Smoking and T cell co-stimulation in rheumatoid arthritis

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ABSTRACT

In this thesis I investigated if smoking limits the co-stimulatory system of CD8\(^+\) T cells in rheumatoid arthritis (RA). I took special interest in the co-inhibitory receptor PD-1 and its ligand PD-L1.

Blood samples from RA patients with known smoking status and experimental models of RA (RA mice) in which orally administered nicotine simulated smoking were used. Additionally, CD8\(^+\) T cells were isolated from human blood and stimulated \textit{in vitro}. Flow cytometry were used to analyze the expression of PD-1. ELISA was used to measure the soluble form of PD-L1 in serum samples from RA patients and healthy controls. Quantitative PCR was used for transcriptional analysis of proteins and microRNAs involved in CD8\(^+\) T cell regulation. Microarray analysis of microRNA was performed in samples of human CD8\(^+\) T cells.

Smokers had fewer activated CD8\(^+\) T cells that expressed PD-1 compared to non-smokers, and human CD8\(^+\) T cells stimulated with nicotine \textit{in vitro} had lower expression of PD-1 messengerRNA. RA mice treated with nicotine had fewer PD-1 expressing CD8\(^+\) T cells in the bone marrow. This was related to the increased production and release in circulation of the onco-protein survivin, a predictive marker for severe RA. CD8\(^+\) T cells of smokers adopted a naïve/memory phenotype and had different expression of several microRNA that are involved in the regulation of memory T cell formation, including the FOXO signaling pathway. Smokers also had lower levels of soluble PD-L1 in serum. The low PD-L1 levels were linked to altered expression of antibody receptors on antigen-presenting cells producing soluble PD-L1. The presence of RA-specific autoantibodies was associated with serum levels of soluble PD-L1.

I conclude that smoking interferes with the PD-1 inhibitory system on CD8\(^+\) T cells, which may contribute to higher risk for RA in smokers. This can occur because of the reduced inhibitory control of the CD8\(^+\) T cells with low PD-1 expression, but also because of a reduced supply of sPD-L1. Furthermore, I suggest that microRNA interfere with the FOXO signaling pathway and influence the phenotype of CD8\(^+\) T cells in smokers.

Keywords: Rheumatoid arthritis, CD8\(^+\) T cell, programmed cell death-1, programmed cell death-1 ligand 1, smoking, microRNA
SAMMANFATTNING PÅ SVENSKA


Vi försökte förstå vilka T-celler som är dominerande när andelen PD-1-uttryckande celler är låg. Vår analys visade att de kan tillhöra en subgrupp av T celler som ännu inte aktiverats eller som har aktiverats men blivit omvandlade till minnesceller. Båda dessa celltyper är mindre specialiserade än de som uttrycker höga nivåer av PD-1, men har stor potential att reagera vid aktivering. Vi fann vidare att rökare hade högre nivåer av microRNA (en molekyl som påverkar cellernas produktion av specifika proteiner) som interagerar med proteiner som är nödvändiga för att utveckla minnescellerna. Vi tror således att rökning kan främja de egenskaper som vi ser hos T-celler med låga PD-1-nivåer genom att påverka microRNA.

PD-1 receptorn skickar hämmande signaler till T-cellen då den binder till ett specifikt signalprotein, en så kallad PD-1-ligand. PD-1-liganden förekommer i löslig form och kan uppmätas i serumprover från patienter. På så vis såg vi att rökning hos RA patienter är associerat med att ha låga nivåer av PD-1-ligand i
serum. Detta kan tyda på att rökare saknar även den hämmande mekanismen hos immunsystemet. Vidare visar vi att sjukdomsspecifika antikroppar kan stimulera produktionen av PD1-liganden och att rökning kan motverka den processen.

Sammanfattningsvis visar jag i denna avhandling att T celler hos rökare förlorar en broms som ska dämpa T cellernas cytotoxiska aktivitet. Det är möjligt att T cellerna i avsaknad av denna broms inte kan stoppas från att angripa kroppens egna celler. Detta har troligen negativa konsekvenser för patienter som lider av ledgångsreumatism, eftersom överdriven aktivitet hos immunsystemet har en central roll i sjukdomen. Det är sedan tidigare känt att rökare löper större risk att drabbas av ledgångsreumatism, möjligen kan dessa fynd bidra till att förklara detta samband.
LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Wasén C**, M Turkkila, A Bossios, M Erlandsson, KME Andersson, L Ekerljung, C Malmhäll, M Brisslert, S Töyrä Silfverswärd, B Lundbäck, and MI Bokarewa
Smoking activates cytotoxic CD8+ T cells and causes survivin release in rheumatoid arthritis
*Journal of Autoimmunity* 2017; 78: 101-10

II. **Wasén C**, MC Erlandsson, A Bossios, L Ekerljung, C Malmhäll, S Töyrä Silfverswärd, R Pullerits, B Lundbäck, and MI Bokarewa
Smoking is associated with low levels of soluble PD-L1 in rheumatoid arthritis
*Frontiers in Immunology* 2018; 9(1677)

III. **Wasén C**, C Ospelt, M Erlandsson, KME Andersson, S Töyrä Silfverswärd, S Gay, MI Bokarewa
Smoking and microRNA regulation of CD8+ T cells in rheumatoid arthritis
*Manuscript*
LIST OF PAPERS NOT INCLUDED IN THE THESIS

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Proceedings of the National Academy of Sciences of the United States of America 2018, 115(51): E12063-e12072

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Autoimmunity Reviews 2017, 16(8): 845-855

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Murine germinal center B cells require functional Fms-like tyrosine kinase 3 signaling for IgG1 class-switch recombination
Proceedings of the National Academy of Sciences of the United States of America 2015, 112(48): E6644-E6653
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCCP</td>
<td>Antibodies against cyclic citrullinated peptides</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ACPA</td>
<td>Anti-citrullinated peptide antibodies</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BT LA</td>
<td>B and T lymphocyte attenuator</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine C-X-C motif receptor</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T cell associated protein 4</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAS28</td>
<td>Disease activity score 28</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMARD</td>
<td>Disease modifying anti-rheumatic drug</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc-γ receptor</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HVEM</td>
<td>Herpes virus entry mediator</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T cell co-stimulator</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-7R</td>
<td>Interleukin-7 receptor</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>ITSM</td>
<td>Immunoreceptor tyrosine-based switch motif</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte activation gene 3 protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
</tbody>
</table>
miR  Micro ribonucleic acid
mRNA  MessengerRNA
mTOR  Mammalian target of rapamycin
nAChR  Nicotinic acetylcholine receptor
OA  Osteoarthritis
PBMC  Peripheral blood monocytes
PD-1  Programmed cell death-1
PD-L1/2  Programmed cell death-1 ligand 1 and 2
PI3K  Phosphoinositide 3-kinase
qPCR  Quantitative polymerase chain reaction
RA  Rheumatoid arthritis
RF  Rheumatoid factor
RNA  Ribonucleic acid
ROR-γt  RAR-related orphan receptor-γt
SF  Synovial fluid
SNP  Single-nucleotide polymorphism
T-bet  T-box transcription factor
TCR  T cell receptor
TIM-3  T-cell immunoglobulin and mucin-domain containing-3
TIGIT  T cell immunoglobulin and ITIM domain
TNF  Tumor necrosis factor
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THE IMMUNE SYSTEM

The human body is immensely complex. It is built up by of trillions of cells and hundreds of different cell types that all need to work perfectly together to ensure our health. Additionally, we are by no means separated from other living organisms; in fact, our bodies are colonized by roughly the same number of bacteria as human cells (Sender, Fuchs, & Milo, 2016). How is it then possible for the human body to ensure that this vast number of our own cells are all functional and work in perfect balance with just as many microorganisms, for an entire lifetime?

The body is constantly surveilled for anything that could threaten its integrity by the immune system. Immune cells are located in multiple organs and tissues: in primary lymphoid organs, i.e. the bone marrow and thymus in which immune cells develop and mature; in secondary lymphoid organs, such as the spleen and lymph nodes that have specialized structures in which mature immune cells can interact; and in peripheral tissues, were immune cells search the body for abnormalities. The immune system is highly potent and has the capacity to effectively kill cells that poses a threat to our health. These threats could be infections of pathogenic microorganisms and viruses or malignant transformations of our own cells, which could develop into cancer. The major challenge for the immune system is to recognize a pathogenic cell in an ocean of healthy cells. The task is solved by a tight collaboration of different cell types and extensive control mechanisms. The immune system is roughly separated into two major divisions: the innate and the adaptive immune system.

The innate immune system

The innate immune system is often referred to as the first line of defense against infectious pathogens. Cells of the innate immune system are always ready to strike when they encounter invading organisms, but lack the ability to remember and react more vigorously toward a previously encountered pathogen. They are equipped with receptors that recognize molecular patterns specific for pathogens (Akira, Uematsu, & Takeuchi, 2006) and give rise to anti-
The process of presenting antigens is essential for the activation of T cells, a major cell type of the adaptive immune system. The DCs pick up antigens that represent the cells of the entire body, including the infected cells. In most cases, the antigen needs to be internalized by the DC and processed into small peptides (Banchereau & Steinman, 1998). The peptides are then associated to proteins called major histocompatibility complex (MHC) class I and II (in mice, in humans they are referred to as human leukocyte antigen). Only antigens bound to MHCs may activate T cells. DCs in tissue are often immature, meaning that they cannot activate T cells, but they can pick up antigen and become mature in doing so. They pick up antigen through various mechanisms including phagocytosis, macropinocytosis and endocytosis. The maturation process transforms the cell from a highly specialized antigen capturer, to a very effective antigen presenter. As part of this process, the DCs migrate to lymphoid organs where they can stimulate and maintain the T cell pool throughout the infection.

The adaptive immune system

The major components of the adaptive immune system are B cells and T cells. They differ from the innate immune system primarily through their specificity and memory. While cells of the innate immune system recognize certain patterns that are associated with many pathogens, B and T cells express receptors that are unique for each cell and recognize a single antigen that distinguishes the pathogen from healthy cells. Recognition of antigens, under the right circumstances, gives rise to a powerful response and the formation of long-lived memory cells that are ready to rapidly react if the body ever again is introduced to the same pathogen. The production of cells with specificity to any infecting pathogen requires an immense diversity amongst these receptors.

In contrast to T cells (see T cell biology), the activation of B cells may lead to the differentiation into plasma cells that produce antibodies. This is a process
of several steps (Stebegg et al., 2018). First, the B cell receptor needs to recognize its antigen. The activated B cell migrates toward the T cell rich zone of secondary lymphoid organs. At the border between B cell and T cell dominated zones the B cell may receive stimulatory signals from T cells that enable it to either start proliferating and enhance its antibody affinity, or differentiate into a short-lived plasma cell that secretes antibodies. B cells remaining in the lymphoid organ migrate from the B-T border into follicles formed by B cells and start producing a structure called germinal centers. Within the germinal center, the B cells undergo clonal expansion and affinity maturation, during which somatic hypermutation enables the B cell to produce antibodies with even higher affinity toward the antigen. The end product is long-lived plasma cell that produces antibodies and leads to immunity against the invading pathogen for the rest of life.
T cell development

T cells originate from bone marrow stem cells and precursors migrate to the thymus via the circulation. Not until the cell enters the thymus does it commit to become a T cell. Within the thymus the T cell undergoes genetic recombination (rearrangement of a certain set of gene segments) that leads to the expression of a T cell receptor (TCR) with a unique antigen-binding variable domain (Moran & Hogquist, 2012). The TCR is a heterodimer of two protein chains, called the α and β chains. These chains build a complex with two clusters of differentiation (CD)3 proteins and form a signal transducing unit. The TCRα gene contains variable, joining and one constant gene segment, and the TCRβ gene contains variable, diversity, joining and two constant segments.

The ultimate goal of the T cell development is to create a pool of T cells that have a diverse repertoire of TCRs, but no TCR that gets activated by proteins expressed by the own body, called self-antigens. Thymus epithelial cells are specialized for the presentation of T cells to self-antigens (Klein, Kyewski, Allen, & Hogquist, 2014). The transcription factor Autoimmune regulator allows the mto express and present genes from the whole genome. The T cell surface proteins CD4 and CD8 stabilize the interaction between the TCR and MHC class II and I, respectively. T cells that successfully go through the thymic development have affinity for MHC-peptide complexes, but only a weak affinity for self-antigens. Other T cells die in the process.

The thymus is made up by lobules, that in turn is constructed by an inner medulla and an outer cortex surrounded by a capsule. The T cell precursors are CD4⁺CD8⁻ and double-negative, and they are located in the subcapsular zone. During the double negative phase, the TCRβ gene undergo rearrangement of the diversity and joining segments, followed by rearrangement of the variable and diversity-joining sequences. When the cells migrate to the cortex, they express the TCRβ chain combined with a surrogate TCRα chain. They gain expression of both CD4 and CD8 and are termed double-positive. These cells go through positive selection, stimulating cells with a sufficient interaction with MHC-peptide complex. The formation of functional receptor will trigger the rearrangement of the TCRα gene and the production of a complete TCR. Cells then go through negative selection, leading to the elimination of cells with too
strong interaction with a self-antigen. Cells that successfully go through this phase lose their expression of either CD4 or CD8 (become single-positive) and differentiate and migrate to the medulla. In the medulla, the cell matures and eventually migrate to the periphery.

**T cell activation and differentiation**

Mature T cell are found in secondary lymphoid organs, the circulation or in other tissues. They are roughly divided into four subsets based on antigen experience, function and location: naïve cells, defined as cells that never have encountered their specific antigen; effector cells, that are found at the site of infection and directly target infected cells; effector memory cells, a population found in circulation after the resolution of the infection; and central memory cells, that are long-lived cells remaining after the resolution of an infection and mostly located within secondary lymphoid organs. T cells subsets are by tradition defined by cell surface markers, called clusters of differentiation. In human, CD27 and CD45RA may be used for this purpose. CD27+CD45RA+ cells are naïve cells, CD27−CD45RA+ cells are effector cells and CD27−CD45RA− cells are memory cells. Memory cells are further subdivided by the markers CCR7 and CD67L; cells that express both are defined as central memory cells, and cells expressing neither are effector memory cells.

The naïve state is a resting and relatively undifferentiated stage of the T cell life cycle. When a T cell recognizes its antigen, via its specific TCR, several processes begin inside the cell. A successful activation via the TCR leads to clonal expansion, a process in which a T cell having the antigen specific TCR starts to rapidly proliferate and after 15-20 rounds of divisions has created a large population of identical effector cells (Kurtulus, Tripathi, & Hildeman, 2012). The viral load of an acute virus infection peaks after approximately 3 days and the infection is cleared after 8 days, at which time the T cell numbers peak (Kurtulus et al., 2012). Within the activated T cell, new gene programs are activated and other transcription factors influence the protein expression, compared to the naïve cell. The effector cells become fully differentiated and gain the ability of producing cytokines and effector proteins.

CD8+ cytotoxic effector T cells produce proteins that are used to kill the target cell. Two major types of effector molecules are released by degranulation of the cytotoxic T cell and act together to ensure the death of the target. Granzymes are serine proteases that induce apoptosis of the target cell (Andrade, Casciola-
Humans express five granzymes named granzyme A, B, H, K and M, among which granzyme A and B is the most extensively studied. Granzyme B induces rapid apoptosis while granzyme A acts slowly. Granzyme A induces single-stand DNA brakes. Granzyme B induce apoptosis by activating caspases 3 or 8, eventually leading to DNA fragmentation and apoptosis. The other type off effector molecule, perforin, is a protein that polymerizes into pores in the targeted cells membrane, disrupts the integrity of the membrane and grants access to the target cells’ cytosol for granzyme B (Figure 1). By the time the targeted cell has repaired its membrane, sufficient amounts of granzyme B diffuse through the pores to ensure the death of the target. The size of the pores differs, and there have been doubts if the size is sufficient to allow the entrance of granzyme B, but pores of 170 Ångström have been reported.

CD4+ helper effector T cells (T_h) use a different approach. Their primary function is to bind to CD8+ T cells or B cells and to stimulate their activation through co-stimulation and cytokine release. Indeed, their activity is essential for a full antibody response by the stimulation of B cells. During the T cell activation process, CD4+ T cells will differentiate into different subpopulations depending on the cytokine environment, which activates master transcription factors.
Transcription factor T-box transcription factor (T-bet) will promote the differentiation into T_h1 cells, characterized by their production of the cytokine interferon-γ (IFN-γ). GATA3 favors the differentiation into T_h2, producing interleukin (IL)-4, IL-5 and IL-13. RAR-related orphan receptor-γt (RORγt) leads to the differentiation into T_h17, producing IL-17, IL-22 and IL-23. Bcl6 regulates the differentiation into follicular helper T cells (T_{FH}), which have a special role in antibody production. FoxP3 drives the formation of regulatory T cells (T_{reg}) that limit the immune response and promote tolerance.

After the infection is resolved, 80-90% of the effector cells die and only a pool of memory cells remain (Kurtulus et al., 2012). The memory cells are antigen experienced, in contrast to the naïve cells, but still hold the potential to proliferate and differentiate when they are reactivated by antigen. The memory cells are the fundamental purpose of the adaptive immune system. They remain for a very long time after an infection and quickly and effectively eliminate the pathogen if we should be infected by the same pathogen again, often without us even noticing the infection.

How the naïve T cells differentiate into effector cells and memory cells after the initial antigen encounter has been debated and at least three models describing this process exist (Ahmed, Bevan, Reiner, & Fearon, 2009; Luca Gattinoni, Klebanoff, & Restifo, 2012). A very usual representation of T cell activation is the linear differentiation model. In this first model, a naïve cell becomes activated, proliferates and differentiates into an effector cell. As the infection resolves most effector cells die but a few cells develop into long-living memory cells. This model, however, assumes that a cell can go from the terminally differentiated effector stage into a memory stage that is less differentiated in the sense that it can be reactivated and produce a second round of effector cells. The second model that is similar, but attempts to deal with this re-differentiation problem, is the bifurcative differentiation model. In this model, the primed T cell will undergo asymmetrical cell division that allows for the formation of a long-lived memory pool and a short-lived effector pool. The third model is the progressive differentiation model, suggesting that after antigen recognition, T cells will go through progressive stages of differentiation; that is the central memory phase followed by the effector memory phase before becoming terminally differentiated effector cells. According to this third model, a subpopulation of T cells stays undifferentiated throughout the infection to form a long-lasting population of memory cells. These cells are antigen experienced, yet closely related to the naïve population.
T cell co-stimulation

Despite extensive control mechanisms in the thymic selection of effective and yet tolerant T cells, additional regulation is essential to avoid autoimmunity. The recognition of antigen is accompanied by the ligation of numerous receptors on the T cell surface that will send both stimulatory and inhibitory signals to the T cell. These receptors are called co-stimulatory receptors. Positive signals will be termed co-stimulation within this thesis, and negative signals will be termed co-inhibition. Stimulation of co-stimulatory receptors is required for complete activation during TCR mediated antigen recognition. Furthermore, the positive signals need to overcome the negative signals. Failing to meet these requirements results in anergy. This additional control system serves the purpose of prohibiting an inappropriate activation of auto-reactive T cells. A large number of co-stimulatory receptors and ligands are well-described in the literature. A selection of co-receptors and their ligands are summarized in Figure 2. Many of these receptors and ligands are members of the immunoglobulin (Ig) and B7 super families, respectively (Sharpe & Freeman, 2002), or the tumor necrosis factor (TNF) receptor super family (L. Chen & Flies, 2013).

The receptors belonging to the Ig super family have an Ig-like extracellular domain and a short cytoplasmic tail. CD28 is probably the best-known co-stimulatory receptor from this family. It interacts with the ligands CD80 (also known as B7-1) and CD86 (B7-2) expressed on the surface of the antigen-presenting cell. However, CD80 and CD86 may also bind the cytotoxic T cell associated protein 4 (CTLA-4 or CD152), which is a co-inhibitory receptor and binds with higher affinity than CD28. Only CD86 is constitutively expressed by the APC, but both ligands are induced by activation and have overlapping functions. Another inhibitory receptor is T cell immunoglobulin and ITIM domain (TIGIT) that interacts with CD155 and has been found in both activated, memory and follicular T cells (Anderson, Joller, & Kuchroo, 2016). Inducible T cell co-stimulator (ICOS) is a stimulatory receptor from the same family, which binds to the ICOS ligand (ICOSL or B7-H2). ICOS is upregulated when the T cell is receiving activating signals from TCR activation and CD28 stimulation. B and T lymphocyte attenuator (BTLA) is a co-inhibitory receptor which is one of the few co-inhibitory receptors among the Ig super family that does not bind ligands of the B7 family, but interacts with the ligand herpes virus entry mediator (HVEM) from the TNF receptor super family. However, the co-inhibitory receptor from the Ig family called lymphocyte activation gene 3 protein (LAG-3) interacts not with a specific ligand, but directly with the MHC complex.
Figure 2. T cell (left) co-stimulatory receptors interacting with their ligands expressed by antigen-presenting cell (right). Red arrows indicate inhibition and green indicate stimulation. CD – cluster of differentiation, MHC I – major histocompatibility complex I, TCR – T cell receptor, CTLA-4 – cytotoxic T cell associated protein 4, ICOS – inducible T cell co-stimulator, ICOSL – ICOS ligand, BTLA – B and T lymphocyte attenuator, HVEM – herpes virus entry mediator, LAG-3 – lymphocyte activation gene 3 protein, PD-1 – programmed cell death-1, PD-L1 and PD-L2 – PD-1 ligand 1 and 2, Gal-9 – galectin-9, TIM-3 – T-cell immunoglobulin and mucin-domain containing-3. The figure is an adaptation of ref: (Mahoney, Rennert, & Freeman, 2015).
T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is an inhibitory receptor belonging to the Tim family of genes. Its primary ligand is galectin-9 but it may also interact with phosphatidylserine.

Several co-stimulatory receptors are found in the TNF superfamily. These include **OX40** interacting with the OX40 ligand (**OX40L**), which supports proliferation and survival of activated T cells (Duttagupta, Boesteanu, & Katsikis, 2009), and **CD27** interacting with **CD70**, which has an important role in regulating the T cell memory response.

**Programmed cell death-1 (PD-1)**

The PD-1 co-inhibitory system has gained a lot of attentions during the past five years due to its huge success as a therapeutic target for immunotherapy of melanoma and other cancers. Its discovery was consequently awarded with the Nobel Prize in medicine to doctors Tasuku Honjo and James Allison in 2018. It was in 1992 that the group led by Dr. Honjo discovered this novel member of the immunoglobulin superfamily. It was identified in search for proteins that were **de novo** synthesized in thymic T cells undergoing programmed cell death, and named after this proposed function (Ishida, Agata, Shibahara, & Honjo, 1992). In 1997, Vibhakar et. al. demonstrated that that PD-1 was upregulated by stimulation of human peripheral blood monocytes (PBMC), and was associated with inhibited proliferation rather than apoptosis (Vibhakar, Juan, Traganos, Darzynkiewicz, & Finger, 1997). This, in addition to structural features of PD-1, led the authors to suggest that PD-1 is a co-inhibitory receptor.

PD-1 is encoded by the gene **PDCD1** on the chromosome 2 and contains 288 amino acids. It exists as a monomer with an extracellular domain built up by a single IgV domain. The binding site contains numerous hydrogen bonds and a lipophilic interaction. 20 amino acids connect the IgV domain to the transmembrane section (Lázár-Molnár et al., 2008). The intracellular part of PD-1 includes an immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIMs and its activating counterpart ITAMs are common features of immunoreceptors, including the T cell receptor and Fc receptors (Finger et al., 1997). The ITIM motif is followed by an immunoreceptor tyrosine-based switch motif (ITSM). Interruption of this site, but not the ITIM, blocks the function of PD-1 (Chemnitz, Parry, Nichols, June, & Riley, 2004). Ligation of PD-1 leads to phosphorylation of the tyrosine residues of the ITSM, and inhibition of T cell receptor signal
transduction is believed to be accomplished by the subsequent docking of the Src homology region 2 domain containing phosphatase-2 (Rota et al., 2018).

The PD-1 ligands

The ligands of PD-1 were discovered by homology to CD80 and CD86 and were named PD-1 ligand 1 and 2 (PD-L1 or B7-H1, and PD-L2 or B7-DC) (Freeman et al., 2000). Similar to CD80, CD86 and ICOSL, PD-1 ligands consist of an IgV and IgC extracellular domain, a trans-membrane domain and a short intracellular domain that is charged. Their expression were induced in antigen-presenting cells after stimulation with IFN-γ. No other co-stimulatory/inhibitory receptor has been shown to interact with the PD-1 ligands, but PD-L1 expressed by T cells may interact with CD80 (Butte, Peña-Cruz, Kim, Freeman, & Sharpe, 2008). This interaction was three times weaker than the PD-1/PD-L1 interaction, and 10 times weaker than the PD-1/PD-L2 interaction, but still stronger than the binding affinity between CD80 and CD28.

Furthermore, ligation of PD-1 gave rise to potent inhibitory signals (Freeman et al., 2000). Adding soluble PD-L1 Ig fusion protein to the cell culture of PD-1 expressing T cells reduced T cell proliferation. Under the condition of sub-optimal levels of αCD3 stimulation, PD-L1 almost completely blocked T cell proliferation. It could, however, be rescued if the cells expressed very high levels of CD28. Similarly, cells exposed to optimal levels of αCD3 were only inhibited by PD-L1 when CD28 stimulation was low or absent, elegantly demonstrating the balancing effect of these two co-stimulatory receptors on the T cell activation status.

Interestingly, the ability of PD-L1 Ig fusion proteins to inhibit T cell proliferation also demonstrates that PD-L1 does not need to be bound to a cell to functionally interact with the PD-1 receptor. Indeed, cell lines produce sPD-L1 to culture media and measurable levels of sPD-L1 were found in human serum samples from healthy individuals (Y. Chen et al., 2011). Furthermore, PD-L1 prepared from human cells had a higher molecular weight than soluble PD-L1, indicating that the soluble form of the protein lacks the trans-membrane domain. Treatment of cultured cells with a matrix-metalloproteinase inhibitor resulted in lower levels of sPD-L1, which supports the notion that PD-L1 is cleaved off the membrane to produce its soluble form.
T cell exhaustion

CD8+ T cells risk becoming unresponsive under condition of prolonged antigen exposure and response. This state is referred to as T cell exhaustion and the concept is illustrated in Figure 3. PD-1 expression was first related to the exhausted state of CD8+ T cells in a model of chronic infection (Barber et al., 2006). Mice infected with Lymphocytic choriomeningitis virus (LCMV) virus of either the Armstrong strain, causing acute infection, or clone 13, causing chronic infection, were used as models to study non-exhausted and exhausted CD8+ T cells, respectively. CD8+ T cells from mice with chronic infection do not respond by reactivation with antigen in vitro; they do not produce cytokines and show little sign of clonal expansion of antigen specific cells.

The mice with chronic infection are unable to produce functional memory cells and are considered exhausted. A microarray analysis demonstrated that the PD-1 expression was notably higher in exhausted CD8+ T cells. Comparing the dynamics of PD-1 in acute and chronic infection showed that both conditions led to increased PD-1 expression in the acute stage. In the chronic setting, PD-1 expression remained, while it was gradually downregulated in the acute infection within 1-2 weeks. Blocking the ligand of PD-1 restored the expansion of antigen specific cells, which increased their effector functions and reduced the viral titers. It was concluded that PD-1 is a central protein in T cell exhaustion, and that releasing this “brake” on the T cell could restore their function (Barber et al., 2006). In the follow-up study, the exhaustion signature of mice with chronic infection was further defined (Wherry et al., 2007). With respect to the gene expression profile, the exhausted cells showed more similarities with effector cells than memory cells, but also upregulated a cluster of genes unique for the exhausted cells. Upregulated genes involved numerous inhibitory receptors, including PD-1, and downregulated genes comprised cytokine receptors, including the IL-7R. Interestingly, there was no enrichment of genes related to anergic cells in the exhausted gene profile.

These initial experiments showed that enhancing T cell function through the blockade of the PD-1 inhibitory systems was very promising. Indeed, when this idea was transferred into the clinic, it was an immense success as an immunotherapy against malignancies. However, PD-1/PD-L1 blocking antibodies do not always produce long lasting results, and many patients eventually progress despite treatment (Nowicki, Hu-Lieskovan, & Ribas, 2018). PD-L1 antibody treatment of chronic LCMV infection in mice eventually fails to improve CD8+ T cell responses. After 8-11 weeks T cell responses was similar to that in infected mice.
without treatment and after 4 months the viral load was similar (Pauken et al., 2016). By the use of an assay for transposase-accessible chromatin with high-throughput sequencing, this lack of the long-term response was attributed to changes in the epigenetic landscape of exhausted cells, which differed from that of effector and memory cells. Notably, mice with chronic LCMV infection were different from the acute setting already during the effector phase of the infection (day 8), during which the increased chromatin accessibility in certain regions and increased enhancer activity allowed the expression of exhaustion related proteins, including PD-1 (Sen et al., 2016). Additionally, chromatin accessibility regions associated with exhaustion in mice overlapped to a large extent with those seen in antigen specific T cells from HIV patients and C63B tetramer+ cells from patients with chronic hepatitis C virus infection.

The idea was born that in order to counteract exhaustion, focus needs to be shifted from the functionally exhausted and terminally differentiated effector
cells, to the loss of memory cell formation. A rare T cell subset called T memory stem cells, that is antigen experienced yet shares properties with hematopoietic stem cells, has recently been put in the spot-light (L. Gattinoni, Speiser, Lichterfeld, & Bonini, 2017). 2-3% of the circulating T cells belong to this sub-population and may be found in what was traditionally defined as the naïve T cell compartment. This population may be differentiated in vitro from naïve cells by activating the Wnt-β-catenin signaling pathway, which is central to its function.

**PD-1 and autoimmunity**

B6 mice lacking the expression of PD-1 spontaneously develop autoimmune disease characterized by glomerulonephritis and arthritis (Nishimura, Nose, Hiai, Minato, & Honjo, 1999). Deleting PD-1 in mice of the Balb/c background leads to autoimmune cardiomyopathy and often to an early death (Nishimura et al., 2001). Interestingly, the consequences of compromised PD-1 function may also be studied in humans, after the approval of anti-PD-1 antibodies for clinical use. Two PD-1 blocking antibodies currently exist on the market, named nivolumab and pembrolizumab. PD-1 blockade as immunotherapy for the treatment of malignant melanoma was first approved by The Food and Drug Administration of USA in 2014, and the side effects observed from this treatment reveal important information of the function of PD-1 in humans. Indeed, Blocking the PD-1 inhibitory system results in immune related adverse events in almost one third of the patients and may affect several organs including the skin, the endocrine system and the digestive system, in which they are the most common (P. F. Wang et al., 2017).

**MicroRNA (miR) regulation of T cells**

MiRs are small non-coding ribonucleic acids (RNA) of only approximately 22 nucleotides. MiRs are expressed by all cells. Their primary function seems to be the repression of transcription or translation of messengerRNA (mRNA). MiRs have affinity for specific mRNA sequences due to complementary binding. Currently, approximately 25 000 miRs are listed in miRBase, a database which annotates miRs described in the literature (Kozomara & Griffiths-Jones, 2014). MiR coding genes are found in-between genes with their own promoters, or in
introns, and sometimes in exons, of other genes. They may be transcribed in clusters and be post transcriptionally spliced into individual miRs. Transcription of miR genes result in primary miRs consisting of a hairpin structure with a poly-A tail and a 5’cap. A protein complex within the nucleus recognizes the double stranded RNA formed by the hairpin loop and the primary miRs are spliced by the ribonuclease III called Drosha. The resulting precursor miRs may be exported from the nucleus and further processed. Outside the nucleus, the RNase III endonuclease Dicer splices away the loop of the hairpin structure, and produces two at least partly complementary strands of miRs. The 5’ and 3’ arms of the precursor are annotated -5p and -3p, respectively.

The mature miRs are loaded into Argonaute proteins to form RNA-induced silencing complexes. The miRs work as guiding strands within these complexes and identify mRNA, often within the 3’untranslated region. If the miR binds with high affinity due to a high level of complementarity with the mRNA, this will induce cutting of the mRNA strand, promoting its degradation. Alternatively, a stable complex will be formed that inhibits translation of mRNA (Jo et al., 2015). There seems to be situations, when miR complexes can also promote translation. It has been demonstrated that miR complexes may interact with transcription factors to influence transcription of genes (O’Brien, Hayder, Zayed, & Peng, 2018).

The impact of miRs on protein expression has been investigated in detail by knocking out miR-223 in mice and analyzing protein expression by a quantitative mass spectrometry based stable isotope labeling with amino acids in cell culture (Baek et al., 2008). Bone marrow hematopoietic cell cells were isolated from miR-223 knock-out and wild-type mice, differentiated into neutrophils in vitro and then analyzed for both mRNA and protein expression. The experiment demonstrated that the protein levels in some cases were reduced without reduced mRNA levels and these were thought to be regulated by miRs on the translational level. The proteins whose expression increased in miR-223 knock-out cells by at least 50% had also increased mRNA levels and demonstrated that miRs suppressed protein levels by destabilizing mRNA. Authors point out that only five proteins were increased by >50% in response to knocking out miR-223, which would correspond to a >33% suppression of these proteins in wild-type neutrophils. Additionally, proteins of highly expressed mRNA showed a higher response to miR repression than those with low expression. The authors conclude that most miR-mRNA interactions result in modest effects on protein expression and primarily have the function of fine-tuning expression.
Even though hundreds of miRs are expressed in T cells, a low number of miRs have a high expression (H. Wu et al., 2007). The miR expression profile differs between cells of different activation stages. Firstly, the global expression of miRs are generally lower in activated T cells compared to both naïve and memory cells (H. Wu et al., 2007). Mice deficient in Dicer are hyper sensitive to TCR stimulation, indicating that miRs play an important regulatory role in suppressing T cell activity in naïve T cells not receiving sufficient stimulation for full activation (Marcais et al., 2014). Yet, Dicer deficiency also results in a poor effector T cell response. MiRs that have been reported differentially expressed after T cell stimulation include miRs -26a/b, -150, -181a, -223, -342-3p (upregulated), -155 and the 17-92 cluster (downregulated) (Rodríguez-Galán, Fernández-Messina, & Sánchez-Madrid, 2018). The immunoregulatory function of individual miRs has been demonstrated in knock-out mice models. Deletion of miR-146a made mice respond more rapidly to the LPS-induced septic shock and spontaneously develop autoimmunity after 6 months of age due to an excess of autoreactive T cells (Boldin et al., 2011). Deletion of miR-142 in T cells, on the other hand, improved survival in a mouse model of graft-versus-host disease (Y. Sun et al., 2015).

MiRs may also regulate T cell immune responses by regulating the expression of co-stimulatory receptors and downstream signaling pathways (Rodríguez-Galán et al., 2018). For example, miR-138 targets both PD-1 and CTLA-4 (Wei et al., 2016). It has also been demonstrated that T cells transfer miRs to each other by the use of extracellular vesicles. Comparing vesicles released from cultures of PBMC from RA patients and healthy controls demonstrated that differentially expressed miRs primarily target co-inhibitory receptors, including both PD-1 and its ligands (Greisen et al., 2017).
Autoimmunity

Despite thorough control mechanisms in the development of immune cells and the expression of co-inhibitory receptors, the immune system could get activated by self-antigens. Because self-antigens are proteins that are expressed by our own tissues, cells presenting these antigens cannot be eliminated and replaced by healthy cells. Instead, chronic autoimmune conditions develop with risk of substantial tissue damage. During these conditions all the brilliant mechanisms that make the immune system so very effective against invading pathogens are redirected toward ourselves. Cytokines are produced that activate and push the immune system toward the sites of inflammation and so-called autoantibodies are produced with activity toward our own cells. Different autoimmune conditions may develop based on what organs are targeted by the immune system.

Disease pathology

Rheumatoid arthritis (RA) is a chronic autoimmune disease. The prevalence of RA is approximately 0.5 to 1% and affects women 2-3 times more often than men. The difference between women and men are largest before menopause. The risk for RA increases with age, but younger individuals can also get the disease. There is a genetic component of RA, but it does not fully explain the etiology of the disease. A first-degree relative of a RA patient have a three times higher risk for developing the disease (Frisell et al., 2013), and a monozygotic twin have a risk of approximately 10% to get RA if the twin sibling has the disease (Svendsen et al., 2013).

It has been postulated that autoimmunity in pre-RA patients develops many years before the disease onset without any evident symptoms of disease. The genetic factors and an accumulation of environmental risk factors over this pre-clinical stage contributes to autoimmunity that eventually lead to RA (Smolen et al., 2018). Although joints are the primary sites of the disease, it is a popular belief that RA originates from organs outside the joints (Catrina, Svensson,
Malmström, Schett, & Klareskog, 2017). This is based on the fact that autoantibodies are found in pre-RA sera long before any inflammation in joints can be clinically detected. Lungs and gums have been suggested to be the sites in which autoimmunity first develop, due to the associations between RA and lung exposure to cigarette smoke or dust.

RA is described as a rather heterogeneous disease. A common way to distinguish between groups of RA patients is serum measurements of autoantibodies. Indeed, titers of autoantibodies are routinely tested in patients with suspected RA for diagnostic purposes. One of these antibodies is rheumatoid factor (RF) that has affinity for the Fc-portion of the human IgG antibody. Another type is antibodies against cyclic peptides containing citrullinated amino acid arginine (ACPA). ACPA differs from RF in that it seems to have a significant role in the pathogenesis, while RF primarily is a biomarker for the disease. ACPA binds to peptides that have undergone the post-transcriptional modification called citrullination. Citrullination is the replacement of a secondary amine group in the amino acid arginine by oxygen, removing the positive charge of arginine. The reaction is catalyzed by peptidylarginine deaminase and the product is an amino acid called citrulline. A vast number of proteins contain citrulline, hence provide plausible target for ACPA. The production of ACPA has been associated with genetic predisposition for RA, including the sequence within allelic variants of the HLA-DR gene referred to as the shared epitope.

During the clinically overt stage of RA, the joints become the primary sites of disease. The joint is the tissue structure connecting two bones. The ends of the connecting bones are covered by a lining of cartilage. Around the bone and cartilage, a capsule is formed that is lined by a membrane called the synovium. A subset of cells within the synovium secretes a lubricant, synovial fluid, to the cavity within the capsule that enables us to move with minimum friction within the joints. The inner layer (intima) of the synovium is built up of fibroblast-like synoviocytes and the outer layer (sub-lining) consists of fibroblasts, adipocytes and blood vessels. During RA, the synovia is infiltrated by inflammatory cells such as macrophages, DCs and lymphocytes. Synoviocytes become activated, they expand, produce pro-inflammatory cytokines and invade surrounding tissues. Proteases produced by fibroblast-like synoviocytes together with the activation of osteoclasts contribute to degradation of bone and cartilage, leading to cartilage degradation and development of bone erosions, which is a common feature of established RA. ACPA may contribute to this process by stimulating osteoclast activity. Meanwhile, the sub-lining layer becomes infiltrated with lymphocytes that further contribute to the inflammatory state of
the joint and may form ectopic germinal centers within the synovium (Smolen et al., 2018).

CD8$^+$ T cells and RA

The frequency of CD8$^+$ T cells in human PBMC is approximately 10-30%, in RA and healthy controls. The frequency of CD8$^+$ T cells is higher in synovial fluid of RA patients compared to peripheral blood, resulting in a higher CD8-to-CD4 ratio in the synovium (Cho et al., 2012). Synovial CD8$^+$ T cells have a larger population of CD27$^+$CD62L$^-$ memory cells than peripheral blood CD8$^+$ T cells, and express more IL-6 and TNF-α (Carvalheiro, Duarte, Silva-Cardoso, Da Silva, & Souto-Carneiro, 2015). Furthermore, synovial CD8$^+$ T cells have a higher frequency of early differentiated (CD27$^+$CD28$^+$) cells, and lower frequency of terminally differentiated (CD27$^-$CD28$^-$) cells (Cho et al., 2012). They have low expression effector cell transcription factor T-bet, activation marker CXCR3, effector molecules granzyme B and perforin, compared to peripheral blood CD8$^+$ T cells. Instead, they have higher expression of proliferative marker Ki-67, high expression of PD-1 and compared to healthy peripheral blood CD8$^+$ T cells; high production of IL-10.

Interestingly, CD8$^+$ T cells that are recruited to, or proliferate within, the inflamed joint of RA patients are enriched in clonal populations with specificity towards virus antigens (Fazou, Yang, McMichael, & Callan, 2001). Indeed, within the T cell population in the RA synovium, a low number of clones take up a substantial part of the entire T cell receptor repertoire, and the same clones may be found in multiple joints. Sequencing of T cell receptors revealed that 27% of the sequences consists of only 6 different clones. This phenomenon was most pronounced in synovium of patients with early, untreated RA, and no highly expanded clones could be found in peripheral blood (Klarenbeek et al., 2012). These results could indicate that T cells are attracted to the joint and undergo clonal expansion due to the presence of autoantigens. A more recent study reported that expanded clones of CD8$^+$, but not CD4$^+$, T cells could be found in blood in 8 out of 65 RA patients, in which one clone represented at least 20% of the total CD8$^+$ T cell pool (Savola et al., 2017). Interestingly, somatic mutations in genes associated with immune cells and proliferation could be found exclusively within these expanded populations.

Increased numbers of CD8$^+$ T cells has been reported peripheral blood of RA patients compared to healthy controls, in early RA but not established RA.
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(Coulthard et al., 2012). The central memory population is slightly less frequent in peripheral blood CD8⁺ T cells of RA patients, instead the effector population is larger. CD8⁺ T cells from RA patients with active disease had higher intracellular expression of cytokines and effector molecules than controls, determined by flow cytometry (Carvalheiro et al., 2015). These include IL-6, TNF-α, IL-17A, IL-10, granzyme B and perforin.

According to one study, there were positive associations between disease activity and expression levels of TNF-α, IFN-γ and IL-17 in CD8⁺ T cells, but a negative association with the expression of CXCR4 that direct T cells to the inflamed joints (Carvalheiro et al., 2015). Another study reported a negative association between the frequency of synovial CD8⁺ T cells and disease activity (Cho et al., 2012). These studies together indicating that CD8⁺ T cells recruited to the joints could have regulatory properties, while the cytotoxic activity of CD8⁺ T cells in peripheral blood contributes to the disease.

PD-1 and RA

Due to the fact that the PD-1 knockout mice develop arthritis similar to human RA, it has long been believed that single-nucleotide polymorphism (SNP) in the PDCD1 gene may be associated with RA. This was first suggested in 2004, when the functional role of PD-1 was emerging, but several years before PD-1 inhibitors were approved for human use (Poole, 2014). In this study on the Chinese population resident in Taiwan, 135 controls and 84 RA patients were tested for an SNP named C+872T after its position in exon 5 (later named PD-1.5). RA was associated with a C/T genotype, while the C/C was more common in the controls. The T/T genotype was only present in approximately 5% of both populations. This SNP did not affect the amino acid composition of the protein, but was thought to have a regulatory function (Lin et al., 2004).

Shortly afterwards, another SNP was investigated in a larger Swedish population of 3404 controls and 1175 RA patients. This SNP was named PD-1.3 and involves a G-to-A alteration of the nucleotide 7809, an enhancer within intron 4. This SNP will disturb the binding affinity of the Runx1 transcription factor and thereby influences gene expression. The frequency of the A allele was 6-9% in all groups, except for the subgroup of RA patients negative for RF and SE, it which it was 12% (Prokunina et al., 2004). The next study on this subject was conducted in 647 control and 180 RA patients of the Hong-Kong Chinese population. In this study, PD-1.3 was investigated in addition to PD-1.1 located
in the promoter and to PD-1.5 in the exon 5. Surprisingly, no polymorphism in PD-1.3 was detected in either control or RA patients within this material. However, the A/A genotype of PD-1.1 was less frequent in RA compared to control (Kong et al., 2005). PD-1.1, PD-1.3, PD-1.5 and PD-1.9 was once again studied in the Chinese population of 309 controls and 320 RA patients. This study confirmed an association between PD-1.1 and RA. PD-1.3 was again shown not to be polymorphic. Importantly, different genotype in the PD-1.1 SNP was translated into different PD-1 expression. Patients with the A/A genotype had low frequency of PD-1 expressing T cells, compared to controls. A/G had intermediate expression and G/G had the highest expression (Liu et al., 2014). An additional Taiwanese study involving 125 controls and 129 RA patients further demonstrated a lack of association between RA and polymorphism in PD-L1 or PD-L2 (S. C. Wang et al., 2007). The SNPs investigated for association with RA are summarized in Figure 4.

The potential association between the PD-1 gene and RA inspired repeated investigations of PD-1 and PD-L1/2 expression in the RA synovium. The first study dated 2010 and demonstrated that the healthy synovium contained no cells expressing PD-1, as determined by immunohistochemistry. In contrast, samples from patients with osteoarthritis (OA) could sometimes be positive, and RA patients had PD-1 expressing cells in 8 of 9 samples (Raptopoulou et al., 2010). This was confirmed in a more recent study, in which 34 of 51 RA synovial samples were positive for PD-1 (Matsuda et al., 2018). Both RA and OA samples were frequently expressing PD-L1 and PD-L2, while controls were always negative. Not surprisingly, high expression of PD-1 and its ligands was the result of high synovial inflammation. PD-1 was expressed by T cells primarily localized within lymphoid aggregates of the sublining layer. Most of the PD-L1 and PD-L2 was expressed by macrophages, but PD-L1 was also expressed by endothelial cells. The frequency of CD4+ T cells expressing PD-1 was around 8 times higher in the synovial fluid (25%) than in the peripheral blood, indicating that T cells with a PD-1+ activated phenotype were accumulated in the RA synovium. A separate study confirmed that synovial fluid CD4+ T cells frequently expressed PD-1 and that the expression level was considerably higher, compared to CD4+ T cells of the peripheral blood. Interestingly, even in the CD4+ population characterized as naïve by classical markers (CD27 and CD45RO), as many as 60% were positive for PD-1, although the mean fluorescent intensity was low (Moret, van der Wurff-Jacobs, Bijlsma, Lafeber, & van Roon, 2014). Other studies reported that besides PD-1, other co-inhibitory receptors including CTLA-4, TIM3 and TIGIT were upregulated on synovial CD4+ T cells (Greisen et al., 2017; Wan et al., 2006). Lastly, CD8+ T cells had higher frequency of PD-
expression in the synovial fluid than peripheral blood, the reported frequency varies between 20% (Greisen et al., 2017) and 80% (Cho et al., 2012).

CD4\(^+\) T cells isolated from the synovial fluid and peripheral blood of RA patients were stimulated with PD-L1 in vitro (Raptopoulou et al., 2010). Interestingly, cells isolated from the synovial fluid were less responsive to PD-L1, measured by inhibition of proliferation and IFN-\(\gamma\) production. It was suggested that PD-1 expression in synovial T cells makes them unresponsive to stimulation by DCs (Moret et al., 2014). Indeed, co-cultures of DC and CD4\(^+\) T cells from either synovial fluid of peripheral blood indicated that only the peripheral blood T cells responded to the interaction with DC. Blocking PD-1 in the peripheral blood CD4\(^+\) memory cells increased proliferation when co-cultured with DC. Additionally, PD-1 blocking antibodies in combination with IL-7 improved proliferation in the synovial fluid CD4\(^+\) T cells. A recent report related PD-1 expression in the synovium to clinical parameters of 51 RA patients. The treatments received by the participating patients did not seem to influence PD-1 or PD-L1. Neither did the RF status or the C-reactive protein (CRP) or synovitis relate to the number of PD-1\(^+\) cells. PD-L1 expression in the synovial lining was, on the other hand, higher in RF-positive patients and had strong positive correlations with CRP and synovitis.

A recent study used mass cytometry of three synovial tissue samples from seropositive RA patients and confirmed that the frequency of PD-1\(^+\)CD4\(^+\) T cells is approximately 25% (Rao et al., 2017). This technique allowed for further characterization of this PD-1\(^+\)CD4\(^+\) population. The majority of these cells co-expressed PD-1, ICOS and MHC class II, which was confirmed with flow cytometry. The proportion of this population was similar in the synovial tissue and synovial fluid, and was closely associated with seropositivity, which could mean they are involved in local antibody production. However, the vast majority of the cells had no expression of CXCR5, neither did they cluster with the population expressing classic T\(_{FH}\) markers. This was an interesting observation since PD-1 expression is a typical feature of T\(_{FH}\) cells and is not considered to reduce their functionality in the way that PD-1 leads to exhaustion in the CD8\(^+\) T cell population. This population, named peripheral helper T cells (T\(_{PH}\)) by the authors, was also found and expanded in the peripheral blood of seropositive patients. Seropositive RA patients whose disease activity was lowered by treatment reduced their T\(_{PH}\) population. A transcriptional analysis to evaluate the functionality of these cells indicated higher mRNA levels of several proteins involved in T\(_{FH}\) differentiation and function, but not the traditional master transcription factor Bcl6, instead Blimp-1 was induced, and IL-2 production was reduced. The cells co-expressed TIGIT, but no other co-inhibitory receptors.
typical for T cell exhaustion. They did, however, express low levels of IL-7R, CD27 and CCR7. *In vitro* co-cultures demonstrated that T\textsubscript{FH} cells had similar ability to induce B cell differentiation as T\textsubscript{FH} cells.

Several reports focused on **PD-1 expression outside the synovium**, with rather conflicting results. A study of 82 RA patients and 90 controls reported lower expression of PD-1 in the CD\textsubscript{4\textsuperscript{+}} and CD\textsubscript{8\textsuperscript{+}} peripheral blood T cells in RA compared to control. Additionally, lower PD-1 expression was associated with high CRP and higher disease activity (Li et al., 2014). Another study containing 37 RA patients and 31 controls reported higher levels of PD-1 expression in CD\textsubscript{4\textsuperscript{+}} cells of the peripheral blood, in addition to higher TIM-3 expression. This study

![Figure 4. Programmed cell death-1 (PD-1) in rheumatoid arthritis (RA). Upper panel: PD-1 gene (*PDCD1*) and polymorphic sites that have been investigated for their association with RA. Lower panel: PD-1 expression in the healthy control (HC, to the left) and RA (to the right) joint and peripheral blood (PB). PD-1 is highly expressed in synovial fluid (SF) T cells and is expressed on the cell surface of peripheral follicular helper T cells (T\textsubscript{FH}) that also expresses T cell immunoreceptor with Ig and ITIM domains (TIGIT), on extracellular vesicles and in soluble form (sPD-1) together with its ligand (PD-L1).](image-url)
still reported a negative correlation between the frequency of PD-1+TIM-3+ CD4+ T cells and disease activity (Koohini et al., 2018). Yet another study including 81 RA patients and 30 controls reported that PD-1 expression in the peripheral blood was indeed higher in RA patients, and found positive correlations between expression frequency/level of PD-1 and inflammation parameters including disease activity, erythrocyte sedimentation rate and CRP.

A third approach to study the PD-1 inhibitory system in RA is to measure the soluble forms of PD-1, PD-L1 and PD-L2. Elevated levels of soluble PD-1 and PD-L1 have been reported in RA serum and synovial fluid (Wan et al., 2006). The soluble variant of PD-1 (sPD-1) is produced through alternative splicing and is preferentially expressed by CD4+ T cells. Surprisingly, when PD-1.Ig or PD-L1.Ig fusion proteins were added to cultures of synovial mononuclear cells, they led to an increase rather than inhibition of the proliferation in the cultures. This fueled a belief that the fusion proteins would inhibit the function of their membrane-bound counterparts. It should be noted, however, that the ELISA used in that study has later been criticized for its cross-reactivity with human IgG antibodies (Nielsen, Barington, Hansen, & Lillevang, 2007). A second study confirmed that sPD-L1 levels were higher in blood samples from RA patients than controls, and even higher in the samples of synovial fluid. However, the association between radiographic progression and sPD-1 was inverse and suggested that sPD-1 protected against erosions (Greisen et al., 2014). High sPD-1 levels were confirmed in RA for the third time, but they were positively correlated to inflammation and disease activity (Hassan, Baraka, & Fouad, 2015). Lastly, PD-1 may also be transferred between T cells by the use of extracellular vesicles (Greisen et al., 2017).

Symptoms and treatments

RA diagnostics is aided by the disease criteria described by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) in 2010 (2010 ACR/EULAR). The criteria are designed to detect the disease at its early stages and focus largely on the number of joints involved, the detection of autoantibodies, the duration of symptoms and the measurement of acute-phase reactants (Aletaha et al., 2010). An older set of criteria, the 1987 ACR criteria, is helpful in defining established RA.

Symptoms of RA can develop quickly or slowly over several months (Lars Klareskog, 2011). It often starts with a general feeling of fatigue and fever with
diffuse pain and stiffness in joints that are worse in the mornings. Joint swelling resulting from synovitis and tenosynovitis usually start in small joints of the hand and then disease progresses by the involvement of more joints in a symmetrical manner. The measurement of autoantibodies can further help diagnosis. However, about a half of RA patients are seronegative for ACPA or RF. Positivity for ACPA predicts a worse prognosis. Other useful serological markers involve erythrocyte sedimentation rate (ESR) and CRP, that indicates systemic inflammation. Radiography of joints is used to identify skeletal damage and bone erosions, which is unusual at the time for diagnosis but may occur over time. Many patients develop extra-articular manifestations in skin, eyes, heart, peripheral nerves and lung, some patients get vasculitis which can lead to serious complications.

To evaluate the disease activity of a patient at a given time, the disease activity score 28 (DAS28) is widely used (Prevoo et al., 1995). For this score, 28 joints are examined, patient’s general health is assessed by the Health Assessment Questionnaire and acute phase reactants are measured. The score is calculated with a formula that takes into account how many joints were tender or swollen, the ESR or CRP and the patient’s general health.

There is no cure for RA. The current goal for RA treatment is to detect the disease at its early stages, suppress inflammation and prevent any irreversible damage to the joints by achieving clinical remission. There are three different groups of pharmacological treatments for RA (Ola Nived, 2015). The first group aim to reduce pain and inflammation, for example non-steroid anti-inflammatory drugs. The second group is the glucocorticoids, steroid anti-inflammatory drugs for symptomatic treatment. The third group includes drugs that offer not only symptomatic relief but also reduce disease activity, called disease modifying anti-rheumatic drugs (DMARD). Within this group we find biological drugs. First choice treatment for RA is methotrexate, which modulates the immune system activity by inhibiting the intracellular access of folic acid. If sufficient effect is not achieved, methotrexate is combined with biological DMARDs targeting the cytokine TNF-α (for example etanercept or infliximab). If this still do not result in sufficient treatment response other biological drugs may be tested. These include abatacept, the binding domain of CTLA4; rituximab, an antibody targeting CD20 expressing B cells; and tocilicumab, an antibody targeting IL-6.
Survivin as a biomarker for RA

Survivin is a member of the *inhibitor of apoptosis* family of proteins with multiple functions related to cell growth and survival in different cellular compartments. It is overexpressed in tumors, but most tissues of healthy adults do not express survivin. All known functions of survivin are intracellular, but it is believed to be transported between malignant cells via exosomes in the tumor microenvironment (Khan et al., 2011).

Survivin measured in serum can predict RA before disease onset (Bokarewa, Brink, Erlandsson, & Rantapää Dahlqvist, 2014). In a case-control study, samples from blood donations were used to study serum levels of survivin in patients several years before their diagnosis. In donated blood from individuals who would not develop RA, 14% had survivin levels that exceeded a cutoff at 450 pg/ml. Blood donated from pre-symptomatic RA patients were positive in 36% of the samples, which was significantly higher. Serum survivin levels at diagnosis were generally higher than the pre-symptomatic samples and 64% of the RA patients were positive for survivin.

Survivin does not only predict who will develop the disease. Being survivin positive at the time for diagnosis may also predict the course of disease. In RA patients followed up within one year of their diagnosis, those who were positive for survivin on base-line would develop more severe disease (Svensson et al., 2010). Already at base-line, survivin positive patients had more often autoantibodies, they had higher levels of CRP and more erosions. Yearly measurements over a five-year follow-up period demonstrated that survivin positive patients developed erosions more rapidly, and the difference in radiological progression between positive and negative patients grew larger over time. Survivin positive patients were more frequently treated with DMARDs, but still, after 5 years, survivin positive patients had an approximately 2 times higher risk of not achieving remission. A different study reported that 80% of newly diagnosed RA patients do not change their survivin status under the following 2 years (Svensson, Hafström, Erlandsson, Forslind, & Bokarewa, 2014). Stably survivin positive patients have higher disease activity with more severe joint damage under the 5 years following diagnosis. Furthermore, a post hoc analysis of clinical trial investigating treatment strategies for RA demonstrated that serum survivin levels could predict treatment response to methotrexate mono-therapy (Levitsky, Erlandsson, van Vollenhoven, & Bokarewa, 2015). Newly diagnosed RA patients were assigned to methotrexate treatment and followed up
for two years. After one year, patients who were stably positive to survivin had higher disease activity and higher functional disability.

Extracellular levels of survivin have also been measured in synovial fluid of RA patients, although at lower levels than serum survivin (Ahn et al., 2010). Higher levels of survivin were found in synovial fluid from RA patients than from patients with osteoarthritis, and RA patients with erosive joints displayed higher levels of extracellular survivin than patients with non-erosive joints. These data indicate that serum survivin might be a product of the RA joints themselves. Survivin has also been investigated for its functional relationship with arthritis using murine models of RA. Inhibiting the expression of survivin reduced both severity and frequency of arthritis in mice with collagen induced arthritis (K. M. E. Andersson, Svensson, Erlandsson, Jonsson, & Bokarewa, 2015). In this setting, synovial expression of survivin seemed to be related to proteins involved in invasive growth and cartilage degradation in the joint. Furthermore, bone marrow expression of survivin was related to joint destruction.

Environmental risk factors

The inability to reverse damage from progressed RA or cure RA put a lot of focus on early prevention of RA progression. But with a diverse and largely unknown disease etiology it is a difficult task to identify individuals at risk. RA can only be partly explained by heredity and genetic risk factors. Thus, substantial efforts have been put on identifying external factors that might lead to RA. Several factors have been identified that could increase the risk for developing RA. The inhalation of toxic substances is one of these factors. Smoking is the most extensively studied, described in more detail below. But exposure to silica dust or carbon-derived nanoparticles could also increase the risk. It has been shown that these agents can activate citrullinating enzymes through toll-like receptors. Additionally, infections may play a role in the disease etiology, for example periodontitis, since certain microbes could also induce citrullination. The gut microbiota could also play a role, and the diet could influence the risk for RA. Hormones are likely to have a major role in the pathogenesis, and the use of contraceptives can influence the risk (Smolen et al., 2018).
Smoking

Smoking is the dominating environmental risk factor for RA. The enhanced risk for RA depends on the exposure to cigarette smoke. Smoking exposure is often reported as pack-years in research reports, calculated by multiplying the number of packs of cigarettes that a person smokes every day with the number of years that person has smoked. A meta-analysis from 2014 reported that the relative risk for RA increases linearly with the smoke exposure but reaches a plateau of approximately doubled risk at an exposure of 20 pack-years (Di Giuseppe, Discacciati, Orsini, & Wolk, 2014). Additionally, an increased risk is only seen in individuals exposed to more than 2.5 pack-years (Hedström, Stawiarz, Klareskog, & Alfredsson, 2018). Interestingly, the duration of smoking has a larger impact on the risk for RA than the number of cigarettes smoked per day, but the age of smoking debut seems to have little impact on the risk. After smoking cessation the risk gradually go down, although patients with a smoking exposure exceeding 20 pack-years, in combination with ACPA-positivity, have an elevated risk even 20 years after cessation (Hedström et al., 2018).

A doubled risk for developing RA may not seem very dramatic. However, smoking has been repeatedly shown to work synergistically with other risk factors to produce much higher risk. For example, having double copies of the shared epitope increases the risk for RA by an odds ratio of approximately 6, still, having both double copies of the shared epitope and a smoke exposure exceeding 20 pack-years result in an odds ratio of almost 40 (Källberg et al., 2011). This increased risk for RA in individuals with shard epitope alleles exist also when the antibody status of the patients is taken into account. However, the risk for RA is further increased when patients are positive for ACPA or RF, in addition to having one or two copies of the shared epitope (Bang et al., 2010). Furthermore, smoking lead to a higher risk to develop ACPA-positive RA than ACPA-negative (Hedström et al., 2018).

Smoking is not only leading to a higher risk of being diagnosed with RA, it is also associated with a worse prognosis in RA patients. Patients who smoke have a three times higher risk for rapid radiographic progression over a 5 year follow-up (Rydell, Forslind, Nilsson, Jacobsson, & Turesson, 2018). Unfortunately, smoking cessation after the diagnosis with RA seems to have little impact on the disease activity (M. L. E. Andersson, Bergman, & Söderlin, 2012).
The association between RA and smoking lead to the conclusion that smoking contributes to the early events that lead to RA, especially ACPA-positive RA. Since the lung has the highest exposure to cigarette smoke, it was speculated that early events that trigger autoimmunity in pre-RA patients are located in the lung. It has been suggested that smoking stimulates the **citrullination** of proteins in the lungs by inducing the expression of peptidylarginine deaminase (Makrygiannakis et al., 2008). A study of patients with early RA, in which the lungs were examined with high-resolution computed tomography, found more frequent parenchymal and airway abnormalities in addition to ground-glass infiltrates and fibrosis in RA patients compared to controls (Reynisdottir et al., 2014). Furthermore, parenchymal changes were primarily identified in ACPA-positive patients and increased citrullination was observed in biopsies from ACPA-positive patients compared to ACPA-negative, indicating that citrullination in the lung could be related to the production of ACPA in RA patients. Another study has demonstrated that ACPAs may be frequently found in sputum from RA patients and to a lesser extent in individuals at risk of RA due to having first-degree relatives with RA or being sero-positive (Demoruelle et al., 2018). Interestingly, no association was found between RA and the use of moist snuff, which is tobacco that is administered under the lip, and not inhaled to the lungs (Carlens et al., 2010).

The active component in cigarette smoke is **nicotine**, but it is not known whether it is nicotine or some other component in cigarette smoke that results in this increased risk for RA. The lack of association between exposure to moist snuff and RA indicates that nicotine may not be the primary component in smoke causing RA (Carlens et al., 2010). Indeed, the role of nicotine in the immune system is rather controversial, since nicotine seems to have both pro-inflammatory and anti-inflammatory properties (Gomes, Watad, & Shoenfeld, 2018). It may interact with the immune system both by activating the cholinergic system including the vagus nerve, and by activating nicotinic acetylcholine receptors expressed by immune cells. It has been repeatedly demonstrated that administration of nicotine prior to induction of arthritis in mice alleviates their symptoms (Lindblad et al., 2009; Van Maanen et al., 2009; S. Wu et al., 2014; Yang et al., 2014). Another study shows that nicotine induces the formation of neutrophil extracellular traps, which associates with ACPA production (Lee et al., 2017)
AIMS

My aim was to investigate how smoking participates in the RA etiology by disrupting the regulation of cytotoxic CD8\(^+\) T cells.

The specific aims of each paper were to investigate:

**Paper I.**
- If CD8\(^+\) T cells are responsible for the release of survivin seen in smokers who develop RA.
- How smoking may break tolerance in CD8\(^+\) T cells.

**Paper II.**
- If the serum levels of PD-L1 are lower in RA patients with a history of smoking.
- How smoking may interact with antigen-presenting cells to limit the production of sPD-L1.

**Paper III.**
- How smoking interacts with PD-1 and the FOXO signaling pathway to alter the phenotype of CD8\(^+\) T cells in RA.
- How miR regulation of CD8\(^+\) T cells participates in this process.
METHODS

Patient materials

The study participants in paper I-III are referred to as patient material 1-4, within this thesis. They are described in detail in Table 1. All collected patients (patient materials 1-4) were recruited from the Rheumatology Clinic at the Sahlgrenska University Hospital in Gothenburg, except the patient material 1 that also comprises of patients recruited from the Northern Älvsborg county hospital in Uddevalla, Sweden. By collaboration, we had an opportunity to use the material of healthy volunteers in patient material 1 that were recruited from the West Sweden Asthma Study, a population study on asthma conducted at the Krefting Research Centre, University of Gothenburg. The control material was carefully selected by not having rheumatic disease and to match the RA patients by age and smoking history. All patients were recruited between 2011 and 2018 and fulfilled the 1987 American Collage of Rheumatology classification criteria of RA. Study participants underwent clinical examination, blood sampling and filled in a questionnaire.

The patients within the patient material 4 were recruited as they initiated their first treatment with infliximab (Remicade). A blood sample was collected before they started treatment, on the day of their very first infusion. A second blood sample was taken the next day from 7 of the patients, after 14 days from 5 patients and after 42 days plus a second infusion from 7 patients.

Information of smoking habits in RA patients were collected by the use of structured questionnaires and healthy controls were interviewed about their smoking habits. Patients who were current smokers, or had smoked within the past 25 years were considered smokers in the statistical analyses. The use of moist snuff was also considered as smoking.

The studies were approved by the Ethical Committee of the Sahlgrenska University Hospital. The studies were carried out in accordance with the Declaration of Helsinki and the participants gave informed written consent prior to participation.
<table>
<thead>
<tr>
<th>Table 1. Patient characteristics.</th>
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<tbody>
<tr>
<td><strong>RA patients</strong></td>
</tr>
<tr>
<td><strong>Total number</strong></td>
</tr>
<tr>
<td>Female, n (%)</td>
</tr>
<tr>
<td>Age, range of years</td>
</tr>
<tr>
<td>Nicotine users within 25 years, n (%)</td>
</tr>
<tr>
<td>Disease duration, range of years</td>
</tr>
<tr>
<td><strong>Autoantibodies, n (%)</strong></td>
</tr>
<tr>
<td>Anti-cyclic citrullinated peptide positive</td>
</tr>
<tr>
<td>Rheumatoid factor positive</td>
</tr>
<tr>
<td><strong>Treatments, n (%)</strong></td>
</tr>
<tr>
<td>Prednisolone</td>
</tr>
<tr>
<td>Methotrexate</td>
</tr>
<tr>
<td>Anti-CD20 antibodies</td>
</tr>
<tr>
<td>Anti-IL-6R antibodies</td>
</tr>
<tr>
<td>CTLR-4-fusion protein</td>
</tr>
<tr>
<td>TNF-α-inhibitors</td>
</tr>
</tbody>
</table>

| **Total number** | 17 | 10 |
| Female, n (%) | 17 (100) | 10 (100) |
| Age, range of years | 45–76 | 49–80 |
| Nicotine users within 25 years (%) | 8 (47) | 4 (40) |
| Disease duration, range of years | 2–44 | NA |
| **Treatments (%)** | | |
| Methotrexate | 14 (82) | NA |
| Anti-CD20 antibodies | 1 (6) | NA |
| CTLR-4-fusion protein | 2 (12) | NA |
| TNF-α-inhibitors | 10 (59) | NA |

| **Total number** | 25 |
| Female, n (%) | 25 (100) |
| Age, range of years | | |
| Nicotine users current/former, n (%) | 5 (20)/9 (36) |
| Disease duration, range of years | <1-30 |

| **Total number** | 16 |
| Female, n (%) | 13 (81) |
| Disease duration, range of years | 1-32 |
| **Treatments (%)** | | |
| Methotrexate | 13 (81) |
| Prednisolone | 6 (38) |

Experimental arthritis

In RA research, the study of RA patients is naturally the most relevant design. However, this method has several major limitations. Firstly, potentially harmful interventions that could help finding causative links to the RA pathogenesis are unthinkable in human studies. While it could be helpful to compare groups of patients with different exposures with respect to their disease status, only associative relationships may be drawn from that study design. Secondly, RA is a heterogeneous disease, and patients recruited to a study will vary depending on disease activity, disease duration and treatment with anti-rheumatic drugs, many of which have potent immune modulating properties. This results in study groups with large variation, meaning that large cohorts are needed. Thirdly, the immune system is a complex system engaging several organs located throughout the body. Measurements made in easily accessible peripheral blood samples from patient can only leave us with guesses of what processes are taking place in other lymphoid organs. For this purpose, several animal experimental models are well-established and frequently used in RA research. In these experimental models we are able to study processes and interventions that are not accessible or possible in humans. The mice are homogenic due to inbreeding methodology which results in little variations between individuals. However, experimental arthritis also has drawbacks. Firstly, the known and unknown differences between mice and humans means that caution is required when extrapolating the findings in mice to RA patients. Secondly, experimental arthritis will cause pain and suffering in the test animals.

There are different methods to study arthritis in mice. The most frequently used collagen-induced arthritis (CIA). In this model, chicken collagen type II is used as an antigen because collagen is a major component in the joints. It is injected subcutaneously into the root of the tail and a second dose is provided on after 21 days. Immunization with collagen triggers joint inflammation with predominant localization in small paw joints and symmetric distribution. This model closely resembles the human RA, involving joint inflammation, bone destruction and the production of antigen-specific and autoantibodies.

Within this thesis mice immunized with collagen was used to model RA in two different experiments referred to as Exp 1 and Exp 2. Details regarding these mice are presented in Figure 5. Mice of strain Balb/c (Exp 1) or DBA1 (Exp 2) were injected with collagen II, however, only the DBA1 mice had the genetic background required to develop full CIA. Mice of Exp 2 would also receive a booster dose after 21 days, while the mice of Exp 1 were sacrificed on day 10,
without receiving the booster. The mice of Exp 2 were sacrificed after a total of 43 days. In both experiments, a subgroup of mice received 0.03% nicotine in their drinking water. In Exp 1, nicotine treatment started 18 days before immunization, in Exp 2 mice received nicotine from the day of the first immunization. The water of all mice was supplemented with sugar to mask the taste of nicotine. At the end of experiment, mice were sacrificed and serum, spleen, lymph nodes and bone marrow were secured for further analysis.
Cell cultures and stimulations

Cell samples from patient materials and experimental models were cultured and stimulated according to Table 2. Lymphoid organs from mice were mashed through a cell strainer to obtain single cell suspensions, peripheral blood monocytes (PBMC) from human blood samples were isolated through gradient centrifugation. Cultures were incubated at 37°C and 5% CO₂ in the complete cell culture media supplemented with 10% heat-inactivated fetal calf serum, β-mercaptoethanol and gentamycin. CD8⁺ T cells were enriched in samples of spleen cells or PBMC by a negative selection, the use of an antibody cocktail binding non-CD8⁺ cells and dextran-coated magnetic beads. Non-CD8⁺ cells were pelleted by the use of magnets and the supernatant including 80-90%

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Healthy mice</th>
<th>Pat. 2</th>
<th>Pat. 3</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells:</strong></td>
<td>BM LN</td>
<td>Spleen cells</td>
<td>CD8⁺ spleen cells</td>
<td>PBMC</td>
<td>CD8⁺ PBMC</td>
<td>CD8⁺ PBMC</td>
</tr>
<tr>
<td><strong>Stimulation:</strong></td>
<td>Unstim.</td>
<td>Unstim.</td>
<td>aCD3 +/- Nic +/- IL-7</td>
<td>aCD3 PMA/IM</td>
<td>PMA/IM</td>
<td>aCD3 Nic</td>
</tr>
<tr>
<td><strong>Duration:</strong></td>
<td>72h</td>
<td>44h</td>
<td>72h</td>
<td>72h 1h</td>
<td>2h</td>
<td>48h</td>
</tr>
<tr>
<td><strong>Analysis:</strong></td>
<td>qPCR ELISA</td>
<td>qPCR ELISA</td>
<td>qPCR Flow cytometry</td>
<td>qPCR microarray</td>
<td>Flow cytometry</td>
<td>qPCR ELISA</td>
</tr>
</tbody>
</table>

Pat. – patient material, Nic – nicotine 10 µM, IL-7 – interleukin-7 10 ng/ml, aCD3 – anti-CD3 antibody coated wells 0.25 µg/ml, PMA – phorbol 12-myristate 13-acetate 30 nM, IM – ionomycin 500 nM, LPS – lipopolysaccharide 10 µg/ml, IgG – immunoglobulin G isolated from RA patients coated plates 10 µg/ml. BM – bone marrow cells, LN – lymph node cells, PBMC – peripheral blood monocytes, qPCR – quantitative polymerase chain reaction, ELISA – enzyme-linked immunosorbent assay.

CD8⁺ cells were cultured and analyzed.
Sample analysis

**Enzyme-linked immunosorbent assay (ELISA)** was used to measure levels of proteins in serum samples and in cell culture supernatants. The protocol used to measure sPD-L1 is described in detail in Figure 6. sPD-L1, IL-1β, IL-6, antibodies against cyclic citrullinated peptides (aCCP), RF and survivin was measured in human serum samples. Survivin was also measured in supernatants of unstimulated cells from mouse lymphoid organs.

![Figure 6. Enzyme-linked immunosorbent assay (ELISA).](image)

Samples of serum and cell culture supernatants were prepared from human and mouse blood samples and cell cultures of mouse secondary lymphoid organs, respectively. The samples were diluted with 1% bovine serum albumin (BSA) in phosphate buffered saline. Multi-well plastic plates were preincubated with capture antibodies with specificity toward the protein of interest. The diluted samples were added to the plate and the protein of interest bound to the capture antibody. All other proteins in the sample were washed away and the biotinylated detection antibody was added. Excess antibody was washed away after incubation and streptavidin conjugated to horse radish peroxidase (HRP) was added. Streptavidin bound to biotin and the excess was washed away. The 3,3',5,5'-tetramethylbenzidine (TMB) substrate and H₂O₂ was added, resulting in a color reaction limited by the amount of HRP enzyme in each well. The reaction was stopped by the addition of sulfuric acid and the color intensity measured with photo spectrometry at wavelengths 450/650 nm. The optical densities of a series of known protein concentrations was used to fit a standard curve from which unknown concentrations could be calculated.
Quantitative polymerase chain reaction (qPCR) was used to measure mRNA levels. RNA was isolated from lysed cells by the use of columns containing a silica-membrane. From the extracted RNA, complementary deoxyribonucleic acid (DNA) was synthesized and used as a template for qPCR (Figure 7). Primers were designed to span an intron to avoid amplification of genomic DNA and melting curves were used to ensure the purity of the amplified product. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene for mRNA and RNU6b as the reference for miR.

Figure 7. Quantitative polymerase chain reaction (qPCR). Initially, cDNA was mixed with forward and reverse primers and a commercial product including nucleotides, DNA polymerase and SYBR® green. The mix was then introduced into a real-time PCR instrument and subjected to repeated shifts in the temperature between 60°C and 95°C, referred to as a cycle. At 60°C the primers bind to the complementary cDNA template and are elongated by the action of DNA polymerase. At 95°C the strands of complementary DNA dissociate from each other, to allow a second round of amplification. SYBR® green bind to double-stranded DNA and emit fluorescent signals. For every cycle, the signal grows stronger as the amount of double-stranded DNA increases. The cycle at which the signal reaches a certain threshold is used to calculate the relative amount of the initial RNA level compared to a reference gene. A reference gene is used to normalize the amount of RNA in every sample. The relative quantity (RQ) of mRNA is calculated in treated samples relative to control samples (assuming that the quantity of the PCR product is doubled for every cycle).
**Flow cytometry** was used to study protein expression on an individual lymphocyte. Single cell suspensions of living cells were incubated with fluorophore-conjugated antibodies targeting cell surface proteins. Cells were then washed, fixed, permeabilized and incubated with fluorophore-conjugated antibodies targeting intracellular proteins. Cells were washed, re-suspended and introduced into a flow cytometer with three lasers and 8 filters. The typical number of cells that entered the flow cytometer was 500 000 to 1 million cells. The concept is explained in Figure 8. Beads stained with antibodies were used to set up the voltages of the detectors and to calculate the compensation for spillover between the channels.

Data analysis was performed in the FlowJo software. Cells expressing the same protein or combination of proteins formed a population. Forward and side scatter was used to identify mononuclear single cells. The “fluorescence minus one” method was used to distinguish between positive signal and background, 1% of the cells were accepted in the negative gate. The minimum accepted number of cells within a gate was 50 cells.

![Flow cytometry diagram](image)

Figure 8. Flow cytometry. Cells have been pre-incubated with antibodies targeting proteins of interest. Each antibody is conjugated to a fluorophore that emits light of a certain wavelength when hit by a laser. The cells pass through a cuvette one at the time and become exposed to the light of three different lasers. The light emitted from the fluorophores is directed to different detectors depending on the wavelength with help of mirrors. The intensity of the light that hits the detector reflect how much of each antibody that has been bound to that cell, which gives a measurement of protein expression. Different sets of proteins define cell populations, and the frequency of cells belonging to a certain population is calculated by the use of two-dimensional plots, in which the intensity of two different proteins are plotted against each other.
Statistical analysis

Comparison between groups and correlation analyses were conducted in GraphPad Prism v.7 for Mac OS X. Because sample sizes were often small or included skewed distributions, non-parametric tests were frequently used within paper I-III. These included the Mann-Whitney U test to compare the medians between two groups or Wilcoxon signed rank test, when the data were paired. The Spearman’s r was calculated to analyze correlations.

Analysis of microarray data in paper III was performed in R using the LIMMA (Linear models for microarray data) package (Ritchie et al., 2015). For all miRs with complete data, the data was log2 transformed and normalized using the cyclic loess method. Pooled samples were stratified on smoke status and memory phenotype in separate analyses. The log fold changes and p-values resulting from this analysis were presented as volcano plots.

Univariate logistic regression and Multiple logistic regression in paper II was performed using IBM SPSS Statistics v.24.0. The dependent variable was set as sPD-L1 dichotomized at the 0.4 quantile. Independent variables included smoking status and other disease related co-variates (described in detail in paper II). Incomplete cases were excluded from the analysis. The full procedure is described in Figure 9. All variables with a significant association with sPD-L1 were entered into the multivariate model that assessed their contribution by the backward Wald method.

Figure 9. Multivariate logistic regression analysis of the association between sPD-L1 levels and smoking and other potential co-variates. The illustration describes how data was transformed and selected for the analysis.
Nicotine facilitates the release of survivin

Survivin is an intracellular protein involved in a broad range of cellular functions important for survival and growth. However, measurable levels of extracellular survivin in serum can be a very early marker for RA, and may appear in serum before a person start presenting symptoms of swollen joints. In Paper I we demonstrated that being a smoker led to an increased risk of having high levels of survivin in serum (being survivin positive). In the present cohort of 252 RA patients and 168 healthy controls (patient material 1, see methods section), the risk for being survivin positive was increased in women smokers both among the RA patients, where the risk was doubled, and in the controls, who had no RA diagnosis at the time for blood sampling. The control women smokers had more than 3-times higher risk to have high levels of survivin compared to non-smokers. Men with diagnosed RA had also an increased risk of having high levels of serum survivin among smokers, although no statistically significant increase was registered. Control men smokers (without RA diagnosis) showed no increased risk of being survivin positive. These observations confirmed and extended earlier results from an independent patient cohort (Svensson et al., 2014).

Learning that exposure to cigarette smoke increased the risk for serum survivin positivity, we wanted to know if nicotine was the substance responsible for the increased risk. To study the phenomenon in a controlled environment, we immunized mice with collagen II to mimic RA (Exp 1 and Exp 2, see Methods: Experimental arthritis). In this experimental setting we could expose the mice to nicotine by adding 0.03% of nicotine to their drinking water, which they drank continuously throughout the experiment. After 43 days, serum levels of survivin were measured and demonstrated that mice exposed to nicotine had higher serum levels of survivin.

Since free survivin in the circulation, to our current knowledge, is not a naturally occurring phenomenon in a healthy individual, we are interested to know what is the source of serum survivin. We speculated that survivin expression was likely to be high in active, differentiating cells. After immunization of the mice, these were likely to be immune cells. We collected lymphoid organs from the nicotine exposed mice, isolated cells from these organs, cultivated them for
72 h and measured the survivin levels in the culture media. Indeed, cultures from every organ produced higher levels of survivin, if the cells were isolated from the mice exposed to nicotine. The increase of survivin levels was significant from the spleen cells and the bone marrow cells. Interestingly, different amounts of survivin were released from cells isolated from different organs. **Bone marrow cells produced 4 times more survivin than spleen cells and 32 times more than lymph node cells.** Indeed, survivin expression is known to be essential for the development of B cells in the bone marrow, and mice lacking survivin expression completely lack mature B cells (Miletic et al., 2016). Survivin is, however dispensable in mature B cells of the spleen. The median level of survivin in the bone marrow cultures from nicotine treated mice was 280 pg/ml and 300 pg/ml was measured in serum of the same mice. There are reports, which suggest that survivin is released from the RA synovium to the synovial fluid, and could also be a source of serum survivin (Ahn et al., 2010). Still, it is unlikely that synovial tissue is the primary source of serum survivin, since levels measured in synovial fluid are many times lower than those measured in serum of RA patients. Based on the results above, we concluded that **survivin was likely to be released from the developing B cells of the bone marrow during early arthritis.**

The precise mechanism of the release was not determined in our experimental setup. It is possible that survivin is simply released through the perforin pores created in the target cell by cytolytic activity of the CD8\(^+\) T cells. Survivin, just like granzyme B, has a diameter of approximately 70Å, which according to some reports is sufficient to diffuse through perforin pores (Lopez et al., 2013). Another suggestion is that the cytotoxic killing takes place at a rate that is difficult to sufficiently clear up, and survivin is released from apoptotic cell debris.

**Nicotine limits the expression of PD-1**

Because survivin has primarily intracellular functions, we hypothesize that survivin has no active secretion from the survivin-rich cells. Instead, we reasoned that over expression of survivin might trigger an attack of cytotoxic T cells (Figure 10). This process is similar to the one described for other onco-proteins that similar to survivin activate cytotoxic killing of malignant cells (Andersen et al., 2001). We therefore put our attention on CD8\(^+\) T cells.
Cytotoxic killing is regulated by the expression of co-inhibitory receptors. In cancer research, PD-1 has been given a prominent place. We analyzed the expression of PD-1 in the patient material consisting of 17 female RA patients and 10 healthy female controls (patient material 2, see Methods: Patient materials). The frequency of PD-1 was lower in the CD8+ T cell population of smokers, and the lowest in smoking RA patients. The presence of degranulation marker CD107 was used to ensure that PD-1 was lost in CD8+ T cells with an activated profile. However, the PD-1 negative population was enlarged both among the cells expressing, and not expressing CD107.

Figure 10. Hypothetic view of how cytotoxic CD8+ T cells attack survivin expressing cells in the bone marrow, and cause the release of survivin to circulation. Nicotine stimulates the expression of survivin in the bone marrow cells. High expression of survivin attracts survivin specific cytotoxic T cells to the bone marrow. Nicotine limits the expression of PD-1, which makes CD8+ T cells less inhibited in the interaction with their target cells. Cytotoxic proteins released by the CD8+ T cells create pores in the membrane of the target cell and force it to undergo apoptosis, a process that also releases survivin to the extracellular space.
The exhausted phenotype of CD8+ T cells is often described as cells expressing high levels of PD-1 and low levels of memory markers such as IL-7R. This exhausted phenotype has been described as favorable in various inflammatory and autoimmune diseases (McKinney, Lee, Jayne, Lyons, & Smith, 2015). Blocking the PD-1 inhibitory system with therapeutic antibodies results in autoimmune conditions with an incidence of approximately 27% according to the recent meta-analysis (P. F. Wang et al., 2017). Treatment of malignant melanoma with the PD-1 blocking antibody pembrolizumab triggered arthritis in 1.8% of the patients. The development of arthritis occurred after approximately 150 days of treatment (Robert et al., 2015). Seropositive RA with production of arthritis-specific antibodies is a rare adverse event, but several new cases of seropositive RA have been reported in patients under the pembrolizumab treatment (Belkhir et al., 2017; Lidar et al., 2018). These reports indicate that reduced PD-1 signaling can be linked to RA etiology. Consequently, the reduced expression of PD-1 that we have observed in smokers could potentially contribute to the increased risk for RA seen in smokers.

On the other hand, abnormally high PD-1 expression has been repeatedly described within the RA synovium. This indicates that high PD-1 expression is a feature of RA pathology (see Introduction: PD-1 and RA). Subsets of CD4+ T cells with important functions to stimulate autoantibody production express high levels of PD-1 without becoming functionally exhausted (Rao et al., 2017).

We hypothesized that nicotine limited PD-1 and made the CD8+ T cells less inhibited. In turn, this increased the risk for autoimmune reactions. To study this hypothesis, we returned to our arthritis model (Exp 1) and analyzed the CD8+ cells in the bone marrow, the lymphoid organ in which we had observed to release the highest levels of survivin. We could see that the CD8+ cells of mice exposed to nicotine were enriched in the bone marrow, suggesting an influx of these cells. We could also confirm that the number of CD8+ cells expressing PD-1 was reduced in the bone marrow. Interestingly, the size of the PD-1-negative population of CD8+ cells has a positive correlation to both mRNA and protein levels of survivin in the bone marrow. This supports the idea that CD8+ cells with specificity toward survivin might be recruited to the bone marrow. Indeed, in a Danish study the authors managed to isolate survivin-specific CD8+ T cells from human lymphoid nodes that effectively lysed melanoma cancer cells (Andersen et al., 2001), and demonstrated that CD8+ T cells identified and targeted cells with high survivin expression.
Nicotine exposure shifts CD8\(^+\) cells toward a naive/memory profile

The IL-7R is believed to be an important feature of the non-exhausted phenotype of CD8\(^+\) T cells (McKinney et al., 2015). It is expressed primarily by naïve or memory cells, and is found exclusively on cells not expressing PD-1. We observed that the bone marrow cells from nicotine treated mice had higher frequencies of CD8\(^+\) cells expressing IL-7R (Paper I), this indicated that these CD8\(^+\) cells were non-exhausted and might have a memory phenotype. However, the CD8\(^+\) bone marrow cells of nicotine treated mice had also increased levels of T-bet transcription factor, which promote the differentiation of effector cells, and CXC chemokine receptor 3 (CXCR3), which directs activated effector T cells to the periphery. A previous study investigating the role of nicotine in memory formation of mouse T cells reported an impaired memory differentiation of OT-I cells stimulated with nicotine in vitro (Z. Sun et al., 2013).

In the patient material 2 (Paper III), IL-7R mRNA expression was similar between smoking and non-smoking RA patients, and the increase seen in smokers of the control group was not statistically significant (Paper I). However, smokers had an increased frequency of CD27\(^-\)CD8\(^+\) T cells (Paper III). CD27 is a co-stimulatory receptor expressed primarily by the naïve and memory populations of CD8\(^+\) T cells. We learned that CD27\(^-\)CD107\(^+\) naïve/memory cells with low PD-1 expression were the most frequent population in the peripheral blood, followed by CD27\(^+\)CD107\(^+\) effector cells. PD-1 was most frequently expressed by the effector population, or cells co-expressing CD27 and CD107.

Taken together, these findings let us to conclude that the population of CD8\(^+\) T cells, which was increased in smokers and nicotine treated mice seemed to have a less differentiated phenotypes and had the surface markers of naïve and memory cells. Recent studies of tumor infiltrating CD8\(^+\) T cells demonstrate that fully exhausted cells are unlikely to regain their full cytotoxic activity, even when the PD-1/PD-L1 interaction is inhibited by pharmaceutical interventions. Instead, the disrupted PD-1 function is believed to have the most prominent impact on a population called stem cell-like memory progenitor cells (Kurtulus et al., 2019; Siddiqui et al., 2019). These cells have the lower level of differentiation compared to exhausted cells, and have the capacity to generate a full response upon stimulation. We speculate that RA patients who have CD8\(^+\) cells with suppressed PD-1 expression due to smoking will also have increased activity of memory-like CD8\(^+\) T cells rather than a population of exhausted cells.
Nicotine interacts directly with the CD8⁺ cell

We were interested to understand how nicotine interacted with the CD8⁺ cell. *In vitro* stimulation of human CD8⁺ cells from the peripheral blood (patient material 3, see Methods: Patient materials) with 10 μM nicotine for 48h demonstrated that nicotine down-regulated PD-1 on the mRNA level (paper III). This suggests that nicotine interacts with the CD8⁺ T cell through a direct stimulation of nicotinic receptors on the cell surface. Nicotine stimulation also reduced the production of IFN-γ, which indicated that these cells were unlikely to be functional effector cells. They also had a significantly reduced mRNA expression of Forkhead box O (Foxo)1, which is normally highly expressed in memory cells. Furthermore, they had no consistent differences in memory markers IL-7R or Bcl6 or effector marker T-bet. Possibly, these were early responses to nicotine exposure of the cells in transition from a population dominated by high PD-1 expressing terminally differentiated effector cells to a population in which the frequency of memory/naïve cells was higher.

In spleen cells of the nicotine-naïve mice, we observed that CD8⁺ T cells expressed nAChRα4 and -α7, whose mRNA levels were induced by activation with nicotine for 72h. Both nicotine and IL-7 had a suppressive effect on the expression of PD-1, although non-significant. Nicotine would, however, stimulate the expression of the IL-7R, both on mRNA and protein levels. To gain a better understanding of how nicotine influenced PD-1, we analyzed several transcription factors that regulated its expression. None of the transcription factors was significantly different in cells exposed to aCD3 and nicotine or aCD3 alone. Indeed, the transcription factors with largest fold change of mRNA were up-regulated rather than down-regulated.

We observed that Foxo1 behaved differently in mouse CD8⁺ cells. FOXO1 mRNA levels were induced by nicotine, in contrast to what we observed in cultures of CD8⁺ T cell of RA patients. But this effect was only observed in the presence of IL-7 stimulation. Theoretically, IL-7 reduces Foxo1 expression by stimulating the PI3K-Akt (Phosphoinositide 3-kinase - Protein kinase B) signaling pathway (Figure 11). The stimulating effect of nicotine on Foxo1 transcription was only evident when this pathway was sufficiently activated. Furthermore, the effector cell transcription factors Blimp-1 and T-bet were suppressed by nicotine in the IL-7R⁺ population, which could indicate the formation of the memory cell phenotype.
Nicotine changes miR-dependent regulation of CD8$^+$ cells

After not finding a conclusive explanation to the question how nicotine was regulated CD8$^+$ cells, we speculated that a shift in the miR environment might underlie the phenotypic changes seen in CD8$^+$ cells exposed to nicotine. To study this, we analyzed RNA isolated from CD8$^+$ cells of the peripheral blood of RA patients and healthy women (patient material 2). RNA was pooled into 16 samples based on smoking status, and analyzed for the expression of 2656 miRs using a microarray (paper III). Two of the samples were not used in the analysis due to the low counts of measurable miRs. The 125 miRs with highest average expression throughout the 14 remaining samples were included in further analysis. 20 miRs had high expression levels ranging between the intensity measurements of 500 to 11000, and included miR-150-5p. The remaining 105 miRs had the mean intensities between 80 and 500. Analysis of the differential expression with the LIMMA method (see Methods: Statistics) demonstrated that 6 miRs were increased in smokers, and had a log fold change higher than 0.5 and a p-value lower than 0.05. These miRs were miR-181a-5p, let-7c-5p, miR-92a-3p, let-7d-5p, let-7e-5p and miR-150-5p. Two miRs were down-regulated in smokers, with a log fold change of less than -0.5. These miRs were miR-3196 and miR-4723-5p.

Using miRPath v.3 (Vlachos et al., 2015) and DIANA-microT v. 5 (Paraskevopoulou et al., 2013) databases, we predicted the potential mRNA targets of these 8 miRs and conducted a pathway analysis to identify which intracellular pathways contained these protein targets. 19 KEGG pathways were predicted to contain the proteins of interest, at a significance level of 0.05. Three of the pathways were focused on the biology of cancer, additionally seven pathways were considered irrelevant to the studied CD8$^+$ T cells, while the remaining 9 pathways were of interest for the studied cell type. Among those were the mTOR (Mammalian target of rapamycin) signaling pathway, the PI3K-Akt signaling pathway, the Wnt signaling pathway and the FOXO signaling pathways. These pathways regulate major cell functions in CD8$^+$ T cells, and are highly integrated (Figure 11). T cell receptor activity, the common-$\gamma$-chain cytokines and insulin-like growth factor-1 (IGF-1) stimulate Akt, which has a central position in the PI3K-Akt pathway, and also in the mTOR and FOXO pathways. The Wnt pathway is indirectly influenced by PI3K-Akt signaling through the Akt-induced inhibition of Glycogen synthase kinase 3 (GSK3) and $\beta$-catenin. Together these pathways orchestrate the memory cell formation by
regulating various transcription factors and signaling proteins required for formation of the memory T cell population (Kim & Suresh, 2013).

To further emphasize the role of miRs in formation of the memory CD8\(^+\) T cells, we divided the samples into those containing predominantly memory/naïve and predominantly non-memory cells, based on their expression of the markers characteristic for these populations. These markers included PD-1 and CD27 protein expression by flow cytometry, and PD-1 and IL-7R mRNA expression by qPCR. Analysis of samples with differential expression between the markers demonstrated that miR-92a-3p and miR-150-5p were significantly up-regulated within the undifferentiated memory/naïve group. Additionally, the memory/naïve group had higher levels of Foxo1 mRNA. These results are divergent with previous reports stating that miR-150-5p inhibits the memory cell formation by directly targeting Foxo1 mRNA in CD8\(^+\) T cells in mice (Ban et al., 2017).

Figure 11. Proteins within the mTOR signaling pathway, PI3K-Akt signaling pathway, Wnt signaling pathway and FOXO signaling pathways were predicted as targets of miRs differentially expressed in smokers. MiRs interacting with the targets in the FOXO signaling pathway are printed next to their targets. Activation of the pathways leads to transcription of proteins involved in the regulation of the memory cell formation. IGF1 – insulin-like growth factor 1, INS – insulin, INSR – insulin receptor, IRS – insulin receptor substrate, PI3K - Phosphoinositide 3-kinase, PTEN - phosphatase and tensin homologue, PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate, AKT – protein kinase B, FOXO1 - Forkhead Box O1, SETD7 - SET Domain Containing Lysine Methyltransferase 7, CBP - CREB-binding protein, SIRT1 - Sirtuin 1, SOD2 - Superoxide dismutase 2, Bcl2 - B-cell lymphoma 2, IL – interleukin, GSK3 - Glycogen synthase kinase 3, B-cat – Betacatenin, TCF7 - Transcription Factor 7, Lef1 – Lymphoid enhancer-binding factor 1, TCR – T cell receptor, CD – cluster of differentiation, PIK3R2 – Phosphoinositide-3-Kinase Regulatory Subunit 2, TSC1 – Tuberous sclerosis 1, mTOR – mammalian target of rapamycin, T-bet - T-box transcription factor.
Smoking limits the levels of soluble PD-L1

Up to this point we investigated the response to nicotine with respect to PD-1 signaling and focused on CD8⁺ T cells. In the real-life situation, RA is a complex system and engages many different cell types with different roles in pathology of this disease. In paper II, we decided to measure the serum levels of a soluble form of PD-L1, which interacted with PD-1 regardless of the cell type. sPD-L1 is a fully functional isoform of the membrane bound ligand of PD-1. By measuring sPD-L1 in serum, we cannot identify its cellular origin, although it is known to be highly expressed by the antigen presenting cells, including dendritic cells and B cells.

sPD-L1 was measured in serum samples from the patient material 1, which also included healthy controls. Surprisingly, the measured sPD-L1 levels were similar in RA patients and healthy controls, in contrast to a previous study that reported that increased levels of sPD-L1 in RA patients (Wan et al., 2006). We collected information on demographic parameters, inflammation parameters, RA characteristics, autoantibody status and RA treatment, in addition to the smoking history of the study participants. Logistic regression was used to assess the associations between sPD-L1 levels and each variable. The variables with a significance level below 0.1 were then included into a multiple logistic regression model, with the purpose of learning if smoking was independently associated to sPD-L1. We learned that smoking was associated with lower serum levels of sPD-L1 in 246 RA patients. This observation suggested that smoking could limit the PD-1/PD-L1 interaction both by inhibiting the expression of the receptor (Paper I) and the ligand (Paper II). The negative association between smoking and serum PD-L1 levels in our cohort of 168 healthy controls was not significant. A previous report analyzed sPD-L1 levels of heavy and light smokers among 96 lung cancer patients and could only detect a slight, non-significant decrease of sPD-L1 in the heavy smokers (Okuma et al., 2017). To explain this discrepancy, we speculate that the effects of smoking are more pronounced in the inflammatory environment of RA. Indeed, in RA patients there were an independent positive association between sPD-L1 and the serum IL-1β levels that indicates systemic inflammation. Additionally, it is possible that the results obtained in the cohorts of non-RA patients were under-powered by the lower number of participants.
Antibodies influence sPD-L1 production

From the multiple logistic regression models, we learned that the aCCP levels had a positive association with the levels of sPD-L1. From this associative analysis we cannot establish the cause and response relationship, but we speculated that antibodies might stimulate the expression of PD-L1 through the activation of Fc-receptors. This assumption was based on the fact that both PD-L1 and Fc-receptors are expressed by antigen presenting cells.

Asking if aCCP directly caused a higher release of sPD-L1, we cultured human PBMC on culture plates coated with IgG isolated from RA patients with different aCCP status. To our surprise, sPD-L1 levels were lower in the cultures exposed to aCCP (paper II). This led us to consider if this difference in acute response to antibody stimulation was due to the shorter exposure to aCCP. We did a rough assumption that the disease duration should reflect the time of exposure to aCCP. In reality, aCCP might be present in serum years before diagnosis, but aCCP-positive patients usually stayed positive. What we could see was that patients with RA diagnosis for 4 years or longer had higher levels of sPD-L1 if they were aCCP-positive. The difference in sPD-L1 levels was statistically significant in patients with a disease duration of at least 7 years.

We could not expose RA patients to aCCP in a controlled setting, but we reasoned that therapeutic antibodies would produce a similar effect. We therefore analyzed the levels of PD-L1 in serum samples collected before and 1 day after the patients’ very first infusion with the TNF-α inhibiting antibody infliximab (patient material 4). We demonstrated that, just as with in vitro aCCP exposure, infliximab would decrease the serum PD-L1 levels. In the association studies performed on the total material of RA patients, we found no correlation between TNFi and sPD-L1 levels. We now stratified the patients into groups based on disease duration. Again, we could see that after 7 years of disease, the patients had indeed higher levels of sPD-L1 after they had received TNFi treatment. The patients with shorter disease duration had significantly lower levels of sPD-L1. We may only hypothesize that, if we would have had the opportunity to measure sPD-L1 levels in patients who had just turned aCCP positive, they would also have lower levels of sPD-L1. It should be noted, that TNF-α stimulation is known to induce the expression of PD-L1 in PBMC of healthy individuals, meaning that neutralization of TNF-α could also explain the reduced PD-L1 expression (Wan et al., 2006).
To explain the different responses to antibodies, we predicted that stimulation of the antigen presenting cells would increase the production of sPD-L1. Additionally, it seemed reasonable to assume that antibodies conveyed their effect through the activation of Fc-γ receptors. We therefore asked if the expression pattern of stimulatory and inhibitory Fc-γ receptors might provide the answer. To test this hypothesis, we divided the IgG/LPS stimulated PBMC into two groups based on their production of sPD-L1 to culture media and measured mRNA levels of the stimulatory Fc-γ receptor FcγRIIIa and the inhibitory FcγRIIb. **The cell cultures with low production of sPD-L1 had a higher relative expression of FcγRIIib.** This occurred because the FcγRIIIa expression was lower. Additionally, we measured the expression levels of these receptors in patient material 2, and found that **smokers had a higher relative expression of FcγRIIib.** We concluded that smoking could shift the expression of Fc-γ receptors towards an inhibitory profile and reduced the activity of PD-L1 producing cells. However, infliximab was shown to induce the expression of FcγRIIib (Belostocki et al., 2008), meaning that infliximab could inhibit PD-L1 expression both by neutralizing TNF-α and by inducing the expression of inhibitory Fcγ-receptors.

Figure 12. Hypothetic view of how nicotine inhibits the production of soluble programmed death ligand-1 (sPD-L1) in the antigen presenting cells (APC). The APCs produce sPD-L1 and are stimulated by the antibodies against cyclic citrullinated peptides (aCCP) and therapeutic antibodies against tumor necrosis factor-α (aTNF). Smoking disrupts a balance in the expression of stimulatory and inhibitory antibody receptors, Fc-γ receptors IIIa and IIb (FcγRIIIa, FcγRIIb), respectively. The reduced stimulatory input results in lower sPD-L1 production and less of sPD-L1 is available for PD-1 inhibition of T cells.
CONCLUSIONS

In this thesis I demonstrate that smoking limits the PD-1/PD-L1 inhibitory system in RA.

The conclusions for each paper are as follows:

**Paper I.**

Nicotine stimulates the production and the release of survivin from the bone marrow during arthritis, which contributes to high levels of survivin in serum. Nicotine stimulates the influx of CD8$^+$ T cells to the bone marrow with a non-exhausted PD-1IL-7R$^+$ phenotype. Inhibition of PD-1 expression seems to be directly mediated by the activation of nicotinic acetylcholine receptors expressed by the CD8$^+$ T cell that induce the transcription of inhibitory transcription factors in the PD-1 expressing population. We suggest that this non-exhausted population of CD8$^+$ T cells target bone marrow cells with high expression of survivin and contribute to the release of survivin to serum.

**Paper II.**

Systemic inflammation stimulates the production of sPD-L1, but this production is limited by smoking. A short-term exposure to therapeutic antibodies and disease specific antibodies suppresses the levels of sPD-L1, but long-term exposure has a stimulating effect. Smoking shifts the expression of inhibitory and stimulatory Fc-receptors toward a higher ratio of inhibitory receptors, negatively regulating the stimulatory effect of antibodies on PD-L1 expressing cells.

**Paper III.**

In CD8$^+$ T cells from smokers, the low PD-1 expression is accompanied by higher expression of CD27, a marker of naïve and memory cells. MiRs with predicted binding to mRNA coding for the proteins within intracellular pathways regulating the formation of memory T cells are differentially expressed in smokers. Nicotine stimulation *in vitro* has a diverse effect on miR expression, but patients with an initial discrepancy between miRs and mRNA expression are more likely to respond nicotine stimulation.
This project provides important insights in how smoking contributes to autoimmunity by limiting the PD-1/PD-L1 inhibitory system in RA. This is based on the assumption that reduced expression of PD-1 and its ligand leads to a situation in which T cells are more susceptible to inappropriate activation. Our conclusions are built upon two previous findings. 1) Therapeutic blockade of PD-1 in cancer patients may cause autoimmune conditions, including RA. 2) Associations have been found between a favorable outcome under autoimmune/inflammatory conditions and the exhausted phenotype in T cells, with high expression of PD-1 (McKinney et al., 2015). Indeed, induced exhaustion in parallel with tolerance, has an potential to treat autoimmune disease (McKinney & Smith, 2016). Inhibiting positive co-inhibition is already a strategy in the treatment of RA, and CTLA-4.Ig fusion protein abatacept binds competitively to CD80 and CD86 to block CD28 positive co-stimulation (Bonelli & Scheinecker, 2018).

However, the function of the PD-1 inhibitory system in RA seems to be more complex. T cells with low PD-1 expression is not a feature of RA pathology. On the other hand, expression of PD-1, PD-L1 and the soluble forms of these proteins are typically high within the RA synovium. Recent studies imply that high PD-1 expression by specific CD4+ T cell subsets within the synovium do not lead to functional exhaustion. Instead, these cells have an important role in the B cell differentiation resulting in the production of disease specific antibodies. Similarly, the function of CD8+ T cells in RA is not fully understood. CD8+ T cells likely contribute to inflammation and tissue destruction, but could also have a regulatory function. Regulatory T cells are yet another population, in which PD-1 seems to contribute to the cells’ function rather than to inhibit its activity. Taken together, it is highly important to further characterize the diversity and similarities of CD8+ and CD4+ PD-1 expressing populations in RA and to understand their functions. This future work will be essential to provide an understanding of the potential of co-inhibitory receptors as therapeutic targets in RA.

We have characterized the influence of smoking and nicotine on the CD8+ T cell compartment using multiple extensively characterized cell surface markers and transcriptional analysis of master transcription factors. However, to completely understand the role of these populations in the RA pathology, further studies focusing on the function of these cells are needed. Autoimmune diseases
resemble chronic infections in the sense that both conditions involve chronic exposure to antigen. These are virus/bacteria in the case of chronic infections, and self-antigens in autoimmunity. It remains an open question if autoimmunity leads to exhaustion of CD8$^+$ T cells in the same fashion as chronic infections do. Exhaustion of CD8$^+$ T cells has been elegantly demonstrated in murine models of acute and chronic infections by transcriptional profiling and functional testing \textit{in vitro} (Wherry et al., 2007). It would be interesting to characterize murine CD8$^+$ T cells after immunization with collagen type II an established antigen in experimental arthritis. Possibly, this experiment will not give a complete answer due to the phenomenon of epitope spreading in human RA leading to the uncertainty of antigen specificity. If we assume, that survivin is a relevant target of auto-reactive CD8$^+$ T cells in arthritis, it is important to isolate survivin specific T cells and to investigate their functional properties \textit{in vivo} and \textit{in vitro}. Nicotine or anti-PD-1 antibodies could be used \textit{in vivo} or \textit{in vitro} to evaluate the effect on proliferative capacity and cytokine production after reactivation with survivin-MHC class I complexes. This will increase our understanding of the naïve/memory population, defined by the expression of CD27 and IL-7R, the receptors gained with nicotine exposure. Furthermore, it would be interesting to compare the profile found in these survivin specific CD8$^+$ T cells from the mouse bone marrow, with CD8$^+$ T cells from human bone marrow and the peripheral blood.

Since RA diagnosis is based on joint inflammation, joints remain being the primary organ of interest in RA research. Our results indicate that CD8$^+$ T cells could play a role in the bone marrow during experimental RA. A substantial part of our investigations has been made in CD8$^+$ T cells isolated from the peripheral blood of RA patients or spleen cells from mice, which is convenient due to the frequency of CD8$^+$ T cells in these compartments. In future studies it is important to learn if self-reactive CD8$^+$ T cells specifically accumulate in the bone marrow. It has been previously demonstrated that treatment with a PD-1 ligand fusion protein has a positive impact on collagen-induced arthritis. It would be interesting to know if mice treated with PD-L1 reduced serum survivin levels, to further establish that the reduced PD-1 signaling is relevant for inducing high serum survivin levels in smokers.

We demonstrated that miRs could be involved in the formation of the CD8$^+$ T cell phenotype seen in smokers. If we could identify a combination of miRs with potential to induce this phenotype, the expression of those miRs could be manipulated to reduce the release of survivin from the bone marrow. To this day, we were unable to confirm our findings from the microarray experiment by a short-term nicotine stimulations \textit{in vitro}. Possibly, further experiments
investigating different experimental settings and conditions could help better understanding a complex interplay between the miRs and nicotine. Furthermore, it would be interesting to put together information gained in next generation sequencing of the complete set of miRs, the mRNA expressed and the protein produced within the same cell, to be able to rule out irrelevant targets within the population of interest in the pathway analysis. This will reveal relevant pathways for intervention in the particular cell type. Once a miR signature is identified, the levels of this miR can be manipulated in vitro to establish the function of this miR in CD8+ T cells. Introducing or inhibiting miR expression could have future therapeutic potential.
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