).

Finally, it has been reported that within VAT of mice receiving HFD, NK cells support the development of obesity-induced insulin resistance, *via* induction of pro-inflammatory/M1-like macrophages, through a mechanism mediated by NK-derived cytokines, including TNF $\alpha$  (121) (**Figure 2E**).

## Risk Factors for ATS: NK Cells in Obesity

Obesity drives a program of a chronic pro-inflammatory state that orchestrates the development of related co-morbidities, including cancer, T2D, CVDs, and ATS (122–125). Several pro-inflammatory cytokines are aberrantly produced in obese individuals and de-regulate the normal homeostasis, such as IL-1 (interacting with insulin signaling) and IL-17 (interacting with adipogenesis) (126, 127). Obesity is widely recognized as a





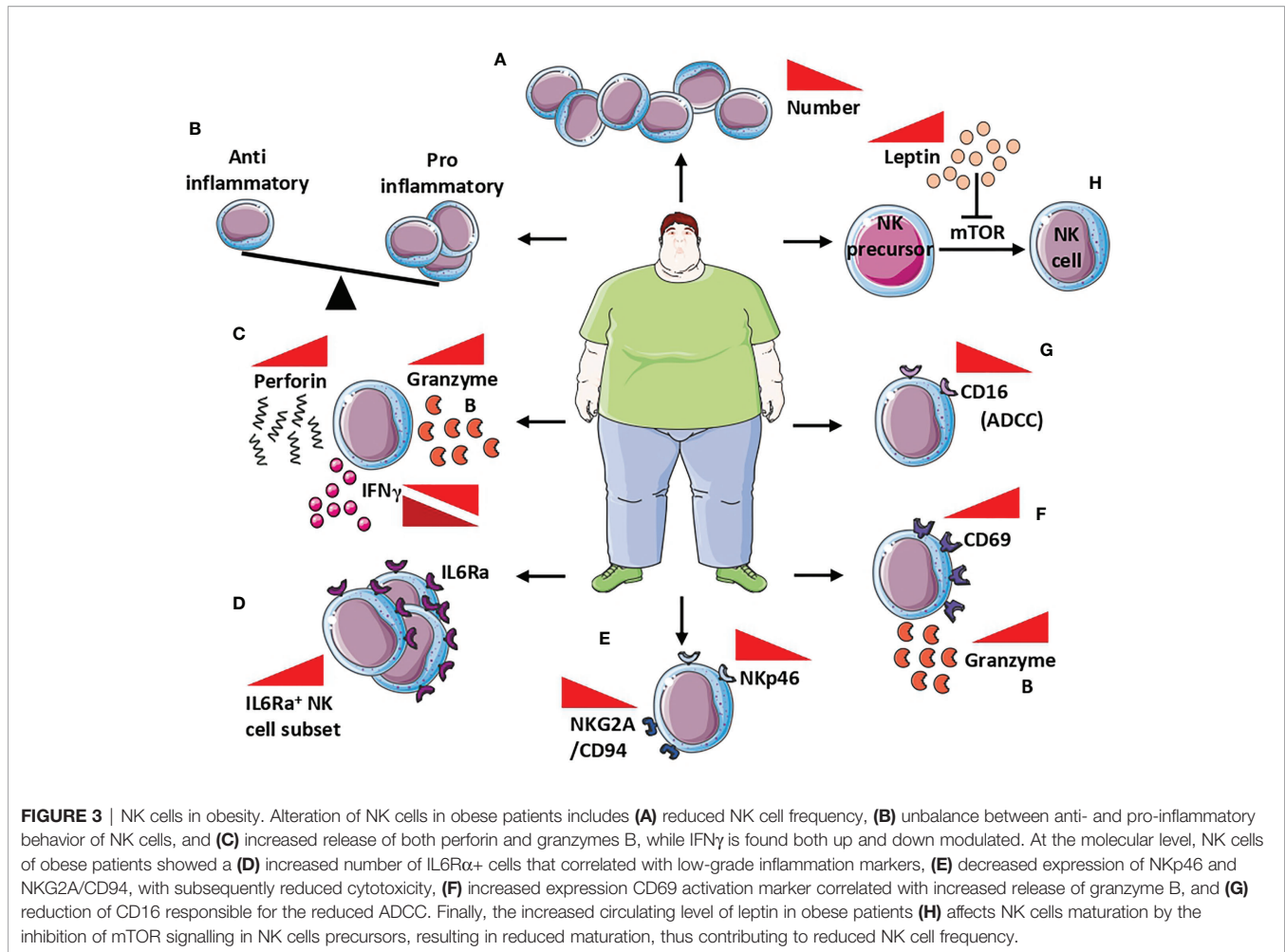
**FIGURE 2 |** NK cells in Type 2 diabetes. In humans, circulating NK cells in T2D subjects have been found to increase in number **(A)** but with decreased expression of both NKp46 and NKG2D activation markers, thus showing a reduced functionality **(B)**. At the molecular level, circulating NK cells in T2D showed an increased mRNA expression of BiP, PDI, and sXBP1, a marker of unfolded protein response (UPR) which in turn is related to ER stress-activated by PERK and IRE1 sensors **(C)**. These mechanisms are related to NKG2D down-modulation in circulating NK cells **(C)**. In addition, NK cells showed an increased general DNA methylation **(D)**. To mimic T2D mice are fed with high fat diet (HFD) **(E)** and within visceral adipose tissue, NK cells through IFN $\gamma$  and TNF $\alpha$  release can induce macrophages polarization toward a pro-inflammatory M1-like phenotype thus promoting inflammation **(E)**.

pivotal risk factor for T2D, as a consequence of the induction of chronic low-grade inflammation in local adipose tissue (128).

Immuno-metabolic alterations characterizing obese individuals significantly impact on NK cell functions. Resting NK cells metabolize glucose, *via* glycolysis, coupled to oxidative phosphorylation, yielding high levels of energy (99, 129–132). Following activation, NK cells rapidly increase their rates of

aerobic glycolytic metabolism, providing the biosynthetic precursors for cytokine and lytic granule production (129–131).

Obese patients showed decreased NK cell frequency **(Figure 3A)**; in these patients, NK are characterized by pro-inflammatory functions **(Figure 3B)**, and unbalance in the equilibrium between inhibitory and activation receptors, lytic capabilities, the release of perforin/granzymes, and altered



release of IFN $\gamma$  (130) (**Figure 3C**). Interestingly, alterations in NK cell frequency (reduced circulating NK cells number) have been found also in obese children, in an insulin resistance-dependent manner (133).

Circulating levels of IL-6 are strongly elevated in obese individuals (134). IL-6 receptor (IL-6R) expression was found in blood, hepatic, adipose tissue, and splenic NK cells, in mice as well as in the human NK-92 cell line and NK cells isolated from blood, spleen, and tonsil of humans (135–139). In a murine model, Theurich et al. (137) demonstrated that obesity promotes the expansion of a distinct IL6R<sup>+</sup>CSF1R<sup>+</sup> NK cell subset, that further supports obesity (**Figure 3D**). Selective ablation of this NK cell subpopulation prevents obesity and insulin resistance. Moreover, conditional inactivation of IL-6R $\alpha$  or STAT3 in NK cells limits obesity-associated induction by NK cells, protecting from obesity, insulin resistance, and obesity-associated inflammation (137). Results from Theurich et al. (137) were also confirmed in humans, where IL6R $\alpha$ <sup>+</sup> NK cells increase in obese patients and correlates with markers of systemic low-grade inflammation (137).

Peripheral blood NKs in obese patients are endowed with phenotype and functional alterations, characterized by reduced expression of the natural cytotoxicity receptor NKp46 and the

NKG2A/CD94 complex, which was correlated with body mass index (BMI) (140) (**Figure 3E**). While expressing high levels of the activation marker CD69 and granzyme B (**Figure 3F**), peripheral blood NKs from obese patients have very low expression of the CD16 antigen, which is responsible for the reduced ADCC (131) (**Figure 3G**). BMI inversely correlates with the impaired capability of peripheral blood NK cells to degranulate or to produce MIP-1 $\beta$ , following co-culture with K562 cells (131).

Leptin and adiponectin production is altered during obesity and these adipokines have been largely reported to modulate NK cell activities (141). Leptin is able to inhibit mTOR, whose activation is necessary for NK cell maturation, by regulating their responsiveness to IL-2 and IL-15 (131, 142, 143) (**Figure 3H**). Since IL-15 is produced by adipocytes (144, 145), it should be hypothesized that leptin impairment, occurring during obesity, is directly involved in NK cell hyporesponsiveness in an mTOR-dependent manner.

Also, adipocytes have been reported to regulate immune response in cancer patients with obesity (123, 146, 147). The connection between adipocyte-derived leptin and immunomodulation in NK cells, has been demonstrated *in vitro* and *in vivo*.



*In vitro*, cytolytic NK cells, exposed to leptin, exhibit decreased ability to release IFN $\gamma$  and were less effective in lysing colorectal cancer (CRC) cell lines (129).

In an *in vivo* model of chemically-induced CRC carcinogenesis by azoxymethane (AOM), mice receiving a HFD showed decreased frequency and functionality of NK cells in the spleen and the livers (129).

In addition, in VAT of obese patients, NLRP3 inflammasome is upregulated and *in vivo* experiments showed that NLRP3 deficient mice are protected from HFD-induced obesity (89), suggesting a crucial role of inflammation in obesity. The link between inflammation and obesity is further confirmed by other evidence as free fatty acids, increased in obesity, can modulate inflammation.

Palmitate, in HFD fed mice, can activate NLRP3 inflammasome in adipose tissue macrophages, increasing IL-1 $\beta$  and IL-18 secretion (148). Also, adipose tissue macrophages are polarized toward M1 phenotype and secrete inflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  that, in turn, can recruit and stimulate NK cells (148).

Obesity arises also from compromised adipose storage that drives inflammation within VAT. This evidence, together with the reported data, support the idea that a vicious loop between obesity and inflammation is crucial for disease onset and progression and, within this loop, NK cells could play a role as a contributor to pro-inflammatory environment development.

These results further corroborate the direct link between obesity, a risk factor for ATS, with NK cell activities.

## CONCLUSIONS AND FUTURE PERSPECTIVES

The immune system, whose native functions are protecting the host, acts as a double-edged sword in the control and insurgence/progression of chronic pathologic disorders, as a consequence of immune cell plasticity. Inflammation is a common hallmark of CVDs, including ATS and ATS-associated risk factors, such as obesity, and T2D. These pathological conditions are characterized by a chronic low-grade inflammation that is involved in regulating immune cell plasticity and adaptation capabilities to the pathophysiological environment(s) of the host organism. Thus modifying/re-educating the immune system, represents a challenging new therapeutic approach. Emerging cancer immunotherapy with engineered T-cells is a successful example of

this clinical translation. Of note, NK cells represent a perfect tool for immunotherapy, due to their natural capability to reach/recognize cells that “have to be eliminated”. Immunotherapies, based on targeting and/or using NK cells, have been explored and include re-education with cocktails of activating (immune)-cytokines (IL-2, IL-12, IL-15), adoptive transfer of modified (BiKe, TriKe) NKs and generation of CAR-NKs. Considering that inflammation and immune cell plasticity are crucial hallmarks of both cancers and CVDs, the relevant question now is whether immunotherapy can be translated to CVDs. Recently, the contribution of diverse subpopulations of immune cells, beyond monocytes and macrophages, to CVDs, has been intensively investigated. Moreover, recent clinical trials have employed biological modifiers of proinflammatory cytokine function, such as targeting TNF $\alpha$  and IL-1 $\beta$ , in heart failure. Surprisingly, little is known about the role of NK cells. We here summarized the current knowledge on NK cells contribution to ATS, and to ATS-associated risk factors, such as T2D, stressing the need of acquiring more information in these fields, to enable designing future NK cells-oriented therapeutic approaches, including re-educating them, according to the CVD pathological setting and environments.

## AUTHOR CONTRIBUTIONS

Conceptualization: MTP, GS, and AB. Text drafting and editing: MTP, MC, MG, FR, LM, GG, GA, GS, and AB. Critical revision: MTP, LM, GG, GA, GS, and AB. Figure preparation: MTP and MC. Funds: AB. All authors contributed to the article and approved the submitted version.

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