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REVIEW ARTICLE

Cytotoxic Lymphocytes and Atherosclerosis. Significance, Mechanisms, and Therapeutic challenges

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Abstract

Cytotoxic lymphocytes encompass natural killer (NK) lymphocytes (cells) and cytotoxic T cells that include CD8+ T cells, NKT cells, gamma, delta (γδ)-T cells and human CD4+CD28- T cells. These cells play critical roles in inflammatory diseases and in controlling cancers and infections. Cytotoxic lymphocytes can be activated via a number of mechanisms that may involve dendritic cells, macrophages, cytokines or surface proteins on stressed cells. Upon activation, they secrete proinflammatory cytokines as well as anti-inflammatory cytokines, chemokines and cytotoxins to promote inflammation and development of atherosclerotic lesions including vulnerable lesions, which are strongly implicated in myocardial infarctions and strokes. Here we review mechanisms that activate and regulate cytotoxic lymphocyte activity, including activating and inhibitory receptors, cytokines, chemokine receptors-chemokine systems utilised to home to inflamed lesions and cytotoxins and cytokines through which they affect other cells within lesions. We also examine their roles in human and mouse models of atherosclerosis and mechanisms by which they exert their pathogenic effects. Finally, we discuss strategies for therapeutically targeting these cells to prevent development of atherosclerotic lesions and vulnerable plaques and the challenge of developing highly targeted therapies that only minimally affect the body's immune system, avoiding complications such as increased susceptibility to infections as they are currently associated with many immunotherapies for autoimmune diseases.

Non-standard Abbraviation

TCR	- T cell receptor
MHC	- Major Histocompablity Complex
NKG2D	- Natural-Killer Group 2, member D
TRAIL	- TNF-Related Apoptosis-Inducing Ligand
DNAM	- DNAX Accessory Molecule-1
RANTES	- Regulated on Activation, Normal T cell Expressed and Secreted
Clec9A	- C-type lectin domain family 9 member A

INTRODUCTION

Atherosclerosis is a disease of large elastic and muscular arteries that is responsible for most myocardial infarctions (MIs) including angina, ischemic strokes, and peripheral vascular disease. Collectively MIs and strokes are the leading cause of global death, responsible for 248 deaths/100,000 persons in 2013, representing 85.4% of all cardiovascular deaths and 28.2% of all mortalities (Barquera et al., 2015; Mortality & Causes of Death, 2015). Without significant new interventions these statistics are predicted to worsen with the world-wide increase in type 2 diabetes mellitus associated with obesity (Dutton & Lewis, 2015; Munnee, Bundhun, Quan & Tang, 2016) as obesity and type 2 diabetes mellitus are independent risk factors for MIs and strokes (Kalofoutis, Piperi, Kalofoutis, Harris, Phoenix & Singh, 2007; Kernan, Inzucchi, Sawan, Macko & Furie, 2013). Atherosclerosis is initiated by subendothelial accumulation of low density lipoproteins rich in cholesterol and apolipoprotein B at sites of disturbed flow, mostly at vessel bends and branch points, where diffuse intimal thickenings develop (Nakashima, Wight & Sueishi, 2008). Apoptotic and necrotic cells are characteristic features of human and mouse atherosclerotic lesions which increase with lesion progression (Otsuka et al., 2015). In vulnerable atherosclerotic lesions the necrotic core is composed of necrotic cells, cell debris and lipid and frequently constitutes more than 40% of a lesion; it is a significant contributor to plaque instability. Necrotic cells are largely the consequence of apoptotic cells undergoing secondary necrosis due at least in part to impaired efferocytosis, with apoptosis initiated by cytotoxins (Froelich, Metkar & Raja, 2004) and cytokines such as TNF-α, largely derived from cytotoxic cells (Tay et al., 2016) and with secondary necrosis recently shown to be mediated by caspase 3 (Rogers, Fernandes-Alnemri, Mayes, Alnemri, Cingolani & Alnemri, 2017). Apoptosis of smooth muscle cells within inflamed fibrous caps covering large necrotic cores is also a significant contributor to lesion instability as their loss results in collagen reduction, leading to fibrous cap thinning (Chen, Huang, Kyaw, Bobik & Peter, 2016; Yahagi et al., 2016).

Recent evidence indicates that cytotoxic lymphocytes play important roles in the pathology of atherosclerosis utilising cytotoxic mechanisms to promote vulnerable plaque development

and progression. Here we highlight the role of cytotoxic lymphocytes in atheroma development, including development of inflamed and unstable atheromas, focusing on the major cytotoxic lymphocyte populations- invariant NKT (iNKT) cells, NK cells, γδ-T cells, CD8+ T cells and human CD4+CD28- T cells. We first review their basic immunological characteristics including their activating and inhibitory receptors and their production of cytotoxic factors and cytokines, highlighting aspects of knowledge which has the potential to advance our knowledge of atheroma development, progression and provide the theoretical basis future therapies. We then review current knowledge of their involvement in atherosclerosis and finally consider pharmacological intervention strategies to prevent atheromas and vulnerable plaque development.

Immunological characteristics of cytotoxic lymphocytes

Major lymphocytes with cytotoxic effector function comprise NK cells, γδ-T cells, NKT cells, CD8 T cells and human CD4+CD28- T cells. Despite having similar hemopoietic origins, NK and γδ-T cells do not require antigen presentation for their activation and effector in function, rather they are activated by innate receptors. Also, γδ-T cells and NKT cells are considered to bridge the innate and adaptive immune systems. Here, we highlight basic aspects of the immunology of cytotoxic lymphocytes (Table 1), much of which has not been applied to atherosclerosis but is likely to impact on our understanding as to how they exert their pro-atherosclerotic effects, with potential for translation.

NK cells. NK cells largely function as part of the innate immune system. These cytotoxic cells develop independently of the thymus and reside in peripheral lymphoid organs. NK cell activity is regulated by activating and inhibitory receptors (Pegram, Andrews, Smyth, Darcy & Kershaw, 2011). Human NK cell inhibitory receptors are mainly killer cell immunoglobulin-like receptors (KIR) recognising MHC-I molecules whereas in mouse, Ly49 receptors perform similar functions. Activating receptors include NKp46, NKp30 and NKp44 as well as activating versions of KIR and Ly49 receptors (Pegram, Andrews, Smyth, Darcy & Kershaw, 2011). The activating receptor NKG2D binds a number of cellular cell surface

ligands induced by stress signals including MICA/B and Rae-1. Other activating receptors include DNAM-1, FcγRIII (CD16) (Watzl, 2014) and NKp80 (Welte, Kuttruff, Waldhauer & Steinle, 2006). Engagement of a single activating receptor is not sufficient to stimulate cytotoxicity or cytokine secretion; rather it is necessary to simultaneously engage at least two different activating receptors to initiate responses, with most effective responses initiated when receptors utilise different signaling pathways (Marcus et al., 2014). Acquisition of cytotoxicity also requires IL-15 (Fehniger et al., 2007; Lucas, Schachterle, Oberle, Aichele & Diefenbach, 2007). NK cells express multiple cytokine receptors and are activated by inflammatory cytokines such as IL-2, IL-12, IL-15 and IL-18. Cytokine "pre-activated" NK cells can be further activated by a single activating receptor, greatly increasing cytokine secretion or cytotoxicity (Tang et al., 2013). Activated NK cells produce multiple cytotoxins including TRAIL (Ochi et al., 2004), FasL (Chua, Serov & Brahmi, 2004) as well as granzyme B and perforin. They also produce proinflammatory cytokines IFN-γ, TNF-α, IL-2 and IL-8 (De Sanctis, Blanca & Bianco, 1997) and secrete chemokines MIP-1α, MIP-1β and RANTES (Fauriat, Long, Ljunggren & Bryceson, 2010). NK cells facilitate the differentiation of naïve CD4+ T cells into IFN-γ secreting Th1 T cells, by providing an early source of IFN-γ within lymph nodes, which is required for Th1 polarisation (Martin-Fontecha et al., 2004). They also promote cross-presentation of antigens to CD8+ T cells (Deauvieau et al., 2015). Like iNKT cells, NK cells are highly migratory, expressing a large number of chemokine receptors including CXCR1, CXCR3, CXCR4, CCR7 and CCR9 enabling them to migrate to sites of tissue inflammation, including atherosclerotic lesions (Berahovich, Lai, Wei, Lanier & Schall, 2006; Peng & Tian, 2014).

γδ-T cells. γδ-T cells are T cells that develop in the thymus and express unique T-cell receptors composed of one γ-chain and one δ-chain. They predominantly reside in epithelial and mucosa layers of the skin, intestine, lung, and tongue where they serve as a first line of defence against infections. Activation, largely but not exclusively by innate mechanisms, initiates or propagates immune responses via cytokine- or cytolytic-dependent mechanisms (Born, Reardon & O'Brien, 2006; Poggi & Zocchi, 2014). Mouse and human γδ-T cells possess many common characteristics that include innate receptor expression, antigen

presentation capabilities, cytotoxicity and cytokine production (Holderness, Hedges, Ramstead & Jutila, 2013; Vantourout & Hayday, 2013). γδ-T cells are composed of a number of subsets. In the mouse they are broadly subdivided into CD27+ and CD27- γδ-T cells and then further subdivided on the basis of different Vγ chains (Pang, Neves, Sumaria & Pennington, 2012). They are highly effective at killing stressed and tumour cells and produce large amounts of pro-inflammatory cytokines (Silva-Santos, Serre & Norell, 2015) . Thy are activated via their γδ-T cell and NK cell receptors but unlike αβ-T cells, antigen recognition by their T cell receptors does not require MHC molecules or CD1 (Chien & Konigshofer, 2007). They express multiple NK cell receptors including NKG2D, DNAM-1, NKp44 and FcγRIII (CD16) and are activated by stressed and/or infected cells expressing MHC I molecules such as Rae-1, nectin, and/or NKp44L (de Andrade, Smyth & Martinet, 2014; Groh, Steinle, Bauer & Spies, 1998). Activated γδ-T cells kill via FasL, TRAIL and granzyme B/perforin (Bonneville, O'Brien & Born, 2010). They are also activated by cytokines IL-1, IL18 and IL-23 and secrete large amounts of IFN-γ, TNF-α and IL-17 as well as Th2 cytokines (Bonneville, O'Brien & Born, 2010). They express chemokine receptors CCR7, CCR10, CXCR5 and respond to multiple chemokines (Kabelitz & Wesch, 2003). Activated γδ-T cells also influence other immune cells, enhancing NK cell mediated cytotoxicity (Maniar et al., 2010). They stimulate monocytes to differentiate into inflammatory dendritic cells (Eberl, Roberts, Meuter, Williams, Topley & Moser, 2009) and promote dendritic cell maturation (Leslie et al., 2002).

iNKT cells. iNKT cells are innate-adaptive hybrid cells expressing NK receptors as well as highly restricted T cell receptors (TCRs) that recognise lipid antigens presented by the transmembrane MHC class I-like CD1d glycoprotein. iNKT cells arise from the thymus, complete maturation in the periphery and are mainly found in the liver and spleen. Their TCRs recognise both bacterial and self-lipid antigen-CD1d complexes presented by antigen-presenting cells such as dendritic cells (Godfrey, Stankovic & Baxter, 2010). Mouse iNKT cells express the semi invariant TCRα Vα14Jα18 whilst human iNKT cells express Vα24Jα18 (Lantz & Bendelac, 1994). iNKT cells are classified into three subtypes depending on expression of co-receptors CD4 or CD8 (Seino & Taniguchi, 2005). Despite an inability to

definitively identify/characterise self-lipid antigens that activate NKT cells (Fox et al., 2009), there is strong evidence for such antigens in atherosclerosis and other inflammatory disorders (Li et al., 2016; Lombardi et al., 2010). iNKT cells can also be activated by non-TCR signals. iNKT cells constitutively express TIM-1 (T cell Ig-like mucin-like-1), a receptor for phosphatidylserine on apoptotic cells which stimulates cell proliferation and cytokine secretion (Lee et al., 2010). These cells express the cell stress ligand receptor NKG2D which directly activates or co-stimulates iNKT cells together with TCRs (Kuylenstierna et al., 2011). Engagement of the Fc gamma receptor (FcγRIII/CD16) also leads to activation, resulting in an antibody mediated inflammation (Kim, Kim & Chung, 2006). iNKT cells express a number of activating or inhibitory killer immunoglobulin-like (Ig) receptors (Patterson et al., 2008), including Ly49 receptors (Skold *et al.*, 2000) as well as natural cytotoxicity receptors NKp30 and NKp46 (Nguyen, Vanichsarn & Nadeau, 2008). Cytokines also activate iNKT cells either alone or in conjunction with TCRs (Kitamura et al., 1999). iNKT cells express receptors for IL-12 (Kitamura et al., 1999), IL-18 (Leite-De-Moraes et al., 1999), IL-21 (Coquet et al., 2007), IL-23 (Rachitskaya et al., 2008) and IL-25 (Terashima et al., 2008). iNKT cells are migratory lymphocytes expressing multiple chemokine receptors (Ho, Denney, Luhn, Teoh, Clelland & McMichael, 2008). Chemokine receptors expressed by these cells include CCR5, CCR6, CXCR3 and CXCR4; CCR4 is predominately expressed by CD4+ iNKT cells (Kim, Johnston & Butcher, 2002; Thomas et al., 2003).

Activated NKT cells produce Th1 and Th2 cytokines including IFN-γ, TNF-α, IL-2 as well as IL-17 and IL-4, IL-10 and IL-13. Factors that pre-determine cytokines secretion include CD4 expression and tissue location (Coquet et al., 2008). Their pattern of cytokine expression is more dependent on the nature of the CD1d+ antigen presenting cell than on the lipid antigen (Bai et al., 2012). Activated iNKT cells are potent killer cells expressing the cytotoxins perforin and granzyme B (Nguyen, Vanichsarn & Nadeau, 2008) , FasL (CD178) (Wingender, Krebs, Beutler & Kronenberg, 2010) and TRAIL (Huang et al., 2014). Their cytotoxic actions are greatly enhanced by IL-4 (Kaneko et al., 2000) and IL-15 (Liu et al., 2012).

Cytotoxic CD8+ T lymphocytes. CD8+ T cells are lymphocytes that express the CD8 coreceptor and recognise antigen peptide-MHC class I complexes presented by antigen-presenting cells such as dendritic cells. CD8+ T cells develop in the thymus and reside in secondary lymphoid organs. They play key roles in many inflammatory diseases (Carvalheiro, Duarte, Silva-Cardoso, da Silva & Souto-Carneiro, 2015; Kyaw et al., 2013; Walter & Santamaria, 2005) as well as in cancers and infections including cytomegalovirus (CMV) infection and Epstein-Barr virus (EBV) infections which can be associated with atherosclerotic lesions (Ahmadzadeh et al., 2009; Brincks, Katewa, Kucaba, Griffith & Legge, 2008; Khanna & Burrows, 2000; Klenerman & Oxenius, 2016). They exist as a number of subsets that include short-lived effectors (with high migratory ability and high capacity to produce cytokines and cytotoxins), effector memory cells (which accumulate in peripheral organs and become effectors upon re-encounter with antigens), central memory cells (which rapidly proliferate and produce abundant cytokines but few cytotoxic molecules upon antigen encounter), tissue resident memory cells (that have very limited migratory capacity, hence permanently reside in peripheral tissue, producing cytokines and cytotoxic molecules upon antigen encounter) (Bisikirska, Colgan, Luban, Bluestone & Herold, 2005; Carvalheiro, da Silva & Souto-Carneiro, 2013; Gupta & Gollapudi, 2007; Mackay et al., 2013; Marzo, Yagita & Lefrancois, 2007) and regulatory cells (Akane, Kojima, Mak, Shiku & Suzuki, 2016; Bisikirska, Colgan, Luban, Bluestone & Herold, 2005). Naïve circulating CD8+ T cells are activated by antigen presenting cells such as CD8α+ dendritic cells presenting peptide antigens on MHC class I molecules through a process called cross-presentation (Joffre, Segura, Savina & Amigorena, 2012). CMV and EBV antigens activate, reactivate and differentiate CD8+ T cells in antigen-specific cytotoxic T cell-mediated responses (Khanna & Burrows, 2000; Klenerman & Oxenius, 2016). Activation can be enhanced by cytokines such as IL-1β (Ben-Sasson, Wang, Cohen & Paul, 2013), IL-2, IL-12, IL-15 and IL-21 (Henry, Ornelles, Mitchell, Brzoza-Lewis & Hiltbold, 2008; Moroz, Eppolito, Li, Tao, Clegg & Shrikant, 2004). Activation can also be initiated in a TCR independent manner (Freeman, Hammarlund, Raue & Slifka, 2012). Like other killer cells, CD8+ T cells express killer-like receptors including NKG2D (Verneris, Karimi, Baker, Jayaswal & Negrin, 2004), Ly49 receptors (McMahon & Raulet, 2001) and activating and

inhibitory KIRs (Bjorkstrom et al., 2012) with inhibitory KIRs mostly confined to effector CD8+ T cells (Arlettaz, Degermann, De Rham, Roosnek & Huard, 2004). However, responses of CD8+ T cells following activation of these receptors is only apparent after activation via T cell receptors (Arlettaz, Degermann, De Rham, Roosnek & Huard, 2004; Marzo, Yagita & Lefrancois, 2007). Other cell surface CD8+ T cell molecules important in regulating activity include programmed cell death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), T cell immunoglobulin and mucin domain-3 (TIM-3) and lymphocyte activity gene-3 (LAG-3) (Gros et al., 2014). Activated effector CD8+ cells can be subdivided based on killer cell lectin-like receptor G1 (KLRG-1) expression with KLRG-1^{hi} expression marking short-lived effector cells and $KLRG^{lo}$ marking memory precursor cells (Ye, Turner & Flano, 2012). They can express a variety of selectins, chemokine receptors and integrins including PSGL-1 and CD44, CCR4, CCR5, CCR7, CCR9, CDR10, CXCR3, VLA-1 and LFA-1 enabling them to traffic and localise in different regions of the body (Nolz, Starbeck-Miller & Harty, 2011). Effector CD8+ T cells secrete pro-inflammatory cytokines IFN-γ and TNF-α, IL-17A, IL-17F, IL-21 and IL-22 (Yu et al., 2013) and may also secrete IL-14, IL-5 and IL-10. Like the other killer cells they express perforin and granzyme (Janas, Groves, Kienzle & Kelso, 2005), FasL (Kilinc, Rowswell-Turner, Gu, Virtuoso & Egilmez, 2009) and TRAIL (Brincks, Katewa, Kucaba, Griffith & Legge, 2008). Highly activated cytotoxic CD8+ T cells also secrete IL-10 to dampen inflammatory responses whilst still exerting potent cytotoxic effects (Noble, Giorgini & Leggat, 2006; Trandem, Zhao, Fleming & Perlman, 2011). In contrast to effector CD8+ T cells, regulatory CD8+ T cells attenuate inflammation by directly killing activated T cells (Akane, Kojima, Mak, Shiku & Suzuki, 2016).

CD4+CD28- T cells. CD4+CD28- T cells are highly differentiated human effector memory CD4+ T cells that have downregulated the costimulatory molecule CD28 due to loss of a CD28-specific initiator complex (Vallejo, Bryl, Klarskov, Naylor, Weyand & Goronzy, 2002; Vallejo, Nestel, Schirmer, Weyand & Goronzy, 1998). Their development and maturation process are similar to CD8 T cells. They are most abundant in in elderly humans over 60 years of age (Vallejo, Nestel, Schirmer, Weyand & Goronzy, 1998) but can also found in

younger adults with chronic inflammatory disorders. Their numbers are increased in humans with rheumatoid arthritis (Bryl, Vallejo, Weyand & Goronzy, 2001), type 2 diabetes (Shi, Du, Wang, Chen & Zhang, 2013; Warrington, Takemura, Goronzy & Weyand, 2001) and following cytomegalovirus infection (van Leeuwen et al., 2004). Unlike other cytotoxic cells, these cells are not expressed in rodents. Despite the loss of CD28 these cells are not anergic and proliferate in response to stimulation. They are autoreactive to ubiquitously distributed autoantigens and exhibit a restricted TCR diversity (Schmidt, Goronzy & Weyand, 1996). Surprisingly, they are resistant to the suppressive actions of CD4+CD25+Foxp3+ regulatory T cells (Thewissen, Somers, Hellings, Fraussen, Damoiseaux & Stinissen, 2007) and also are resistant to activation induced apoptosis (Vallejo, Schirmer, Weyand & Goronzy, 2000) due to high expression of the anti-apoptosis factor Bcl-2 (Schirmer, Vallejo, Weyand & Goronzy, 1998).

CD4+CD28-T cells express multiple chemokine receptors including CCR5, CCR7, CXCR4 and CX3CR1 enabling them to home to lymphoid organs and sites of tissue inflammation including atherosclerotic lesions (Maly & Schirmer, 2015; Zhang, Nakajima, Goronzy & Weyand, 2005). Cytokines such as IL-12 regulate their pattern of chemokine receptor expression (Zhang, Nakajima, Goronzy & Weyand, 2005). CD4+CD28- T cells are proinflammatory and cytotoxic, expressing IFN-γ and TNF-α (Pieper et al., 2014) as well as perforin and granzyme B (Betjes, Huisman, Weimar & Litjens, 2008; Namekawa, Wagner, Goronzy & Weyand, 1998). They respond to IL-15 by upregulating granzyme B and perforin expression, increasing their cytotoxicity (Alonso-Arias et al., 2011). In many ways these cells mimic the effects of other cytotoxic lymphocytes, expressing cell surface markers CD11b and CD57 found on NK cells (Chapman et al., 1996; Schmidt, Goronzy & Weyand, 1996). They also express NK cell activating receptors which markedly increase their activity when T cell activation is suboptimal; receptors expressed include DNAM-1 and CRACC (Fasth, Bjorkstrom, Anthoni, Malmberg & Malmstrom, 2010), NKG2D (Groh, Bruhl, El-Gabalawy, Nelson & Spies, 2003) and the killer cell immunoglobulin-like receptor (KIR) KIR2DS2 (Yen et al., 2001). Detailed studies of their significance in inflammatory disorders including atherosclerosis has been greatly hampered by the lack of such cells in mice.

Together these basic immunology studies on the different cytotoxic lymphocytes indicate that they are highly migratory and their accumulation in lesions during development of atherosclerosis is most likely dependent on chemokines. Their ability to influence vulnerable lesions is largely but not exclusively dependent on their presence in lesions, where they have the potential to influence development of vulnerable atherosclerotic lesion by a number of common mechanisms involving cytotoxins. In lesions, cytotoxic lymphocytes are also very likely activated or co-activated by a number common killer cell receptor dependent mechanisms. However, knowledge of the relative importance of precise mechanisms in atherosclerosis is still rather limited (see Cytotoxic Lymphocytes and Development of Atherosclerosis) and further studies are warranted to more precisely define the best therapeutic targets to effectively prevent their deleterious actions.

Cytotoxic lymphocytes and development of atherosclerosis

In the very early stages of development of atherosclerosis, circulating leukocytes including lymphocytes migrate into intimal layers via vascular adhesion molecules upregulated as a result of endothelial dysfunction. Subsequent chemokine upregulation in atherosclerotic lesions may also contribute to lymphocyte recruitment. With progression, tertiary lymphocyte organs that develop in adventitial layers may also contribute to lymphocyte recruitment and activation. Antigens implicated in atherosclerosis are thought to be multiple in origin, but current understanding on antigens involved in atherosclerosis is limited, with the exception of modified LDL and heat shock protein60. Necrotic materials are thought to be important, yet their role in atherosclerosis remains to be elucidated.

Human atherosclerotic lesions are histologically divided into 6 categories; type I, presence of foam cells in the intimal layer, type II, fatty streak formation, type III, pre-atheroma, type IV, atheroma, type V, fibrous cap formation with or without calcification and type VI, rupture with thrombus formation. Mechanistic insights as to how cytotoxic lymphocytes influence development and progression of established atherosclerotic lesions requires animal models. Several genetically modified mouse models have been developed including $ApoE^{-/-}$ mice and

LDLR$^{-/-}$ mice, transgenic ApoE3-Leiden mice and HuBTg$^{+/+}$ LDLR$^{-/-}$ mice (Kapourchali, Surendiran, Chen, Uitz, Bahadori & Moghadasian, 2014). Among these genetically modified mouse models, ApoE$^{-/-}$ and LDLR$^{-/}$ atherogenic mouse models are the most widely used as the lesions that develop in both mouse models are morphologically similar to human atherosclerotic lesions. Both stage IV and V lesions will take 14-20 weeks of high fat diet feeding to generate in mouse models and stage. Stage VI lesions are only seen in the innominate artery, however mouse lesions, unlike human lesions, appear to be more resistant to rupture Therefore recently a model of plaque rupture has been developed using these mice (Chen et al., 2013). LDLR$^{-/-}$ mice have an advantage over ApoE-/- mice in that it is much easier to generate mixed bone marrow chimeric mouse models with specific gene deletions in immune cells.

Cytotoxic lymphocytes accumulate in both mouse and human atherosclerotic lesions, and many appear to be involved in nearly all stages of atherosclerosis- development, progression of established lesions and vulnerable plaque development; their roles in plaque rupture are yet to be elucidated. It is also important to investigate where and how these immune cells are activated and their site of action during development/progression of advanced atherosclerosis as this information is not available currently. This knowledge will provide important insights as to how best to therapeutic target these cells. Too frequently pre-clinical studies have focused only on early development of atherosclerosis whilst clinical studies based on results of pre-clinical studies have focused on progression of vulnerable lesions and plaque rupture-myocardial infarctions and/or strokes. Cytotoxic lymphocytes including NK cells, iNKT cells and CD8+ T cells have the potential to not only influence early development of atherosclerotic lesions but also advanced atherosclerotic lesions, particularly vulnerable lesions and plaque rupture, frequently acting locally within lesions or within lymph nodes and producing proinflammatory cytokines, chemokines and/or cytotoxins.

NK cells. NK cells have been strongly associated with atherosclerosis development atherosclerosis in humans and genetically modified mice. They are present in human and mouse atherosclerotic lesions (Bobryshev & Lord, 2005b; Whitman, Rateri, Szilvassy,

Yokoyama & Daugherty, 2004), and are recruited to developing lesions by chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) and fractalkine (CX3CL1) (Allavena et al., 1994; Yoneda et al., 2000) to promote atherosclerosis development (Aiello et al., 1999; Lesnik, Haskell & Charo, 2003) . In humans with atherosclerosis, expression of the activating cell receptor CD160, which triggers cytotoxicity and cytokine secretion, is increased on circulating NK cells and suggested to contribute to atherosclerosis (Le Bouteiller et al., 2011; Zuo, Shan, Zhou, Yu, Liu & Gao, 2015). Also, NK cells expressing the activating receptor NKG2C are increased in seropositive patients for human cytomegalovirus and associate with high-risk carotid atherosclerotic plaques (Martinez-Rodriguez et al., 2013). Other studies indicate that patients with severe atherosclerosis have greater numbers of circulating NK cells (Clerc & Rouz, 1997); elderly patients with peripheral artery disease also have greater numbers of circulating NK cells but with reduced cytotoxic capability (Bruunsgaard, Pedersen, Schroll, Skinhoj & Pedersen, 2001). Immediately after non-STEMI myocardial infarction NK cell numbers are low, and then increase over the ensuing 12 months possibly contributing to myocardial infarction accelerated atherosclerosis; their failure to increase in some patients is associated with persistent low grade inflammation (Backteman, Ernerudh & Jonasson, 2014). In other studies circulating but not lymph node CD56+ NK cells are reduced in patients with acute coronary syndrome compared to patients with stable angina (Backteman, Andersson, Dahlin, Ernerudh & Jonasson, 2012). Given that NK cells are activated in periodontitis (Kramer et al., 2013; Wang, Zhang, Xu & Jin, 2016) and periodontitis has been associated with cardiovascular disease (Tonetti, Van Dyke & Working group 1 of the joint, 2013), it is surprising that the role of NK cells in periodontitis accelerated atherosclerosis has not been investigated. Similarly whether NK cells contribute to cytomegalovirus aggravated atherosclerosis has not been investigated (Beziat et al., 2013; Vliegen, Duijvestijn, Grauls, Herngreen, Bruggeman & Stassen, 2004).

In contrast to these association studies in humans, mechanistic studies defining the precise role of NK cells in atherosclerosis are more limited. Early studies in mice with a beige mutation indicated that NK cells might be atheroprotective (Schiller, Boisvert & Curtiss, 2002). However these mice have a complex phenotype with defects in cell function not only

restricted to NK cells but also affecting neutrophils and other cells and this could have affected outcome (Getz, 2002). Subsequently, Ly49A transgenic mice were used. These mice express the Ly49A inhibitory receptor under the control of the granzyme A promoter and whilst the authors concluded that NK cells contribute to development of atherosclerosis, the possibility that Ly49A affected other proatherogenic cells such as cytotoxic T lymphocytes cells was not excluded (Whitman, Rateri, Szilvassy, Yokoyama & Daugherty, 2004); Ly49A is known not only to inhibit NK cells but also to prevent CD8+ T cell activation (Oberg, Eriksson, Fahlen & Sentman, 2000). More recent studies using anti-Asialo-GM1 antibodies to deplete NK cells in hyperlipidemic ApoE-/- mice also indicate that NK cells promote development of atherosclerosis, studies supported by gain of function experiments (Selathurai et al., 2014). As anti-Asialo-GM1 antibodies might deplete other immune cells, we carried out a gain of function experiment where adoptive transfers involving transfer of wild type NK cells and NK cells deficient in IFN-γ, granzyme B and perforin into triple knockout mice (i.e., T, B and NK cell-deficient ApoE-/- mice) indicated that cytotoxic effects of NK cells are pro-atherogenic and promote necrotic core development. However, given that lymphocyte deficient mice were used, a pro-atherogenic role for NK cells involving secretion of IFN-γ could not be excluded. In immune competent mice NK cell derived IFN-γ promotes CD4+ Th1 priming (Martin-Fontecha et al., 2004). Thus in immune competent mice NK cells might also promote atherosclerosis via a CD4+ T cell dependent mechanism. How NK cells are activated during development of atherosclerosis is unknown but given the fact that macrophage foam cells express ligands for NKG2D receptors (Ikeshita, Miyatake, Otsuka & Kasahara, 2014), activation within lesions via NKG2D receptors is highly likely.

γδ-T cells. To date few studies have addressed the role of γδ-T cells in atherosclerosis despite their identification in human atherosclerotic lesions more than 20 years ago (Kleindienst, Xu, Willeit, Waldenberger, Weimann & Wick, 1993). In ApoE-/- mice hyperlipidemia increases γδ-T cells but aortic lipid accumulation is unaffected, suggesting no role in early lipid lesion/fatty streak development (Cheng, Wu & Hedrick, 2014). Others have shown that γδ-T cells are the most abundant T cell within atherosclerotic lesions despite being a very minor T cell population and their deletion reduces atherosclerotic lesion size (Vu, Tai, Tatro, Karas,

Huber & Beasley, 2014). It has been suggested that γδ-T cell derived IL-17 contributes to atherosclerosis. Their role in progression of established lesions and plaque rupture has not been investigated.

iNKT cells. iNKT cells migrate to developing atherosclerotic lesions and are present as a minor cell population in mouse atherosclerotic lesions (To, Agrotis, Besra, Bobik & Toh, 2009). In human atherosclerotic lesions, iNKT cells are also a minor population and originally identified as CD161+ T cells (Bobryshev & Lord, 2005a). This however does not distinguish iNKT cells from CD161+ Foxp3+ T cells or other CD161+ T cell subtypes (Gonzalez, Herrera, Juarez, Salazar-Lezama, Bobadilla & Torres, 2015; Pesenacker, Bending, Ursu, Wu, Nistala & Wedderburn, 2013), but more recent studies using anti-TCR Vα24 antibodies have definitively demonstrated their presence in human lesions (Kyriakakis et al., 2010). Early studies using loss and gain of function provide strong evidence that iNKT cells are important for development of atherosclerosis. Loss of function studies involving hyperlipidemic NKT cell-deficient CD1d-/- chimeric LDLR-/- mice as well as CD1d-/-ApoE-/- mice demonstrated smaller lesion development in the absence of iNKT cells (Nakai et al., 2004; Tupin et al., 2004); mice deficient in invariant Vα14 NKT cells also exhibit reduced atherosclerosis (Rogers et al., 2008). Increasing atherosclerosis by administering pharmacological doses α-GalCer to activate NKT cells to provide evidence that iNKT cells promote atherosclerosis (Tupin et al., 2004), is complicated by extensive bystander activation of T, B, NK and γδ-T cells (Kitamura et al., 2000; Paget, Chow, Duret, Mattarollo & Smyth, 2012; Smyth, Wallace, Nutt, Yagita, Godfrey & Hayakawa, 2005; Tupin et al., 2004); these lymphocytes also exert iNKT cell independent pro-atherogenic effects (Perry & McNamara, 2012; Selathurai et al., 2014; Tse, Tse, Sidney, Sette & Ley, 2013; Vu, Tai, Tatro, Karas, Huber & Beasley, 2014). More recent studies indicate that iNKT cells promote atherosclerosis largely independently of bystander T, B or NK cell activation (Li et al., 2015). CD4+ iNKT cells have been identified as the proatherogenic subtype in mice. This subtype expresses lower concentrations of Ly49 inhibitory receptors-Ly49A, Ly49C/I and Ly49G2 compared to other subtypes, possibly explaining their greater pro-atherogenic activity (To, Agrotis, Besra, Bobik & Toh, 2009). In contrast human CD4+ iNKT cells exhibit a somewhat

different pattern of killer receptors with increased expression of activating receptors NKp30 and NKp46. These cells are also highly cytotoxic, killing CD4+CD25^{hi}CD27$^{lo/-}$ regulatory T cells to promote inflammation (Nguyen, Vanichsarn & Nadeau, 2008). Although early studies suggested that pro-inflammatory cytokines such as IFN-γ promote iNKT cell mediated atherosclerosis (Tupin et al., 2004), more recent studies indicate a major role for cytotoxins (Li et al., 2015). CD4+ iNKT cells promote atherosclerosis and development of large necrotic cores via mechanisms dependent on perforin and granzyme B rather than cytokines (Li et al., 2015). The cytotoxic actions of iNKT cell increase lesion apoptotic cell numbers and necrotic cores which in turn augment inflammation and atherosclerosis development via a sterile inflammatory response (Li et al., 2016). iNKT cell activation during development of atherosclerosis is at least in part dependent on lipid antigens activating TCRs, indicated by findings that a CD1d-dependent lipid antagonist to iNKT cells attenuates both development and progression of established atherosclerosis (Li et al., 2016). Although the lipid antigens have not been identified, some appear to be carried by lipoproteins in the circulation and may also reside within atherosclerotic plaques (VanderLaan et al., 2007). iNKT cells are also important in lipopolysaccharide (LPS) accelerated atherosclerosis (Ostos, Recalde, Zakin & Scott-Algara, 2002), a model resembling infection associated atherosclerosis. Bacterial infections involving *Chlamydia pneumoniae*, *Porphyromonas gingivalis* and *Helicobacter pylori* have been associated with accelerated atherosclerosis in humans (Ameriso, Fridman, Leiguarda & Sevlever, 2001; Campbell & Rosenfeld, 2014; Hussain, Stover & Dupont, 2015). iNKT cells constitutively express TLR4 on their cell surface and direct engagement of TLR4 on iNKT cells promotes inflammatory disorders (Kim, Kim, Kim, Oh & Chung, 2012). Recently iNKT-derived IFN-γ has been shown to induce apoptosis of marginal zone B cells, suggesting a regulatory iNKT subset. The authors implicate expansion of marginal zone B cells in relation to loss of iNKT-derived IFN-γ in increased atherosclerosis in long-term high fat feeding (Soh et al., 2016).

Cytotoxic CD8+ T lymphocytes. Multiple lines of evidence indicate that CD8+ T cells contribute to atherosclerosis and vulnerable plaque development. Correlative studies in humans with coronary artery disease imply important roles for cytokine and cytotoxin

producing CD8+ T cells in advanced coronary artery atherosclerosis (Bergstrom, Backteman, Lundberg, Ernerudh & Jonasson, 2012; Hwang et al., 2016; Kolbus et al., 2013; Longenecker et al., 2013). In advanced human lesions, CD8+ T cells predominate over CD4+ T cells (Gewaltig, Kummer, Koella, Cathomas & Biedermann, 2008; Paul, Paul & Kuruvilla, 2016; Rossmann et al., 2008) and concentrate around shoulder regions and fibrous caps (Paul, Paul & Kuruvilla, 2016). They are also abundant in mouse atherosclerotic lesions (Kyaw et al., 2013). Oxidised LDL and heat shock protein peptides have been implicated in their activation (Kolbus et al., 2010; Rossmann et al., 2008; Wu, Giscombe, Holm & Lefvert, 1996). Activation does not appear to involve antigen cross presentation by CD8α+ dendritic cells (Legein et al., 2015), but may involve other antigen presenting cells such as γδ-T cells which are present in lesions. Despite such associations, early studies in mice led to conflicting results on the significance of CD8+ T cells (Elhage et al., 2004a; Fyfe, Qiao & Lusis, 1994), with conclusions largely based on poorly understood complex mouse models (Araujo et al., 1995; Schaible, Collins, Priem & Kaufmann, 2002). An atheroprotective role was suggested by increased atherosclerosis in β2m deficient mice. But β2m deficient mice disrupt CD8α/α, not CD8α/β T cell development and develop iron overload aggravating atherosclerosis (Araujo et al., 1995). While genetic knockouts of CD8 and tap1 showed no change in lesions (Elhage et al., 2004b), it is likely that CD4 T cell expansion during development compensated for the CD8 T cell deficiency. More recent independent studies using specific CD8+ T cell depleting antibodies indicate pro-atherogenic roles for CD8+ T cells (Cochain et al., 2015; Kyaw et al., 2013). Activated CD8+ T cells promote atherosclerosis and vulnerable plaque development by cytotoxic mechanisms involving perforin and granzyme B as supported by adoptive transfer studies with CD8 T cells deficient in perforin and granzyme B that failed to promote atherosclerosis development (Kyaw et al., 2013). These adoptive transfer studies suggest that CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques by perforin and granzyme B mediated apoptosis of macrophages, smooth muscle cells and endothelial cells that in turn leads to secondary necrosis and necrotic core formation. These studies also suggest that CD8 T cell-mediated cell death initiates a sterile inflammatory response (Chen & Nunez, 2010) as the transfer of CD8 T cells deficient in perforin and granzyme B led to reduction of inflammatory MCP1, IL1β, IFNγ and VCAM-

1. A role for TNF--α produced by CD8 T cells is also supported by adoptive transfer studies with CD8 T cells deficient in TNF-α that failed to promote atherosclerosis development (Kyaw et al., 2013). While adoptive transfer of CD8 T cells deficient in IFN-γ suggest that CD8 T cell derived IFN-γ has no role in atherosclerosis (Kyaw et al., 2013), other studies indicate a role for CD8+ T cell derived IFN-γ in atherosclerosis development, regulating monopoiesis and circulating inflammatory Ly6C^{hi} monocytes (Cochain et al., 2015). A role for CD8 + T cells has been suggested in Chlamydia pneumoniae-accelerated atherosclerosis (Zafiratos, Manam, Henderson, Ramsey & Murthy, 2015). It is also possible that CMV and EBV antigen-specific CD8+ T cells may contribute to pathogen-enhanced atherosclerosis as such viral DNAs have been detected in atherosclerotic lesions (Ibrahim et al., 2005), limited data is available linking CMV and EBV infections to atherosclerosis. Recently PD-1 and TIM-3 have been implicated in regulating CD8+ T cell function in atherosclerosis in humans, by affecting TNF-α and IFN-γ production (Qiu, Wang, Dai, Wang, Ou & Quan, 2015). In contrast to these pro-atherogenic effects of CD8+ T cells, CD8 T cell cytotoxicity increased by ApoB-100 targeted immunization modulates the functions of dendritic cells, monocytes and macrophages (Chyu et al., 2012; Cochain & Zernecke, 2016; Honjo et al., 2015), suggesting a possible favourable effect in atherosclerosis, but their relative relevance in vivo is uncertain.

Hypertension, hypercholesterolemia and diabetes mellitus are major risk factors for plaque development and rupture (Bentzon, Otsuka, Virmani & Falk, 2014). Hypertension elevates activated CD8+ T cell numbers in human subjects (Itani et al., 2016; Youn et al., 2013) and increases CD8+ T cell accumulation in mouse aortas, increasing augmented perivascular inflammation and augmented endothelial dysfunction (Itani et al., 2016; Mikolajczyk et al., 2016). Together with early CD8+ T cell activation in hypercholesterolemic mice (Kolbus et al., 2010) and CD8+ T cell-induced macrophage accumulation in metabolic diseases (Nishimura et al., 2009), cytotoxic CD8+ T cells may contribute, at least in part, to the mechanisms by which these risk factors promote plaque development and rupture.

CD4+CD28- T cells. Association studies suggest a role for CD4+CD28-T cells in human atherosclerosis (Liuzzo et al., 2000; Liuzzo et al., 1999; Nakajima et al., 2002). These cells express multiple cytotoxins including granzymes A and B, perforin and granulysin as well as proinflammatory cytokines IFN-γ and TNF-α (Teo et al., 2013). They are highly resistant to apoptosis (Kovalcsik, Antunes, Baruah, Kaski & Dumitriu, 2015) and appear to accumulate in vulnerable coronary atherosclerotic plaques (Nakajima et al., 2003). Activation appears to be triggered by heat shock protein 60 (HSP60) antigens (Zal et al., 2008; Zal et al., 2004) and by the co-stimulatory molecules Ox40 (CD134) and 41BB (CD137) present on CD4+CD28- T cells in acute coronary syndromes (Dumitriu et al., 2012). Cytotoxic CD4+ T cell responses have been reported in latent and chronic viral infections (Walton, Mandaric & Oxenius, 2013), but whether there is any role for virus-specific CD4+ CD28- T cells in atherosclerosis is not known. CD4+ CD28- T cells are also activated by IL-12 (Zhang et al., 2006). Cytotoxic CD4 T cells have been reported to be stimulated by plasmacytoid dendritic cell-derived interferon-α to induce expression of TRAIL and kill vascular smooth muscle cells in carotid atheromas (Niessner, Sato, Chaikof, Colmegna, Goronzy & Weyand, 2006). Despite these associations their role in atherosclerosis and vulnerable plaque development remains to be defined.

Collectively cytotoxic cells can effectively target and kill lesion cells by inducing apoptosis and necrosis via three mechanisms, i.e. (1) cytotoxins such as perforin- and GranzymeB-mediated, (2) Fas-FasL or TRAIL-mediated and (3) cytokine-induced mechanisms (Figure 1). Macrophages, major constituent of lesion cellular contents are major target cells killed by cytolytic mechanisms, suggesting an important role of cytotoxic cells in generating of necrotic core and vulnerable plaques. As vascular smooth muscle cells and endothelial cells can also be targeted by cytotoxic cells, cytotoxic cells are also important in destabilising plaque and inducing plaque rupture leading to MIs or strokes. Thus targeting against cytotoxic cells may be therapeutically beneficial in preventing premature atherosclerosis-related deaths.

Pharmacologically targeting cytotoxic lymphocytes in atherosclerosis

Specific cytotoxic lymphocyte depletion could theoretically be considered as one therapeutic approach to limit their pro-atherogenic actions during atheroma and vulnerable plaque development. However, such an approach is difficult to justify in essentially healthy immune competent subjects as it would make individuals highly susceptible to life threatening viral and bacterial infections. Rather, more specific approaches that target specific receptors on individual cell types or even unique cell types may be more appropriate to attenuate atherosclerosis and vulnerable plaque development. Towards this aim pharmacological targeting could involve the use of either small molecules or long acting biologicals (e.g. antibodies) which are becoming increasingly accepted in atherosclerosis therapy (Stein et al., 2012). Targeting iNKT cell and CD8+ T cell activation may be an effective therapeutic strategy (Figure. 2A). Recently a CD1d lipid antagonist was shown to prevent iNKT cell activation in atherosclerotic mice and to reduce lesion inflammation and necrosis; the antagonist was also highly effective in preventing not only lesion development but also progression of established lesions (Li et al., 2016). Targeting antigen presentation with biologicals such as anti-CD1d antibodies may also be an effective therapeutic strategy to prevent iNKT activation in atherosclerosis (Duthie, Kahn, White, Kapur & Kahn, 2005); an anti-human CD1d inhibitory antibody has recently been developed (Nambiar et al., 2015). Such approaches to limit activation of killer cells seems to impact on immune defence against infectious agents, but killer cells are able to respond against pathogens microbes via various innate receptors without utilising TCR- or CD1d-dependent activation. Therefore targeting against activation of iNKT and CD8+ T cells will not be expected to compromise host defence systems. β2-adrenoceptors have recently been shown to be elevated on human CD8+ effector memory T cells and β2-adrenoceptor activation decreases IFN-γ and TNF-α secretion as well as cytotoxic activity of human and murine CD8+ T cells (Figure. 2A). Also, long acting β2-agonists such as salmeterol are effective in vivo in suppressing cytokine secretion by CD8+ T cells (Estrada, Agac & Farrar, 2016). Whether treatment with β2-agonists is effective in preventing CD8+T cell activation and its consequences in atherosclerosis remains to be determined. Necrotic cells are abundant in advanced lesions and very likely contribute to the cytotoxic actions of CD8+ T cells with lesion dendritic cells utilising Clec9A to cross-present necrotic cell remnant antigens to CD8+ T cells. It is tempting to speculate that

preventing necrotic cell sensing by dendritic cells expressing Clec9A may also be an effective strategy to prevent CD8+ T cell activation in advanced lesions (Figure. 2A); Clec9A favours antigen cross presentation to cytotoxic CD8+ T cells (Zelenay et al., 2012). Preventing migration of cytotoxic lymphocytes to atherosclerotic lesions could also be an effective therapeutic strategy to attenuate atherosclerosis (Figure. 2B), but will require definition of the chemotactic factors that are responsible for migration of cytotoxic lymphocytes to lesions. A large number of receptor antagonists to G-protein coupled chemokine receptors have been developed including CCR2, CCR5, CXCR3, CXCR4, CCR1 and CCR3 but have not been assessed in atherosclerosis (O'Boyle et al., 2012; Suzaki et al., 2008; Zweemer et al., 2013). The findings that NKG2D ligands are upregulated in human plasma and in human and mouse atherosclerotic lesions together with the findings of NKG2D deletion studies in mice indicate that NKG2D receptors are a viable therapeutic target (Figure. 2C) (Xia et al., 2011). Anti-NKG2D inhibitory antibodies are available (Kjellev, Haase, Lundsgaard, Urso, Tornehave & Markholst, 2007; Steigerwald et al., 2009) but their effects on development and progression of established atherosclerosis and on vulnerable plaque development have not been assessed. One potential limitation of targeting NKG2D is that receptor expression may not be restricted to a single cell type but rather expressed on multiple cytotoxic lymphocytes in the periphery. Similarly KIR activating and inhibitory receptors could be targeted to limit proatherogenic effects (Figure. 2C). Such receptors have been targeted to increase the cytotoxicity of lymphocytes in cancer (Benson et al., 2011); antibodies could be developed to activate inhibitory receptors or inhibit activating receptors suppressing cytotoxic lymphocyte activity and attenuating atherosclerosis and vulnerable plaque development.

Given that cytotoxic lymphocytes accumulate within atherosclerotic lesions, more specific targeting of cytotoxic lymphocytes residing within lesions might also be considered as such an approach would not affect cytotoxic lymphocyte activity in other tissues or in the circulation. There is now a strong body of evidence for tissue resident memory CD8+ T cells and NK cells with unique gene expression patterns and receptor profiles characteristic of a particular tissue (Melsen, Lugthart, Lankester & Schilham, 2016; Park & Kupper, 2015;

Sojka et al., 2014; Wakim et al., 2012). Clearly additional studies will be required to determine whether such cytotoxic lymphocytes with unique protein expression profiles are present in atherosclerotic lesions and developing vulnerable plaques. Such an approach offers unique pharmacological opportunities to suppress atherosclerosis and vulnerable plaque development without significantly affecting other components of the immune system, minimising the possibility of any unwanted immune suppressive effects such as increased susceptibility to infections.

Summary and conclusions

Vulnerable atherosclerotic plaques characterised by large necrotic cores and increased lesion apoptosis are an important concern in atherosclerosis management because their rupture initiates thrombotic occlusion of vital arteries causing heart attacks and strokes. Cytotoxic lymphocytes in human and mouse atherosclerotic lesions are of interest because of their ability to induce apoptosis that leads to secondary necrosis. Further research is warranted to precisely and definitively define the roles of each cytotoxic lymphocyte in development, progression and rupture of vulnerable atherosclerotic plaques. Clearly global depletion of a cytotoxic lymphocyte is not an option, suggesting instead a targeted therapeutic strategy that specifically affects their activation or trafficking pathways. While approaches to target lipid-antigens such as CD1d antagonists will impact on NKT cell effector functions, this will not completely abolish effector functions of other cytotoxic cells against infections that recognise pathogenic antigens presented by MHC molecules. In conclusion, it is more beneficial and clinically feasible to target cytotoxic lymphocytes through either their activation/trafficking pathways or targeting resident cytotoxic lymphocytes within lesions. More studies are needed to better understand the roles of the different cytotoxic lymphocytes in atherosclerosis, particularly in vulnerable plaque formation and rupture so that new therapeutic targets can be defined for controlling activated cytotoxic lymphocytes and their effector functions.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015a; Alexander et al., 2015b).

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TABLE 1. Comparison of general characteristics for different cytotoxic lymphocytes

	NK cells[1]	γδ-T cells[2]	iNKT cells	CD8+ T cells[4]	CD4+ CD28- T cells[5]
Immune response	Innate	innate/?adaptive[2]	Adaptive	Adaptive	Adaptive
Antigen	Not Required	Not Required	Lipid	Peptide	Peptide
Tissue residence	SLO, Spleen	Mucosa, Epithelium	SLO, Liver/spleen	SLO	SLO
Signature surface markers	NK1.1, TCR$^-$	TCRγδ	TCR Vα24-Jα18 (h) TCR Vα14-Jα18 (m) NK1.1	TCRαβ CD8	TCRαβ CD4
Activating or inhibiting	NKG2D, NKp46, NKp30, NKp44, KIR (h), Ly49 (m), DNAM, FcγRIII	NKG2D, NKp44, DNAM, FcγRIII	NKG2D, NKp30, NK046, KIR (h), Ly49 (m), FcγRIII	TCR-dependent antigens, NKG2D, KIR (h), Ly49 (m),	TCR-dependent antigens, NKG2D, DNAM
Chemokine Receptors	CXCR1, CXCR3, CXCR4, CCR7, CCR9	CCR7, CCR10, CXCR5	CCR4, CCR5, CCR6, CXCR3, CXCR4	CCR4, CCR5, CCR7, CCR9, CDR10, CXCR3	CCR5, CCR7, CXCR4,, CX3CR1
Effector functions *cytotoxins *Fas *TRAIL *cytokines	 + + + +	 + + + +	 + + + +	 + + + +	 + ? ? +
Cell-to-cell interaction	CD4 T cells	NK cells, Monocytes	MZ B cells	Monocytes, Dendritic cells, Macrophages	NA

1. (Vivier, Tomasello, Baratin, Walzer & Ugolini, 2008), 2. (Vantourout & Hayday, 2013), 3. (Brennan, Brigl & Brenner, 2013), 4. (Zhang & Bevan, 2011), 5. (Marshall & Swain, 2011). See text for detail.

h; human, m; mouse, NA; Not available.

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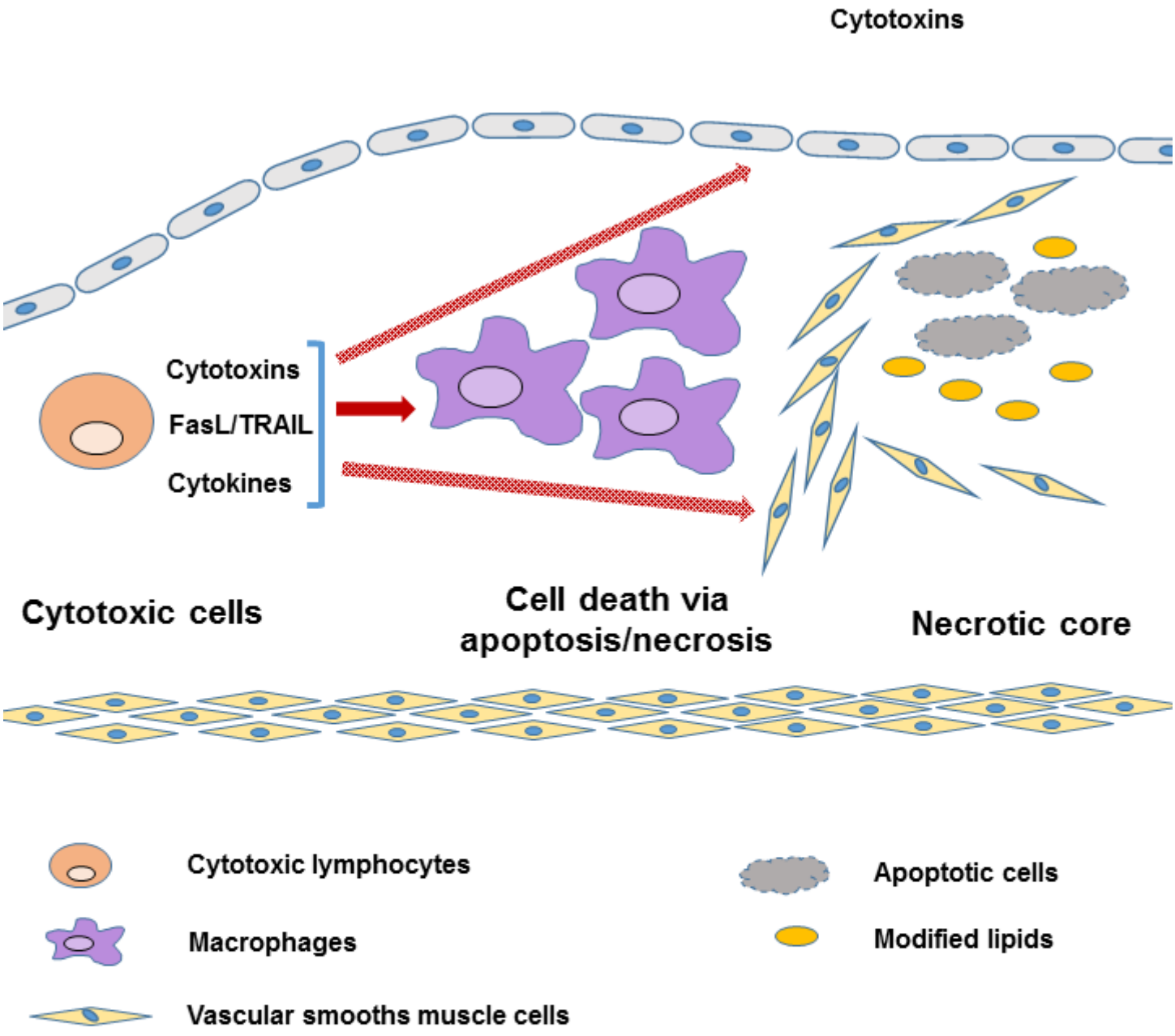
FIGURE LEGENDS

Figure 1. Cytotoxic lymphocytes promote lesion apoptosis and necrosis via cytotoxin-, FasL/TRAIL- or cytokine-mediated mechanisms. Lesion macrophages are major apoptotic or necrotic cells in lesions and increased lesion apoptosis and necrosis generated larger necrotic cores, a predominant feature of vulnerable atherosclerotic plaques. Cytotoxic lymphocytes also induce apoptosis and necrosis in vascular endothelial or smooth muscle cells that may contribute to rupture of vulnerable plaques.

Figure 2. Molecules expressed by cytotoxic lymphocytes that may be targeted to attenuate atherosclerosis and vulnerable plaque development. (A) CD1d on antigen presenting cells, e.g. dendritic cells to prevent TCR activation of iNKT cells and Clec9A on dendritic cells to prevent uptake of necrotic cell remnants and presentation on MHC I to activate CD8+ T cells. Also, activation of β2-adrenoceptors (β2-AdR) by β2-adrenoceptor agonists (β2-AdR-Ag) to inhibit activated CD8+ T cells. (B) Inhibiting chemokine receptors expressed by cytotoxic lymphocytes to prevent their migration to developing/developed atherosclerotic lesions. (C) Targeting NK activating and inhibitory receptors/co-receptors to inhibit/attenuate activation of cytotoxic lymphocytes to attenuate atherosclerosis and vulnerable plaque development with activating receptors inhibited and inhibitory receptors activated.



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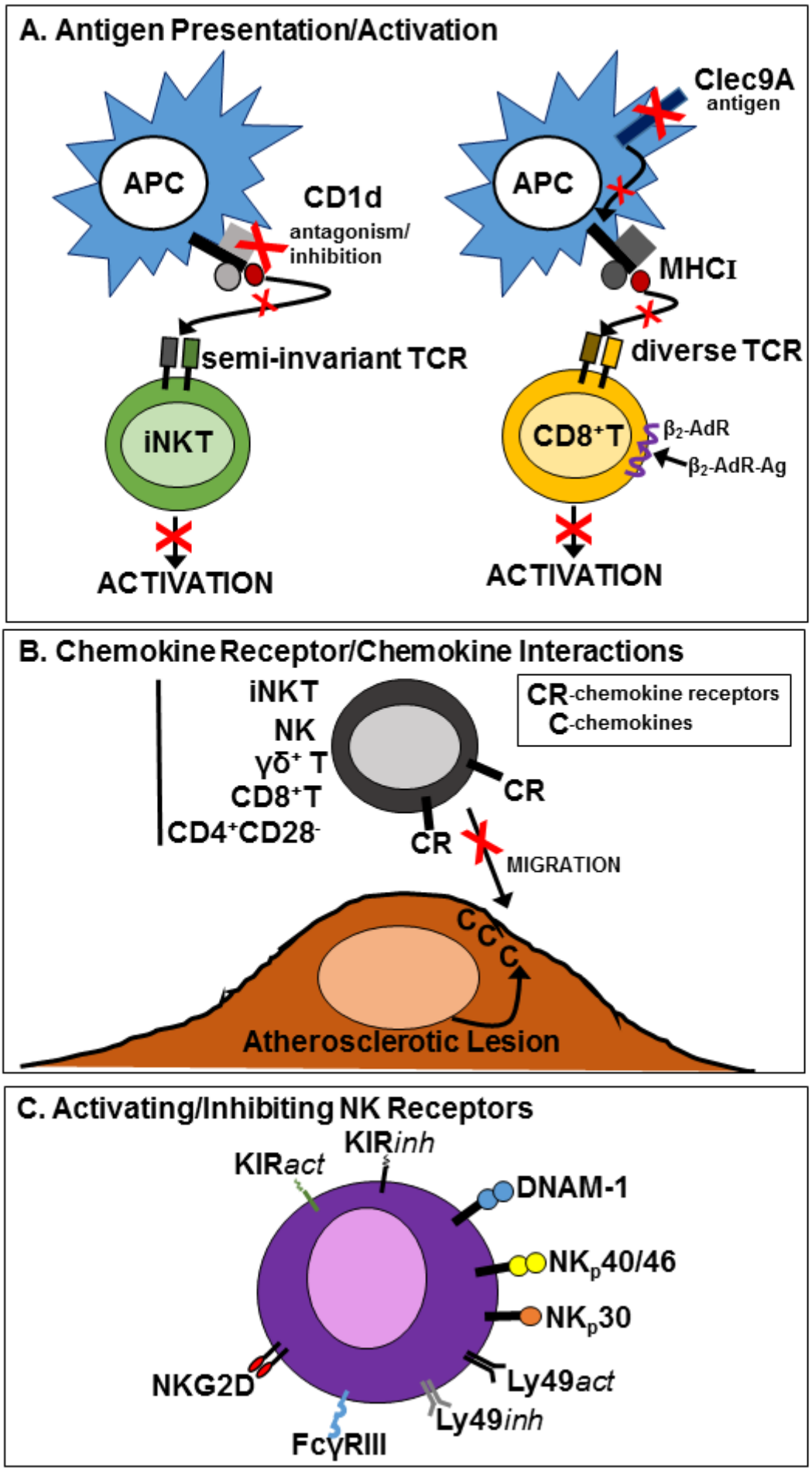

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