Regulation of T cells in Atherosclerosis

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Megan Mulholland

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DOCTORAL DISSERTATION

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Abstract:

Atherosclerosis, the underlying pathology of cardiovascular diseases such as myocardial infarction and ischemic stroke, is characterized by chronic inflammation. T cells are present in atherosclerotic plaques and have been implicated in its pathogenesis. This thesis describes studies exploring both the regulation of T cells in atherosclerosis as well as immunomodulatory therapeutics.

To study the role of IL-2 signaling, we selectively delivered IL-2 to T effector memory cells via administration of IL-2 complexed to the anti-IL-2 clone S4B6 (IL-2/S4B6) in atherosclerotic mice. IL-2/S4B6 administration resulted in increased levels of effector memory T cells and NK cells and their capacity to produce IFN-γ, increased pro-inflammatory cytokines in plasma, and increased regulatory T cells, but no effects on subvalvular plaque size or composition. Additionally, IL-2/S4B6 treatment decreased total plasma cholesterol and lipid-lowering effects were contingent on the presence of adaptive immune cells.

We next set out to characterize interferon-γ (IFN-γ)-producing aortic T cells. Utilizing an atherosclerotic IFN-γ-reporter mouse model, we identified that IFN-γ$^+$ T cells in the atherosclerotic aorta are characterized by elevated PD-1 expression. These cells are not functionally exhausted and respond to anti-PD-1 antibody treatment by increased IFN-γ production, possibly providing a mechanism behind the increased cardiovascular risk in patients after receiving anti-PD-1 therapies. Furthermore, we show that administering anti-LAG-3 therapies in combination with anti-PD-1 exacerbates T cell infiltration into the plaque.

Lastly, we tested a novel monoclonal antibody blocking the IL-1 receptor accessory protein (IL1RAP) in atherosclerotic mice. Administration of anti-IL1RAP antibodies, which disrupt the signaling of IL-1α/β, IL-33, and IL-36α/β/γ concomitantly, resulted in decreased plaque burden and reduced immune cell infiltration into both the atherosclerotic plaque and surrounding adventitia. We propose these results were in part mediated by reduction of gene expression for the adhesion molecules ICAM-1 and VCAM-1 as well as reduced CXCL1 chemokine production by macrophages and fibroblasts.

Our results support further studies into anti-IL1RAP as a therapeutic for atherosclerosis. Furthermore, we provide mechanistic insight into how immune checkpoint inhibitor therapy increases cardiovascular risk, by "releasing the brake" off IFN-γ$^+$ T cells in the plaque, potentially leading to increased plaque vulnerability and adverse events.

Key words: Atherosclerosis; T cells; vascular inflammation; immunomodulation; ICI; immune checkpoint inhibitors; PD-1; LAG-3; IL-2Rβγ; IL1RAP

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It all seems so very arbitrary. I applied for a job at this company because they were hiring. I took a desk at the back because it was empty. But, no matter how you get there or where you end up, human beings have this miraculous gift to make that place home.

-Creed Bratton

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Preface

After about two weeks of dedicated writing of my “kappa”, the portion of this book before the papers, I had an overwhelming feeling wash over me of “I’m doing this entirely wrong.” Just as I was finally starting to feel the needle move and progress being made, it all felt wrong. Like I used the wrong tonality, the wrong words, the wrong approach. Granted, there is a lot of liberty given when writing a kappa- so could it really be wrong? Well, I hope not, because if you’re reading this sentence, it clearly already made it to print and there’s no turning back now.

One thing I really strived for while writing was trying to retain what I feel like is my “regular” writing voice. I also really wanted to make even the kappa (relatively) digestible to those without an advanced science degree. Of course, the subject matter is still advanced, but I hope by providing a background immunology chapter and some fun anecdotes and facts along the way that those who wish to follow along can. For some reason, I kept envisioning my dad trying to read this book as I typed (sorry y’all, he is the only “reader” in the family). Each time I have visited home throughout these past four years, I have been asked by family and friends about my research and what it is we do. Realizing I now have the opportunity to hand them one compact answer, I wanted it to be as understandable as possible while still providing the necessary information.

Furthermore, I have always said I study a disease that highlights the cruciality of preventative medicine, a facet of medicine that hinges upon education. So, in addition to displaying all of the really cool research we’ve accomplished, I am hoping that anyone can pick up this book and learn something. Learn one thing, form one question, make one change, from topics that I personally find extremely interesting and have dedicated a good deal of time and energy to, but also topics that are pertinent to all of our health and wellbeing.

Acknowledgements

My path towards gaining a PhD I think was unconventionally conventional. There have truly been so many individuals from so many different experiences I have had over the past decade that have landed me in the position I am in today. I am grateful for every single interaction and influence that has gotten me here, and the following acknowledgements do not encompass them all.

I never had any intention in completing a PhD until my master thesis supervisor put the idea in my head, and well, here we are, **Daniel**, four (five?) years later. I have often questioned why, exactly, you thought I would be good at this. I tell myself it's the mixture of Office trivia knowledge and sassiness that I bring to the table that made you know we would make a good team. You have offered endless amounts of support and consideration throughout these years, never letting me talk down on myself or get discouraged, and always being available for questions, help, and advice. You not only provided me the stepping stones for a career I never saw for myself on my own, but also helped my friends find their opportunities. Thank you for the continual guidance.

To my permanent officemate and fellow PhD student, **Gabriel**- I have "joked" throughout the years that I wouldn't have been able to do any of my projects or lab work without you- I hope you know they were never really jokes. I still remember being wary of you joining me and Daniel for your master's project because I thought you two would form some impenetrable "Swedish" duo and I'd be left out; the reality couldn't have ended up more opposite. Thank you for, quite literally, almost always lending a helping hand, encouraging all my event planning ideas, and being a friend as well as a colleague.

I want to thank **Harry** for being an amazing co-supervisor, for always being available for my "after-hours" questions held over the threshold of your office door, and for teaching and helping with logistical issues. Your gift of having such a pedagogical approach is valued by all of us students.

Thank you, **Lena**, for teaching me animal welfare and procedures, introducing me to histology, and not making me feel weird for talking to the mice. And of course, **Irena**, the true powerhouse behind all of our experiments; our small lab "of two" has really always been a lab of three and you have been an invaluable part of this thesis. To **Alex**, **Eva**, and **Jan** for always providing thoughtful feedback and advice,

and listening to our countless ideas, experiments, and projects over the years, as well as **Pernilla**, the final piece to the triad of our PhD student class.

To **Andrietta**, **Samuel**, and **Anthi**- you all made coming into the office so much more enjoyable. Thank you all, as well as **Welsey** and **Jenifer**, for always entertaining my babbling and distracting ways.

I want to also thank **Sara** and **Cat** for all the support and discussions with our IL1RAP project. It goes without saying that without you two there would be no "Paper IV".

Thank you, **Ana**, for always making me feel welcomed and included, and letting me tag along to surgery. And thank you, **Linda**, **Miheala**, **Fong**, **Annelie**, **Tomi**, and **Filiz** for the many chats, laughs, guidance in lab, and fresh pots of coffee. And to **Gertrud**, for the 4-years of lab days, julfikor, and administrative support.

I'd also like to acknowledge and thank **all of the mice** over the years for the ultimate sacrifice in the name of education and scientific progress, as well as the wonderful CRC animal facility staff for caring for them.

To my high school homie, college roommate, and recurrent Baltimore host, **Travis**- thanks for sticking it out with me all these years, inviting me into your family and homes, and always reminding us of the importance of spooky season. It's been a gift to grow and learn with you as a friend. To my longest friend, **Cassie**- I want to thank and acknowledge your continual friendship, support, and validation over the years, and for always making time for me when I visit home.

To **Linn**, the cool-looking girl I approached at orientation 6+ years ago- thank you for the worldwide adventures, for the translation services, for teaching me to pay more attention to the world around us and not settle, for picking me up when I was knocked down or when I literally just needed a ride, for your unwavering support. Thank you for being my home away from home.

To **Alex**, my real long-distance relationship and international alarm clock, it's been an absolute pleasure being in the trenches with you during our decade-long education endeavors. There's truly too many memories and not enough words for your impact on me and my life. Thank you for being a constant source of joy, laughter, safety, comfort, support, and inspiration.

Thank you, **Kara**, for continual support and encouragement of my life over here while also taking care of things for us both on the homefront. And to you and **Andrew** for the constant morale boosts via Kona pictures. A thank you to **Michelle**, for the many thoughtful and reflective conversations as well as new baking recipes to stay creative (and full) over the years; to **Dad** for always cheering me on, for reminding us of the importance of perseverance (they don't give out medals after the first mile ran in a marathon, right?), and letting me win Quiddler at least once.

And also, a thank you to **Grandma** and **Papa** for always supporting the family and us in our education- next time you see me, you can finally call me Dr.

And finally, **Mom**. I didn't even tell you I was thinking of applying to schools abroad. I walked into your room one afternoon and told you I had been accepted into a school and your motherly instincts just knew to ask if it was in the country. You immediately offered support, guidance, and assistance in moving me across an entire ocean (as you have with anything Kara and I have wanted to do) and have continued to do so throughout this journey. Everything I am today and have been able to accomplish stems from your never-ending selflessness and generosity. Thank you for helping me get here. Thank you for everything.

Original Papers

I. IL-2Rβγ signalling in lymphocytes promotes systemic inflammation and reduces plasma cholesterol in atherosclerotic mice.

Mulholland M, Jakobsson G, Lei Y, Sundius L, Ljungcrantz I, Rattik S, Tietge U, Engelbertsen D.

Atherosclerosis. 2021 Jun;326:1-10. ePub 2021 Apr.

II. PD1-expressing T cells produce IFN-γ in the aorta and are associated with subclinical coronary atherosclerosis.

Mulholland M, Depuydt M.A.C., Andersson S, Yu Y, Lin S, Jakobsson G, Ljungcrantz I, Schiopu A, Lichtman A.H., Björkbacka H, Slütter B, Gisterå A, Engelbertsen D.

Manuscript.

III. LAG3 regulates T cell activation and plaque infiltration in atherosclerotic mice.

Mulholland M*, Kritikou E*, Katra P, Nilsson J, Björkbacka H, Lichtman A.H., Rodriguez A[#], Engelbertsen D[#].

JACC CardioOncology. 2022 Dec 20;4(5):635-645. eCollection 2022 Dec.

**shared first authors, [#]shared last authors*

IV. IL1RAP blockade limits development of atherosclerosis and reduces plaque inflammation.

Mulholland M, Depuydt M.A.C., Jakobsson G, Ljungcrantz I, Grentzmann A, Jaensson Gyllenbäck E, Grönberg C, Rattik S, Liberg D, Schiopu A, Björkbacka H, Kuiper J, Bot I, Slütter B, Engelbertsen D.

Manuscript, in resubmission for *Cardiovascular Research*, Oct 2023.

List of Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
Apo	Apolipoprotein
Apoe$^{-/-}$	Apolipoprotein-E knock-out
ASCVD	Atherosclerotic cardiovascular disease
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcome Study
CCR	C-C chemokine motif receptor
CD	Cluster of differentiation
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL	C-X-C chemokine motif ligand
CXCR	C-X-C chemokine motif receptor
DC	Dendritic cell
FIMCOD	Functional IMmunity and CardiOvascular Disease
HCD	High-cholesterol diet
HDL	High-density lipoprotein
ICI	Immune checkpoint inhibitor
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL1RAP	Interleukin-1 receptor accessory protein
IL-2Rα	Interleukin-2 receptor alpha
IL-2Rβγ	Interleukin-2 receptor beta-gamma
LAG-3	Lymphocyte-activation gene 3

LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
Ldlr$^{-/-}$	Low-density lipoprotein receptor knock-out
MHC	Major histocompatibility complex
MI	Myocardial infarction
NK	Natural killer
oxLDL	Oxidized low-density lipoprotein
PD-1	Programmed cell-death protein 1
PD-L1	Programmed death-ligand 1
TCR	T-cell receptor
Th cell	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell

Popular Science Summary

I know I prefaced this book by saying that I hope anyone who picks up my thesis will be able to learn something from it. While there are definitely components I tried to write more simplistically, this *Popular Science Summary* is truly geared towards everyone understanding the underlying concepts and results of my thesis work. As I have chosen to put this section and the *Acknowledgements* at the beginning of the book, I realize there is a very solid chance that the majority of you will not make it past page 23 of this book. And that's okay! I hope you do, but if not, here is an "easy to read" summary.

The sentence that starts nearly every article I have read during the past four years goes something like, "Atherosclerosis is a chronic inflammatory disease that affects large to medium sized arteries and is responsible for majority of underlying cardiovascular disease." If you do make it past this section of the book, you'll see I myself have fallen victim to writing such a sentence, and definitely more than once. But it is a good place to start.

Atherosclerosis is the process of "plaque" formation within the arterial wall. This is often confused, understandably, with the term arteriosclerosis, which is the stiffening of arteries. Arteries are the blood vessels that carry blood away from your heart to the rest of your body, providing tissues with oxygen and nutrients. While there are many factors contributing to plaque formation, having high levels of "bad cholesterol," or rather low-density lipoprotein (LDL), initiates this process. When there is an oversaturation of LDL in the circulation, some LDL particles enter into the layers of the artery and become trapped. This trapped LDL will initiate an immune response. This immune response amps itself up over time, resulting in non-resolving inflammation and an atherosclerotic plaque.

This process, the initiation and growth of plaques, goes undetected for large periods of time. The problem with this is you usually do not know you have a dangerous plaque until, well, it becomes dangerous. These plaques can become vulnerable to rupture over time, and ruptured plaques can lead to heart attacks or strokes. Therefore, the name of the game in atherosclerosis treatment is prevention and management. Having some degree of atherosclerosis is nearly impossible to avoid as a human. But by understanding the underlying mechanisms of pathology, we can continue to create better preventative therapeutics and mitigate cardiovascular risks. And here is where my research and thesis work come in.

As I mentioned, immune cells are a big component in driving atherosclerosis. One of the key immune cells at play is T cells. T cells are a part of the adaptive immune system, meaning they mount extremely specific and targeted responses against whatever pathogen is ailing you. T cells come in many different "flavors," and which flavor T cell is present in the plaque can cause either "pro-atherogenic" (i.e., promoting atherosclerosis) or "anti-atherogenic" effects. Despite many studies already conducted to investigate the role of T cells in atherosclerosis, we felt there are still some missing pieces.

A really important molecule for T cell survival and activity is IL-2 (interleukin-2) and the receptor for this molecule comes in different forms. A previous study demonstrated that selectively delivering IL-2 to a specific flavor of T cell reduced atherosclerosis in mice. We were curious to examine the opposite- instead of targeting T cells with the IL-2α receptor as this study did, we wanted study effects of delivering IL-2 to T cells with the IL-2βγ receptor. However, when we did this, we induced both pro- and anti-atherogenic effects (increased inflammatory and anti-inflammatory cells, increased systemic inflammation, decreased plasma cholesterol). Ultimately, selectively delivering IL-2 to these T cells via the IL-2βγ receptor had no overall effect on atherosclerotic plaque burden.

We next examined the influence of immune checkpoint inhibitors (ICIs) on T cells in atherosclerosis. If immune cells were turned on and could never be turned off, we'd have a serious problem. In fact, we call that chronic inflammation, and it causes a lot of damage to the body. Fortunately, your body has developed many mechanisms to avoid this, and one way is by having receptors that act as a "brake" on some immune cells. On T cells we call these checkpoint inhibitors, and when these receptors are engaged with their ligand, they "inhibit" the activity of the T cell. Oncologists take advantage of these ICIs with what is called ICI therapy. Cancer is the opposite problem of chronic inflammation in that we want to generate a more robust immune response rather than dampen it. ICI therapy releases these "brakes" on T cells, allowing your own immune system to fight back harder against the cancer. While "releasing the brake" can be helpful at tumor sites it may be harmful at other locations, like the plaque, where we want the "brakes" functioning normally.

Recent studies have shown that cancer patients treated with these ICI therapies have a 5-fold increase in risk for atherosclerosis cardiovascular disease. In this thesis, we investigated the effects of the checkpoint inhibitors PD-1 and LAG-3, both of which are FDA-approved ICI therapy targets, on T cells in atherosclerosis. What we found was administration of PD-1-targeted ICI therapy to atherosclerotic mice increased T cell infiltration into the atherosclerotic aorta and plaque. In a separate study, we also demonstrated LAG-3 can modulate T cell presence in plaques, and administration of combination LAG-3/PD-1-targeted ICI therapy increased plaque T cells greater than administrated of PD-1-targeted ICI therapy alone. Furthermore, we also revealed that T cells that produce a molecule called interferon-γ (which has

been shown repeatedly to be pro-atherogenic) also express PD-1 and respond to PD-1-targeted ICI therapy. Our results from these studies may provide mechanistic insights into how ICI therapy increases cardiovascular risk.

Lastly, we were able to test a potential new anti-inflammatory therapeutic for atherosclerosis. This treatment targets a co-receptor called IL1RAP. IL1RAP is necessary for signaling of multiple molecules (IL-1α/β, IL-33, IL-36α/β/γ) that have previously been suggested to have pro-atherogenic implications. By blocking IL1RAP with anti-IL1RAP antibodies we disrupt signaling of all of these molecules to their target cells with only one antibody. We show that administration of anti-IL1RAP antibodies to atherosclerotic mice decreased plaque size and reduced immune cells in the plaque and surrounding vasculature (including T cells). Our results support further investigation into using anti-IL1RAP antibodies as an atherosclerosis treatment.

By further understanding T cells and their regulation in atherosclerosis, this thesis has provided possible mechanistic insights as to how ICI therapy exacerbates cardiovascular disease and potentially a new therapeutic agent for atherosclerosis.

Populärventenskaplig Sammanfattning

Jag vet att jag inledde denna bok med att säga att jag hoppas att alla som läser min avhandling har möjligheten att lära sig något från den. I min avhandling finns det absolut delar som jag har försökt att skriva om och beskriva så enkelt som möjligt, men denna populärvetenskapliga sammanfattning är än mer till för att alla ska kunna förstå koncepten och resultaten som framkommit genom min avhandling. Eftersom jag valt att lägga både denna sammanfattning och min tack-sektion i början av boken, så inser jag att det med stor sannolikhet är många som inte kommer läsa längre än sidan 27. Och det är trots allt helt okej! Jag hoppas att ni läser vidare, men om inte, kommer här en ”lättläst” sammanfattning.

Den första meningen av majoriteten av de artiklar jag har läst under de senaste fyra åren är oftast något i stil med ”Ateroskleros är en kronisk inflammations-sjukdom som påverkar stora till medium-stora artärer, och är ansvarig för majoriteten av underliggande hjärt- och kärlsjukdomar.” Om du efter denna sammanfattning läser vidare i min avhandling så kommer du se att jag själv också har fallit offer för att beskriva ateroskleros i en liknande mening, och definitivt mer än en gång. Det finns dock en bra anledning; det är ett bra ställe att börja på.

Ateroskleros är processen där så kallade aterosklerotiska ”plack” formas i artärväggen. Denna process blandas oftast ihop med den liknande termen arterioskleros, vilket i själva verket är förhårdningen av artärer. Artärer är de blodkärl som för blodet från ditt hjärta till resten av din kropp och förser dina vävnader med syre och näringsämnen. Det finns många faktorer som bidrar till formationen av aterosklerotiska plack, men höga nivåer av det ”onda kolestrolet” närmare bestämt low density lipoprotein (LDL), initierar processen. Förhöjda mängder av LDL i cirkulationen, leder till att LDL-partiklar läcker in i artärer, fastnar och börjar ansamlas. Dessa ansamlade LDL-partiklar leder sedan till att immunförsvaret inleder en immunrespons. Immunresponsen växer sen i storlek över tid, vilket leder till en konstant inflammation och bildandet av en aterosklerotisk plack.

Denna process, bildandet och tillväxandet av plack upptäcks sällan utan pågår o-detekterat under långa perioder. Problemet är att man oftast inte vet att man har

farliga plack förrän det blir just farligt. Plack kan försvagas över tid, och då bilda sprickor och rupturer. Dessa så kallade plackrupturer kan leda till hjärtattacker och stroke. På grund av detta, är det viktigaste inom aterosklerosbehandling prevention och underhåll. I princip alla människor får ateroskleros i någon mån under sin livstid, men genom att öka vår förståelse av de underliggande mekanismerna av sjukdomen så kan vi fortsätta att skapa bättre preventiva behandlingar och genom dessa minska risker för hjärt-och kärlsjukdomar. Och det är här min forskning och avhandling kommer in i bilden.

Som jag nämnde tidigare, så är immunförsvaret och immunceller en stor drivande komponent av ateroskleros. En av de immunceller som bidrar mest till processen är T-celler. T-celler är en del av det adaptiva immunförsvaret, vilket innebär att de driver ett extremt specifikt och målinriktat försvar mot den patogen som invaderar. T-celler kommer i många olika ”smaker”, och beroende på vilken smak av T-cell som är närvarande i placken kan den ha antingen en effekt som gynnar eller missgynnar ateroskleros-processen. Det finns många studier som undersöker effekten av T-celler i ateroskleros, men vi kände att det fortfarande saknades bitar för att fullt ut förstå denna effekt.

En väldigt viktig molekyl för överlevnad och aktivitet av T-celler är interleukin-2 (IL-2), och receptorn för denna molekyl kommer i olika former. En tidigare studie har sett att selektiv leverans av IL-2 till en specifik smak av T-cell reducerade ateroskleros i möss. Vi var nyfikna på att undersöka motsatsen – i stället för att specifikt ha T-celler som mål med IL-2α-receptorn som gjordes i studien, så ville vi studera effekterna av att leverera IL-2 till T-cellerna med IL-2βγ-receptorn. När vi gjorde detta, så såg vi effekter som var både pro-ateroskleros och anti-ateroskleros (ökat antal både inflammatoriska och anti-inflammatoriska celler, ökad systemisk inflammation, och minskad mängd kolesterol i plasman). I slutändan så hade selektiv leverans av IL-2 till dessa T-celler via IL-2βγ-receptorn ingen effekt på mängden aterosklerotiska plack.

Vi ville även undersöka effekten av så kallade immunkontrollpunktshämmare (ICIs) på T-celler i ateroskleros. Om immunceller alltid är påslagna utan möjlighet att ”stängas av” innebär det stora problem för oss. Det är detta som kallas kronisk inflammation, och detta skapar stora skador i kroppen. Som tur är har kroppen utvecklat många mekanismer för att undvika kronisk inflammation. Ett sätt är genom att ha receptorer som fungerar som en ”broms” på vissa immunceller. På T-celler kallas dessa immunkontrollpunktshämmare eller ICI:s efter dess engelska namn, och när dessa receptorer är bundna till sitt ligand så hämmar de aktiviteten av T-cellen. Onkologer använder sig av denna effekt med något som kallas ICI-terapi. Med cancer vill vi, till skillnad mot under kronisk inflammation, förhöja immunresponsen snarare än att hämma den. Så, ICI-terapi släpper på dessa ”bromsar” på T-celler, så att ditt immunförsvar kan kämpa hårdare mot cancern. Att

”släppa på bromsarna” kan alltså vara hjälpsamt vid tumörer, men kan skada på andra ställen, till exempel vid en plack där vi vill ha ”bromsarna” fungerande som vanligt.

Nyligen gjorda studier har visat att cancerpatienter som behandlas med dessa ICI-terapier har en 5 gånger högre risk att utveckla ateroskleros. I min avhandling undersökte vi effekter av immunkontrollpunktshämmarna PD-1 och LAG-3, som båda två är FDA-godkända mål av ICI-terapier, på T-celler i ateroskleros. Vi fann att administration av ICI-terapi med PD-1 som mål i möss med ateroskleros ökade infiltrationen av T-celler till den drabbade aortan och placken. I en separat studie demonstrerade vi även att LAG-3 kan modulera närvaron av T-celler i placken, och administration av en kombinerad ICI-terapi med LAG-3/PD-1 som mål ökar antalet T-celler i placken i en högre omfattning än terapi med endast PD-1 som mål. Dessutom såg vi att T-celler som producerar interferon-γ - en molekyl som har bevisade effekter som gynnar ateroskleros – även uttrycker PD-1 och svarar på ICI-terapi med PD-1 som mål. Våra resultat från dessa studier kan ge mekanistisk insikt i hur ICI-terapier ökar risken för hjärt-och kärlsjukdomar.

Slutligen testade vi även en potentiell ny anti-inflammatorisk terapi för ateroskleros. Denna behandling har en co-receptor kallas IL1RAP som mål. IL1RAP är nödvändig för kommunikationen av flertal molekyler (IL-1α/β, IL-33, IL-36α/β/γ) som tidigare har indikerats ha effekt på ateroskleros. Genom att blockera IL1RAP med anti-IL1RAP antikroppar kan vi störa kommunikationen av alla dessa molekyler med de celler de har som mål med hjälp av en enda typ av antikropp. Vi har visat att administration av anti-IL1RAP antikroppar till möss med ateroskleros förminskade storlek av plack, och reducerade immunceller i placken och omgivande vaskulatur (inklusive T-celler). Våra resultat stödjer fortsatt undersökning av användandet av anti-IL1RAP antikroppar som en behandling för ateroskleros.

Genom att öka vår förståelse av T-celler och dess reglering i ateroskleros, så har denna avhandling bidragit med möjliga mekanistiska insikter för hur ICI-terapi ökar risken för hjärt-och kärlsjukdomar och potentiellt visat på möjligheter för en ny terapi för behandling av ateroskleros.

The Immune System

Our bodies require constant protection. In addition to physical and chemical barriers, our bodies have the immune system comprised of specialized cells and the chemicals they secrete that work to protect us from both external and internal threats as well as mediate healing processes. External threats include pathogens such as viruses, bacteria, or parasites, and internal threats arise from non-infectious agents, such as tissue injury, cell turnover, and cancer cells.[1, 2]

The original pillars of inflammation included signs of rubor (redness), calor (heat), tumor (swelling), and dolor (pain); the word "inflammation" itself derived from the word "flame."[3] However, after the invention of the microscope and advances in cellular and molecular biology, the term inflammation has broadened significantly.[4] Ultimately, a beneficial and healthy inflammation response is a balancing act. For example, inflammation is necessary to clear harmful pathogens, but can also cause unwanted damage to surrounding tissues. At the same time, eliminating the initial "pro-inflammatory" responses can risk losing subsequent "anti-inflammatory" responses, thus attenuating the positive healing efforts involved in inflammation resolution. The topic of inflammation as a whole is extremely nuanced and complicated, and it remains to be decided just what exactly "inflammation" is and if it is always a negative thing.[3, 5, 6]

The immune system is divided into two main branches- innate and adaptive immunity. The innate immune system is comprised of non-specific "first responders," providing a rapid, general response to infections and injuries. The adaptive immune system takes longer to mount a response; however, this response is tailored specifically to the imposing threat, making it a more efficient line of defense. The adaptive immune system also provides immunological memory to support stronger and more robust responses to re-exposure.[1, 2]

Cytokines and Chemokines

Cytokines are small chemicals that help mediate and regulate immune responses. Cytokines can be secreted by nearly every cell in the body and are extremely pleiotropic, exhibiting different functions depending on the cell(s) they affect. There

are various names for cytokines, such as **chemokines**, which is just a cytokine that induces chemotaxis (cell movement), interleukins, interferons, and growth factors. Further, cytokines can be pro- or anti-inflammatory, contributing to both initiation and regulation of immune response, and can act synergistically or antagonistically with other cytokines, or even other signaling peptides, on cells.[7-9]

Innate Immune Cells

The key differentiating factor between the innate and adaptive immune systems is specificity. The innate immune system is non-specific, although I find this to be a bit of a misnomer. The innate immune system will respond differently based on the overall type of infection, such as virus versus bacteria versus parasitic, but that's about as far as it goes. As such, the innate immune system consists of components that provide general defense against pathogens like physical barriers, such as the skin and mucosal membranes, and physiological factors, such as temperature, pH, and chemical mediators, and the innate immune cells.[2, 10]

As alluded to, the immune cells of the innate immune system are able to sense some features of the pathogens they are up against, which they do via pattern recognition receptors (PRRs). PRRs can detect shared features and molecules of pathogens, called pathogen-associated molecular patterns (PAMPs), as well as detect signs of tissue damage or cellular stress, called danger-associated molecular patterns (DAMPs).[11] In response to PAMPs and DAMPs, the innate immune cells will initiate an inflammatory response, releasing chemical mediators (e.g., cytokines, chemokines) and recruiting more immune cells within hours of detection to deliver a rapid response.[11]

The granulocytes

The granulocytes are a group of innate immune cells comprised of neutrophils, basophils, and eosinophils, named for the many granules they contain in their cytoplasm. **Neutrophils** are the most abundant immune cells in the human body, constituting 70% of white blood cells in circulation. With an extremely short lifespan (12-24 hours in circulation), constant production and release of neutrophils from the bone marrow is required daily.[12-14] Due to their abundance and constant patrolling, neutrophils provide the first line of defense for the body. Neutrophils are recruited to sites of inflammation by chemoattractants in masses, responding to pathogens by release of cytotoxic granules and reactive-oxygen species (ROS), release of neutrophil extracellular traps (NETs), and phagocytosis. Neutrophils will also amplify the immune response via release of inflammatory mediators, activating

and recruiting other immune cells, among other functions.[12-14] Neutrophils are best suited to respond to bacterial and fungal infections, but they also have been shown to play an important role in the resolution phase of inflammation. **Basophils** and **eosinophils** are related to type 2 immune responses, which are targeted at helminth infections and allergy responses.[14] **Mast cells**, loaded with pre-formed histamine granules, are also involved in type 2 responses, although are not classified as granulocytes by lineage.[15] Aside from a personal vendetta as a life-long asthmatic and seasonal allergy sufferer,[16] further expansion on these cell types is out of scope for the focus of this thesis.

Monocytes and macrophages

Similar to neutrophils, **monocytes** are derived from the bone marrow and spend the majority of their time circulating in the blood. Historically, monocytes are best described for their plasticity, as upon migration into inflamed tissues, monocytes can differentiate into dendric cells (monocyte-derived dendritic cells, moDCs) or macrophages.[17, 18] However, it has recently been appreciated that monocytes can contribute to steady-state surveillance within tissues and can participate in antigen presentation without differentiation.[19] Monocytes that transmigrate into tissues can be distinguished by their expression of Ly6C in mice and CD14 in humans, while the monocytes that remain patrolling are $Ly6C^{low}$ or $CD14^{low}CD16^{+}$, respectively.[17, 18] Migratory monocytes are highly dependent on the chemokine receptor CCR2 for both attraction to site of inflammation and egress from bone marrow, and this migration is heavily increased during inflammation.[20, 21]

Macrophages are tissue-resident cells. Macrophages can be derived from migratory monocytes or from embryonic hematopoiesis, meaning these macrophages were generated and migrated to their tissue location before birth. Thus, the origin of adult macrophages will vary between tissues. Further, the permanence of macrophages varies with origin, with monocyte-derived macrophages generally being a more transient population (although not always).[17, 22, 23]

The main job of a macrophage can be easily deciphered from its name- it's a big cell (macro) that likes to eat (phagocytose). This includes removal of foreign pathogens as well as cellular debris. In addition to being highly phagocytic, macrophages also contribute to tissue repair and remodeling, metabolism, antigen presentation, and produce pro- and anti-inflammatory cytokines, and thus can be found during homeostasis, acute inflammation, and resolving inflammation. Macrophages come in many subtypes, the most well-known polarization being an M1 (pro-inflammatory) or M2 (anti-inflammatory/reparatory) macrophage. However, it is well agreed upon now that this binary categorization is extremely over-simplified, and macrophages constitute a very functionally diverse cell type.

Macrophages are also one of the antigen presenting cells (APCs) of the immune system, along with dendritic cells and B cells. **APCs** present foreign antigen peptide on a major histocompatibility complex (MHC) molecule, in addition to co-stimulation and cytokines, to activate T cells, providing a bridge between the innate and adaptive immune systems.[24]

Dendritic cells

Dendritic cells (DCs) are the most efficient of the APCs- their main role is to phagocytose and present antigens to T cells. As such, DCs are found throughout the body, especially at interfaces between the external and internal environments (e.g., skin, intestines) and in the lymphoid organs (thymus, spleen, lymph nodes).[25, 26] Immature DCs are very endocytic, making them extremely efficient at capturing antigens. Upon changes in homeostasis or local microenvironment (e.g., cancer, autoimmunity, vaccinations, etc.) or recognition of PAMPs or DAMPs, DCs mature. DC maturation results in increases in motility, expression of the chemokine receptor CCR7, MHC II, and costimulatory molecules, and cytokine production.[25, 26] All of these maturation features are necessary for DCs ability to find and activate T cells.

DCs have the special ability to present exogenous proteins on MHC I molecules without being infected themselves by a process called cross presentation. Briefly, **MHC I molecules** express peptides derived from endogenous proteins (i.e., produced inside the cell itself), including virus-derived peptides upon cell infection, while **MHC II molecules** express peptides from exogenous proteins (i.e., captured from the extracellular environment). The ability of DCs to cross-present is key in many immune responses, such as priming naïve CD8 T cells without CD4 T cell help as well as immune surveillance and tolerance.[27, 28]

DCs derived from hematopoietic stem cells in the bone marrow are divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs), and as mentioned previously, DCs can also be derived from monocyte precursors (moDCs). Upon stimulation of their PRRs (TLR7 and TLR9) pDCs secrete large amount of type 1 interferons (IFN-α, -β, -ω, and -τ) and are extremely important in anti-viral responses.[26] "Interferons" was another fantastic name choice by immunologists, as these cytokines "interfere" with viruses and their multiplication. cDCs are divided again into two categorizations: cDC1, important for priming naïve CD8 T cells and skew towards providing a type 1 immune response; and cDC2, which activate and polarize CD4 T cells by release of cytokines (skewing towards Th2, Th17, Tregs) as well as have regulatory and tolerogenic roles.[26, 29, 30] The subdivision of cDCs can become even more confusing when also taking into consideration the expression patterns of cellular markers, such as CD11b, CD103, CD8α, and CD4 in mice.[29] moDCs are difficult to distinguish between cDCs, both phenotypically and functionally, although they are often referred to as "inflammatory DCs" due to their

increase in production of TNF-α (tumor-necrosis factor α) and iNOS (inducible nitric oxide synthetase) upon infection.[25, 30] Ultimately, DCs are a very diverse and plastic cell population that constitute a fundamental bridge between the innate and adaptive immune responses.

Natural killer cells and innate lymphoid cells

The main function of **natural killer (NK) cells** is also quite apparent in its name. NK cells can directly target and kill cells, and thus are especially important in protection intracellular pathogens like viruses and cancer. NK cells are interesting in that they are categorized as innate immune cells, but their lineage is closer to that of the lymphocytes (B and T cells). While NK cells received their name due to the notion that they could kill cells without any kind of immunization, is now known that NK cells actually have rather low levels of killing activity without some kind of activation from inflammatory stimuli.[31, 32] NK cells regulate their cytotoxicity via various activating and inhibitory receptors, determining which cells to target based on three different types of recognition (i) "non-self" (detection of PAMPs) (ii) "missing self" (iii) and "distressed self."[31, 33] For example, MHC I molecules are expressed on the surface of all nucleated cells in the body and upon recognition of this molecule, NK cells' cytotoxicity is repressed, but if MHC I is lacking/downregulated the NK cell will become uninhibited. The cytotoxic functions of NK cells include release of cytotoxic granules (perforin, granzyme B) that can perforate and kill cells as well as directly killing cells via antibody-dependent cell cytotoxicity (ADCC). NK cells are known for producing large quantities of IFN-γ, which, in addition to providing anti-viral properties,[34] contribute to maturation and polarization of macrophages, DCs, and T cells.[32, 33]

Lastly, I want to briefly mention **innate lymphoid cells (ILCs)**. Like NK cells, ILCs are a part of the innate immune system but their lineage stems from the common lymphoid progenitor, although the non-cytotoxic ILCs are still distinct from cytotoxic NK cells developmentally.[35] There are three types of ILCs (ILC1, ILC2, and ILC3), dictated by expression of transcription factors, effector functions, and cytokine production. Overall, this largely corresponds to which type of immunity they participate in (type 1, 2, or 3). As such, ILCs are extremely diverse cells and play a role in a variety of immune functions, from combating infectious pathogens to promoting tolerance against commensal bacterium to even engaging in tissue repair mechanisms.[35]

Adaptive Immune Cells

In contrast to the innate immune system, the adaptive immune system is characterized by its ability to create a specific response. While the innate response may be quicker, mounting within hours of infection or injury, a specific response is more efficient and also supplies immunological memory. The generation of memory cells is how we are able to respond quicker and more robustly against secondary challenges and is the basis for immunizations. The adaptive immune system is comprised of the two lymphocytes, B cells and T cells, which provide specificity via recognition of antigens. Simplistically, an **antigen** is basically anything a lymphocyte can recognize and mount a response to, for example, the spike protein of the SARS-CoV-2 virus.

B cells

B cells are derived from and develop in the bone marrow. The characteristic feature of B cells is their ability to provide **humoral immunity** via production and secretion of antibodies. During development, B cells generate a B cell receptor (BCR), which due to somatic recombination of the constituent light and heavy chains, are unique to each B cell clone and capable of binding to a specific antigen.[36, 37] As APCs, B cells can also present antigen peptide on MHC II. B cells become activated when their corresponding antigen binds to the BCR and a second activating signal is received, which varies depending on the nature of the antigen. Often times, T cell help is required to deliver this second signal. Antigens that require T cell help to activate B cells receive this help when B and T cells meet in secondary lymphoid organs and (i) the T cell receptor recognizes the antigen peptide:MHC II complex on the B cell and (ii) CD40 (B cell) binds to CD40 ligand (T cell).[36, 37] There are some B cells that do not require help from T cells and, instead, the antigen concomitantly activates toll-like receptors (TLRs) on the B cell to provide the second signal for activation.[37-39]

Antibodies, also called immunoglobulins (Ig), are actually just secreted BCRs. Antibodies serve several functions, including neutralizing infectivity of pathogens directly, opsonizing pathogens and infected cells (e.g., tag them for removal via phagocytes or ADCC), and activating the complement system. There are four main classes, or isotypes, of antibodies (IgG, IgM, IgA, and IgE) whose functionality are tailored towards different types of pathogens.[40] B cells receiving T cell help result in more robust and versatile antibody production, as some of these B cells will be able to undergo processes in germinal centers that increase antibody affinity and allow antibody class switching.[37, 38] B cells that do not receive T cell help produce "natural antibodies," which are functionally less versatile (IgM only) and tend to have lower affinity; however, these natural antibodies are rapidly available.[39]

Long-lived antibody-secreting B cells (long-lived plasma cells) and memory B cells are B cells that persist after initial antigen exposure, trained by natural infection or immunization. Upon secondary antigen challenge/infection, memory B cells are able to quickly proliferate and differentiate into plasma cells themselves, producing new antibodies in addition to the pre-existing antibodies produced by the long-lived plasma cells.[37, 41, 42]

T cells

Given its title, I believe it will come as no surprise that the T cell section will be the most in-depth immunology review within this book. The following section will cover an overview of T cell development, subtypes, activation, memory and regulation, and end with a brief touch on T cell exhaustion.

T cell development

Like B cells, **T cells** are generated in the bone marrow. However, their maturation occurs in the thymus (hence the T in T cell). When these precursors arrive to the thymus, they are missing many defining markers of mature T cells, including the CD3 complex, CD4 and CD8 co-receptors (double-negative), and a rearranged **T cell receptor (TCR)**.[43] Also similar to B cells with their BCR, the TCR is composed of two chains, which undergo somatic recombination during development in the thymus, such that every TCR is, largely, unique from one another.[44] The majority of mature T cells are α:β T cells but there is a minority population of γ:δ T cells, each named for the chains that make up their TCR.[43] The contents of this thesis refers almost exclusively to α:β T cells.

After TCR rearrangement, immature T cells will become double-positive for CD4 and CD8 expression. These TCRs are tested by specialized thymic epithelial cells that express unique self-antigens in both MHC I and MHC II complexes, as well as bone-marrow derived DCs and macrophages.[45-47] Which T cells survive this selection process is a bit of a goldilocks situation, or *lagom* if you're Swedish. T cells that engage too strongly with peptide:MHC complexes will undergo negative selection and be eliminated, while those that do not engage strongly enough, or at all, face "death by neglect." Thus, to receive survival signals and continue their development to become a naïve T cell (positive selection), the affinity of TCR interaction with peptide:MHC complex needs to lie somewhere in between.[46, 47] T cells that react too strongly towards self-antigens need to be removed, otherwise they can lead to autoimmunity. At the same time though, some of these T cells that respond stronger, but not strongest, against self-antigen can be well-suited to regulate other T cells. Generation of such regulatory T cells (Tregs) can help restrict auto-reactive T cells in the periphery and maintain homeostasis.[48, 49] Concurrently during these negative and positive selection processes, the developing T cell

commits to one of the co-receptors in relation to which MHC molecule it interacted with during positive selection.[47] At this point, the naïve T cell is matured, expressing a functional TCR and either CD4 or CD8 co-receptor (single positive), and will emigrate from the thymus into circulation.

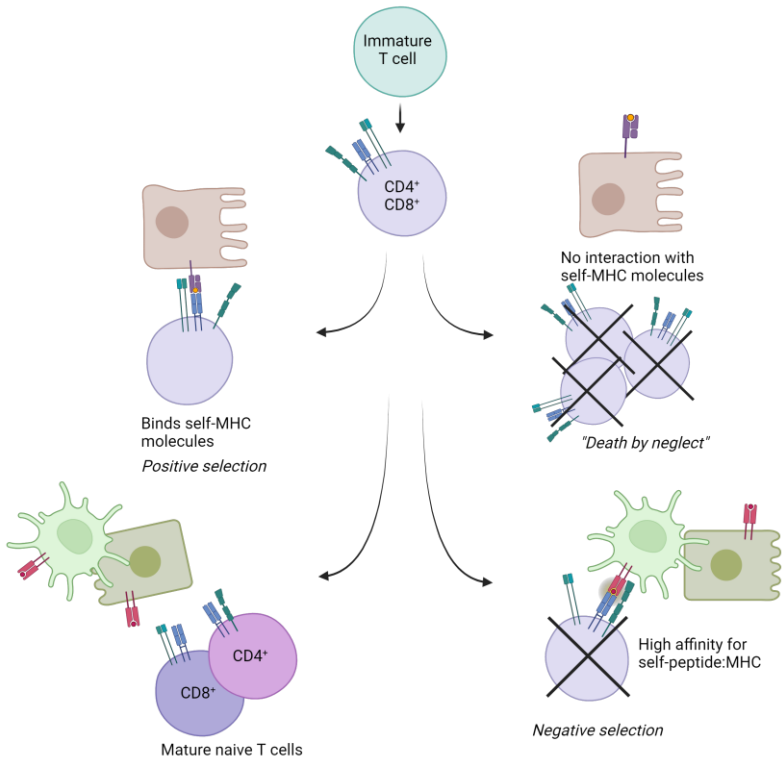


Figure 1: Overview of thymocyte development. During maturation in the thymus, immature T cells undergo T cell receptor (TCR) rearrangement and gain expression of CD4 and CD8 (double positive) co-receptors. The double positive T cell then undergoes positive and negative selection processes. T cells with TCRs that do not recognize self-MHC molecules with undergo death by neglect, while those that do will receive survival signals (positive selection). The TCR will also be tested for affinity towards self-peptide:MHC complexes, and those with very high affinity will be removed (negative selection). At the end of these processess, a single positive CD4 or CD8 mature naïve T cell is generated and will leave the thymus. Created with BioRender.com.

T cell subsets

Similar to every immune cell you've read about thus far, T cells are divided into different subsets based on their function and expression of receptors and transcription factors. The main division is based on which co-receptor the T cell expresses, CD4 or CD8.

CD4 T cells are also known as the **helper T cells (Th cells)**, as their activation aids the functionality of other immune cells, namely through the cytokines they secrete. Th cells are polarized based on the type of infection and the surrounding cytokine milieu.[50, 51] **Th1 cells** are identified by expression of the transcription factor Tbet and produce cytokines that contribute to type 1 immune response, notably **IFN-γ** (type 2 interferon). Th1 cells help enhance the microbicidal activity of macrophages and B cell class-switching to opsonizing IgG antibodies. **Th2 cells** express the transcription factor GATA3, produce the cytokines interleukin (IL)-4, IL-5, and IL-13 to contribute to type 2 responses by promoting responses from mast cells and eosinophils as well as IgE secretion. **Th17 cells** express the transcription factor ROR-γt and their classical cytokines include their namesake IL-17 as well as IL-21 and contribute to type 3 immune responses by amplifying neutrophilic responses and promoting IgG2 and IgG3 class-switching. Further, Th17 cells are important in protection in epithelial barriers, inducing epithelial cells to produce antimicrobial peptides.[50, 51] Additionally, T follicular helper cells (**Tfh cells**) present in B cell follicles are an integral component of humoral immunity, providing antigen-specific B cells with proliferation and differentiation signals via IL-21, IL-4, and CD40 ligand (CD40L). Tfh cells express the transcription factor Bcl-6, which is required for the expression of the chemokine receptor CXCR5, an essential receptor for trafficking and retention in the follicle.[50-52]

Regulatory T cells (Tregs) are CD4 T cells that function to minimize, or regulate, effector T cell responses by secreting anti-inflammatory cytokines (e.g., IL-10, TGF-β), outcompeting other T cells for activating mediators and metabolites, direct cytolytic activity (e.g., production of granzymes), or suppressing DC maturation.[53] Tregs are characterized by their transcriptional regulator FoxP3 and constitutively high expression of CD25 (IL-2Rα) and CTLA-4 (cytotoxic T-lymphocyte associated protein 4). Tregs can be generated "naturally" during thymic maturation (natural Tregs)[49] or can be induced in the periphery (peripheral Tregs) when a naïve CD4 T cell encounters its antigen in the presence of IL-2 and transforming growth factor (TGF)-β, although the latter constitutes a much small proportion of the total Treg population.[54] Overall, CD4 T cells are extremely heterogeneous, plastic, and dynamic, continuously adapting to their environment, and can adopt expression profiles and functions associated with different lineages.[51]

Lastly, there are **CD8 T cells**, also termed cytotoxic T cells or cytotoxic T lymphocytes (CTLs), due to their high cytolytic capacity. CD8 T cells can directly kill target cells either by release of preformed cytotoxic granules (granzymes, perforin, granulysin) or receptor-mediated apoptosis (Fas/FasL binding).[55] Further, activated CD8 T cells produce large amounts of IFN-γ as well as the cytokines TNF-α and LT-α (lymphotoxin-α). These features make CD8 T cells extremely proficient at targeting virus-infected cells and cancer cells.

T cell activation

Naïve T cells circulate the body in a quiescent state in search of their antigen. Activation of naïve T cells requires three signals, typically coming from DCs.[56, 57] (i) The TCR binds to its specific antigen peptide in the context of presentation on an MHC molecule. CD4 T cells engage with peptide:MHC II complexes and CD8 T cells engages with peptide:MHC I complexes. (ii) Upon receiving activating signals and maturation, DCs increase expression of costimulatory molecules (CD80/CD86) that must be engaged by CD28 on the T cell. (iii) The last signal comes from DC cytokines which support T cell differentiation, as discussed above.

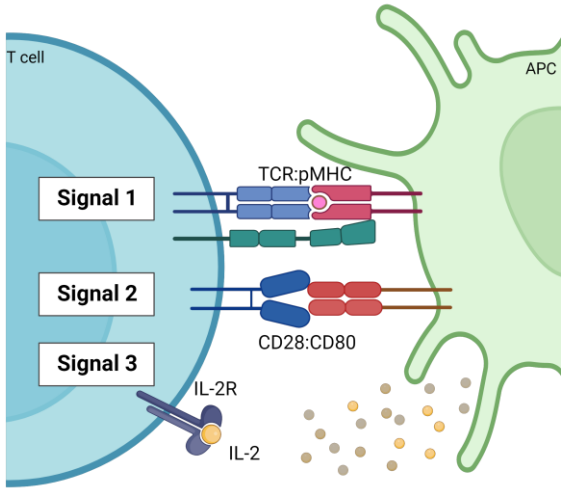


Figure 2: Naïve T cell activation requires 3 signals. Signal 1= T cell receptor binds to peptide:MHC complex (TCR:pMHC) on an antigen presention cell (APC). Signal 2= The costimulatory receptor CD28 on the T cell binds to costimulatory molecules (CD80, CD86) on the APC. Signal 3= APC secretes cytokines to support T cell survival, proliferation, and differentiaion (pictured: interleukin-2 [IL-2] binding to the IL-2 receptor). Created with BioRender.com.

Downstream signaling of CD28 costimulation results in secretion of IL-2 as well as expression of the high-affinity IL-2 receptor (IL-2Rα , or CD25).[58] This autocrine IL-2 signaling promotes the T cell's survival, differentiation, and proliferation and its importance in this thesis is discussed further in *Immunomodulation with Monoclonal Antibodies*. Further, naïve CD8 T cells often also require CD4 T cell help to receive proper costimulation to become activated. Activated CD4 T cells can provide this help via their IL-2 production nearby, as well as by "licensing" cognate DCs via CD40/CD40L interactions, which results in increased costimulatory molecule expression on the DC to sufficient levels to activate the CD8 T cell.[59]

Effector T cells

The exact trajectory of an activated naïve T cell is still debated, although the immediate subsequent stage seems to be either an effector T cell or a memory T cell.[60] **Effector T cells** are identified by losing receptors and ligands that assist in trafficking and retention in secondary lymphoid organs. This includes loss of or downregulation of CCR7, S1PR1, and CD62L, receptors that aid in naïve cell maintenance like CD127 (IL-7Rα), and CD45RA. Effector T cells are also demarcated by increased expression of CD44 and other markers of activation, such

as CD69, PD-1, and CD25.[61, 62] Upon resolution, the majority of effector T cells will die but a small percentage (5-10%) will live on as memory T cells,[63] primed for rapid response after secondary challenge.

Memory T cells

Memory T cells are T cells that are antigen-experienced and persist long-term. Memory T cells may arise either from effector T cells that persist after contraction of the immune response or from naïve T cells after activation.[60, 63, 64] There are two main factions of memory cells, central memory T cells (T_{CM}, $CD44^+CD62L^+CCR7^+CD45RO^+$) and effector memory T cells (T_{EM}, $CD44^+CD62L^-CCR7^-CD45RO^+$). **$T_{CM}$** cells circulate within blood, lymphatics, and secondary lymphoid organs, which can be deduced based on their expression of CD62L and CCR7, and are functionally defined by their high proliferation capacity and IL-2 production. **T_{EM}**, on the other hand, circulate throughout the blood and are recruited to sites of inflammation, where they produce effector cytokines rapidly, and it has been suggested that CD4 T_{EM} cell retain some plasticity upon rechallenge.[63, 64]

In addition to quicker responses, memory T cells differ from naïve T cells in that they do not require tonic MHC stimulation for survival and can be reactivated via cytokines alone, a phenomenon called **bystander activation**.[65, 66]

Regulation of T cells

All immune responses require regulation. A healthy immune response is one that can be turned on when needed and off once the job is done, and unregulated immune responses lead to autoimmunity and chronic inflammation. T cells begin being screened for autoreactive clones in their early developmental stages in the thymus and regulatory T cells can be induced both in thymus and periphery to modulate T cell (and other leukocytes) activity.[46, 54] Additionally, upon activation, T cells will upregulate expression of **coinhibitory molecules**, such as **PD-1** (programmed cell-death protein 1), **CTLA-4**, and **LAG-3** (lymphocyte activation gene 3).

Coinhibitory molecules inhibit effector T cell responses via various mechanisms, such as out-competing CD28 for costimulatory molecules on APCs (CTLA-4), providing intracellular inhibitory signals upon TCR-ligation (PD-1, Figure 3),[67] or interfering with MHC binding (LAG-3).[68-70] There are more coinhibitory molecules than these three receptors (e.g., Tim3, TIGIT)[71, 72] but these three receptors are currently the only ones with clinical utility via **immune checkpoint inhibitors (ICI)**. ICI therapy will be discussed further in *Immunomodulation with Monoclonal Antibodies*.

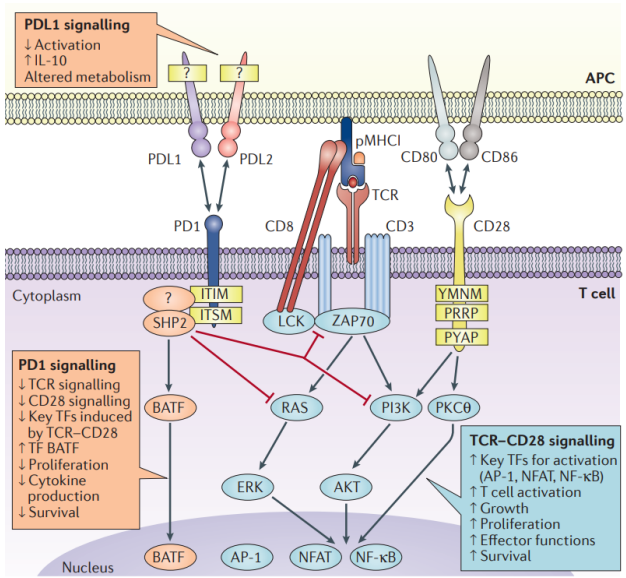


Figure 3: Intracellular signaling of PD-1. The cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). Phosphorylation of the ITIM and ITAM upon PD-1 ligation with its ligands (PD-L1, PD-L2) results in SHP2 recruitment. Phosphorylation of SHP2, a phosphatase, results in inhibition of several downstream TCR and co-stimulatory receptor signaling molecules (e.g., LCK, ZAP70, PI3K, RAS). This ultimately reduces the translocation of transcription factors necessary for T cell activation (e.g., NFAT, NF-KB). The same cell presenting the PD-1 ligand must also present peptide:MHC complex to the T cell to deliver these inhibitory signals. Reproduced with permission from (67) ©Springer Nature.

T cell exhaustion

Upon repeated antigen exposure, such as in chronic infections and cancer, T cells become exhausted. However, like most things in immunology, it's not as simple as that. The path of becoming an exhausted T cell is quite nuanced and still being understood.

It is largely agreed upon, though, that exhausted T cells will display increased expression of coinhibitory molecules, reductions in proliferation and cytokine producing capacities, and undergo transcriptional and metabolic changes.[73, 74] That being said, the process is seen more as a progressive gradient, and T cells that are "not all the way exhausted" may retain some of their functionality. These T cells often are called **progenitor exhausted T cells** or stem-like T cells and can be identified by expression of Slamf6, PD-1, and the transcription factor TCF1. These progenitor cells are still committed to exhaustion and pass this phenotype on to their

progeny.[74] However, it has been demonstrated that these TCF1^{+} T cells are also responsible for sustained immune responses in chronic infection and are the T cells that can be "rescued" by ICI therapy to enhance immune responses.[75-77] At the end of this exhaustion spectrum are **terminally exhausted T cells**, identified as PD-1^{high}Slamf6^{-}TCF1^{-} as well as Tim3 expression and increased expression of the transcription factor Tox. Tox has been shown to be needed to generate the dysfunctional responses associated with exhaustion and correlates to PD-1 expression.[78]

T cell exhaustion has sort of picked up a negative connotation, as it is often discussed in the context of providing suboptimal responses to cancer. But exhaustion and negative regulation of T cells is important in maintaining control on immune responses and can be beneficial in organ transplantation and autoimmunity.[79] It is also important to point out that expression of many of the markers present on exhausted T cells are also upregulated upon activation, or re-activation, of T cells, making it difficult to distinguish recently activated T cells and exhausted T cells based on markers alone.

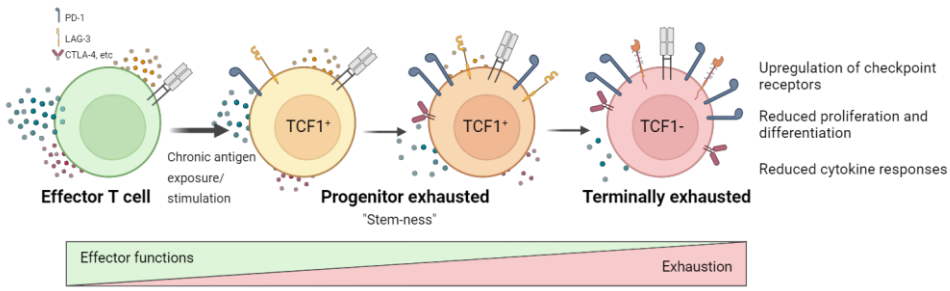


Figure 4: Progression from T effector to exhausted. Upon chronic antigen stimulation, T cells will start progressing towards a state of exhaustion, increasing expression of coinhibitory molecules (e.g., PD-1, LAG-3, CTLA-4), alter their metabolism and transcriptional programming, and lose proliferation and polyfunctionality capabilities. Created with BioRender.com.

Cardiovascular Disease

The cardiovascular system is responsible for circulating blood throughout the body and is comprised of the heart and blood vessels (arteries, veins, capillaries). **Cardiovascular disease (CVD)** is the leading cause of death worldwide, accounting for approximately 18 million deaths annually.[80] There are several forms of CVD, however the top two culprits for CVD-related deaths are ischemic heart disease and ischemic stroke.[80-82]

Ischemia is the term for when a tissue is not receiving an adequate amount of blood. If the tissue is starved for oxygen long enough, tissue death occurs, and this process is an infarction. The driver behind this interruption in blood flow and ischemia is typically the formation of a thrombus or embolus caused by **atherosclerosis cardiovascular disease (ASCVD)**. When this ischemia and infarction occurs within the coronary vessels, the blood vessels that supply the heart muscle itself, it is a myocardial infarction (MI; "heart attack"). If this occurs in a blood vessel that feeds the brain, it can result in an ischemic stroke.

CVD-related deaths are costly not only in lives but also to society. Between the years 2018 and 2019 alone, direct costs of CVD in the United States alone were estimated to be over $250 billion (USD).[82] For all of these reasons, it is imperative research continues to be done to determine new therapeutics and preventative measures as well as continuing our efforts to educate on lifestyle modifications in pursuit of better outcomes and a healthier world.

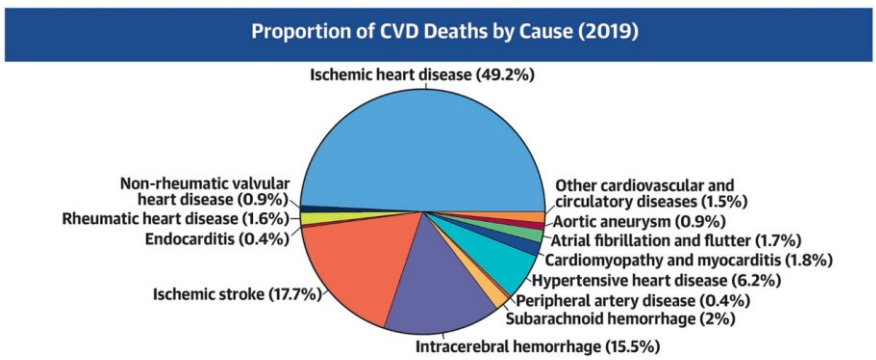


Figure 5: Proportion of cardiovascular deaths by cause worldwide, 2019 (80).

Atherosclerosis

The Healthy Artery

Arteries are responsible for delivering oxygenated blood from the heart to the rest of the body. There are three layers of an artery: the tunica intima, tunica media, and tunica adventitia (Figure 6).

The **intima** faces the lumen of the vessel and is lined with a monolayer of endothelial cells called the **endothelium**. The endothelium serves several homeostatic properties, providing a dynamic barrier between circulation and the vessel and regulates vascular tone and thrombosis. Disruption of this barrier functions leads to various disease settings, including atherosclerosis.[83-85]

One of the characteristic features of an artery is its thick **media** layer, comprised predominantly of contractile vascular smooth muscle cells (VSMCs). These VSMCs lie on an endothelial matrix composed of collagen, elastin fibers, and proteoglycans. Between the intimal and medial layers lies the internal elastic lamina and between the media and adventitia is the external elastic lamina, both of which are composed of elastin fibers. This thicker contractile and elastic medial layer is necessary for arteries and arterioles to compensate for and withstand the high blood pressure of the arterial system.[83, 84]

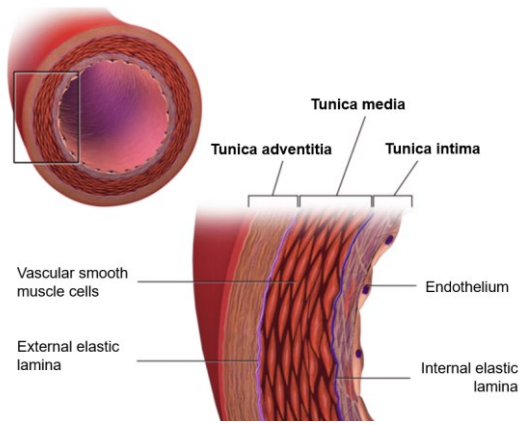


Figure 6: Healthy arterial vessel. Adapted from: Point-based Registration of Vascular Structures: Applications for Diagnostic and Interventional Procedures. Common creative licensed image, reprinted from [Anat 19].

The most external layer is the **adventitia**, consisting of connective tissues, namely collagen and elastin, that helps provide anchoring of the vessel to nearby tissues. The adventitia is predominated by fibroblasts, but macrophages, DCs, T cells, and

B cells can also be found in the adventitia along with lymphatic vessels and nerves.[83, 84]

Lipoproteins

Cholesterol and triglycerides are essential macromolecules for the body. Cholesterol is necessary for cell membrane structure and fluidity and serves as the precursor to several vital biological substances, such as steroid hormones, vitamin D, and bile salts. Meanwhile, triglycerides functions as a source of dense energy storage in the body. As lipids, both cholesterol and triglycerides are insoluble in water and must be coupled to water-soluble proteins in order to be transported in circulation.[86]

Lipoproteins allow for the transportation of cholesterol and triglyceride molecules in blood by surrounding these lipids with phospholipids and **apolipoproteins** (apo). There are several classes of lipoproteins based on size, lipid composition, and apolipoprotein composition. Lipoprotein nomenclature derived from the increase in lipoprotein density as the protein-to-triglyceride ratio increases with concomitant decreases in size (Figure 7).[87]

Chylomicrons are large, very triglyceride-dense, and have **apoB-48** as the core structural apolipoprotein. Chylomicrons transport dietary triglycerides and cholesterol to peripheral tissues and delivery/removal of triglyceride molecules results in production of **chylomicron remnants** enriched with cholesterol. These remnants can be removed from circulation via binding of **apoE** to hepatocyte receptors.[87]

Very low-density lipoproteins (**VLDL**) are produced in the liver, are rich in triglycerides, and use **apoB-100** as their core apolipoprotein. As triglyceride is removed from these particles, intermediate-density lipoproteins (**IDL**) are produced. Further removal of triglycerides from VLDL and IDL results in formation of **low-density lipoproteins (LDL)**, the predominant carrier of cholesterol in circulation. Of note, apoB-100 in the ligand for the **LDL receptor (LDLR)** and thus play a vital role in lipid clearance. Several features of LDL are pro-atherogenic, including capability of transcytosis across endothelium, propensity for arterial retention due to apoB-100 interactions with proteoglycans, and high susceptibility to oxidation (**oxLDL**).[87, 88]

High-density lipoproteins (HDL) are a little different in that they function in reverse cholesterol transport, taking cholesterol from peripheral tissues to the liver. Additionally, HDL and its apoA-I core have anti-inflammatory and antioxidant properties. As a result, HDL has been regarded to be anti-atherogenic.[87, 89]

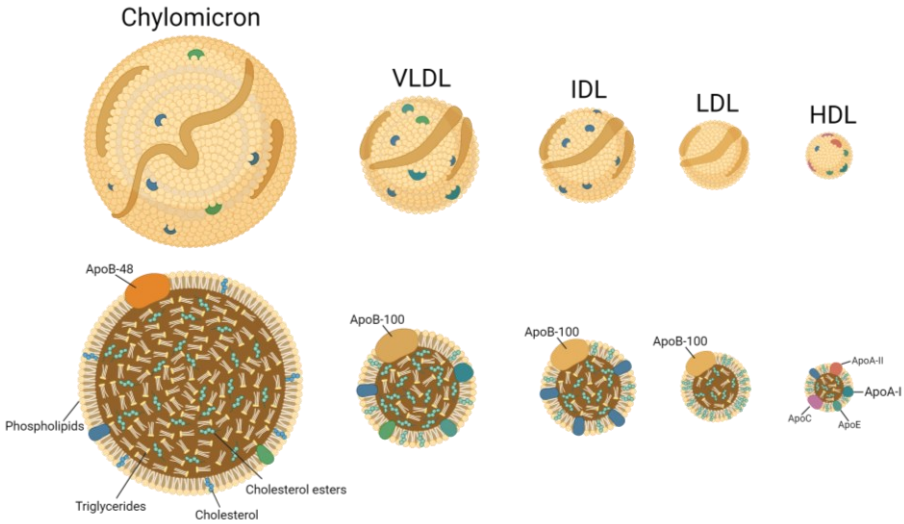


Figure 7: Classes of lipoproteins. Lipoproteins are utilized by the body to transport water-insoluble cholesterol and triglycerides in circulation from the small intestine and liver to peripheral tissues. Lipoproteins are characterized by their size, density, and composition of lipids and apolipoproteins (Apo). VLDL: very low-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; HDL: high-density lipoprotein. Created with BioRender.com.

Pathogenesis

The main underlying pathology of both MI and strokes is **atherosclerosis**, the process of lipid-rich plaque formation within the walls of medium to large sized arteries. Formation of the atherosclerotic lesion begins with the accumulation of LDL within the intimal layer of the arterial wall. The initial connection of excess cholesterol and atherosclerosis came from studies over 100 years ago, in which rabbits fed a high-cholesterol diet (HCD) developed hypercholesteremia and subsequently atherosclerotic lesions in their arteries.[90] Its contribution to atherosclerosis is why LDL received the moniker "bad cholesterol" and why your physician may harp on it during your annual bloodwork.

Perturbations to the endothelial layer are, at least in part, responsible for entry of excess LDL particles into the arterial intima. Factors including increased shear stress, such as at sites of non-laminar flow, and increased expression of adhesion molecules (e.g., VCAM-1) from an atherogenic diet have been suggested to impact endothelial homeostatic function.[91, 92]

Once within the intima, LDL particles can be retained in various ways. Proteoglycans secreted within the intimal have been shown to bind to apoB100-containing particles and vascular smooth muscle cells (VSMCs) can take up these aggregated LDL particles.[93-95] Further, LDL can undergo different modifications within the intima. In particular, LDL can become oxidized (oxLDL) and this oxLDL can be taken up by scavenger receptors on macrophages.[95] Monocytes, along with other immune cells, are recruited to the intima via the upregulation of adhesion molecules previously mentioned, as well as by secretion of chemokines by vascular cells.[96] Upon entering the vascular tissue, these recruited monocytes will differentiate into macrophages and excessive uptake of oxLDL by intimal macrophages creates lipid-laden **foam cells**- the hallmark sign of early plaque development.[95]

Increased accumulation of intracellular cholesterol leads to upregulation of toll-like receptors (TLRs) and activation of NLRP3 (nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin-domain containing 3) inflammasome.[97, 98] Activation of the NLRP3 inflammasome leads to secretion of the pro-inflammatory cytokines IL-1β and IL-18, and has also been suggested to cause phenotypic switching of VSMCs.[99] As the capacity for lipid-uptake is exceeded, extracellular lipid pools begin to arise. Similarly, the formation of necrotic cores occurs, due to defective efferocytosis (clearance of apoptotic debris) and increased necrosis of these lipid-laden cells.[95, 100, 101] A feed-forward cycle of inflammation takes over, potentiating recruitment of more immune cells to the growing atherosclerotic lesion, including B cells and T cells.[102] The role of T cells in atherosclerosis is covered in more detail in *T cells in Atherosclerosis*.

There are regulatory mechanisms trying to combat the inflammatory milieu and to stabilize the growing lesion. A fibrous cap will form on top of the growing necrotic core, so that its thrombogenic material remains separated from the lumen.[103] This fibrous cap consists of VSMCs and extracellular matrix they secrete, namely collagens. A thin fibrous cap is associated with unstable or "vulnerable" plaques, prone to rupture. Fibrous cap thinning can occur due to several factors, such as blunted collagen synthesis by VSMC or breakdown of extracellular matrix by proteolytic enzymes (e.g., matrix-metalloproteinases).[103-106] In addition to a thin fibrous cap, atherosclerotic plaques can be categorized as vulnerable due to: large size, extensive outward remodeling, intraplaque hemorrhage and neovascularization, and adventitial inflammation.[104]

Of note, there are competing theories that suggest the vascular inflammation in atherosclerosis development actually begins from the "outside-in," such that the initial stages occur in the adventitia instead of the intima.[107, 108] Upon activation, adventitial fibroblasts secrete inflammatory cytokines and chemokines and express adhesion molecules, increasing immune cell recruitment to the adventitia.

Adventitial fibroblasts will also differentiate into motile myofibroblasts in an inflammatory environment, which participate in extracellular matrix turnover and cardiac remodeling.[107-111]

ASCVD progresses largely subclinical- that is, the majority of the processes I have outlined above happen without the person showing any symptoms. While it is possible the plaque can grow so large that it can critically occlude the artery, the majority of clinical complications stem from plaque rupture. Among other things, a ruptured plaque exposes thrombogenic material, initiating the coagulation cascade, and creating a thrombus (blood clot). This thrombus can then either occlude the vessel locally or break off and cause blockage at a distance vessel (embolism), ultimately causing an acute ischemic event.

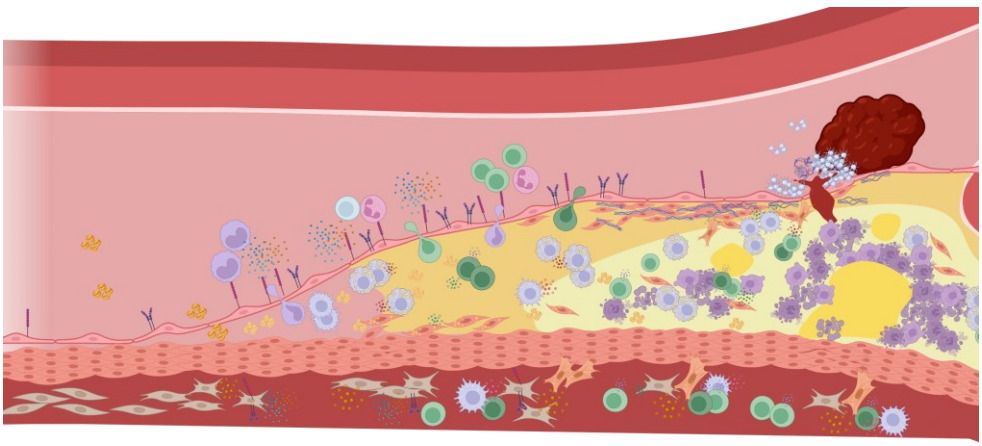


Figure 8: Disease progression of an atherosclerotic plaque. Overview from initiation to rupture. Created with BioRender.com.

IL-1 in atherosclerosis

IL-1 is an extremely pleotropic cytokine and has repeatedly been shown to promote atherosclerosis. While macrophages are the major source of IL-1, multiple other cells produce cytokine, including monocytes, neutrophils, endothelial cells, VSMCs, fibroblasts, keratinocytes, and platelets. There are two isoforms of IL-1, IL-1α and IL-1β, and both are expressed as pro-forms. Canonical cleavage of pro-IL-1-β into active IL-1β requires activation of caspase-1 and thus also the assembly of the NLRP3 inflammasome, however, IL-1α has biological effects even in its pro-form.[112, 113] In addition to being produced in response to PRR signaling, previous studies have indicated that cholesterol crystals and oxLDL can also initiate NLRP3 assembly and IL-1 production in macrophages.[97, 114, 115] Additionally, endothelial

cells and VSMCs are capable of producing IL-1, and necrotic and apoptotic VSMCs may release IL-1 that triggers subsequent IL-1 release by surviving VSMCs.[116-119] Taken together, the atherosclerotic plaque contains the cells and molecular triggers needed to produce IL-1 throughout course of the disease.

The effects of IL-1 signaling vary depending on which cells are affected. IL-1 can induce its own secretion, providing a positive-feedback loop.[120, 121] IL-1 also increases leukocyte adhesion and trafficking via increased expression of adhesion molecules on endothelial cells and chemokine release (e.g., CXCL8, MCP-1).[122-124] Additionally, IL-1 increases granulopoiesis[125] and NET formation and NETosis,[126] and neutrophil degranulation, which can further process IL-1 family cytokines (IL-1α, IL-33, IL-36α/β/γ).[127-129] IL-1-β can also affect T cell differentiation directly (Th17)[130] as well as T cell activation indirectly via maturation effects on DCs.[131, 132] Multiple mouse studies have confirmed pro-atherogenic effects of IL-1, with studies in which IL-1 signaling is hindered resulting in reduced lesion size, less adhesion molecules in the aorta, and increased plaque vulnerability (increased macrophage content and decreased smooth muscle cells). [97, 115, 133-136]

Treatments targeting IL-1 signaling have shown promise in preventing ASCVD events, and these therapies are discussed further in *Current Treatments* as well as throughout the discussions of **Paper IV**.

Risk Factors

Classical risk factors

The have been several risk factors identified for ASCVD and these can be split into nonmodifiable and modifiable factors. **Nonmodifiable risk factors** include age, male sex, race, and genetic components. **Modifiable risk factors** for ASCVD are hypertension, dyslipidemia (elevated LDL, reduced HDL), smoking, obesity and sedentary lifestyle, and diabetes.[137-142]

Risk assessment

The idea of cardiovascular risk factors originates from **The Framingham Heart Study**.[137] The history of this study is actually quite interesting. The study began officially in 1948 in the quintessential small- town of Framingham, Massachusetts, during a time when 1 in 2 deaths in the U.S. were from cardiovascular disease. Included in these deaths was President Franklin Delano Roosevelt, whose passing in 1945 led to the National Heart Act, which ultimately ended up funding the study. Since then, there have been multiple follow-ups and a few offshoots from the original study, conducted by cooperation of Boston University and the National Heart, Lung, and Blood Institute.[143] To this day, the **Framingham risk score** (10-year risk assessment based on age, sex, total cholesterol levels, HDL levels, systolic blood pressure, smoking, and diabetes) is still extensively used, especially in cardiovascular research.

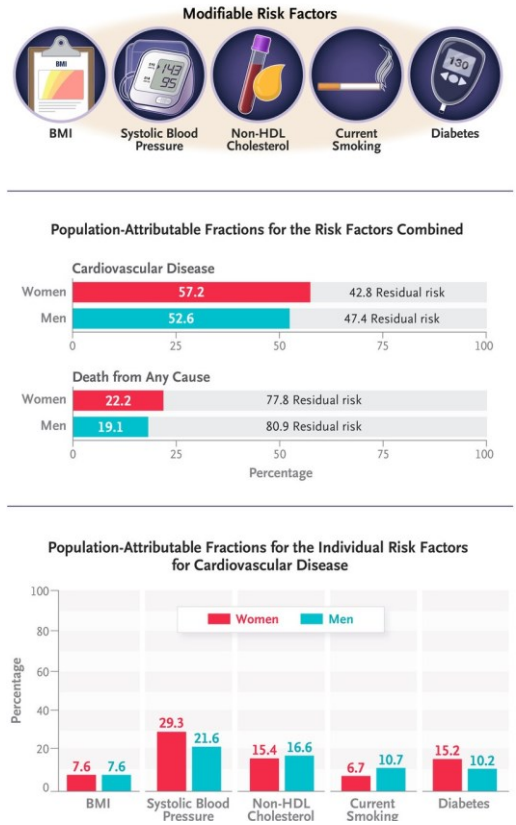


Figure 9: Attribution of five modifiable CVD-related risk factors on cardiovascular disease and all-cause mortality over 10 years. Measured in 112 cohort studies, spanning 34 countries. Modifiable risk factors are combined in the top pane and then stratified individually in the bottom pane. Reproduced with permission from (142) ©Massachusetts Medical Society.

Additional algorithms have been implemented to assess a person's 5-year and 10-year cardiovascular risk, in both those with present risk factors and apparently healthy people (no established ASCVD, no type 2 diabetes, no other severe comorbidities).[141] Prevention guidelines employed by physicians intensify with increasing risk, and, in general, those with the highest risk stand to receive the most benefit from treatment.

Current Treatments

I want to preface this section with this: atherosclerosis therapies are focused on prevention and maintenance, not on a "cure," so to speak.[144] The deposition of lipids and formation of fatty streaks can be observed in early adolescence, with some studies suggesting occurrence even as early as childhood and infancy.[145, 146] However, a fatty streak is far from morphology and clinical implications of an advanced atheroma. Indeed, it has been shown having low levels of risk factors before mid-life age can reduce the risk of cardiovascular events later in life, irrespective of post mid-life lifestyle.[147, 148] Still, at any age, it has been shown that duration of ideal cardiovascular health is important for mitigating both subclinical disease and risk of cardiovascular events.[149, 150]

Thus, at the forefront of atherosclerosis treatment is **lifestyle modification**. Examples include smoking cessation, regular physical activity, healthy diet (e.g., encouraging fruits, vegetables, fiber; limiting salt, alcohol, sugar-sweetened beverages, red meat, trans unsaturated fatty acids) and mental healthcare (e.g., stress management). The goal of such strategies is to attain/maintain guideline values of blood cholesterol, blood glucose, and blood pressure. Failure to meet such guidelines leads to pharmacological intervention.[141]

Lipid-lowering therapies

The first-choice drug to reduce LDL levels, and thus cardiovascular risk, are statins. **Statins** are a group of drugs that disrupt cholesterol synthesis in the liver (HMG-CoA reductase inhibitor). Disruption of cholesterol synthesis in hepatocytes decreases intracellular lipid levels, causing an increase in expression of the LDL receptor and uptake of LDL from the blood into the cell.[151] Although the first statin (lovastatin) was approved for lowering blood cholesterol by the FDA in 1986, it took until almost the turn of the century for statin therapy to solidify its role in primary and secondary prevention of coronary heart disease.[152-156] Nowadays, statins are one of the top prescribed medications, with reports of 35% of the general U.S. population taking any kind of statin in 2019.[157]

However, there is the possibility of residual cholesterol risk or intolerability with statin use. In these cases, other lipid-lowering therapies may be added to or replace statin regiments. One such example is ezetimibe, a cholesterol absorption inhibitor, which may be suggested either when statins cannot be used, or the therapeutic goal is not reached with a statin alone.[141] Another option is PCSK9 inhibitors (proprotein convertase subtilisin/kexin type 9). PCSK9 inhibitors are monoclonal antibodies that result in upregulation of the LDL receptor on hepatocytes by inhibiting their degradation by the enzyme PSCK9.[158] Similarly, PCSK9 inhibitors can be considered as a monotherapy or given in adjunct to either statin or ezetimibe; but, as an antibody therapy, is quite costly.[141] Recently, bempadoic acid has been shown to reduce major adverse cardiovascular events in specifically statin-intolerant patients, possibly providing another treatment avenue for these patients.[159]

Statins are very pleiotropic drugs and have also been recognized for their anti-inflammatory properties, in particular, their dose-dependent and LDL-independent effects on lowering C-reactive protein (CRP; biomarker of systemic inflammation).[160] Multiple studies have confirmed this dual-mechanism of statins is beneficial for cardiovascular outcomes and highlighted the importance of addressing what has been termed this **residual inflammatory risk** in treating ASCVD.[161-163]

Anti-inflammatory therapies

It is clear that atherosclerosis is both a lipid- and inflammatory-driven disease. Levels of the inflammatory cytokines IL-6 and TNF-α, as well as the downstream inflammatory marker CRP, have been shown to be correlated with future adverse cardiovascular events.[161, 164]

Providing the first proof-of-concept for targeting inflammation to reduce cardiovascular risk, the **CANOTS trial** showed treatment with a monoclonal antibody that neutralized IL-1β (**canakinumab**) decreases risk of recurrent cardiovascular events in individuals with previous MI, with dose-dependent reductions in CRP and IL-6 but irrespective of lipid lowering.[165] Trials investigating the effects of **colchicine**, an anti-gout drug that also targets the NLRP3 inflammasome and maturation and release of IL-1β, have also yielded positive results as secondary prevention.[166-169] In fact, just this year (2023) the FDA has approved reformulated low-dose colchicine (**Lodoco**) for the indication of ASCVD, making it the first anti-inflammatory agent with this approved indication. Targeting IL-6 signaling has also shown promise. A subfraction of participants from the RESCUE trial (included based on chronic kidney disease and elevated CRP levels) treated with anti-IL6 monoclonal antibody (**ziltivekimab**) demonstrated a 77-92% decrease in CRP levels, as well as many additional biomarkers for atherosclerotic risk.[170] These promising results have led to studying the effects ziltivekimab on

major adverse cardiovascular events in this patient population at a much larger-scale in the ongoing **ZEUS trial**.[171]

Still, other anti-inflammatory medications have been investigated in the treatment of ASCVD. **Anakinra**, a recombinant form of the IL-1 receptor antagonist (IL-1Ra) , has been shown to decrease lesion size and inflammation in atherosclerotic mice.[172, 173] Further, patients with acute ST-segment-elevation myocardial infarction (STEMI) treated with anakinra exhibited reduced inflammation and reduced incidence of heart failure and hospitalizations for heart failure.[174] However, administration of low-dose methotrexate was shown to have no effects on cardiovascular event prevention nor attenuating levels of inflammatory markers (CRP, IL-1β , IL-6) in patients with stable atherosclerosis.[175]

Mo' inflammation, mo' problems

Further supporting the importance of inflammation in ASCVD, it has also been documented over the years the connection between chronic inflammatory diseases and increased ASCVD risk. The connection between rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and increased ASCVD risk have been appreciate for quite some time, with potential pathogenic mediators being attributed to elevated TNF-α, IL-1, and IL-6 in RA and elevated interferons in SLE.[176-178] Other inflammatory conditions that have been associated with increasing ASCVD and CVD risk include systemic sclerosis, vasculitis, psoriasis, gout, and inflammatory bowel diseases.[178-181] These associations further highlight the inextricable link between ASCVD and inflammation.

T cells in Atherosclerosis

It remained unappreciated that T cells were present in atherosclerotic plaques until the mid-1980s.[182] Now, T cells are known to be one of the most abundant immune cells within the plaque, with studies reporting somewhere between 25-38% of all leukocytes are $CD3^+$ and with $CD4^+CD3^+$ cells constituting 10%.[102] Plaque T cells are predominantly memory CD4 T cells and accumulate in the shoulder region of the plaque.[182-184]. Recent studies utilizing single cell sequencing, CyTOF (Cytometry by Time Of Flight), and CITE-seq (Cellular Indexing of Transcriptome and Epitopes sequencing) of human carotid plaques have demonstrated that plaque T cells are more activated and differentiated compared to circulating T cells in the blood, as well as display signs of clonal expansion.[185, 186] In mice, studies have shown that ablation of CD4 T cells decreases plaque burden in atherosclerotic mice while transfer of CD4 T cells to immunodeficient atherosclerotic mice potentiated atherosclerosis.[187, 188]

Autoimmunity

The presence of T cells (and B cells), detection of MHC II expression by plaque vascular smooth muscle cells, and indications of T cell clonal expansion have supported the notion that atherosclerosis might be driven by autoimmunity, that is, adaptive immune cells responding to self-antigen.[186, 189-192] As such, the hunt for "the" atherosclerotic antigen has been on for quite some time. The most sought-after candidate has been modified LDL (oxLDL), with several reports supporting the idea of it being an autoantigen in atherosclerosis.[102, 193, 194] However, there have been other candidate antigens, including heat shock proteins[195, 196] and foreign-derived antigens from agents such as cytomegalovirus (CMV) and *Chlamydia pneumoniae*.[190, 197, 198] While vaccination attempts against atherosclerosis showed promise in experimental models, it has been a challenge to translate them clinically, although trials are still ongoing.[199]

Helper T cells

As previously discussed, T cells are a very heterogenous cell population and thus their implication in atherosclerosis varies with subtype. Multiple studies have identified Th1 cells as pro-atherogenic. Atherosclerotic mice deficient in Th1 transcription factors (Tbet)[200] and homing-receptors (CXCR6, CCR5)[201, 202] display reduced atherosclerotic burden while those receiving Th1-potentiating cytokines (IL-12, IL-18) promoted plaque development and inflammation.[200, 203, 204] At heart of Th1 cell's proatherogenic effects is their production of IFN-γ. Early histological staining of human plaques revealed that IFN-γ^+ T cells were present.[205] Since then, numerous studies have confirmed IFN-γ as pro-atherogenic, including attenuation of atherosclerosis in mice deficient in IFN-γ or its receptor [206, 207] and acceleration when administered injections of IFN-γ.[208]

Reports on Th2 and Th17 cells in atherosclerosis are less clear-cut. Studies on the Th2 cytokine IL-4 reveal conflicting results. Atherosclerotic mice deficient in IL-4[209] or receiving T cells deficient in IL-4[210] have reduced plaques. However, early injections of IL-4 reduced lesion size in atherosclerotic mice[211] and higher levels of circulating Th2 cells and capacity for IL-4 production was negative associated with carotid IMT (intima-media thickness) and acute MI in patients.[212] Furthermore, the other prototypical Th2 cytokines, IL-5 and IL-13, appear to be protective against atherosclerosis.[213-215]

Similarly, the role of Th17 cells in atherosclerosis has largely been deduced by the actions of IL-17 and results have also been conflicting. Some studies indicate a protective role for IL-17, such that expression of IL-17 in lesions has been associated with more stable plaques in atherosclerotic mice[216, 217] and low serum levels of IL-17 in patients is associated with increased risk of acute MI.[218] Conversely, other studies have shown that loss of IL-17 function reduces lesion development and stability in atherosclerotic mice, suggesting pro-atherogenic implications of IL-17 signaling.[219, 220]

The role of Tfh cells in atherosclerosis is also still being discerned. Tfh cells have been shown to expand under hypercholesteremic conditions and loss of regulation of the cells accelerates atherosclerosis in mice.[221, 222] Further, atherosclerotic mice deficient in Tfh cells (*Bcl6*$^{-/-}$*Ldlr*$^{-/-}$) demonstrated a decrease in lipid area and percent lesion area in aortas compared to Tfh-competent mice.[223] Conversely, studies utilizing apoB-100-specific T cells identified that T cells recognizing LDL particles can develop into Tfh cells that induce B cell activation and produciton of anti-LDL antibodies, suggesting an athero-protective role of Tfh cells instead.[224]

CD8 T cells

CD8 T cells have been identified in human carotid and femoral plaques and displayed a more activated phenotype compared to those in blood.[225] However, manipulation of these cells in mouse models have revealed conflicting results. Transfer of CD8 T cells into lympho-deficient atherosclerotic mice (*Rag2*$^{-/-}$*Apoe*$^{-/-}$) increased lesion size as well as macrophage infiltration and necrotic cores, attributed to CD8-derived TNF-α, perforin, and granzyme B.[226] This same study showed depletion of CD8 T cells via anti-CD8 antibodies reduced plaque size and macrophages infiltration.[226] Yet, in a separate study, CD8 antibody-mediated depletion did not change plaque size and instead increased macrophage content and necrotic cores and decreased collagen content.[227] One potential important variable to keep in mind when comparing these studies is the duration of hypercholesteremia. CD8 T cells have been suggested to play a role in early responses to hypercholesteremia and athero-protective role in advanced lesions.[227, 228] Still, having a higher fraction of CD8 T cells in circulating PBMCs has been shown to be associated with increased incidence of coronary events[229] and it appears overall that more research supports a pathogenic role of CD8 T cells than not.

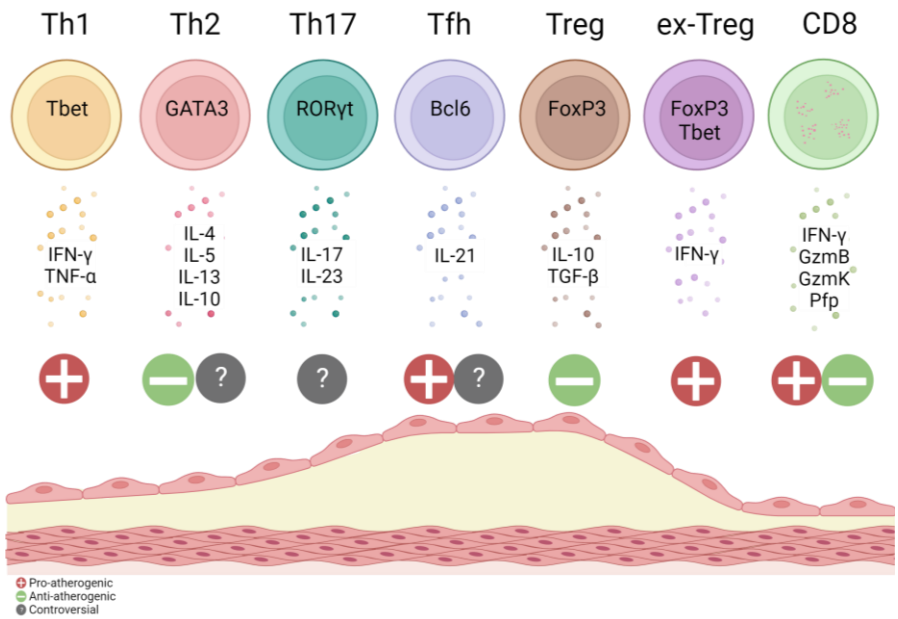


Figure 10: Summary of T cell phenotype and affect on atherosclerosis. Created with BioRender.com.

Regulation and Dysregulation

Regulatory T cells

Tregs have been shown time and time again to be protective against atherosclerosis. Potentially as a way to combat increasing inflammation with hypercholesteremia, Treg populations have been shown to be expanded and induced as well when atherosclerotic mice are fed a high-cholesterol diet and can migrate to the atherosclerotic aorta.[230, 231] Loss of Tregs, either by antibody depletion (anti-CD25)[232] or utilizing DEREG mice (with diphtheria toxin injection),[233] or disruption of their signature cytokines IL-10[234] and TGF-β,[235] results in increased plaque size in atherosclerotic mice. Clinical utility of measuring circulating Tregs in patients to predict risk has been shown to be limited, although this does not discredit the therapeutic potential of Treg-targeted therapies in ASCVD.[236]

Atherogenicity of plasticity

Additionally, the plasticity of Tregs has demonstrated atherogenic implications. Tregs may convert into Tfh- and Th1-like "ex-Tregs" with high-cholesterol diet feeding. These ex-Tregs have been shown to be pro-atherogenic and blocking their conversion limits plaque development in mice.[223] Similarly, $CCR5^{+}IFN\text{-}\gamma^{+}FoxP3^{+}$ cells are elevated in the aortas of *Apoe*$^{-/-}$ mice and display a loss of suppressive capacity.[237, 238] These results are in line with an earlier study that showed increasing duration of hypercholesteremia in mice leads to a reduction of Tregs in the lesion while simultaneously increasing lesional effector T cells. Further, Treg numbers could be maintained by switching back to a cholesterol-free diet.[230]

Coinhibitory molecules

Pro-atherogenic consequences from interruption of immune regulatory components aside from Tregs have been described. Of particular interest in this thesis is the effects of PD-1 in atherosclerosis. Single cell sequencing of human carotid plaques has revealed a subset of T cells expressing *PDCD1* (gene encoding PD-1) along with other markers of T cell exhaustion.[185, 186] Indeed, atherosclerotic mice deficient in PD-1 (*Pd1*$^{-/-}$*Ldlr*$^{-/-}$) or its ligands (*Pdl1/2*$^{-/-}$*Ldlr*$^{-/-}$) displayed increased T cell activation and accumulation in the plaques as well as larger lesions,[239, 240] while *Ldlr*$^{-/-}$ mice administered stimulating agonistic anti-PD-1 antibodies had opposite effects.[241] Atherosclerotic mice deficient in costimulatory molecules (*B71/2*$^{-/-}$*Ldlr*$^{-/-}$) also showed reduced plaque burden and decreased amounts of IFN-γ-production upon restimulation of T cells from these mice.[242] Finally, recent data has revealed

an association between increased cardiovascular risk, including atherothrombotic events, in patients treated with immune checkpoint inhibitor (ICI) therapy.[243-245] Administration of combined anti-CTLA-4 and anti-PD-1 antibodies to atherosclerotic mice resulted in increased infiltration of T cells into the heart and lesion as well as adhesion molecules (VCAM-1, ICAM-1), but did not increase plaque size.[246] Further, one autopsy study (non-cardiovascular related death) revealed an increase in the ratio of $CD3^+$: $CD68^+$ (macrophages) cells in coronary plaques in those that had recently undergone ICI therapy.[247] Taken together, these studies suggest an important regulatory role for coinhibitory molecules in controlling T cell responses in atherosclerosis.

Women and Men: Not created equal

And why we should stop acting like it

Show of hands- how many of you reading this book have a designated "office sweater"? You know, the cardigan/sweater/sweatshirt/hoodie, whatever you want to call the extra layer of clothing you need to wear while at work, inside, because the building is kept at a temperature much lower than your comfort. Personally speaking, I have had the most stylish rolling chair in the office for the past five years. It adorns my beige, knit cardigan year-round, aside from the (too few) occasions I take it home to wash it and promptly return it to my naked (and probably chilly) chair. I'm quite certain I learned the phrase "office sweater" from my mom and know many other women who know (and do) exactly what I'm talking about, but not nearly as many men.

Office temperature settings were based off a study from the 1960s that investigated metabolic rate while working. The subjects? Well, it was actually a subject, as in singular, and was one 40-year-old man. It has been shown since then that this may overestimate the metabolic rate of a woman by 35%.[248, 249] But who cares, right? Just put on the office sweater. Well, in addition to energy consumption concerns of over-conditioning offices, it has also been shown that women perform worse in these overly chilled environments, and this impairment in performance is greater and statistically significant compared to the, statistically insignificant, effects of a warmer environment on men.[250] *

* If you were wondering why I possibly spent so much time reading up on office temperatures and rambling about my professional knitwear when I had an entire scientific thesis to write: I read all of these articles over a year ago, while actively shivering inside a NYC office, wearing 3 upper-body and 2 lower-body layers of clothing, thick socks, and boots, and I could not focus on work.

Male-Centric Research

This seemingly trivial sex difference in optimal office temperatures is just one example out of the many where operating under the assumption that women are just smaller men is not only incorrect but also results in the negative impact being shouldered by the women.

Women have historically been underrepresented in clinical trials.[251] Although representation is generally improving, and varies per trial, it has been reported that still only about 38% of CVD clinical trial participants are women.[252] Perhaps unsurprisingly then, women also continue to have worse outcomes and disproportionate CVD-related deaths compared to men, especially younger women.[253, 254] Similarly, preclinical atherosclerosis research disproportionately uses male mice, with a meta-analysis showing that, of the 81% of researchers who did report which sex was used, over 50% used exclusively male mice. The remaining roughly-half of these experiments were split between female-only (20.4%) or mixed sexes (24.1%).[255]

I do not want to diminish the many social factors and influences that contribute to differences in representation, treatments, and outcomes between women and men, and there are several review articles that propose solutions to the issues brought up here.[256] My aim in including this section in my thesis to further highlight biological differences that are relevant to CVD, immune responses, and the work in and surrounding my thesis.

Cardiovascular Disease in Women and Men

Several differences in the presentation of cardiovascular diseases and risks have been identified between women and men. Men are more likely to develop CVD at an earlier age, but overall lifetime risk does not differ between women and men.[257] Other differences include the type of primary CVD incident (coronary disease in men vs. cerebrovascular event or heart failure in women), atherosclerotic plaque phenotypes and pathophysiology (plaque rupture vs. erosion), and risks from smoking.[257-259] Women are also known to present with acute MI differently than men, which, along with other societal influences, results in their symptoms being more likely to be dismissed by their providers (and themselves).[260]

One study showed that when women receive the similar standardized cardiovascular work-up and secondary prevention therapy given to men, their long-term survival is

more favorable than that of men.[261] A news article from the British Heart Foundation as recent as 2015[262] was released in attempt draw attention to that women do, in fact, respond to statin therapy for primary and secondary prevention. The fact this news bulletin was necessary, despite results from studies in the late 1990s already calling for statin therapy in "virtually all patients" with coronary heart disease[263, 264] as well as in 2008 for primary prevention in both women and men,[163] is, to put it nicely, upsetting.

These differences highlight the importance of not only including women (and other minorities) in clinical research, but making sure research is designed to actually investigate potential differences between the sexes. While it is apparent there are differences in CVD between women and men, there are clearly also similarities. The point is we don't know which is a difference and which is a similarity without testing it. And testing for differences does not matter if we do not listen to the results.

Sex-related Differences in Immune Responses

Differences in immune responses based on biological sex are well-reported. In general, it has been shown that women mount stronger immune responses than men, which can result in faster clearance of certain pathogens and more robust responses to vaccination, but also increases susceptibility to autoimmune diseases. Meanwhile, men are, in general, at a greater risk for non-reproductive malignant cancers and less efficacious results from immunizations. The reasons for these differences are multifactorial, including influences from sex chromosomes (X vs Y) and sex hormones (estrogen, progesterone, and testosterone), as well as differences in gender and societal roles.[265]

Recent studies have proposed that the androgen receptor on T cells contributes to CD8 T cell dysfunction in mouse models of bladder cancer and liver cancer.[266, 267] Another recent study in a glioblastoma model also reports CD8 T cells in the tumors of male mice become exhausted quicker than in female tumors. Further, this study reports that the male mice are more responsive to anti-PD-1 treatment.[268]

Such results in man are controversial. Discerning whether the "greater" benefit of ICI therapy lies with women or men is complicated by the plethora of parameters that must be considered, such as the type of cancer, ICI agent, use of adjunct therapies, and which metric to examine (e.g., overall survival vs. progression-free survival).[269-272] However, most studies in this area are relatively fresh (within ~6 years) and reported differences are more inconsistent than absent. I think it will be

extremely interesting to keep an eye on this phenomenon as more sex-related T cell differences are being understood.

Considering Biological Sex in Preclinical Atherosclerosis Studies

> Is this justification for avoiding the study of female animals an extension of paternalistic thinking, or even of remnants of scientific misogyny persisting from times of yore? Rather than regarding the fluctuations as confounders, and thus excluding study of female mice or mixing the sexes willy-nilly, we should embrace the study of differential responses between the sexes to derive insight into biologic mechanisms of capital importance to half of our population. We must ask ourselves critically if excluding 1 sex or another in the design of experimental studies has a strong biological justification or merely represents a convenient extension of habit or of traditional practices.[273]
>
> "*Taking Sex Seriously: An Oft-Overlooked Biological Variable" Dr. Peter Libby, 2020*

I included the excerpt above not only due to my appreciation of its message and its delivery, but also because of how close to home it hits. At the beginning of my research career, I used to hate being asked if we considered sex-related effects in our results. We almost exclusively used female *Apoe*$^{-/-}$ mice, as they experience plaque growth more rapidly and could reduce experimental timelines slightly. It also reduced variability in the data, which, as Dr. Libby points out,[273] is less of a supporting argument and more of a confirmation of intrinsic differences between the sexes (which may have clinical implications).

In the latter half of my thesis work, we did start regularly incorporating both female and male mice and our lab as a whole has discussed this concern several times. And as I learned more about the repercussions, I personally wanted to start advocating for and supporting the call for preclinical and clinical researchers to include both sexes. We did end up identifying sex-related differences in T cell exhaustion/activation in both atherosclerotic mice and FIMCOD participants. These results and further discussion on potential sex-related differences in T cells and atherosclerosis implications can be found in regard to **Paper II**.

Aims

Overall Aim:

The overall aim of my PhD studies was to further elucidate mechanisms regulating T cells in atherosclerosis.

Specific Aims:

- To study the atherogenic effects of IL2Rby signaling in T cells (**Paper I**)
- To characterize the phenotype of IFN-γ-producing T cells in the atherosclerotic vessel (**Paper II**)
- To unravel how immune checkpoint inhibitor (ICI) therapies contribute to increased atherosclerosis cardiovascular (ASCVD) risk (**Papers II and III**)
- To study the therapeutic potential of IL1RAP blockade in atherosclerosis (**Paper IV**)

To address these aims, I utilized various mouse models of atherosclerosis, including different transgenic reporter models, human blood and carotid plaque samples, and various immunomodulatory monoclonal antibodies. I have outlined these main methodological approaches in the following *Methods* chapter.

Methods

Mouse Models of Atherosclerosis

The two mouse models most extensively used for atherosclerosis research are low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice and apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice. Chylomicron remnants, VLDL, and LDL can be removed from circulation via receptor-mediated internalization by binding to the LDL receptor (LDLR).[87, 274] The LDLR ligands are apoB-100 and apoE and LDLR in the liver plays a major role in regulating plasma cholesterol levels.[87] Thus, *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice have increased levels of circulating plasma cholesterol due to impaired clearance from missing the LDLR or its ligand.

There are some differences between these two models, though. *Apoe*$^{-/-}$ mice will spontaneously develop hypercholesteremia while remaining on a chow diet while *Ldlr*$^{-/-}$ mice require a high-cholesterol diet to induce hypercholesteremia. Further, the distribution of lipoproteins varies between models, with *Apoe*$^{-/-}$ mice accumulating predominantly apoB-48-containing VLDL and chylomicron remnants and *Ldlr*$^{-/-}$ mice accumulating apoB-100-contianing LDL primarily.[275, 276] Of note, utilization of bone marrow transplantation (BMT) requires *Ldlr*$^{-/-}$ mice due to the ability of transplanted macrophages to reconstitute apoE in *Apoe*$^{-/-}$ recipient mice, thus interfering with hypercholesteremia and the atherosclerotic phenotype.[277]

Limitations exist in both models when translating to the human setting. This includes differences in location of atherosclerotic lesion, differences in lipoprotein metabolism and lipid profile, rate of disease progression, and atherosclerotic lesions in the mouse models will not rupture or form thrombi.[275, 278] Although, coronary atherosclerosis and ischemia has been shown to be possible in a double knock-out model (*Apoe*$^{-/-}$ *Ldlr*$^{-/-}$).[279] Laboratory mice are also genetically identical, which, with the exception of monozygotic twins, is obviously not the case for the populations we aim to treat. Moreover, laboratory mice are kept in overly sanitized, unnatural environments and it has been shown that mice exposed to natural pathogens and microbiota more closely resemble immune responses seen in humans.[280]

The *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mouse models were established in the early 1990s. Since then, a newer model has been developed utilizing adeno-associated virus engineered to carry a gain-of-function mutation of the gene encoding PCSK-9, driven by a liver-

specific promoter (AAV-PCSK9^{D374Y}). PCSK-9 is a protein that binds to the LDLR and, once internalized, catalyzes the destruction of the receptor instead of it recycling back to the cell surface.[87] Mice injected with AAV-PCSK9^{D374Y} will have increased activity of PCSK-9 in hepatocytes, thus increasing levels of plasma cholesterol when fed a high-cholesterol diet.[281, 282] Effectively, these mice become hypercholesteremic due to a reduction in LDLR, similar to *Ldlr*$^{-/-}$ mice. An advantage of this model is that wild-type mice can become hypercholesteremic via a single *i.v.* injection of the PCSK-9 vector versus extensive breeding required with genetic knockouts.

Collectively, our studies have utilized each of these atherosclerotic mouse models. However, the majority of our studies use the *Apoe*$^{-/-}$ model due to its rapid induction of hypercholesteremia and plaque growth when fed a high-cholesterol diet (0.21% cholesterol, 21% butter fat). Female *Apoe*$^{-/-}$ mice will generate large plaques quicker than their male counterparts,[275, 283] and for this reason our earlier studies used only female mice in focal experiments (**Papers I** and **IV**). However, with the increasing interest in and acknowledgement of the role of biological sex in CVD,[257, 258] atherosclerosis research,[273, 284] and immune responses, [265, 271, 272, 285] we began incorporating both female and male mice throughout our experiments in our later works (**Papers II** and **III**).

Why use mice at all?

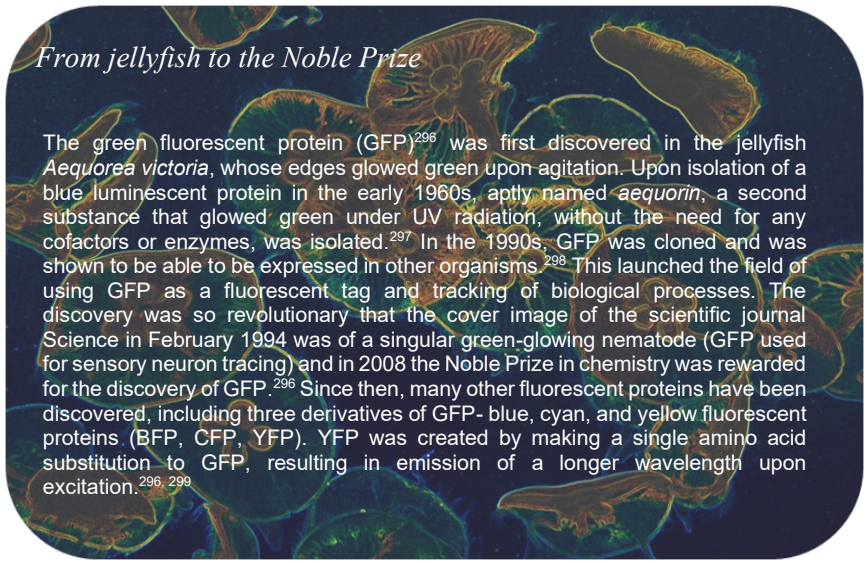
The use of animal models in scientific research dates back to ancient Greece.[286] Early animal studies of atherosclerosis used rabbits and pigs, which had many advantages such as susceptibility to atherogenesis and translatability to human presentation of the disease.[278] In fact, mice originally were not good candidates for atherosclerosis research because they simply do not develop the disease. The only mouse strain prone to plaque development, when fed a high-cholesterol diet, is the C57Bl/6 strain.[278, 287] This is the background strain that the *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice we use today are on. Ultimately, the "perfect" animal model when translating to humans does not exist. Humans are not rabbits are not mice are not swine. Choice of animal model varies with each disease and comes down to a cost-benefit analysis. Utilizing mice has many advantages, such as relatively low maintenance costs, quick breeding time, ease in generating large cohorts, and, with a high-cholesterol diet, quick plaque development.[278, 287] Of course, one must always keep in mind the ethical implications of using live animal models for research. The studies we conduct to investigate atheosclerosis in mice are largely non-invasive, with interventions usually consisting of a switch to a high-cholesteorl diet and intraperitoneal or intravenous injections. Furthermore, all animal studies are approved by ethical committees and mice receive daily monitoring and care.

Transgenic Mice

One of the highlights of our work in Paper II is our utilization of transgenic reporter mice. The lab of Dr. Richard Locksley developed the novel interferon-γ (IFN-γ)-YFP reporter mouse model, in which an internal ribosomal entry site (IRES)-enhanced yellow fluorescent protein (eYFP) was inserted after the stop codon of the *Ifng* gene.[288, 289] IRES allow co-expression of more than one gene under the same promoter. In this case, the result is IFN-γ and eYFP will be translated from the same mRNA, allowing us to observe *in vivo* IFN-γ production by measuring eYFP expression. We purchased these "GREAT" mice (Jackson Laboratories) and bred them in-house with *Apoe*$^{-/-}$ mice to generate the *Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice that were the focal point of our experiments in Paper II. Utilizing this reporter model allowed us to not be reliant on artificial re-stimulation of cells to measure cytokine-producing capacity, like previous atherosclerosis research has.

Additionally, we utilized the Nur77-GFP reporter mouse, which relies on the fusion of green fluorescent protein (GFP) cDNA to the start site of the *Nr4a1* (Nur77) gene. Nur77 is immediately upregulated after T cell receptor (TCR) signaling,[290, 291] thus by measuring GFP, we can detect which T cells have undergone recent TCR signaling. Furthermore, we can also correlate the level of GFP expression with strength of TCR signaling.[292] As with our cytokine-reporters, we purchased these Nur77-GFP mice (Jackson Laboratories) and bred them in-house with *Apoe*$^{-/-}$ mice to generate *Nur77*$^{GFP/wt}$*Apoe*$^{-/-}$ mice. Taking it one step further, we also bred *Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice with *Nur77*$^{GFP/wt}$*Apoe*$^{-/-}$ mice to generate dual-reporter *Nur77*$^{GFP/wt}$*Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice, with which we could measure TCR-signaling and IFN-γ production *in vivo* in the same cell at the same time.

It must be noted that for all of these fluorescent models there is the major limitation of the relatively long half-life of both GFP and YFP. GFP is reported to have a half-life of about 26 hours,[293] and it is assumed, being almost structurally identical, that the half-life of YFP is similar. This means that when we record GFP/YFP expression, we could be reporting events that are not actively occurring since detection of the fluorescent protein can last for days. It has also been noted that retaining GFP or YFP fluorescent during fixation procedures can be difficult. In our own studies (**Paper II**), a good amount of time went into re-optimizing established flow cytometry fixation protocols to address this issue. We have also decided to use anti-GFP antibodies for future histological analysis, as others have done.[294] Further, in our dual-reporters, it can be complicated to fully differentiate between GFP-and YFP-positive cells via flow cytometry, as the fluorescent proteins share very similar emission wavelengths.[295] However, we believe this is still a superior method compared to the artificial setting created upon re-stimulation of T cells *ex vivo*.



From jellyfish to the Noble Prize

The green fluorescent protein (GFP)[296] was first discovered in the jellyfish *Aequorea victoria*, whose edges glowed green upon agitation. Upon isolation of a blue luminescent protein in the early 1960s, aptly named *aequorin*, a second substance that glowed green under UV radiation, without the need for any cofactors or enzymes, was isolated.[297] In the 1990s, GFP was cloned and was shown to be able to be expressed in other organisms.[298] This launched the field of using GFP as a fluorescent tag and tracking of biological processes. The discovery was so revolutionary that the cover image of the scientific journal Science in February 1994 was of a singular green-glowing nematode (GFP used for sensory neuron tracing) and in 2008 the Noble Prize in chemistry was rewarded for the discovery of GFP.[296] Since then, many other fluorescent proteins have been discovered, including three derivatives of GFP- blue, cyan, and yellow fluorescent proteins (BFP, CFP, YFP). YFP was created by making a single amino acid substitution to GFP, resulting in emission of a longer wavelength upon excitation.[296, 299]

Immunomodulation with Monoclonal Antibodies

The immune system can be manipulated in many ways. Classical modulation comes in the form of common pharmaceuticals such as corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDS), histamine antagonists, and cell-signaling inhibitors (e.g., calcineurin inhibitors). All of these drugs dampen immune responses either by interfering with pro-inflammatory molecules, their receptors, or intracellular signaling that results in immune cell activation.[300] Nowadays, immunomodulation encompasses also biologics (e.g., monoclonal antibodies), genome editing, and stem cell therapies.[300] In every paper included in this thesis, we have modulated the immune system in some way to further unravel the role of T cells in atherosclerosis (**Papers I-III**) and investigate how immunomodulation can affect disease burden (**Paper IV**).

IL-2 complexes

In **Paper I**, we used immunocomplexes to selectively deliver the cytokine interleukin-2 (IL-2) to different immune cells based on which IL-2 receptor (IL-2R) complex was expressed. IL-2 is the prototypical T cell cytokine and is crucial for activation, proliferation, and survival of T cells. The IL-2R exists as either a moderate-affinity dimer (IL-2Rβγ, also known as CD122) or a high-affinity trimer

(IL-2Rαβγ, also known as IL-2Rα or CD25). The high-affinity IL-2Rα is expressed constitutively on regulatory T cells as well as on recently activated effector T cells and, as a result, these cells typically outcompete IL-2Rβγ-expressing cells (memory T cells and natural killer cells) for IL-2.

When bound to IL-2, the anti-IL-2 antibody clone S4B6 interferes with IL-2 binding to IL-2Rα. Therefore, administration of IL-2:anti-IL2 complexes made with the S4B6 clone (**IL-2/S4B6**) shifts the delivery of IL-2 to cells with high expression of IL-2Rβγ (Figure 11).[301] Alternatively, another anti-IL-2 clone, JES6-1, augments IL-2 delivery to IL-2Rα-expressing cells instead. In this way, we can manipulate delivery of IL-2 (i.e., which T cell populations will expand) by choosing which clone of anti-IL-2 antibody we administer.

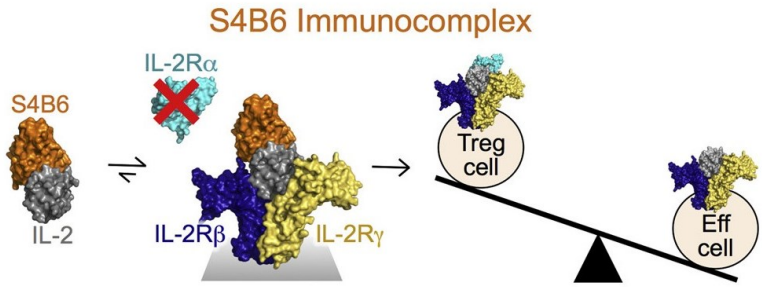


Figure 11: Mechanism of action of IL-2/S4B6 immunocomplex. When complexed to interleukin-2 (IL-2), the anti-IL-2 clone S4B6 sterically hinders IL-2 interaction with the alpha chain of the IL-2 receptor (IL-2Rα). This hindrance results in skewing of IL-2 delivery towards immune cells expressing the IL-2Rβγ (T effector cells; Eff cell) and away from IL-2Rα-expressing cells (regulatory T cells; Treg). Adapted and reproduced with permission from (301) ©Elsevier.

In **Paper I**, IL-2 complexes were prepared according to Boyman *et al*.[302] In brief, 1.5 µg of recombinant IL-2 was combined with 50 µg anti-IL-2 (clone: S4B6 or JES6-1) in phosphate buffered saline (PBS). For comparison of effects of different anti-IL-2 clones, we administered intraperitoneal (*i.p.*) injections of either IL-2/S4B6, IL-2/JES6-1, or PBS vehicle to female wild-type (C57Bl/6) mice for a total of two injections spaced seven days apart. In the atherosclerosis experiments, we used female *Apoe*$^{-/-}$ mice and fed them a high-cholesterol diet (0.21% cholesterol, 21% butter fat) for six weeks before starting weekly *i.p.* injections of IL-2/S4B6 or PBS for a total of four weeks (total 10 weeks on diet). Mice were 8-12 weeks old at the start of experiments and randomly assigned to treatment groups.

Immune checkpoint inhibitors

Immune checkpoint inhibitor (ICI) therapy has revolutionized the field of oncology. The first FDA approved ICI was an anti-CTLA-4 monoclonal antibody (ipilimumab), launched in 2011 for the treatment of unresectable or metastatic melanoma.[303] Since then, several more ICI therapies have been produced and approved, including antibodies targeting PD-1 and PD-L1, and have been approved in several solid tumor indications, including non-small cell lung cancers, pancreatic cancers, and bladder cancers. Anti-PD-1 inhibitors (e.g., nivolumab, pembrolizumab) are the most common type of ICI therapy given, either as monotherapy or the foundation of combination therapies (i.e., in concert with chemotherapy, radiation therapy, etc.).[303] Anti-LAG-3 therapy was FDA approved just last year in 2022, although only in a fixed-dose combination with nivolumab (Opdualag), validating the potential for more coinhibitory molecules to become therapeutic targets.[304] In **Papers II** and **III**, we investigated the effects of administration of anti-PD-1, anti-LAG-3, and combination of anti-PD-1/anti-LAG-3 monoclonal antibodies in atherosclerotic mice.

In **Paper II**, we used both female and male *Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice, aged 10-11 weeks at start of the experiment, and fed them a high-cholesterol diet (0.21% cholesterol, 21% butter fat) for 20 weeks before starting injections. Mice were randomly assigned in treatment groups and received *i.p.* injections of either anti-PD-1 antibody (clone: RMP1-14) or isotype control IgG2a (clone: 2A3), biweekly for three weeks at 10mg/kg,[240] for a total of 6 injections and 24 weeks of diet.

In **Paper III**, female and male *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice, aged 8-12 weeks at start of experiments, were administered either anti-LAG-3 antibodies (clone: C9B7W), anti-PD-1 antibodies (clone: 29F.1A12), or combination anti-LAG-3/anti-PD-1 antibodies. Isotype control IgG1 (clone: HRPN) was used for anti-LAG-3, isotype IgG2a (clone: 2A3) for anti-PD-1 controls, and combination of both isotypes for anti-LAG-3/anti-PD-1 controls. Antibody dosage was 0.2mg/injection, administered biweekly for 3-4 weeks,[240] and duration of total high-cholesterol diet feeding was between 5-10 weeks.

Anti-IL1RAP antibodies

In **Paper IV**, we investigate immunomodulation as a potential therapeutic for atherosclerosis. In collaboration with the Lund-founded biotechnology company Cantargia AB, we were able to utilize their monoclonal antibody that targets the IL-1 receptor accessory protein (IL1RAP) in the context of atherosclerosis.

IL1RAP is a co-receptor necessary for signal transduction through the IL-1 receptor (IL-1R1), the IL-33 receptor (ST2), and the IL-36 receptor (IL-36R; Figure 12). Thus, inhibition of IL1RAP colocalization interferes with signaling of multiple inflammatory cytokines simultaneously- respectively, IL-1α, IL-1β; IL-33; IL-36α, IL-36β, IL-36γ (Figure 13). Nadunolimab (CAN04, Cantargia AB), an anti-IL1RAP monoclonal antibody with enhanced antibody-dependent cellular cytotoxicity (ADCC) properties is currently in Phase II/III trials in multiple solid tumor indications, including non-small cell lung cancer, triple-negative breast cancer, and pancreatic cancer (in combination with chemotherapy: NCT05116891, NCT04990037, NCT03267316, NCT05181462; in combination with pembrolizumab: NCT04452214).

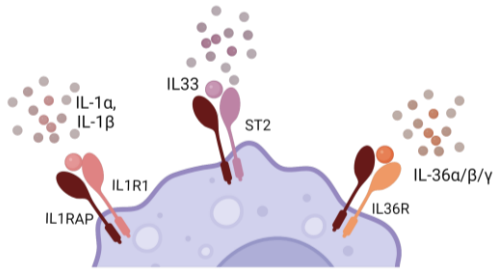


Figure 12: IL1RAP signaling. The co-receptor IL1RAP co-localizes with the IL-1 receptor (IL1R1), IL-33 receptor (ST2), and IL-36 receptor (IL36R) to mediated signaling by the IL-1 family cytokines, IL-1, IL-33, and IL-36. Created with BioRender.com.

Another **anti-IL1RAP antibody** (CAN10) has had this ADCC-capacity removed (LALA-PG mutation) and has recently entered a Phase I clinical trial to investigate safety and dosing in healthy volunteers as well as in 16 patients with psoriasis to provide initial proof-of-concept. The murine version of the anti-IL1RAP antibody from the CAN10 project has shown reduced disease burden in models of several inflammatory conditions including myocarditis, systemic sclerosis, psoriasis, and psoriatic arthritis (unpublished posters)[305, 306] and this is the antibody we have utilized in our experiments in **Paper IV**.

The CANTOS trial validated targeting inflammation and IL-1 as a therapeutic approach for treating ASCVD.[165] Thus, while the pro-atherogenic role of IL-1 signaling has been appreciated for some time,[307] the roles of IL-33 and IL-36 are less clear. Previous studies have identified a possible pro-atherogenic role for IL-36 via promoting foam cell formation and activation of the NLRP3 inflammasome in macrophages in *Apoe*$^{-/-}$ mice.[308, 309] Although IL-33 seems to be either anti-atherogenic, or irrelevant, when examined in Apoe mice,[310, 311] IL-33 can also activate inflammatory responses directly from endothelial cells, mast cells, and neutrophils, and scrutiny when evaluating IL-33 implications in CVD has been called for.[312, 313] Therefore, it stands to reason that limiting signaling of all IL1RAP-related cytokines rather than individually may provide greater ASCVD benefits.

Optimal dosing of anti-IL1RAP was predetermined by pharmacokinetic studies performed by Cantargia AB to be 20 mg/kg loading dose and 10mg/kg biweekly subsequent doses. Female *Apoe*$^{-/-}$ mice, aged 10-11 weeks at start of the experiment, were randomly assigned into treatment groups. Mice were administered biweekly *i.p.* injections of either anti-IL1RAP (clone: 3A9) or isotype control (anti-HEL IgG2a-LALA-PG) during the final six weeks of 10 weeks of high-cholesterol diet (0.21% cholesterol, 21% butter fat) feeding.

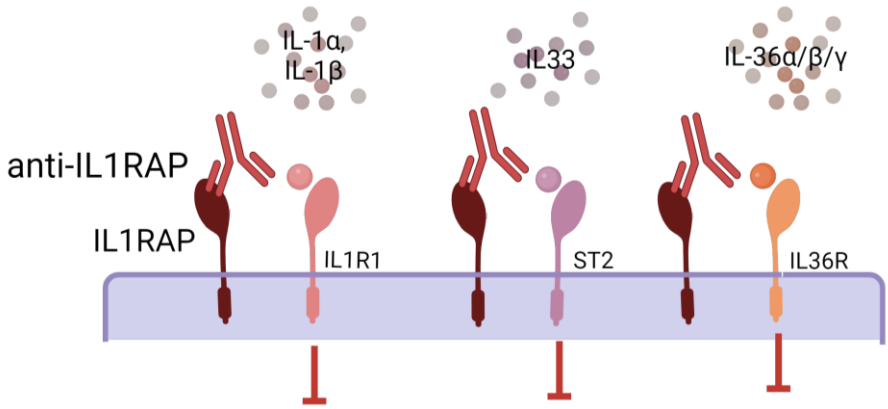


Figure 13: Mechanism of action of anti-IL1RAP antibody (CAN10, Cantargia AB). Created with BioRender.com.

Human Samples

While animal models can be a significant source of information and I thoroughly enjoyed gaining the skills I now have to work with mouse models, it's no secret to anyone who knows me reading this book that my personal research interests lie in translational science. At the end of the day, we're aiming to treat a human disease, and the utility of clinical translation is the biggest value.

In **Paper II** we were able to utilize blood samples from individuals participating in the Functional IMmunity and CardiOvascular Disease (FIMCOD) study. Participants in the FIMCOD study are women and men between the ages of 65 to 72 and who previously participated in the Swedish CArdioPulmonary bioImage Study (SCAPIS; www.scapis.org) in Malmö, Sweden. SCAPIS is a general population-based prospective study that analyzed cardiovascular and pulmonary health in recruited women and men aged 50 to 64.[314] Between 2014 and 2018, SCAPIS participants received a coronary computed tomography angiography (CCTA), which can be used to evaluate degree of subclinical coronary

atherosclerosis.[315] Upon inclusion into the FIMCOD study (January to June 2022), participants blood was drawn, from which peripheral blood mononuclear cells (PBMCs) were isolated and stored (in-house) and metabolic profile (plasma levels of LDL, HDL, triglycerides, non-fasting glucose) was assessed (Skåne University Hospital). Access to PBMCs from the FIMCOD study allowed us to pair analysis of circulating T cell phenotype (flow cytometry) with associations of subclinical atherosclerosis (CCTA).

Through collaboration with researchers at Leiden University, The Netherlands, we were also able to include single-cell sequencing and flow cytometry data from both human carotid plaques and matched PBMCs in two of our studies. In **Paper IV**, single-cell RNA sequencing on carotid plaque cells were from samples collected from 18 patients (4 female, 14 male) enrolled in the ongoing biobank AtheroExpress,[316] at the University Medical Centre Utrecht. Also in **Paper IV**, flow cytometry was performed on carotid plaque and whole blood samples collected from 4 patients undergoing carotid endarterectomy (blood collected via venipuncture prior to surgery) at the Haaglanden Medical Center, Westeinde, The Hague, The Netherlands. Similarly, the single-cell RNA and TCR sequencing of plaque and circulating T cells in **Paper II** was performed on samples from 3 patients undergoing carotid endarterectomy enrolled in the ongoing biobank AtheroExpress,[316] at the University Medical Centre Utrecht.[186] Incorporating human samples and translation is something our small, budding lab-of-two continued to struggle with at the beginning of my PhD studies. So, the addition of this collaboration with Leiden University, in particular with **Marie Depuydt**, midway was truly valuable in many ways.

Flow Cytometry

The main laboratory technique used throughout this thesis and that I have become the most skillful in is **flow cytometry**. As we know by now, cells express many different things that can be used to identify them, including extracellular receptors, transcription factors, specific genes, and cytokines and intracellular vesicles. Flow cytometry uses fluidics and lasers to quantify and characterize cells on a single-cell basis based on these identifiers, which are labeled with different fluorescent tags.

To simplify the physics for all of us, a flow cytometer utilizes what is called hydrodynamic focusing to force a suspension of cells to flow in a single-file-line past a laser beam. Different cytometers have varying amounts of lasers, filters, and detectors that emit, pass, and detect various wavelengths in order to determine the fluorescent tags on each cell. These tags are actually antibodies that are conjugated to a fluorophore (excitable fluorescent molecule) and each fluorophore has a unique

excitation and emission spectrum. We can also use features such as size and granularity to identify cell populations, and these features can be determined by light-scattering properties (forward scatter, side scatter, respectively) without the need for any addition of fluorophores. Further, cells from fluorescent reporters, like the GFP- and YFP-reporter mice we used in Paper II, can be detected in the flow cytometer without additional antibody staining.

We can identify specific immune cell populations by staining our cell sample with multiple fluorescent antibody tags at once, each of which bind to known, or possibly exploratory, features of our cells of interest. For example, if we wanted to identify a Th1 cell we would use tags against basic T cell markers, such as anti-CD45 (leukocyte), anti-CD3 (T cell), anti-CD4 (helper T cell), and tags specific for Th1 lineages, such as against Tbet or IFN-γ. We then continue including and excluding cells based on expression, or lack of thereof, of the features we included in our antibody panel design until we get to our target cell population. We also stain our cell suspensions with a fluorescent viability dye. The viability stain is a bit counterintuitive. There are two main types of viability dyes, amine-reactive dyes and fluorescent DNA dyes, both of which operate under the concept that what this dye binds strongly to should not be as available on a healthy, living cell. Thus, these dyes will give the strongest single on dead or dying cells and we exclude this population at the beginning of our analyses. An example of such a "gating strategy" can be seen below (Figure 14).

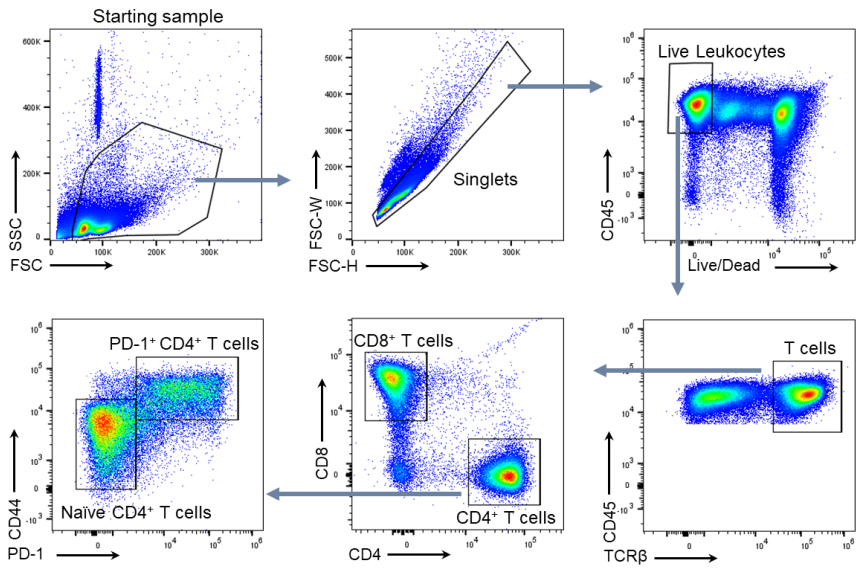


Figure 14: Representative flow cytometry gating strategy. Example pictured here is from splenocytes and can be used to identify T cell populations and expression of effector markers, such as CD44 and PD-1.

Objectives and Key Findings

Paper I

Objective: Study the atherogenic effects of IL-2Rby signaling in T cells by administering IL-2 complexed to the anti-IL-2 antibody clone S4B6 (IL-2/S4B6).

Key findings: Atherosclerotic mice treated with IL-2/S4B6 had increased levels of effector memory T cells and NK cells, increased systemic inflammation, increased levels of Tregs, and decreased total plasma cholesterol, ultimately resulting in no net change in atherosclerosis burden.

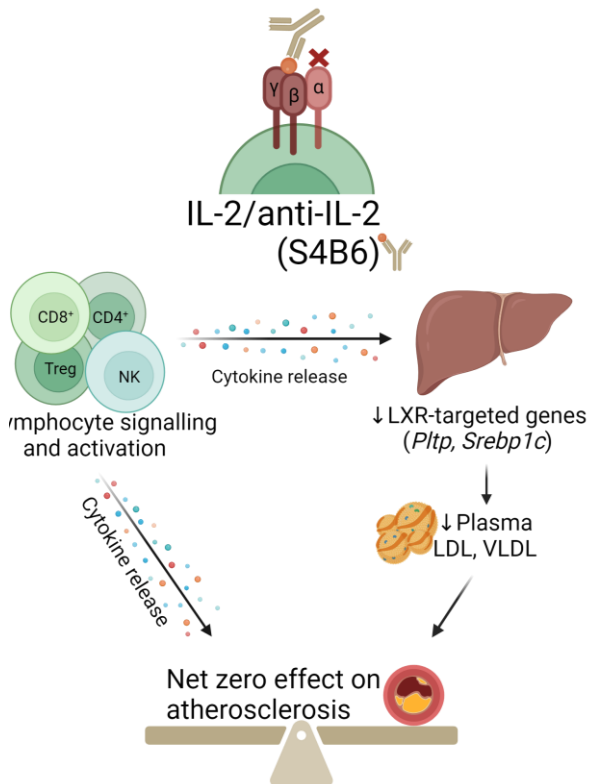


Figure 15: Graphical abstract, **Paper I**.

Paper II

Objective: Characterize the phenotype and regulation of IFN-γ^{+} T cells in atherosclerosis.

Key findings: Murine aortic IFN-γ^{+} T cells are characterized by a mixture of progenitor and terminally exhausted T cells that express PD-1. These PD-1^{+} IFN-γ^{+} T cells respond to anti-PD-1 therapy and levels of circulating PD-1^{+} T cells associates with subclinical coronary atherosclerosis in man.

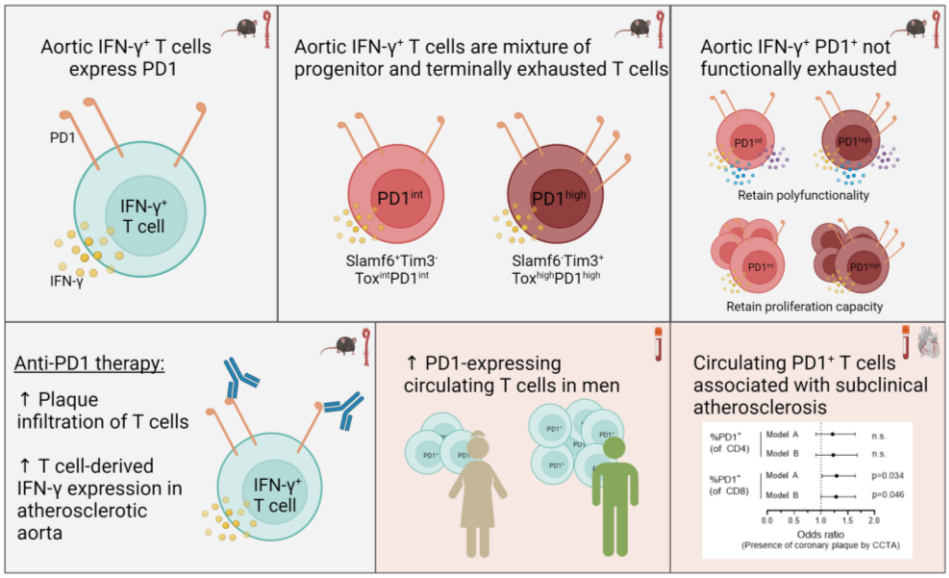


Figure 16: Graphical abstract, **Paper II**.

Paper III

Objective: Determine if LAG-3 modulates T cells in atherosclerosis.

Key findings: Disruption of LAG-3 signaling resulted in increased infiltration of T cells into the atherosclerotic aorta in mice and treatment with combination of anit-LAG-3/anti-PD-1 antibodies potentiated this infiltration compared to anti-PD-1 monotherapy.

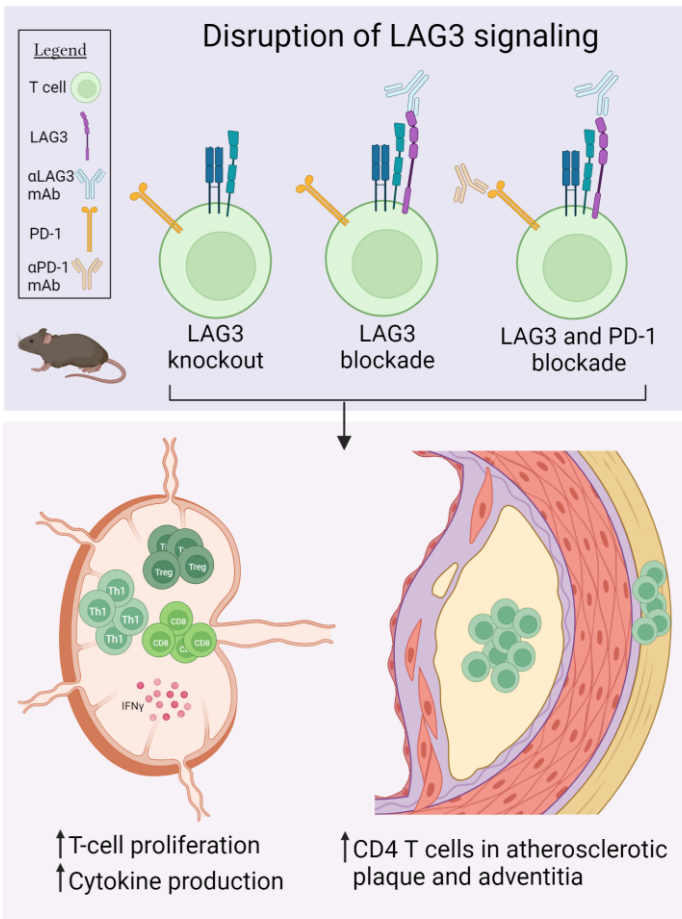


Figure 17: Graphical abstract, **Paper III**.

Paper IV

Objective: Investigate IL1RAP as a therapeutic target in atherosclerosis.

Key findings: Administration of anti-IL1RAP antibodies to atherosclerotic mice reduced plaque size and plaque inflammation, possibly due to decreased leukocyte recruitment mediated by reductions in adhesion molecules and chemokine secretion.

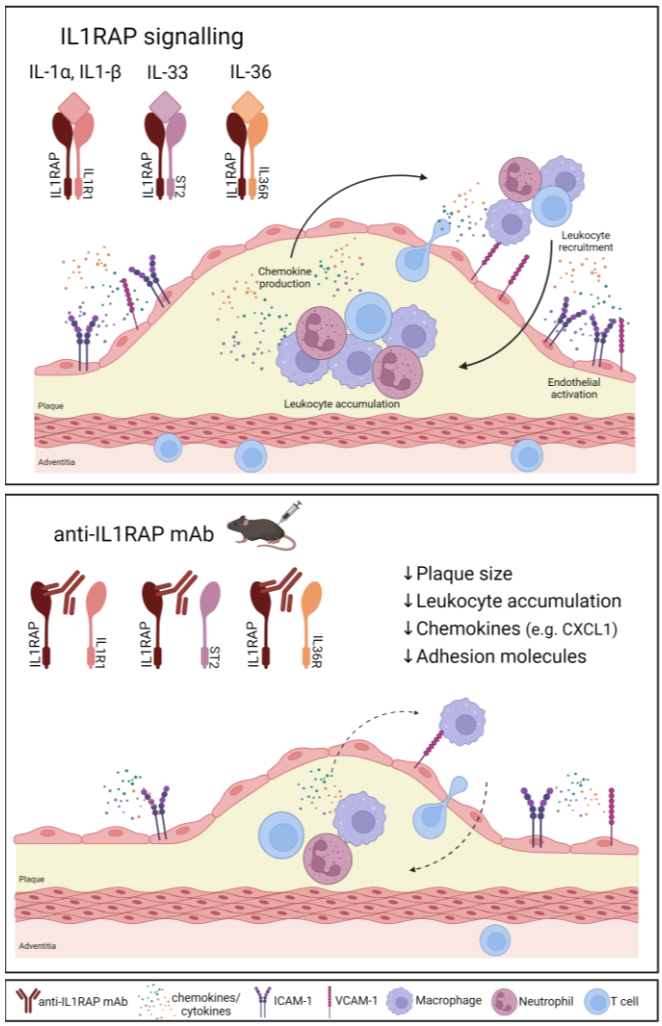


Figure 18: Graphical abstract, **Paper IV**.

Results and Discussion

Counteracting effects of IL-2Rβγ signaling

It was previously shown that immunomodulation via selective delivery of IL-2 delivery to cells expressing IL-2Rα (CD25) by complexing IL-2 to the anti-IL-2 antibody clone JES6-1 resulted in a decrease in atherosclerotic lesion size in mice. This phenotype was attributed to expansion of regulatory T cells (Tregs), which constitutively express high levels of IL-2Rα, and a reduction in plasma cholesterol levels.[317] Conversely, it was not known how IL-2Rβγ signaling, predominantly expressed on effector memory T cells and NK cells, affected atherosclerosis.

To this end, we treated atherosclerotic mice with injections of IL-2 complexed to the anti-IL-2 clone S4B6 (IL-2/S4B6), which sterically hinders IL-2 interaction with the alpha chain of the IL-2 receptor. In line with previous studies,[301, 318] IL-2/S4B6 injections led to expansion of central memory ($CD44^+CD62L^+$) and effector memory ($CD44^+CD62L^-$) CD4 and CD8 T cells, although this expansion was more pronounced in CD8 T cells (Figure 19). These expanded CD8 T cells and NK cells also demonstrated significantly increased capacity to produce IFN-γ in the spleen and in the aorta for NK cells. Further, mice receiving IL-2/S4B6 complex displayed elevated systemic inflammation, with levels of several pro-inflammatory cytokines as well as IL-10 increased in serum.

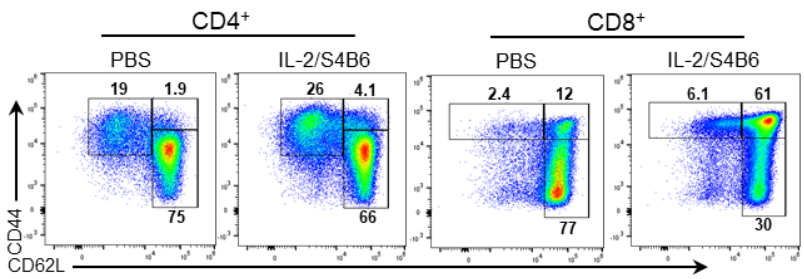


Figure 19: Expansion of central memory ($CD44^+CD62L^+$) and effector memory ($CD44^+CD62L^-$) CD4 and CD8 T cells in spleen following treatment with IL-2/S4B6.

Interestingly, there was an 18% reduction in total plasma cholesterol in mice treated with IL-2/S4B6 compared to controls. However, no effects on subvalvular plaque size or composition were observed. Thus, IL-2/S4B6 treatment may result in no net changes in atherosclerosis due to eliciting both pro- and anti-atherogenic mechanisms concomitantly.

What the cholesterol?

We then performed several more experiments in **Paper I** to understand the cholesterol-lowering effects of IL-2/S4B6 complex. As CD8 T cells and NK cells demonstrated the most pronounced response to IL-2/S4B6 treatment, we investigated if this expansion contributed to the cholesterol phenotype we observed. Antibody depletion of neither CD8 T cells nor NK cells altered the effects on subvalvular plaques or plasma cholesterol from IL-2/S4B6 treatment. However, treating lymphopenic atherosclerotic mice (*Rag1*$^{-/-}$*Apoe*$^{-/-}$) with IL-2/S4B6 complex did not yield any difference in total plasma cholesterol, indicating some component(s) of the adaptive immune system was contributing to the reduction in plasma cholesterol, but not CD8 T cells or NK cells individually.

In our final attempt to solve this cholesterol mystery, we analyzed macrophage content and gene expression in the livers of IL-2/S4B6 treated atherosclerotic mice. Indicating hepatic inflammation, administration of IL-2/S4B6 complexes increased Ly6C^{high}MHCII^{+} macrophages in the liver 3-fold as well as increased gene expression of serum amyloid a (SAA; homolog for CRP in human) while decreasing expression of *Il10*. Further, gene expression of several liver X receptor (LXR)-target genes, including *Pltp* and *Srebp1c*, were reduced in the livers of mice treated with IL-2/S4B6. Srebp-1c and PLTP (phospholipid transfer protein) have been shown to augment hypertriglyceridemia and produce large VLDL particles in *Ldlr*$^{-/-}$ mice and PLTP expression was reliant on Srebp-1c.[319] It is possible that the systemic and hepatic inflammation induced with IL-2/S4B6 complexes reduces expression of LXR-target genes leading to decreased total plasma cholesterol.

It is also possible that the expansion of Tregs with IL-2/S4B6 treatment contributed to reduced plasma cholesterol levels.[233] While the anti-IL-2 clone S4B6 does prevent IL-2/IL-2Rα interactions, Tregs do still express the beta and gamma chains of the IL-2 receptor and thus are still able to respond to IL-2/S4B6, just not at an advantage as with IL-2/JES6-1. Of note, while the decrease in total plasma cholesterol was statistically significant with IL-2/S4B6 treatment, it is relevant to acknowledge that these mice were still severely hypercholesteremic.

Aortic IFN-γ$^+$ T cells: activated or exhausted?

In **Paper II**, we utilized atherosclerotic IFN-γ-YFP reporter mice (*Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$) to characterize the phenotype of IFN-γ-producing T cells in the atherosclerotic aorta. Th1 cells and IFN-γ are largely regarded as pro-atherogenic. However, the vast majority of the previous studies that led to this conclusion rely on whole-body genetic knockouts or artificial restimulation of cells *ex vivo*, both of which do not reflect the natural environment of the atherosclerotic plaque.[205-208] Moreover, many other cell types can either produce IFN-γ or respond to upstream regulators of Th1 induction (IL-12, IL-18).[320, 321] Utilizing atherosclerotic IFN-γ-YFP reporter mice allowed us to directly measure which cells in the atherosclerotic aorta were producing IFN-γ *in vivo* via detection of YFP.

Our initial results surprised us, in that IFN-γ-producing CD4 and CD8 T cells expressed markedly elevated levels of PD-1 compared to those that were not producing IFN-γ (Figure 20). We were then able to categorize these IFN-γ$^+$ PD-1$^+$ T cells further by expression level of PD-1: PD-1$^-$, PD-1^{int}, and PD-1^{high}.

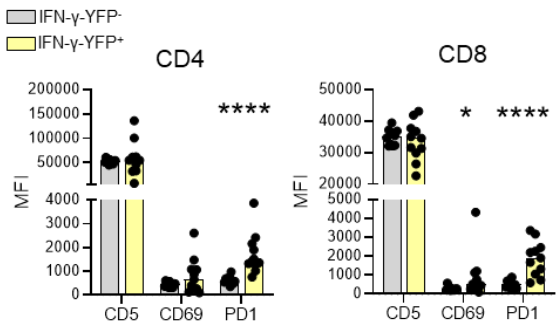


Figure 20: Comparison of mean fluorescent intensity (MFI, geometric mean) of T cell activation markers on IFN-γ-producing (yellow bars) and IFN-γ- non-producing (grey bars) T cells. Measured by flow cytometry of aortic T cells from *Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice.

PD-1^{high} differed from PD-1^{int} T cells in that a greater proportion of PD-1^{high} T cells were Slamf6$^-$Tim3$^+$ and Tox$^+$ and displayed increased levels of LAG-3. However, both PD-1^{int} and PD-1^{high} T cells retained proliferation capacity and polyfunctionality in the atherosclerotic aorta, suggesting these aortic IFN-γ$^+$ PD-1$^+$ T cells were not functionally exhausted. Further, aortic IFN-γ$^+$ PD-1$^+$ T cells displayed a predominantly memory phenotype in both CD4 and CD8 populations (PSGL1$^+$Ly6C$^-$ CD4; KLRG1$^-$IL-7α$^+$ CD8). We also demonstrated that apoB-100-specific T cells increased expression of PD-1 in the presence of LDL and clonally expanded T cells taken from human carotid plaques also exhibited increased expression of genes related to memory phenotype and exhaustion signatures (*TOX, TBX21, EOMES, LAG3*). Taken together, these results suggest that IFN-γ-producing

T cells in the atherosclerotic aorta can be defined by their expression of PD-1, and that these cells are a mixture of progenitor exhausted and terminally exhausted T cells.

IFN-γ^+ T cells are modulated by PD-1

In light of the increasing reports of adverse cardiovascular events occurring in cancer patients treated with ICI therapies[243-245] and identifying apparent PD-1 expression on IFN-γ-producing T cells in our atherosclerotic mice, we wanted to test how anti-PD-1 therapy may affect plaque inflammation. We fed *Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice a high-cholesterol diet for 20 weeks before starting biweekly injections of anti-PD-1 monoclonal antibodies or isotype control (n=11-12/group). Mice treated with anti-PD-1 antibodies had an increase in T cells in the atherosclerotic aorta and subvalvular plaques, shown by flow cytometry and immunohistochemistry (Figure 21). These T cells were also higher in expression of YFP (IFN-γ production) and PD-1. Our results suggest that treatment with anti-PD-1 antibodies augments production of IFN-γ in the atherosclerotic plaque, possibly providing a mechanism for the increased risk of ASCVD in patients receiving ICI therapies.

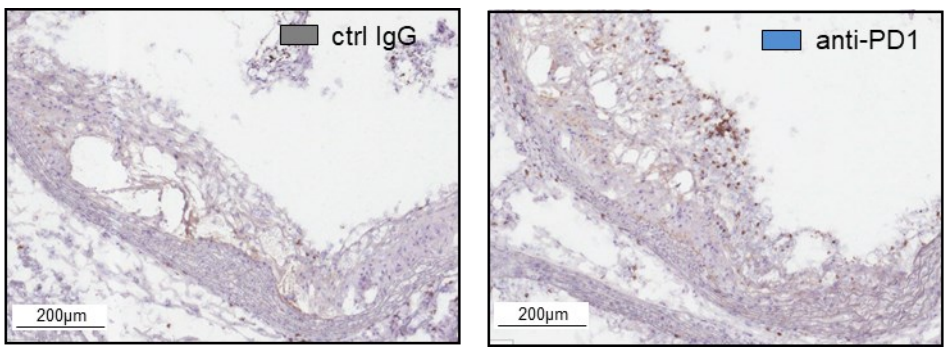


Figure 21: Immunohistochemical staining of T cells (CD3, brown staining) in aortic root cross-sections of atherosclerotic mice treated with isotype IgG (left pane) or anti-PD-1 antibodies (right pane).

PD-1 associates with subclinical coronary atherosclerosis

In **Paper II**, we revealed an association of T cell PD-1 expression with subclinical coronary plaques. We utilized blood samples from FIMCOD participants, of whom were also originally a part of the Swedish Cardiopulmonary BioImaging Study (SCAPIS). As such, the majority of the participants we had blood samples for also received a coronary computed tomography angiography (CCTA; n=611), which evaluates the presence of atherosclerotic plaques within the coronary arteries. From this, a segment involvement score (SIS) was determined, which is just an enumeration of the number of vessels affected by at least one plaque. We stratified participants by absence (SIS=0) or presence (SIS≥1) of coronary plaques and showed that percent PD-1$^+$ CD4 and PD-1$^+$ CD8 T cells in circulation is associated with subclinical coronary atherosclerosis. This association remained statistically significant for PD-1$^+$ CD8 T cells after adjustment for classical risk factors (p=0.04, OR= 1.3; Figure 22).

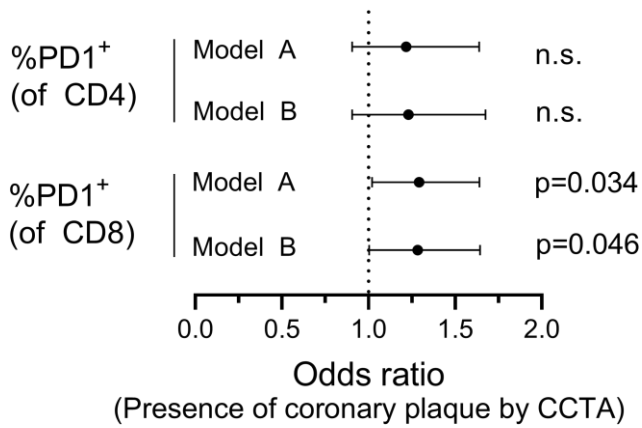


Figure 22: Association of levels of PD-1$^+$ circulating CD4 and CD8 T cells and presence of subclinical coronary plaque (segment involvement score ≥1). Analyzed by logistic regression and adjusted for classical risk factors. Model A: adjusted for age and sex. Model B: adjusted for age, sex, non-fasting blood glucose, LDL, HDL, and triglycerides.

LAG-3 regulates T cells in atherosclerosis

Previous studies have indicated that disruption of checkpoint molecules PD-1 and CTLA-4 can exacerbate disease progression in atherosclerotic mice.[239-241, 246] Just last year, in 2022, anti-LAG-3 therapy became the fourth FDA approved ICI therapy, but only in fixed-dose combination with anti-PD-1 (Opdualag). In **Paper**

III, we sought to investigate if LAG-3 blockade may also impact the atherosclerotic lesion.

Atherosclerotic mice deficient in LAG-3 (*Lag3*$^{-/-}$ BMT to *Ldlr*$^{-/-}$; AAV-PCSK9 injections in *Ldlr*$^{-/-}$) experienced an increase in CD4 T cell infiltration in subvalvular plaques and these T cells expressed a more activated phenotype (CD44^{high}). Subvalvular plaque size and collagen content, descending aorta plaque area, and total plasma cholesterol did not differ between LAG-3-deficient and LAG-3-competent mice. Administration of anti-LAG-3 antibodies yielded similar results in both *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice. Splenic T cells from anti-LAG-3 treated mice displayed increased levels of proliferation, an effector memory phenotype, and increased propensity to produce IFN-γ and IL-2, but still there was no effect on plaque size.

Anti-LAG-3 antibodies potentiate T cell plaque infiltration

Apoe$^{-/-}$ mice treated with anti-LAG-3 antibodies also displayed an increase in CD44^{high}PD-1^{int} CD4 and CD8 T cells in the spleen. These results coupled with the then ongoing clinical trials investigating anti-LAG-3/anti-PD-1 therapy led us to test the potential atherosclerotic effects of combination antibody treatment compared to anti-PD-1 monotherapy. Indeed, we saw a stepwise increase in T cell activation (%CD44^{high}CD62L^{-}, %IFN-γ^{+}) in the spleen of *Ldlr*$^{-/-}$ mice treated with isotype controls versus anti-PD-1 antibodies versus anti-LAG-3/anti-PD-1 combination (Figure 23A-B). We also reported an increase in lesional and adventitial CD4 T cells in mice treated with anti-LAG-3/anti-PD-1 combination compared in isotype controls (Figure 23C-D). Once again, no difference in subvalvular plaque size nor plasma cholesterol was observed. Similar to **Paper II**, our results from **Paper III** suggest the increased ASCVD risk associated with ICI therapies may come from the loss of regulation of inflammatory T cells that can infiltrate the lesion and contribute to plaque progression. Of note, the percentage of Tregs in secondary lymphoid organs also increased with treatment of anti-LAG-3 monotherapy and anti-LAG-3/anti-PD-1 co-therapy. However, we cannot make any inferences on the functionality of these regulatory cells nor their presence in the atherosclerotic plaque from our study.

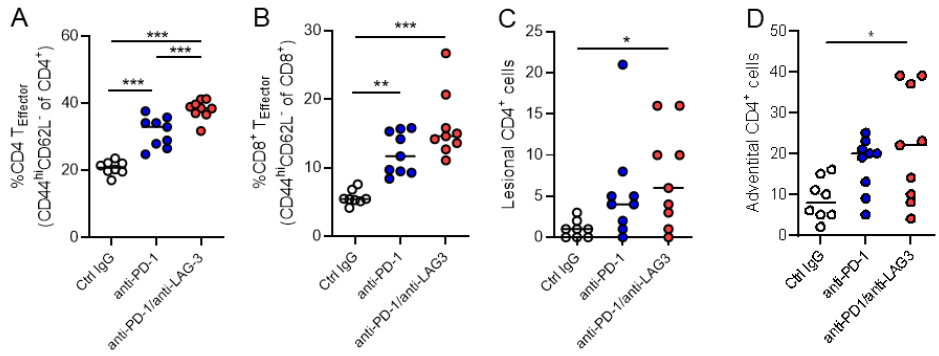


Figure 23: Atherosclerotic mice were treated with either anti-PD-1/anti-LAG-3 co-therapy, anti-PD-1 monotherapy, or control isotypes. (A-B) Quantification of effector CD4 and CD8 T cells in the spleen by flow cytometry. (C-D) Quantification of CD4 T cells in the atheroslcerotic lesion and surrounding adventitia by immunohistochemistry.

Sexual dimorphisms of PD-1 expression

Differences in the immune system of women and men has been appreciated for some time, with women in general having more robust immune responses.[265] We pooled all of the untreated *Ifng*$^{YFP/YFP}$*Apoe*$^{-/-}$ mice used in **Paper II** and evaluated differences in IFN-γ production and PD-1 expression on female (n=29) and male (n=42) T cells. CD8 T cells in male mice displayed a significant increase in IFN-γ production in the atherosclerotic aorta compared to female, as well as higher percentage of aortic PD-1^{high} CD4 and CD8 T cells and aortic PD-1^{int} CD8 T cells in males.

Blood samples from FIMCOD participants revealed a similar finding in **Paper II**. Circulating PD-1^{+} CD4 and CD8 T cells were significantly elevated in samples taken from men than women (n=675). It has been suggested that sex may influence ICI therapy efficacy, although such studies remain inconclusive. Our results of higher levels of PD-1^{+} T cells in male mice and men may support those that report a male-bias response to ICI therapy, particularly anti-PD-1 therapy.[270, 271, 285]

Moreover, these results reiterate the importance of including both sexes when conducting both preclinical and clinical research.

ILRAP blockade reduces atherosclerotic burden in mice

In **Paper IV**, via collaboration with Cantargia AB, we were able to administer a murine version of anti-IL1RAP antibodies to atherosclerotic *Apoe*$^{-/-}$ mice. IL1RAP is a co-receptor necessary for the signaling of the cytokines IL-1α/β, IL-33, and IL-36α/β/γ. Therefore, blocking of IL1RAP may provide ASCVD benefits by reducing inflammatory and subsequent pro-atherogenic effects of these cytokines.[307-309, 312, 313] Biweekly injections of anti-IL1RAP antibodies or isotype IgG (n=14/group) were started after six weeks of high-cholesterol diet and continued for four weeks. Mice treated with anti-IL1RAP antibodies experienced an average of a 20% reduction in subvalvular plaque area (Figure 24) and volume independent of total plasma cholesterol levels.

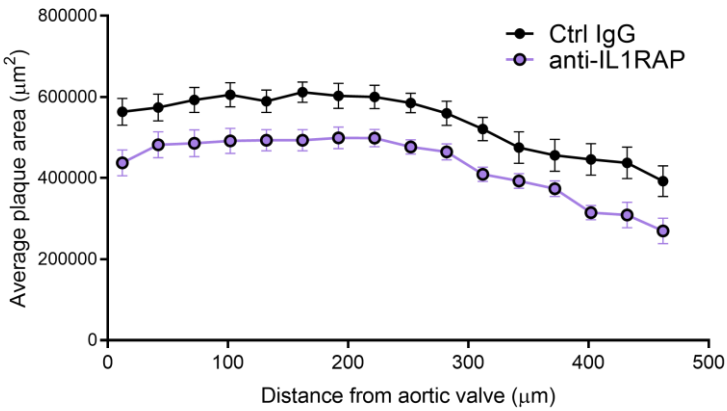


Figure 24: Average subvlavluar plaque area progressing through the aortic valve in *Apoe*$^{-/-}$ mice treated with anti-IL1RAP antibodies (purple circles) or istoype control (black circles). Dots denote the average of all mice in each treatment group at indicated distance.

Reduced plaque inflammation with anti-IL1RAP treatment

Immunohistochemistry analysis of subvalvular plaques form *Apoe*$^{-/-}$ mice treated with anti-IL1RAP antibodies revealed a decrease in neutrophil (Ly6G^{+}) area and macrophage (CD68^{+}) content within the plaque as well as reduced T cells in the adventitia. Similarly, flow cytometry of whole aortas displayed trends of decreasing neutrophils (Ly6G^{+}CD11b^{+}) and total T cells (TCRβ^{+}CD11b^{-}) in treated mice.

Quantitative polymerase chain reaction (qPCR) analysis of pooled carotid arteries from anti-IL1RAP treated mice identified a reduction in expression of the genes for the chemokines CXCL1, CXCL2, and CXCL5 and for the adhesion molecules VCAM-1 and ICAM-1 compared to controls. VCAM-1 and ICAM-1 are adhesion molecules found highly expressed on activated endothelial cells and VSMCs, facilitating the transmigration of leukocytes across the vessel wall and have both been implicated in the development of atherosclerosis.[322-324] The chemokines CXCL1, CXCL2, and CXCL5 can induce cell-trafficking of various leukocytes via their common receptor, CXCR2. Of particular interest, CXCL1 has been reported to regulate inflammasome activation in macrophages and promote monocyte and neutrophil accumulation in atherosclerotic lesions in mice.[325-328]

Anti-IL1RAP antibodies decrease CXCL1 production

Single-cell RNA sequencing of cells from human carotid plaques revealed that $CD68^+$ myeloid cell clusters had highest relative levels of expression of *CXCL2* and *CXCL8*, the functional homolog of murine CXCL1. Additionally, fibroblasts have been shown to secrete CXCL1 (and induce VCAM-1 and ICAM-1 expression) in response to IL-1.[327] Therefore, we decided to test the response of bone marrow-derived macrophages (BMDM) from wild-type mice and fibroblasts from a murine cell-line (NIH3T3) stimulated with IL-1α, IL-1β, IL-33, and IL-36 and subsequently treated with anti-IL1RAP antibodies *in vitro*. Both BMDMs and NIH3T3 fibroblasts treated with anti-IL1RAP produced less CXCL1 in response to stimulation with IL1RAP family cytokines. Taken together, our results suggest blockade of IL1RAP signaling may reduce plaque size and inflammation by reducing chemotaxis and infiltration of pro-atherogenic immune cells to the atherosclerotic vessel.

Conclusions and Clinical Perspectives

Atherosclerosis is a chronic disease characterized by unresolving inflammation. T cells accumulate in the atherosclerotic plaque and are believed to be key players driving local inflammation. The work in this thesis has aimed to help further unravel the ways in which T cells contribute to plaque pathology and also possibly identify novel anti-inflammatory therapeutics against atherosclerosis.

In **Paper I**, we showed that administration of IL-2 complexed to the anti-IL-2 antibody S4B6 (IL-2/S4B6) results in no change in atherosclerotic burden in mice. Although treatment with IL-2/S4B6 increased abundance of memory T cells and NK cells and their propensity to produce pro-atherogenic cytokines, it also boosted conventionally anti-atherogenic properties, including increased percentage of Tregs and decreased total plasma cholesterol. A clinical trial assessing the use of low-dose IL-2 to reduce vascular inflammation in patients with acute coronary syndrome is now in Phase II (IVORY study). By administrating IL-2 at low doses, one will preferentially expand Tregs due to their constitutive expression of the high-affinity alpha chain of the IL-2 receptor (IL-Rα, CD25). Although Tregs are highly sensitive to IL-2, there is still the possibility that some IL-2Rβγ-expressing cells or recently activated non-Treg cells that express CD25[329] may respond to low-dose IL-2 treatment. However, our results from **Paper I** suggest that even if this did occur, it likely would not compromise treatment goals of expanding Tregs and their effects nor negatively affect patients' atherosclerosis risk.

Immune checkpoint inhibitor (ICI) therapy has truly revolutionized the field of oncology and, as of 2019, 66% of active immuno-oncology clinical trials are investigating T cell-targeting immunomodulatory agents.[330] With the advent and expansion of these therapies and their use, appreciation of the immune system and the environment of the tumor when treating cancer has grown. Among many differences, treatment with ICI therapy has required the consideration of adverse effects not associated with other anti-cancer drugs, namely immune-related adverse events (irAEs). Patients receiving ICI therapy have a 5-fold increased risk of experiencing a cardiovascular event after three years. This increased CVD risk

includes risk of atherothrombotic events (MI, ischemic stroke, coronary revascularization),[243] but the underlying mechanisms have not been fully elucidated.

In **Papers II** and **III**, we demonstrated that PD-1 and LAG-3, two targets of FDA-approved ICI therapies, regulate inflammation in atherosclerotic aortas and lesions in mice. We also show in **Paper II** that IFN-γ-producing T cells in the aorta of atherosclerotic mice express pronounced levels of PD-1 and aortic T-cell increase IFN-γ production in response to anti-PD-1 antibodies. As IFN-γ has previously been shown to contribute to plaque formation/destabilization,[185, 206-208, 331] it is possible that systemic delivery of ICI therapy increases cytokine secretion by PD-1^+ plaque T cells, possibly providing a mechanism for the increased risk of ASCVD with ICI therapy. Speculatively, if plaque pathogenesis is partially driven by autoreactive T cells, removing systemic regulation with ICI antibodies may also increase activity of these T cells that might have been kept partially at bay otherwise.

As more and more cancer patients receive ICI therapies and long-term survival continues to increase,[332] long-term adverse reactions, including cardiovascular risk, must not only be considered but understood in order to mitigate subsequent cardiovascular events. It is difficult to speculate exactly how this might be achieved. New delivery technologies are being investigated such that ICI therapy can be administered locally instead of systemically, in hopes of reducing off-target effects and irAEs.[333-335] Updating treatment guidelines may also help reduce cardiovascular risks. It is currently speculated that statins may act synergistically with ICI therapy,[336] and we know statins lower ASCVD risk. Perhaps adjusting guidelines so that patients either start or remain on their statins during (and after) ICI therapy may be a route taken eventually. Further, if novel biomarkers are found that predict or correlate with ICI-related adverse cardiovascular effects, guidelines can require monitoring of these and suggest alterations in treatment plan if applicable, similar to how corticosteroids may be prescribed and/or ICI therapy discontinued with high grades of irAEs.[337] Monitoring for advancing lesions, such as by CCTA or carotid ultrasound, may also be relevant to add in conjunction to follow-up cancer screenings for high ASCVD-risk patients.

Finally, in **Paper IV**, we evaluated the possibility of anti-IL1RAP monoclonal antibodies as a therapeutic for atherosclerosis. We demonstrated that mice treated with anti-IL1RAP antibodies had decreased plaque size and inflammation, suggesting anti-IL1RAP as a potential therapy to reduce residual inflammatory risk by reducing vascular inflammation. The antibody we used and tested in **Paper IV** is a murine version of the CAN10 antibody (Cantargia AB). A Phase I clinical trial testing the safety and dosing of CAN10 in both healthy volunteers and psoriasis patients began in September 2023 and initial data is expected by 2024.

The anti-IL1RAP antibody nadunolimab (CAN04), differing from CAN10 in that it is not LALA-mutated (i.e., able to promote ADCC), is currently being tested in multiple Phase II/III clinical trials as an anti-cancer drug. As we demonstrate in **Paper IV** the ability of CAN10 to reduce vascular inflammation locally, it is interesting to consider if treatment with CAN04 in cancer patients might circumvent the increased ASCVD risk associated with ICI therapy.

As with most results from scientific research, we are ultimately left with more questions than answers. The goals of future studies will include investigating the impact of T cell exhaustion in atherosclerosis in man, elucidating the mechanisms underlying ICI-induced adverse cardiovascular events, and further exploring IL1RAP blockade as an atherosclerosis therapy in clinical trials.

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