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THE DNA REPAIR ENZYMES MTH1 AND OGG1 AS TARGETS TO TREAT INFLAMMATION

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THE DNA REPAIR ENZYMES MTH1 AND OGG1 AS TARGETS TO TREAT INFLAMMATION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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“All we have to decide is what to do with the time that is given us.”

-J.R.R. Tolkien

POPULAR SCIENCE SUMMARY OF THE THESIS

Our immune system guards the body from disease by killing bacteria, infected cells, and cancer cells. A weak immune system can lead to infections and cancer, whereas a too strong system can result in allergies and inflammatory conditions like the nerve-destroying disease multiple sclerosis (MS) and the skin-inflammatory disease psoriasis. Sometimes the immune system fails to keep bacteria or viruses at bay, and desperate to fight off the disease, the immune cells can generate an exaggerated reaction. Just like using nuclear weapons to kill mosquitoes would probably cause more damage than value, this excessive reaction called *sepsis* is not efficient, but instead one of the world's leading causes of death. One of the medical causes of death by the disease Covid-19 was through sepsis and lung failure.

MS and psoriasis are so called autoimmune conditions, where a specific type of immune cells – the T cells – attack healthy tissue. These diseases are lifelong and incurable, although there are medicines that lessen the symptoms and improve life quality. Sepsis is not a lifelong disease for the survivors, but there are no specific treatment options except for an array of symptomatic and life-supporting alternatives. Therefore, new treatment options are urgently needed to save lives and improve life quality of patients with sepsis and autoimmune conditions.

One treatment approach that is quite well-established within the field of cancer, but not so much in inflammation, is disturbing the repair mechanisms of the DNA in disease-associated cells. It has long been considered harmful to have any kind of damage in the genome, but lately different kinds of DNA modifications have been thought to potentially play a role in the normal functions of the cells, for example in the immune system. By altering the DNA repair mechanisms, it could be possible to treat disease in new ways.

In this thesis I investigated the possibilities to treat the life-long T cell driven diseases MS and psoriasis, as well as acute inflammation like that of sepsis, by altering the DNA repair mechanisms.

In **Paper I**, we propose a mechanism for the inhibition and thus anti-inflammatory effect of the DNA repair enzyme OGG1. We use both a cell model and a mouse model to prove that inflammation is alleviated with the OGG1 inhibitor TH5487. It could thus be a new promising treatment option against acute inflammation.

In **Paper II-III**, we show that inhibition of another DNA repair enzyme, MTH1, can kill T cells and ease the symptoms in MS and psoriasis in mouse models. T cells have more MTH1 when they are activated and drive disease, and we show that this correlates with treatment efficacy of MTH1 inhibition. We also show a correlation between MTH1 levels and psoriasis in patients, and suggest that not all activated T cells have high levels of MTH1.

Conclusively, I investigated new treatment strategies for inflammatory diseases by disturbing the DNA repair machinery of the cells. The significance of this is further discussed in the thesis, where it is suggested that MTH1 and OGG1 inhibitors could be new promising drug candidates to treat severe inflammatory diseases.

POPULÄRVETENSKAPLIG SAMMANFATTNING AV AVHANDLINGEN

Vårt immunsystem försvarar kroppen från sjukdom genom att döda bakterier, virusinfekterade celler och cancerceller. Ett svagt immunförsvar kan leda till infektioner och cancer, men ett alltför starkt försvar kan i stället leda till allergier och inflammatoriska tillstånd, såsom den nervnedbrytande sjukdomen multipel skleros (MS) och den inflammatoriska hudsjukdomen psoriasis. Ibland misslyckas immunförsvaret med att stoppa en attack från bakterier eller virus, och i desperation kan en alltför stark immunreaktion skapas i syfte att försvara kroppen. Liksom att använda kärnvapen mot mygg sannolikt skulle orsaka mer skada än nytta, kan en sådan överdriven immunreaktion, *sepsis*, leda till döden. Sepsis är en av världens ledande dödsorsaker och var även tillsammans med associerad lungsvikt en ledande orsak till många av dödsfallen orsakade av sjukdomen Covid-19.

MS och psoriasis är så kallade autoimmuna sjukdomar och drivs av en särskild typ av immunceller – T-cellerna. Sjukdomarna är obotliga, även om det finns livskvalitetshöjande mediciner med symtomlindring. Sepsis är inte obotligt för den som överlever, men det finns ingen specifik behandling, endast en rad lindrande och livsuppehållande åtgärder. Det finns alltså ett stort behov av nya behandlingsalternativ för både sepsis och autoimmuna sjukdomar.

Ett behandlingssätt som etablerats inom cancerforskning, men ännu knappt förekommer inom immunologi, är att påverka reparationen av DNA i cellerna. Längre har DNA-skador setts som något ovillkorligt skadligt, men på senaste tiden har man uppmärksammat att vissa typer av DNA-förändringar kunde vara en del av cellens normala funktion, exempelvis i immunceller. Genom att påverka reparationsmekanismerna i DNA kunde man potentiellt behandla immunologiska sjukdomar på ett nytt sätt.

I denna avhandling undersökte jag därför om man genom att hämma DNA-reparationen skulle kunna behandla T-cellsdrivna sjukdomar såsom MS och psoriasis, samt sepsis.

I **Delarbete I** föreslås en modell över mekanismen och därmed den anti-inflammatoriska effekten för hämning av DNA-reparationsenzymet OGG1. Vi använde både en cellmodell och en musmodell för att bevisa att inflammationen kan dämpas med OGG1-hämmaren TH5487. Den kunde således utgöra en ny lovande behandling mot akut inflammation.

I **Delarbete II-III** visar vi att om man hämmar ett annat DNA-reparationsenzym, MTH1, så kan man döda T-celler och dämpa symtomen för MS och psoriasis i musmodeller. T-celler har mer MTH1 när de aktiveras och driver på sjukdom, och vi visar att detta korrelerar med effekten av MTH1-hämning. Vi påvisar också ett samband mellan MTH1-nivåer och psoriasis i patienter, och föreslår att inte alla aktiverade T-celler har höga MTH1-nivåer.

Sammanfattningsvis undersöker jag i denna avhandling nya sätt att behandla inflammatoriska tillstånd genom att påverka DNA-reparationen i cellerna. Betydelsen av detta diskuteras vidare i avhandlingen, där det föreslås att hämmare av MTH1 och OGG1 kunde vara nya lovande sätt att behandla allvarliga inflammatoriska sjukdomar.

ABSTRACT

Chronic and acute inflammatory diseases, such as multiple sclerosis (MS), psoriasis and sepsis, account for vast disability and morbidity in the world. Several new immunomodulating treatment alternatives have been developed over the past decades, but there is still an urgent need for new options.

Reactive oxygen species (ROS) are tightly bound to inflammation. They can cause oxidized DNA lesions, which are commonly considered to be detrimental. However, these modifications could potentially also constitute an important part of inflammatory signaling. In this thesis, we thus wanted to determine whether inhibition of two DNA repair enzyme, MTH1 and OGG1, could have immunomodulating effects.

MTH1 sanitizes the nucleotide pool from oxidized dNTPs and thus prevents oxidized bases, such as oxidized guanine (8-oxoG) from entering the DNA. OGG1 is a DNA glycosylase excising 8-oxoG from the DNA. MTH1 has been described as a promising target for cancer, as many cancers rely on an up-regulation of MTH1 due to elevated ROS pressure, but its role in inflammation has not been investigated. OGG1 was known to be involved in inflammation from before, but this had mainly been validated with knockout models and few inhibitors. Hence, we wanted to investigate novel small-molecule inhibitors of OGG1 and MTH1 for acute and T cell driven inflammation, respectively.

In **Paper I**, we demonstrate an anti-inflammatory effect of the OGG1 inhibitor TH5487 in both *in vitro* models and an *in vivo* model of acute pneumonia. We propose that TH5487 prevents OGG1 from binding to 8-oxoG-rich promoter regions of pro-inflammatory genes, further preventing transcription factors from binding to the DNA. We show that the effect is comparable to OGG1 knockout, and that TH5487 has an effect in the pneumonia model both prophylactically and when given after inflammatory stimulation. In preliminary data, we also propose that the effect is comparable to dexamethasone, but without having a T cell suppressing effect, which could be a major advantage in sepsis and pneumonia.

In **Paper II-III**, we show proof-of-concept of MTH1 inhibitors as anti-inflammatory drug candidates in mouse models of psoriasis and MS, respectively. We show that psoriatic tissue from patients have elevated MTH1 levels, and that the inhibitor TH1579 suppresses T cell activation and kills activated T cells by inducing DNA damage, cell cycle arrest and mitotic disruption. We further discovered some new T cell biology findings, proposing that activated T cells exhibit a heterogeneity in MTH1 levels, where a subgroup of T cells can proliferate despite low MTH1 and ROS levels. The toxicity among other immune cells was generally low.

Conclusively, we propose these novel inhibitors of the DNA repair enzymes OGG1 and MTH1 to be promising drug candidates for acute and T cell driven inflammation. Other indications, as well as the role of ROS and DNA repair in inflammation, are discussed further in the thesis.

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- I. Visnes T., Cázares-Körner A., Hao W., Wallner O., Masuyer G., Loseva O., Mortusewicz O., Wiita E., Sarno A., Manoilov A., Astorga-Wells J., Jemth AS., Pan L., Sanjiv K., **Karsten S.**, Gokturk C., Grube M., Homan EJ., Hanna BMF., Paulin CBJ., Pham T., Rasti A., Berglund UW., von Nicolai C., Benitez-Buelga C., Koolmeister T., Ivanic D., Iliev P., Scobie M., Krokan HE., Baranczewski P., Artursson P., Altun M., Jensen AJ., Kalderén C., Ba X., Zubarev RA., Stenmark P., Boldogh I., Helleday T. **Small-molecule inhibitor of OGG1 suppresses proinflammatory gene expression and inflammation.** *Science* **362**, 834–839 (2018).
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- IV. Bräutigam L., Pudelko L., Jemth AS., Gad H., Narwal M., Gustafsson R., **Karsten S.**, Carreras Puigvert J., Homan E., Berndt C., Berglund UW., Stenmark P., Helleday T. **Hypoxic Signaling and the Cellular Redox Tumor Environment Determine Sensitivity to MTH1 Inhibition.** *Cancer Research* **76**, 2366 (2016).
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- VI. Chen Y.*, Hua X.*, Huang B*, **Karsten S.**, You Z., Li B., Li Y., Li Y., Liang J., Zhang J., Wei Y., Chen R., Lyu Z., Xiao X., Lian M., Wei J., Fang J., Miao Q., Wang Q., Warpman Berglund U., Tang R.,[#], Helleday T.[#], Ma X[#]. **MTH1 inhibitor Karonudib Attenuates Autoimmune Hepatitis by Inhibiting DNA Repair in activated T Cells.** *Accepted to Hepatology Communications*, doi 10.1002/hep4.1862 (2021).

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- VII. Bonagas N., Gustafsson N.M.S., Henriksson M., Wiita E., Gustafsson R., Marttila P., Borhade S., Green A.C., Vallin K., Sarno A., Svensson R., Göktürk C., Pham T., Jemth A.S., Loseva O., Cookson V., Kiweler N., Sandberg L., Rasti A., Unterlass J.E., Haraldsson M., Andersson Y., Scaletti E.R., Bengtsson C., Paulin C.B.J., Sanjiv K., Abdurakhmanov E., Pudenko L., Kunz B., Desroses M., Iliev P., Färnegårdh K., Krämer A., Garg N., Michel M., Häggblad Sahlberg S., Jarvius M., Kalderen C., Palombini A., Almlöf I., **Karsten S.**, Zhang S.M., Häggblad M., Eriksson A., Liu J., Glinghammar B., Nekhotiaeva N., Klingegård F., Koolmeister T., Martens U., Llona Minguez S., Moulson R., Nordström H., Parrow V., Dahllund L., Sjöberg B., Vargas I.L., Vo D., Wannberg J., Knapp S., Krokan H.E., Arvidsson P.I., Scobie M., Meiser J., Stenmark P., Warpman Berglund U., Homan E.J., Helleday T. **Targeting MTHFD2 kills cancer via thymineless-induced replication stress.** *Accepted in principle to Nature Cancer* (2021).

- VIII. Michel, M.*, Benítez-Buelga, C.*, Calvo, P.†, Hanna, B.M.F.†, Mortusewicz, O.†, Masuyer, G.†, Davies, J.†, Calvete, O.†, Rajagopal, V.†, Wallner, O.†, Sanjiv, K., Zhenjun, Z., Danada, A.N., Castañeda-Zegarra, S., Albers, J.J., Müller, S., Homan, E.J., Marimuthu, K., Visnes, T., Jemth, A.S., Chi, C., **Karsten, S.**, Sarno, A., Wiita, E., Komor, A., Hank, E.C., Varga, M., Scaletti, E.R., Martilla, P., Rasti, A., Mamonov, K., Pandey, M., Von Nicolai, C., Ortis, F., Schömberg, F., Loseva, O., Stewart, J., Koolmeister, T., Henriksson, M., Michel, D., de Ory, A., Sastre-Perona, A., Scobie, M., Hertweck, C., Vilotijevic, I., Kalderén, C., Osorio, A., Stolz, A., Perona, R., Stenmark, P., Warpman Berglund, U., De Vega, M., Helleday, T. **Small-molecule activation of OGG1 increases base excision repair by gaining a new enzymatic function.** *Manuscript in revision.*

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LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APCs	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
ATM	Ataxia telangiectasia mutated
ATR	ATM and RAD3-related
AZA	Azathioprine
BRCA1	Breast cancer 1
CHK2	Checkpoint kinase 2
DAG	Diacylglycerol
DAMPs	Damage Associated Molecular Patterns
DDR	DNA damage response system
DSBs	Double strand breaks
EAE	Experimental autoimmune encephalomyelitis
EdU	5-Ethynyl-2'-deoxyuridine
ERK1/2	Extracellular signal-regulated kinase 1/2
FMO	Fluorescence minus one
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
GvHD	Graft-versus host disease
HIF-1 α	Hypoxia inducible factor-1-alpha
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin-
IP ₃	Inositol-1,4,5-triphosphate
IRs	Inhibitory receptors
KO	Knockout
Lck	Lymphocyte specific protein tyrosine kinase
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cells
MHC-I or MHC-II	Major histocompatibility gene complex I or II
MS	Multiple Sclerosis
MTH1	Human MutT homologue 1
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer cells
NOXs	NADPH oxidases
OGG1	8-oxoguanine DNA glycosylase 1
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated patterns
PAMs	Protospacer adjacent motifs
PARP1	Poly(ADP-ribose) polymerase-1

PLC	Phospholipase C
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
sgRNA	single-guide RNA
STAT	Signal transducers and activators of transcription
TCR	T cell receptor

1 BACKGROUND

The immune system is crucial for health and homeostasis, to avoid infections and erroneous cells that ultimately can lead to diseases like cancer. The natural and healthy form of the human body can contain more bacteria than human cells, that risk ending up in sites of the body where they cause infectious disease. Every day thousands of mutations occur in humans due to erroneous DNA replication and DNA damage, such as oxidation and methylation (1), also highlighting the importance of well-functioning immunity. Already during fetal development, when the immune system of the pregnant mother impressively tolerates the foreign cells of the unborn child for several months, the fetal immune system develops and monitors all the numerous cell divisions and cell migrations forming the new life. The plasticity of the immune system is thus both extraordinary, but also volatile, with high demands on the correct fine-tuning of the immunity.

Inflammatory diseases account for a large part of morbidity and disability in the world. Although inflammation plays an important role in many physiological events in the body, like wound healing and clearance of pathogens and dysfunctional cells, it is detrimentally associated with conditions such as cancer and autoimmune diseases like psoriasis (2-4) and multiple sclerosis (MS) (5-9). Acute inflammation initiated by the innate immune system, culminating in conditions like sepsis, also puts a serious strain on the health and healthcare systems globally, with limited treatment options (10, 11).

The field of immunology is growing, with many new immunological therapeutics being presented every year, but there is still an urgent need for new treatment options against pathologic inflammation and cancer. In this project, we seek to find new therapeutics targeting the DNA repair system and redox balance of the immune cells, with inhibitors of Human MutT homologue 1 (MTH1) and 8-oxoguanine DNA glycosylase 1 (OGG1), both involved in DNA repair and described further below.

1.1 AN OVERVIEW OF THE IMMUNE SYSTEM

The immune system is classically divided into the two categories “innate” and “adaptive”, although current findings blur the categorical border (12, 13). The innate immune system responds rather non-specifically to pathogens and damage, and the adaptive system relies on pathogen specific recognition. The innate immune system includes everything from the skin and stomach acid barriers, antimicrobial peptides and complement factors, to leukocytes like neutrophils, monocytes, macrophages, innate lymphoid cells and Natural Killer (NK) cells. These cells can ingest and destroy microbes, or induce cell death of infected host cells. Innate cells are found both at tissue-specific sites and circulating in the blood and lymph systems. $\gamma\delta$ T cells are in the grey zone of being adaptive and innate, as they are not fully dependent on antigen-specific activation in the way that the adaptive cells are (14). The adaptive and antigen-dependent immune system consists of B and T lymphocytes, mainly found in the blood and lymph system.

Subfunction of different parts of the immune system leads to infections and cancer, which can be empirically observed in medically immunosuppressed patients, HIV positive patients (15) and older patients, the latter suffering from an age related decline in adaptive immunity and chronic non-productive activation of innate immunity (16, 17). On the contrary, an overly active immune response can lead to conditions like autoimmune diseases, allergic reactions and chronic inflammation, depending on what kind of immunological malfunction the patient suffers from. ROS signaling, DNA damage response (DDR) and metabolic reprogramming all affect the polarization and functions of the immune cells.

1.2 T CELL SUBTYPES

T cells are considered as powerful immune cells in both health and disease. Different subtypes are associated with different conditions, making specific T cell subsets potential therapeutic targets. T cells are divided into two major subsets: CD8⁺ cytotoxic cells and CD4⁺ T helper (Th)/regulatory cells. CD4⁺ T cells are central in conducting the adaptive immune response to efficiently clear the body from invading pathogens but at the same time maintain self-tolerance. Naïve CD4⁺ T cells differentiate into specific CD4⁺ T cell subsets, of which Th1, Th2, Th17 and regulatory T cells (Tregs) constitute the most studied and established types (18-20). Other subtypes, like Th22 among others, are suggested to either constitute unique Th subsets or different differentiation stages of the more established ones (21).

The subsets are characterized by their different cytokine profiles, with their different roles in inflammatory diseases (21, 22). The cell fate is affected by core transcription factors, like Nuclear factor of activated T cells (NFATs) and Activator protein 1 (AP-1), and signal transducers and activators of transcription (STAT) proteins. Core transcription factors also activate master transcription factors, necessary for the distinguished subtypes, with T-bet, GATA3, ROR γ t and Foxp3 specific for the subtypes Th1, Th2, Th17 and Treg, respectively (21). Below is a brief description of the 4 most established CD4⁺ subtypes, summarized in Table 1.

Th1 T cells are characterized by their production of interleukin-2 (IL-2) and interferon-gamma (IFN- γ), but they do also produce tumor necrosis factor (TNF), lymphotoxin and granulocyte-macrophage colony-stimulating factor (GM-CSF). IFN- γ increases the expression of toll-like receptors (TLRs) in innate immune cells, promotes immunoglobulin (Ig) G class switching, increases antigen presentation by major histocompatibility gene complex (MHC) I and II, increases phagocytosis and macrophage activation, enhances immunogenicity of tumor cells and induces secretion of several chemokines (21, 22). Many inflammatory diseases and disease models, like experimental autoimmune encephalomyelitis (EAE), used to be considered Th1 driven, but were later proven to be Th17 driven (23). However, there do exist some conditions where dysregulated T-bet leading to strong Th1 response causes disease, like Crohn's disease. IFN- γ deficient mice developed a more severe form of EAE, possibly due to an increased amount of pro-inflammatory Th17 cells. In addition, IFN- γ /STAT1 signaling has been suggested to maintain and generate anti-inflammatory Foxp3⁺ regulatory T cells. It also serves

as an autocrine/paracrine anti-inflammatory regulator of T cells, activating GTPase 1 which promotes oxidative killing during viral infections, but also inhibits TCR signaling and IL-2 production (21-25).

Conclusively, both pro-inflammatory and anti-inflammatory effects of Th1 cells and IFN- γ have been described. The role of Th1 cells in autoimmune diseases is not yet fully understood, whereas a suppression of Th1 cells readily causes immunosuppression and severe infections (21-25).

Table 1. CD4⁺ T cell subsets.

CD4⁺ subset	Polarizing agents	Transcription factors	Secreted cytokines	Physiological functions/targets	Associated diseases
Th1	IFN- γ IL-12	STAT1 STAT4 T-bet	IFN- γ , IL-2	Intracellular pathogens, cancer cells	MS, COPD, DM1, IBD, RA among others
Th2	IL-4	STAT5 STAT6 GATA3	IL-4, IL-5, IL-9 IL-13	Helminths and other multicellular parasites	Asthma, allergy, promote regulative macrophages in the tumor microenvironment
Th17	TGF- β IL-6, IL-21, IL-23	SMADs STAT3 ROR γ t	IL-17, IL-21, IL-22, IL-25, IL-26	Extracellular pathogens	MS, Psoriasis, IBD, RA, asthma, allergy
Treg	TGF- β	SMADs STAT5 Foxp3	IL-10, TGF- β	Immunoregulation, suppression	Suppresses antitumor response, controversial association with many autoimmune diseases

MS = multiple sclerosis; COPD = chronic obstructive pulmonary disease; DM1 = diabetes mellitus type 1; IBD = inflammatory bowel disease; RA = rheumatoid arthritis;

Th2 T cells are thought to promote antibody-driven autoimmune diseases and allergies, but the secreted cytokines can also suppress inflammation by suppressing Th1 and Th17 responses. The Th2 subtype is also important for the defense against multi-cellular parasites, like helminths. They function mainly in epithelial tissues, like the lungs and intestinal tract, and are extensively regulated by epithelial cells and innate immune cells. IL-4 is important for antibody class switching to IgG1 and IgE and serves as a survival factor. Since parasite infections can cause extensive tissue damage, Th2 cells also promote the function of tissue repairing regulative macrophages through IL-4, which can have adverse effects in the tumor microenvironment (21, 22, 26).

Th17 T cells are considered to promote and enhance inflammation, including auto-inflammation. They can be induced in multiple tissues but are most commonly found at barrier sites like the lungs, skin and intestines, providing protection against fungi and bacteria. IL-17 is also secreted by a few other cell types, like macrophages and NK cells, and can recruit neutrophils, activate innate immune cells, enhance B cell function, and induce secretion of cytokines like TNF and GM-CSF. IL-17 is overexpressed in many inflammatory conditions, like MS, psoriasis, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus (SLE) and airway inflammation (21, 22). In the tumor microenvironment, IL-17 produced by both Th17 cells and $\gamma\delta$ T cells has been suggested to suppress the cytotoxic CD8⁺ T cells by attracting myeloid-derived suppressor cells (MDSCs) and regulative macrophages, and thus inhibit the antitumoral immune defense (27). Th17 cells are uniquely functionally coupled to Tregs by requiring Transforming growth factor beta (TGF- β) for their development, which makes the cells express both ROR γ t and Foxp3 at the same time. The presence of TGF- β , IL-6 and IL-23 in the microenvironment further determines the fate of the cells. Many transcription factors, like hypoxia inducible factor-1- α (HIF-1 α), also stabilizes the Th17 transcriptional program (21, 22).

Tregs are important regulators of the immune system, suppressing excessive immune responses against self and foreign antigens. They are also thought to play an important role in diseases like asthma and MS but can have adverse effects suppressing the antitumoral responses. Tregs express Foxp3 and can be derived in the thymus (natural Tregs) or induced via post-thymic maturation (iTregs). They can further be Foxp3⁺ or Foxp3⁻, where Foxp3 plays a critical role for the suppressive functions. TGF- β suppresses IL-17 production and is critical for the induction of Foxp3 and maintaining peripheral tolerance. IL-10 downregulates MHC-II expression and co-stimulatory molecules, and reduces pro-inflammatory cytokines from the innate immune cells. It also suppresses Th2 mediated allergic responses. On the contrary, TGF- β and IL-10 enhance the survival of CD8⁺ cells and increase production of IL-17 and IFN- γ (22).

Although the different CD4⁺ cell subsets have been described over the past decades and specific subtypes have been proposed to drive certain diseases, it has also been shown that T cells have a certain instability and plasticity regarding different subtypes – Tregs and Th17 share many similar properties despite the view of them being on opposite sides of the anti-/pro-

inflammatory spectrum as described above (21, 28). Anti-inflammatory subsets like Tregs can convert into IFN- γ producing Foxp3⁺Tbet⁺ cells and pro-inflammatory Th1 cells can convert into IL-10 producing cells. Thus, there might not be a single subtype or cytokine that drive a certain disease or is exclusively pathological. Instead, it could rather be a question about immunologic homeostasis and cytokine profile, due to the plasticity of the immune cells.

CD8⁺ cytotoxic T cells act by migrating to peripheral sites of infection upon stimulation via MHC-I and clonal expansion, where they control the pathogens by direct cytotoxic activity and the production of cytokines like IFN- γ and TNF- α . After the clearance, the effector cells rapidly die, whereas another subpopulation of the CD8⁺ T cells, the memory precursor effector cells, survive for mediating future antigen-specific long-term protection against secondary challenge (29).

Persistent antigen stimulation, altered co-stimulation/co-inhibition by cell surface receptors and chronic inflammation can all affect T cells polarization and lead to the development of T cell exhaustion. This is a problem in cancer and inflammatory diseases, where the exhausted T cells are suppressed, leading to inhibition of the clearance of pathogenic cells, as described more below. Viruses or tumors can drive hyperactivation of T cells and eventually lead to sustained co-expression of multiple inhibitory receptors (IRs) and their ligands on antigen presenting cells (APCs), virally infected cells and tumors. The surrounding cells also contribute further to the exhaustion, by producing pro-inflammatory cytokines like IFN and inhibitory cytokines like IL-10 and TGF- β (30).

1.3 ROS AFFECT DIFFERENT PARTS OF THE T CELL ACTIVATION PATHWAYS

Oxidative stress and ROS play a key role in both physiological and pathological immune signaling, and can both prevent and promote cell death, inflammation or ageing (31-33). ROS consist of small, reactive signaling molecules that can arise both from within and outside the cells. They can be generated by NADPH oxidases (NOXs), the mitochondrial respiratory chain, lipoxygenases, cyclooxygenases, cytochrome P450s, nitric oxide synthases and free copper or iron ions, to mention a few sources. Inflammatory signaling, like TNF, GM-CSF and complement component 5a binding to their receptor, will physiologically lead to ROS production by NOXs. NOXs are highly associated with innate immune cells, but T cells are also dependent on them since T cell receptor (TCR) engagement will trigger ROS production (34-37).

In order to become activated, expanded and differentiated, naïve T cells require three signals: antigen presentation, co-stimulation and cytokines or ROS (38). The TCR form a complex with the APC and its presented antigen, and the co-receptors CD4 or CD8 facilitate the colocalization of tyrosine kinases, with lymphocyte specific protein tyrosine kinase (Lck) and Zeta-chain-associated protein kinase 70 (Zap70) in the frontline, phosphorylating the immunoreceptor tyrosine-based activator motifs, further activating adaptors and scaffold proteins, phospholipids and GTPases. Ca²⁺ ions are released into the cytoplasm, induced by

phospholipase C (PLC) dependent pathways, generating inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Calmodulin then captures free Ca²⁺, activating the phosphatase calcineurin, which dephosphorylates multiple serine residues in NFATs, translocating it to the nucleus (39, 40). DAG signaling also results in the transcription factors AP-1, through activation of Ras and mitogen-activated protein kinase (MAPK), and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) via activation of PKC-θ and phosphorylation of IKKγ (NEMO), which further stimulates ubiquitination of IκB, releasing NF-κB. It can thus be summarized that the CD3/CD28 pathway leads to three signal transduction pathways – the IP₃/calcium-calcineurin-NFAT pathway, the DAG/RAS-MAPK-AP-1 pathway and the DAG/PKC-θ-NF-κB pathway. NFAT, AP-1 and NF-κB all promote T cell activation and IL-2 production, inducing proliferation, which also require nucleotide synthesis, and different immune responses (36).

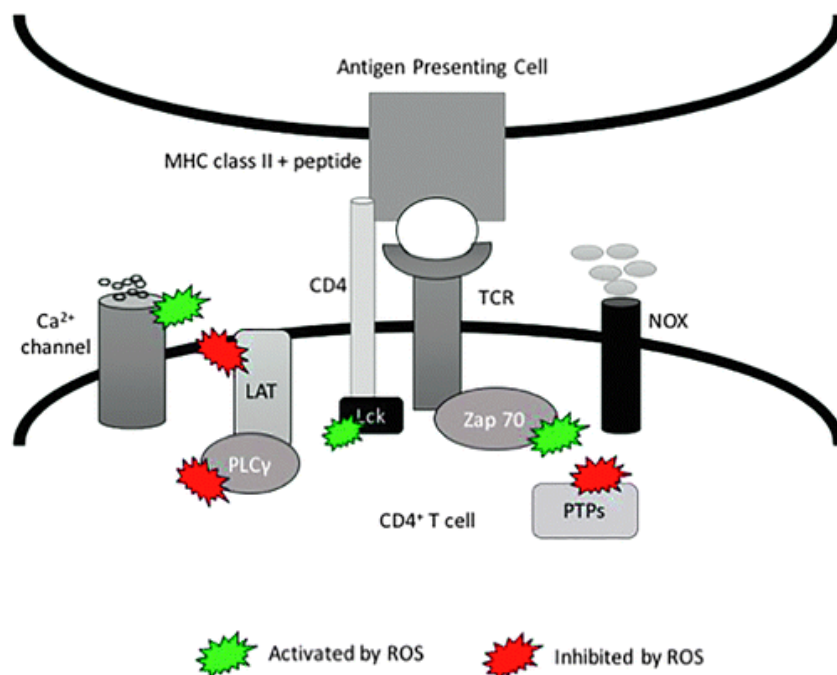


Figure 1.1 TCR stimulation is ROS dependent. Many of the steps in T cell activation upon TCR stimulation are redox-dependent. The steps that are inhibited by ROS are marked in red, and the activated steps in green. Reprinted with permission from Mary Ann Liebert Inc, Antioxidants & Redox Signaling, Previte, Piganelli 2018 (38).

The transcription factors described are highly redox dependent, overviewed in Fig. 1.1. T cells appear to produce ROS mainly via NOX and mitochondrial leakage (36), generating O₂^{•-} and further H₂O₂ that has been suggested as a regulator of NF-κB through tyrosine kinases. ROS are thus considered important for T cell activation, but the complete mechanism is yet to be elucidated. For example, antioxidants can inhibit IL-2 expression, but H₂O₂ seems to have a both inhibiting and stimulating role (36). Glutathione (GSH) has been shown to promote proliferation, whereas ROS producing macrophages seem to be able to induce T cells apoptosis via Extracellular signal-regulated kinase 1/2 (ERK1/2) activation and DNA damage response (41). Furthermore, ERK1/2 is required for the activation of AP-1 and is suggested to be regulated in a redox-dependent manner (38, 41). Calcium channel signaling, Lck and Zap70

are all activated by ROS, whereas PLC and protein tyrosine phosphatases are suggested to be inhibited by ROS (38).

Hence, the role of ROS in T cells remains intricate, with suboptimal or excessive amounts of ROS resulting in anergy or DNA damage and cell death, respectively (24, 38).

1.4 ROS ORCHESTRATE IMMUNE SIGNALING

Although ROS are important for T cell activation, mouse studies suggest that a global NOX deficiency results in a Th17 skewed phenotype, rather than immunosuppression, supporting the theories that maturation into different subtypes is highly redox dependent (24, 38). Studies have also shown that effector cells are more resistant to oxidative stress than naïve cells, which affects their survival in highly inflamed and hypoxic tissues. Naïve cells normally remain in the lymph nodes and lymphatic systems.

It has also been suggested that TGF- β dependent immunosuppression by Tregs is highly ROS dependent, where Tregs in NOX deficient mice were less capable of suppressing T effector cells. Furthermore, treatment with the antioxidant N-acetylcysteine has been shown to reduce TGF- β expression in other cells (24, 38) and hypoxic conditions to increase the yield of Tregs *in vitro* (38, 42). Thus, the absence of ROS or a surplus of reducing agents can indirectly result in a delayed response, but still a pro-inflammatory Th17 skewed response, since ROS themselves are needed for normal activation of both effector cells and Tregs (24, 38)

Macrophages can become activated by an environmental condition, like ROS or LPS. Upon this, they produce high levels of ROS through NOX-2 expression, which triggers MAPK and NF- κ B, resulting in pro-inflammatory signaling with cytokines such as TNF and IL- β . They form a tight inflammatory synapse with the T cells, enabling H₂O₂ to pass over the cell membranes and activate the T cells through the MAPK and NF- κ B pathways (38). The extracellular source of ROS by macrophages, but also neutrophils and other immune cells, might not have the same effect as intracellular alterations of redox state in the T cells. However, both intrinsic and extrinsic sources affect the T cells, with endogenous production being enough by itself for activation (36, 38).

1.5 METABOLIC COORDINATION OF T CELLS

The redox environment is tightly connected to cellular metabolism, and studies suggest different metabolic profiles for different types of T cells (43, 44), as overviewed in Fig. 1.2. Naïve CD4⁺ T cells predominantly rely on oxidative phosphorylation (OXPHOS) and memory T cells have been described to rely on OXPHOS and fatty acid oxidation (45), whereas activated T cells undergo metabolic reprogramming by transitioning towards aerobic glycolysis. A similar transitioning is well described in tumors, known as the Warburg effect (43, 44, 46-48).

Mammalian target of rapamycin (mTOR), HIF-1 α and Myc are critical for the glycolytic switch, whereas overexpressed AMP-activated protein kinase (AMPK), as a known inhibitor of mTOR, suppresses effector differentiation and causes anergy (49). Many of the metabolic regulators are redox-dependent, and ROS scavengers have been shown to inhibit clonal expansion of T cells by inhibiting Myc and mTOR (43). Skewing the metabolism towards the pentose phosphate pathway is another example of reducing ROS in the cells, leading to a Th1/Th17 polarization in RA patients (43, 50-52).

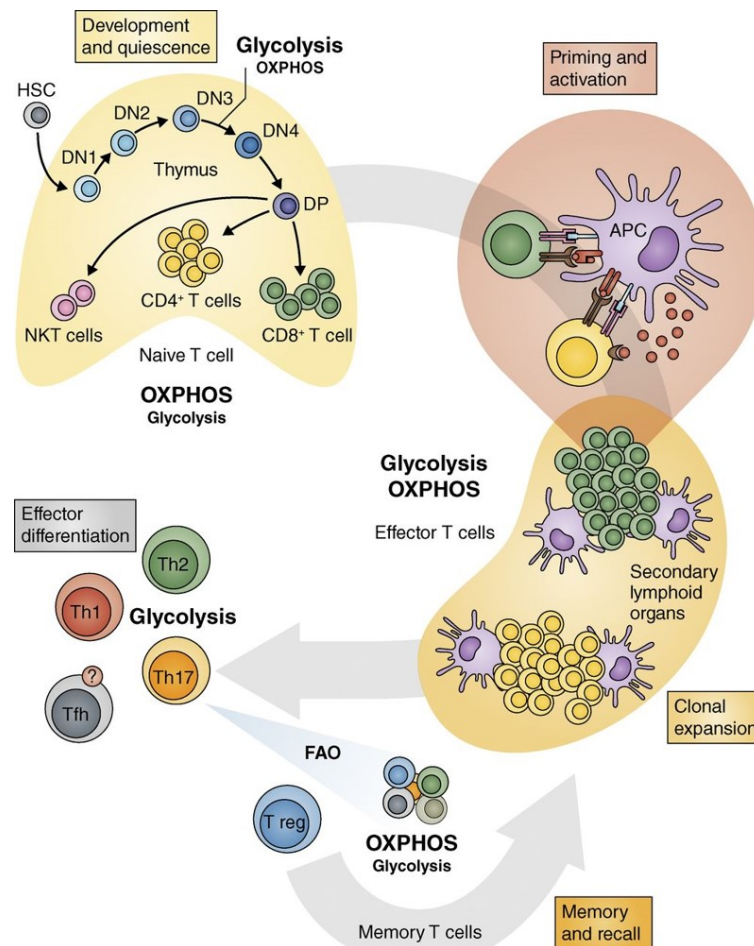


Figure 1.2 The metabolism differs between different T cell subsets. Th1, Th2, and Th17 effector cells (and possibly Tfh cells) undergo different metabolic stages during development and activation, and are metabolically distinguished from Treg cells and memory cells by glycolytic metabolism. Reprinted with permission from Rockefeller University Press, J Exp Med, Buck et al 2015 (53).

In summary, studies suggest a potential for metabolic manipulation as a therapeutic strategy (50, 54-56). Myc and HIF-1 α regulate metabolic programming at transcriptional level, and AMPK and mTOR at posttranscriptional level, constituting important metabolic checkpoints of the T cells (57). We have previously shown that activation of HIF-1 α sensitizes cancer cells to inhibition of the DNA repair enzyme MTH1, which could indicate that the metabolically transitioned activated T cells could be sensitive to MTH1 inhibition, like the cancer cells (58).

1.6 DNA REPAIR AND IMMUNITY

In addition to the ROS signaling pathways, the DDR system plays a critical role in immune signaling and modification, both in the innate and adaptive system (59, 60). The DDR is a signaling pathway system with damage sensors, mediators, transducers and effectors that act differently depending on cell cycle phase and type of damage. Excessive DNA damage accumulation or defects in the repair system eventually leads to cellular senescence or apoptosis. Aging of the immune system leads to a higher amount of accumulated DNA damage, as does chronic systemic inflammation, such as rheumatoid arthritis, where this immune aging is accelerated. The level of DNA damage is typically higher in aged individuals, as well as differentiated memory cells compared to naïve T cells (59, 60).

Described below are several examples of how DDR affects immunity, as the main focus of this thesis is to modulate the DNA repair system as a tool for immunomodulation.

Inhibiting the DNA repair system must not always lead to cell damage and death, but can instead result in more sophisticated signaling consequences (59, 60). Poly(ADP-ribose) polymerase-1 (PARP1), activated by the presence of DNA breaks, induces the translocation of NF- κ B into the nucleus upon genotoxic stress, but also during T cell stimulation in the absence of DNA damage, giving rise to pro-inflammatory signaling and ultimately apoptosis via excessive ROS induction followed by ERK1/2 phosphorylation (41, 61, 62). Experimental results suggest that PARP1 inhibitors could have a protective role in not only cancer, but also inflammatory diseases, like acute and chronic airway inflammation, as a regulator of NFAT (62, 63). It has been suggested that PARP1 knockout (KO) in T cells can disrupt the Th1/Th2 balance by increasing IFN- γ and other Th1 associated chemokines, and by suppressing IL-4 and Th2 (64). PARP1 is also tightly associated with caspase-independent cell death, parthanatos, which is important in many diseases such as stroke, heart attack, Parkinson's disease, diabetes and ischemia-reperfusion injury (65-67).

Other key players in DNA damage sensing and repair are the DNA repair and cell cycle kinase Ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR). Both can be activated by DNA damage, like double strand breaks (DSBs), and replication stress. ATM phosphorylates many substrates, like BRCA1, CHK2 and p53 and has been shown to be activated by ROS. Thus, low levels of ATM in T cells from RA patients, who already have metabolically altered T cells with a disbalance in ATP and NADPH leading to a consumption of ROS, is correlated with a Th skew towards Th1 and Th17 (52). Patients with mutations in ATM typically suffer from systemic chronic inflammation with autoimmunity, neurodegeneration and accelerated aging (68). ATM modulates NF- κ B in a multifaceted manner, both in health and disease: It assembles with IKK γ (NEMO) and further stimulates NF- κ B during physiological DSB-induced V(D)J recombination of the immunoglobulin loci. It also plays a role for mediating both homologous recombination (HR)-mediated repair as well as non-homologous end joining (NHEJ) (60).

Another form of DNA wear is telomeric shortening, eventually leading to cellular senescence. However, normal human T cells maintain telomeric sequences over 5000 kb, never entering telomeric senescence. Nevertheless, chronic inflammation, like RA, has been shown to lead to age-inappropriate shortening of the telomeres also in T cells (52). Short telomeres are detected through ATM and ATR, which in turn phosphorylate several nuclear targets, including Histone 2 at serine 139, forming γ H2AX, which further recruits ATM complexes as a positive feedback-loop. This initiates cell-cycle arrest via p53 and p21. Studies show that DSBs and DDR-induced γ H2AX expression in circulating CD8⁺ T cells is overrepresented in patients with chronic inflammation caused by hepatitis C, and that these T cells have an impaired response to IFN- γ and hence a functional deficit (69). Thus, persistent DNA damage within the cytotoxic T cells does not seem to have a beneficial role for the cytotoxicity. Likewise, age-related DNA damage (“inflammageing”) is associated with both a decline in adaptive immunity and low-grade inflammation (59, 70).

When it comes to DNA damage within any cell in the body, mononuclear phagocytes are trained to clear the body from these damaged cells via Damage Associated Molecular Patterns (DAMPs) that can trigger an innate immune response via pattern recognition receptors (PRRs). DAMPs give rise to signals of danger, like DNA damage or intracellular proteins in the extracellular space. They can be sensed via specific receptors, like TLRs, causing inflammation via downstream signaling pathways (Fig. 1.3).

Many different types of cells in addition to the monocytes and macrophages are involved in DAMP sensing, such as dendritic cells, granulocytes, NK cells and lymphocytes, but also non-immune cells, like epithelial cells, endothelial cells and fibroblasts (71). ATM and ATR with downstream p53 play a key role activating the NKG2D ligand on DNA-damaged cells, resulting in the recruitment of NK cells and cytotoxic CD8⁺ T cells, and thus clearance of damaged cells together with the phagocytes. DDR is also important for triggering antigen-presenting-like functions in fibroblast and activating cytotoxic T cells (60). Furthermore, ATM is modulating the IFN system that gets activated upon DNA damage via the cGAS-STING pathway, enhancing the microbial response upon DNA damage (72).

Exogenous sources of DNA damage, such as ionizing radiation, have been shown to induce an inflammatory response via IL-6, TNF and IL-1 β . Although radiation therapy in cancer treatment is meant to induce DNA damage in the cancer cells, one of the therapeutic effects of ionizing radiation as part of cancer treatment is also thought to be due to an induced immune response, with increased expression of MHC-I and APCs (60). Induced DNA DSBs are detrimental for proliferating cells, but also single strand breaks (SSBs), oxidized bases and abasic sites trigger cytokines, chemokines and ROS signaling (59, 60), highlighting the complicated role of DNA damage for inflammation.

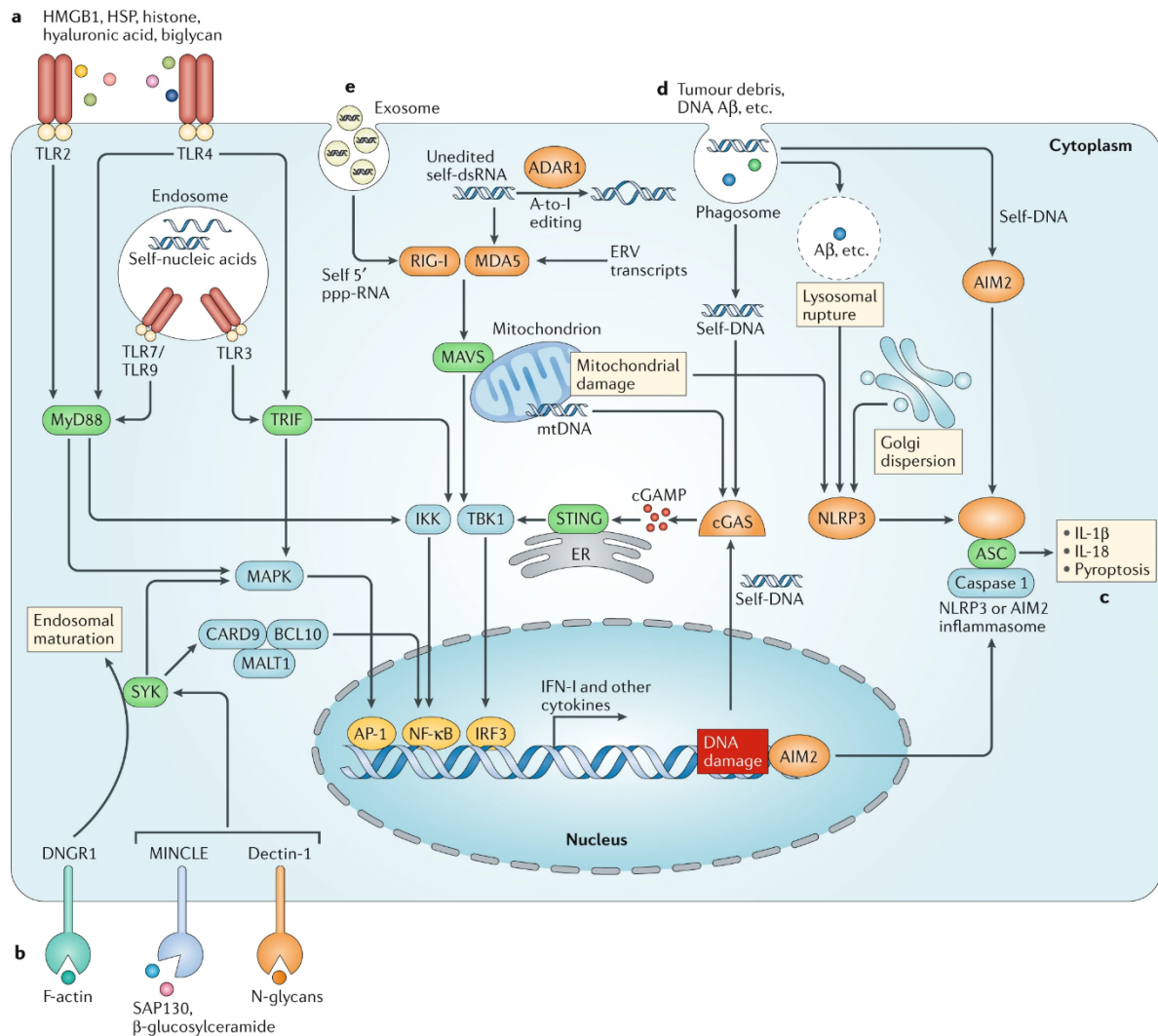


Figure 1.3 Overview of DAMP-induced pro-inflammatory patterns. There are several types of DAMPs recognized by innate immune cells both via intracellular and surface receptors. DNA damage is an important trigger of the immune response, both as damaged DNA inside the nucleus, and as self-DNA outside the nucleus or cell. Reprinted with permission from Springer Nature, Nature Reviews Immunology, Gong et al 2019 (71).

1.7 THE TWO TARGETS

The two DNA repair enzymes central in this thesis are MTH1 and OGG1 (Fig.1.4). High levels of ROS modulate any type of biological macromolecules, including DNA itself (73). Since guanine (G) is the DNA base with the lowest redox potential, it is particularly vulnerable to oxidation. 8-oxo-7,8-dihydroguanine (8-oxoG) is thus one of the most common DNA oxidation products (74) and particularly interesting in the context of inflammation. 8-oxoG in the DNA readily leads to mutations, since the cytosine (C) in the original base pair G·C can be converted to an adenine (A) via mismatch repair, due to the ability of 8-oxoG to mimic thymine (T), making the 8-oxoG·C pair look like a T·C mismatch pair (75). Large amounts of incorporated 8-oxoG can also result in SSBs and cell death (76-78). OGG1 is a DNA glycosylase/AP lyase that removes 8-oxoG from the DNA through base excision repair (74, 79, 80).

The nucleotide pool is another source of oxidized G, and to prevent oxidized nucleotides from entering the DNA, MTH1 sanitizes the dNTP pool by turning oxidized dNTPs into dNMPs (81-83). In this way the cells avoid mismatch, DNA breaks and cell death. Although dGTPs are not the most abundant form of dNTPs, and although MTH1 does not hydrolyze oxidized dGTPs selectively, the sanitizing effect of MTH1 on the guanine pool is still highly relevant due to the vulnerability of guanine as compared to other DNA bases (84).

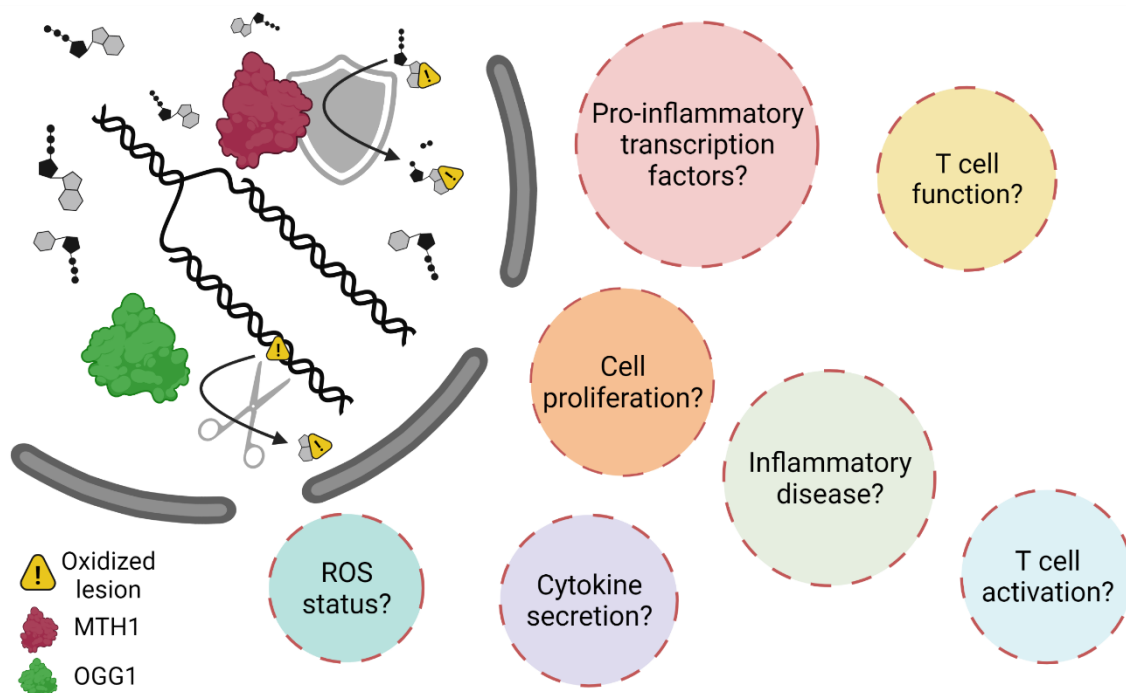


Figure 1.4 MTH1 and OGG1 protect the DNA from oxidized lesions. MTH1 hydrolyses oxidized dNTPs to dNMPs, thus preventing 8-oxoG and other oxidized bases from entering the DNA. OGG1 excises 8-oxoG from the DNA. The role of OGG1 and MTH1 in inflammation is not fully known yet but considering the many ways that ROS and DNA damage affect immune signaling, inhibition of OGG1 or MTH1 could affect the immune system in many ways. *Figure created with BioRender.com*

It has been suggested that these types of addiction enzymes are important for the survival of cells that suffer from oxidative stress, like cancer cells, to sustain normal function and survival (74, 79, 80, 85, 86). Since many immune cells both suffer from oxidative stress and use ROS for their normal signaling, MTH1 and OGG1 might be directly involved in many parts of immune signaling. Targeting these enzymes could have immunomodulatory effects yet to be discovered (Fig. 1.4). Furthermore, OGG1 levels are elevated in many inflammatory diseases such as MS and inflammatory bowel disease, whereas MTH1 expression remains somewhat unclear (87-89).

1.7.1 Inhibition of OGG1

It has been suggested that OGG1 KO mice are inherently resistant to different types of inflammatory conditions, while still being viable – inflammatory models suggest OGG1 KO to be beneficial in the animals, like a sepsis model with LPS, where the KO mice were more viable than the WT mice (90-94). We and others have developed small-molecule inhibitors

against OGG1 over the past years, with promising results in a *Pseudomonas* sepsis model in 2020, complementing the LPS/TNF- α model used in **Paper I** of this thesis (95-98).

The mechanism of OGG1 being involved in inflammatory signaling is not completely understood, but it has been proposed that OGG1 interacts with 8-oxoG in regulatory gene regions, facilitating gene expression, affecting the expression of several cytokines and transcription factors like NF- κ B, Myc and vascular endothelial growth factor (VEGF) (99-108). Interestingly, the promoter regions of many inflammation-associated genes are rich in guanine (109, 110). Studies also propose free 8-oxoG as a pro-inflammatory molecule, that after being excised from the DNA binds to OGG1, activates small GTPases like K-Ras, Rac1 and RhoA, and thus activates immune cells like dendritic cells (79, 99, 106-108, 111, 112).

The role of OGG1 in different inflammatory diseases and cancer thus remains to be clarified, yet small molecule inhibitors and genetic KO of OGG1 have dramatic effects *in vivo* in several inflammatory models (90-92, 97).

1.7.2 Inhibition of MTH1

It has been demonstrated that MTH1 is essential for cancer survival due to the large amount of ROS found in cancer cells (86, 113-118), whereas MTH1 KO mice are viable and healthy (119). It could therefore be speculated that MTH1 is essential for activated T cells too, due to their elevated ROS pressure.

TH1579 (other names karonudib and OXC-101) is a small molecule inhibitor of MTH1 with favorable pharmacokinetic and pharmacodynamic properties (117). It is currently undergoing clinical trials for solid tumors and leukemia (NCT03036228 and NCT04077307). However, little was known about the role of MTH1 and its inhibition in inflammation at the initiation of this thesis.

In 1997, Oda et al. showed that MTH1 is up-regulated in activated peripheral blood mononuclear cells, but not to the same extent as in the immortalized leukemia variant, Jurkat cells (120). If MTH1 would be essential for the activation and survival of T cells due to their cancer-like ROS levels and metabolism, it could be speculated that they, like the cancer cells, should be sensitive to TH1579. However, Einarsdottir et al. show that patient derived tumor infiltrating cells (TILs) are insensitive to TH1579 regarding their cytotoxic function (121). Both degranulation and IFN- γ secretion upon challenging with tumor cells were sustained, as well as tumor clearance with or without anti-CTLA-4, indicating that TH1579 treatment does not impair the function of TILs.

Conclusively, the potential effects of MTH1 on T cells, and its inhibition by TH1579 in inflammatory settings had not been fully investigated before. Considering the known effects of 8-oxoG in inflammation and the cancer-like glycolytic switch activated T cells undergo when they up-regulate MTH1, we considered MTH1 a highly relevant target to study in inflammation.

1.8 THERAPEUTIC IMMUNOLOGICAL APPLICATIONS

1.8.1 T cell driven diseases

Many autoimmune diseases are T cell driven, like MS and psoriasis (2-9). For MS, the treatments available mainly consist of disease modifying agents that reduce the inflammatory activity, and symptomatic treatment. IFN- β , glatiramer acetate, teriflunomide and dimethyl fumarate are examples of first-line MS treatment, followed by monoclonal antibodies inhibiting CD52, CD20, cell adhesion molecules or the sphingosine-1-phosphate receptor, all inhibiting lymphocytes. The biological drugs are often more effective than the traditional ones, but they also come with the risk of severe immunosuppression. High dose corticosteroids are also used for acute relapses (7). Regardless the generous spectrum of treatment options for MS, there is still a 50% risk of being permanently dependent of a wheelchair 25 years after the disease onset, which is typically around the age of 30 (7, 8).

For psoriasis, affecting 2-3% of the world's population (2), the treatment options span from topical agents, like vitamin D analogues, retinoids, glucocorticoids and phototherapy, to systemic treatment, like Methotrexate (MTX), Cyclosporine A (CsA) and Acitretin. In some cases, inhibitors of IL-17, IL-23 or TNF- α can also be tested, but just as for MS and other diseases, the use of biological drugs increases the risk of severe immunosuppression and infections (122, 123). Like many other systemic inflammatory diseases, psoriasis is not only affecting life quality by increasing depression and anxiety, but also increasing the risk of comorbidities like nonalcoholic fatty liver disease, cardiovascular disease, obesity, diabetes mellitus, psoriatic arthritis and inflammatory bowel disease (2).

Emerging evidence show that many commonly used immunosuppressive drugs, like mTOR inhibitors (sirolimus, everolimus), calcineurin inhibitors (tacrolimus, CsA), purine/pyrimidine synthesis inhibitors (Azathioprine (AZA)), mycophenolic acid, and MTX not only work by inhibiting the activation and proliferation of T cells, but also by targeting metabolic checkpoints, like HIF-1 α , Myc and AMPK (57, 124-134). This is curious considering the role of metabolism for T cell differentiation described above.

Taken together, the complete mechanisms of the immunosuppressive pathways for these classical drugs are only partly understood, and it could be that drugs used for the past decades for both cancers and immunological disease, actually have acted through more polarizing and immunomodulating mechanisms in the patients than previously thought.

1.8.2 Sepsis and acute inflammation

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (135). It is the leading cause of death in intensive care units in industrialized countries. In 2017, it was shown that 54 % of patients admitted to intensive care units worldwide had a suspected or proven infection, with a mortality rate of 30 % (136). At the same time sepsis-related deaths resulted in 54.4 % (48.9-59.7, 95 % UI) of total global deaths (137). Then came the Covid-19 pandemic, not lowering the prevalence of sepsis.

The pathogenesis of sepsis (Fig. 1.5) is both due to an initial inflammation, activated by pathogen-associated patterns (PAMPs), DAMPs and crosstalk between innate and adaptive immunity. At a later stage, a dysregulated immunosuppression occurs, with apoptotic depletion of B, T and dendritic cells, increased Tregs and a Th2 skew, T cell exhaustion and regulative macrophages. Some patients die already in the early acute phase as a direct result of shock, organ failure and coagulopathies, but a majority of septic deaths seem to occur after several days during the immunosuppressive stage due to multiorgan failure (138, 139). All organs are affected by sepsis, but as a respiratory failure is one of the most acute medical urgencies and more complicated to treat than a circulatory failure, pneumonia and acute respiratory distress syndrome (ARDS) are highly relevant to study when it comes to finding cures for sepsis.

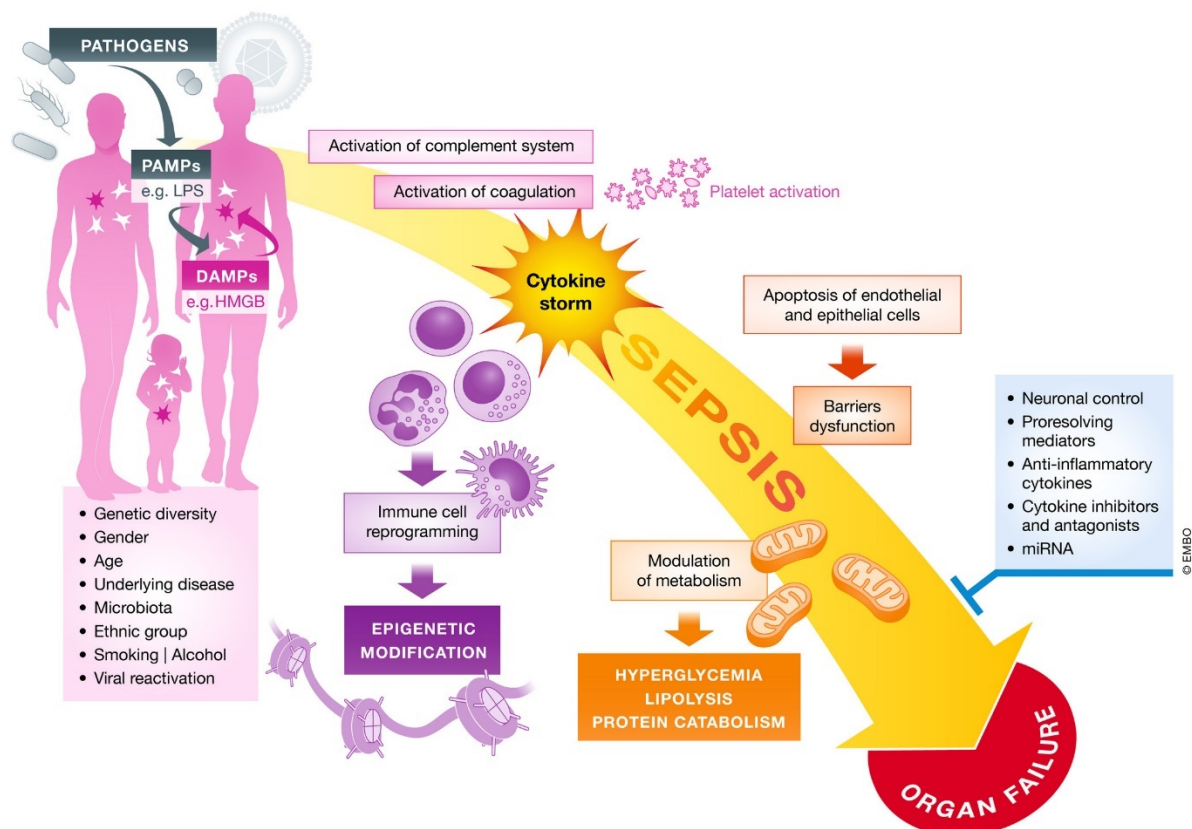


Figure 1.5 The pathogenesis of sepsis. Pathogens enter the body and induce an activation of the immune system. The character of the immune response is affected by internal host factors and external circumstances. If the activation escalates in strength and time, a systemic dysregulation of immunity and physiology occurs, ultimately leading to organ failure and death. Reprinted with permission from John Wiley and Sons, EMBO Molecular Medicine, Skirecki et al 2020 (140).

For bacterial sepsis, quick administration of antibiotics is of great importance, but for virally induced sepsis, broad-spectrum antibiotics can even have adverse effects. Virally induced sepsis has traditionally not gotten as much attention as bacterial sepsis, although 42 % of sepsis was culture-negative in 2018 (141), suggesting viral components. Therefore, it would be a great advantage to establish medications against sepsis that work on both bacterial and viral sepsis, without adverse immunosuppressive effects. The Covid-19 pandemic worked as a strong reminder of the urge for treatment options against viral sepsis and ARDS.

Except for the anti-microbial and symptomatic life-supporting treatment, there are no efficient treatment options against sepsis today (11, 138, 142, 143). The role of glucocorticoids is controversial, with a potential role in severe sepsis (144-146). Severe illness and pro-inflammatory cytokines can cause peripheral glucocorticoid resistance, and possibly only a subgroup of patients with an inadequate inflammatory inhibition of the hypothalamic-pituitary-adrenal axis would benefit from external corticosteroids during sepsis (146). There is in general moderate evidence that corticosteroids reduce 28-day and hospital mortality in sepsis, and high evidence that it reduces the length of hospital days, but the effect on major complications and long-term mortality is still uncertain (147). Drugs like dexamethasone have shown some beneficial effects on mortality among Covid-19 patients with oxygen therapy (148), but the use of glucocorticoids remains controversial as it suppresses T cells and induces apoptosis, potentially enhancing viral replication (149-151).

1.9 INFLAMMATION AND CANCERS

This thesis is focused on inflammation, but as cancer and inflammation are tightly connected, and as the inhibitors investigated are very relevant for cancer, this last introductory section briefly focuses on the cancer perspective.

1.9.1 Avoiding immune destruction – a hallmark of the cancer cell

T cells play a crucial role in the defense against tumors. However, due to continuous antigen exposure, T cells can become dysfunctional during chronic inflammation and cancer (152-157), resulting in T cell exhaustion (30) and expression of IRs like PD-1 and CTLA-4 among others. PD-1 can regulate the level of TCR signaling (158, 159) whereas CTLA-4 compete with CD28, with a higher affinity to CD80/86 of the two (152, 160). The tumor microenvironment plays a critical role for the fate of the immune response, where inhibiting ligands and cytokines of both APCs and cancer cells modulate the immune response and IRs (26, 152).

Immunosuppressive cells in the tumor microenvironment include Tregs (secreting TGF- β and IL-10 and up-regulating receptors associated with T cell dysfunction), tumor-associated macrophages (supporting Tregs and dysregulate the vasculature), MDSCs (promoting T cell dysfunction together with TAMs, secreting nitric oxide, ROS and arginase-1), endothelial cells (secreting VEGF, improving production of prostaglandin E2, suppressing vascular cell adhesion molecule 1, thus promoting T cells dysfunction), cancer-associated fibroblasts (shaping the tumor microenvironment, secreting TGF- β and VEGF) and cancer associated adipocytes (metabolic and paracrine regulation of the immune cells in favor of the cancer) (152). In addition, emerging evidence also indicate that subsets of $\gamma\delta$ T cells are crucial for tumor development (27). Targeting these suppressive cells might improve the anti-tumor response (152, 161-166). Even genetically engineered CAR-T cells can up-regulate their inhibitory receptors due to the tumor microenvironment, and thus lose their function (167-172).

Furthermore, the metabolic programming by the tumor microenvironment also plays a role for the anti-cancer immunity, where the T cells compete with the cancer cells to obtain nutrients due to the common metabolic pathways, and glucose deprivation in T cells result in impaired function (46, 173, 174). CD28 can facilitate the metabolic switch to glycolysis but CTLA-4 and PD-1 can restrict this switch. In addition, PD-1 can promote fatty acid oxidation (152, 175). Hypoxia is also a hallmark of the tumor microenvironment, but its role for the immune response is controversial (152, 176-178).

Conclusively, the tumor microenvironment and immunosuppressing receptors and cytokines are tightly involved in cancer, and also here ROS, T cell polarization and DNA damage are highly relevant. Different parts of the tumor microenvironment could work as targets for anti-cancer therapies, although the complete pathways and significance remain to be mapped (179-185). The extent of which successful treatments of today also affect the tumor microenvironment as an additional mechanism of action remains to be discovered.

1.9.2 The Return of the Immune surveillance theory?

Despite great advances and resources within cancer research and healthcare, enabling extensive sequencing on single cell level and personalized medicine, the success of new drugs has been modest in relation to the number of interventions, and cancer still constitutes one of the leading causes of death (186-188). The origin of cancer is widely accepted to be explained by mutagenesis of cells, leading to uncontrolled proliferation of cells with the *Hallmarks of cancer*, and the inability to clear them out leads to the disease (189, 190). However, what matters for the clinical outcome is not how the single cancer cell behaves, but how and if a disease with symptoms develops. The question is – is *the disease cancer* caused by mutations in cells that become cancer cells, or is it caused by an inability by the immune system to clear out the cancer cells that would appear anyways, explaining why immunosuppressed patients eventually get cancer (15)? Both factors might be involved, and from a pragmatic point of view, the origin is not as important as finding treatments that work regardless the mechanism. But from a research-, healthcare system-, preventive care- and drug development perspective it is of great importance, to allocate the resources right.

The theory of Immune surveillance was mentioned already over 100 years ago, suggesting that aberrant cells from the fetal development stay latent thanks to immune surveillance, and that cancer can develop when this fails (191). Later, tumor antigens were proposed to exist, as an explanation to the fact that natural tumors are typically rejected from syngeneic hosts as opposed to normal transplanted tissues, suggesting a role of the immune system rather than the tumor (191). With immune therapy advancing, oncoimmunology has received great interest over the past decades. It has become clear that no cancer is like the other, and both the intra- and intertumoral heterogeneity is vast (189, 190, 192), but still a lot of focus is aimed at the cancer cells and the close tumor microenvironment. At the same time, the cells of the immune system, like NK cells, ILCs and T cell subsets, are evolutionarily primed to be able to eliminate both cancer cells and microbes, regardless of if they have encountered them before, questioning how important it is to find specific traits for every cancer cell of every patient.

There are however no clear immunological markers that would prove that all cancer patients have systemically dysregulated immunity as the reason for the origin of cancer, but with improved methodology and access to other immunological tissues than blood, it is more and more accepted that the variation between seemingly healthy individuals is large (193). Men and women also seem to have different immune signatures, and even though both are considered “normal”, men are still overrepresented in cancer (194), as are women in many autoimmune diseases (193). If DNA damage and DDR affects these differences in immunity remains to be investigate further.

The induction of DNA damage and mutations is generally accepted as the mechanism of action to the origin of cancers. However, most known risk factors of cancer can be traced to a dysregulated immunity. Examples of this are high age (17, 70), obesity and metabolic syndrome (195, 196), and smoking, which downregulates NK cells in the lungs (197). Also hereditary deficiencies in DNA repair enzymes linked to cancer affect the T cells directly, like BRCA1 (198), VHL (176) and ATM (52). Autoimmune conditions are controversial, as they are often treated with immunosuppressives that can increase the cancer risk (15), but milder hyperinflammatory conditions that are not treated with immunosuppressants, like atopy, has an inverse correlation to cancer (199, 200). On the other hand, chronic inflammation caused by infections, autoimmune diseases or irritants in selected organs promote cancer (61), but not necessarily only by induction of mutations – the rise of the disease could be due to the downregulation of immune clearance. The cytokines that the DNA damaged cells excrete can suppress the immune clearance via IRs and exhaustion described above (17, 201). Many viruses downregulate MHC-I on the cells (202-204), which could contribute to the oncogenesis in addition to any mutations they cause in the cells.

All factors above are tightly connected to DNA damage and repair, whether it is in the cancer cells, immune cells or systemic immune dysregulation (Fig. 1.6). Increased DNA damage and oxidative stress is immunogenic, and the DDR plays a crucial role in inflammatory signaling (59-61, 205-207), making it an interesting target for immunomodulation.

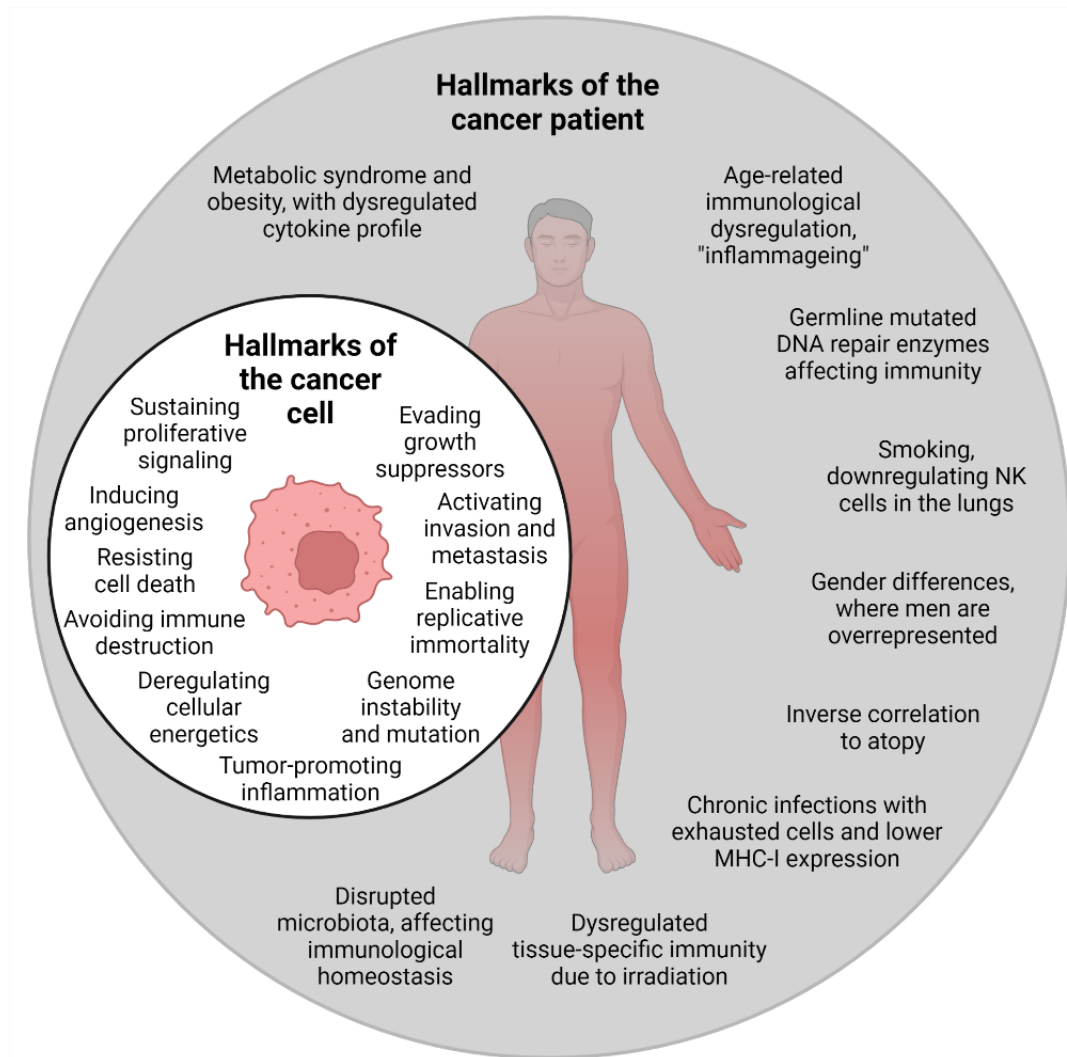


Figure 1.6 The hallmarks of the cancer patient from an immunological point of view. Many of the risk factors of cancer are tightly associated with immunology and could suggest a more systemic phenotype as the cause to cancer, by a discrete dysregulation of the immune system. By only investigating the cancer cells, important components might be missed. *Figure created with BioRender.com*

In this thesis, I explore new drug candidates for inflammatory disease, by investigating DNA repair inhibitors originally created for the fight against cancer. To have both anti-cancer and immunomodulating effects is not unique among established drugs, and by allowing immunology to acquire a bigger part of the cancer-centered DNA repair field, there might be many valuable scientific findings to obtain in the fight against disease.

2 RESEARCH AIMS

The overall aim of the thesis was to contribute to the battle against inflammatory diseases by generating new basic biology knowledge about the targets MTH1 and OGG1, and by investigating the novel drug candidates TH1579 and TH5487. DNA repair has long been studied in the context of cancer research, and several current and experimental anti-cancer drugs affect DNA repair, whereas it has received limited attention in the efforts against inflammatory diseases.

The association between inflammation and OGG1 has been known for decades, but there have only been a few drug candidates targeting OGG1 (95-98). For MTH1, great strides have been made within the field of cancer (58, 86, 88, 96, 113-117, 119, 208-217), with ongoing clinical trials for TH1579, but little was known about the role in inflammation, both when it comes to the basic biology and to MTH1 inhibitors.

In **Paper I**, the aim of this thesis was to assess TH5487 as an anti-inflammatory drug candidate both *in vitro* and *in vivo*. The effect of the inhibitor was also to be compared to knocking out OGG1 in the inflammatory *in vitro* models using the CRISPR/Cas9 method. The aim was also to compare the inhibitor to dexamethasone, an established drug that is currently used for indications where OGG1 inhibition could play a role in the future. However, the latter remained non-published preliminary data presented in the Result section.

Paper II and **III** focus on MTH1. As the advantage of MTH1 inhibition in cancer is believed to be due to elevated ROS and redox pressure in cancer cells (86), and as T cells too have been described to have an altered ROS status (35, 36, 43, 218, 219), we hypothesized that T cells would be sensitive to MTH1 inhibition, just like cancer cells. In **Paper II** and **III**, we thus investigated MTH1 inhibitors for the treatment of the T cell driven diseases psoriasis and MS, respectively.

We also examined MTH1 levels in patients in **Paper II**, and verified the ROS induced sensitization to MTH1 inhibition previously shown in cancer cells (58) in the skin cells. The preliminary results made us hypothesize that different T cell subsets could be differently sensitive to MTH1 inhibition, and thus IL-17 producing $\gamma\delta$ T cells and IL-17 downstream signaling was investigated, in addition to assessing other immune cells relevant to psoriasis.

In **Paper III**, we investigated the T cells specifically, by elucidating their sensitivity to MTH1 inhibition. As we surprisingly found that not all T cells were sensitive to inhibition when treated and activated simultaneously, we also measured MTH1 levels and ROS status of the cells per cell generation. For the sensitive cells, we elucidated the mechanism of action by investigating cell cycle, DNA damage and 8-oxoG incorporation. The effect on memory T cells and other immune cells from a toxicology perspective was also explored.

The specific research questions per paper from the perspective of this thesis were:

Paper I

- Can TH5487 suppress pro-inflammatory gene expression *in vitro* in inflammatory cell models?
- Can TH5487 suppress inflammation (neutrophil infiltration) *in vivo* in a pneumonia model?
- Is the effect of TH5487 comparable to CRISPR/Cas9 KO of OGG1?
- Does TH5487 impair the interaction between OGG1, NF- κ B and the guanine-rich promoter regions, affecting the downstream inflammatory signaling?

Paper II

- Is MTH1 up-regulated in psoriatic patient samples?
- Is there a correlation between oxidative stress and sensitivity to MTH1 inhibition in skin cells?
- Can MTH1 inhibition alleviate psoriasis *in vivo*, regarding skin thickness, cell infiltration and pro-inflammatory gene expression in the skin?
- Can MTH1 inhibition affect the pathological shift in cell constitution of the cells in the spleen and lymph nodes of mice upon induction of psoriasis?
- How are IL-17 producing $\gamma\delta$ T cells and IL-17 signaling affected by MTH1 inhibition in the psoriatic mice?

Paper III

- Does TH1579 kill activated T cells and how selective and potent is the compound as compared to the established drugs MTX and AZA?
- Is there a correlation between ROS status and MTH1 expression?
- Does the amount of MTH1 vary over the cell generations upon activation in untreated T cells?
- Is there a target engagement of TH1579 to MTH1 in human T cells?
- Does TH1579 inhibit proliferation, induce apoptosis or both?
- Is there any effect on the cell cycle, mitosis, DNA damage and 8-oxoG incorporation in T cells upon treatment with TH1579?
- Can activated T cells have low levels of MTH1, and is there in general a heterogeneity in MTH1 expression among activated T cells? Can TH1579 select for these MTH1^{low} cells?
- Are there activated T cells with lower ROS, and can TH1579 select for these cells?
- Does TH1579 impair the function of other immune cells?
- Does TH1579 have a therapeutic role in a murine model of MS?
- Is the toxic effects on the T cells reversible?
- Are memory T cells affected more or less than naïve T cells?

3 METHODOLOGICAL CONSIDERATIONS

The key methods of this thesis, as well as the methodological strengths and limitations, will be described and discussed below. Multidisciplinary methods were used including cell lines, primary cells, and *in vivo* studies. For a detailed description of the methodological procedures, please see the Materials & Methods sections of the papers.

3.1 CRISPR/CAS9 KNOCKOUT OF OGG1 IN HEK293T CELLS

In order to create a complete OGG1 KO cell model, the CRISPR/Cas9 method was used following the protocol by Ran et al. from 2013 (220). CRISPR engineering has developed into a common and important tool for gene editing over the past years, and the method has rapidly improved since the experiments in **Paper I** were performed. Today there are different available Cas proteins from several species as well as engineered Cas9 proteins, making the method less dependent on protospacer adjacent motifs (PAMs) (221).

As described in Fig. 3.1, the protocol (220) briefly included finding a suitable 20-basepair sequence upstream of any PAMs close to the gene of interest, in this case OGG1. Then an expression construct of single-guide RNA (sgRNA) was created, using two separate OGG1 targeting sequences inserted into plasmid vectors containing the Cas9 protein of *S. pyogenes* and resistance to puromycin. HEK293T cells were used in the inflammation assays, as HEK293 cells had been successfully used for studying OGG1 and inflammation before (106).

Single cell clones were isolated using limiting dilution cloning. Relevant clones among the transfected cells were selected with puromycin, and then suspended to 0,5 cells per well-volume, theoretically giving a single cell in every second well. This was a more blunt and time-consuming way to select the cells than sorting with GFP and flow cytometry. The reason for that was to save resources and to not transfect the cells with GFP, as we intended to later transfect them with OGG1 mutants containing GFP. The clones were validated with the SURVEYOR assay, that recognizes indel mutations via the formation of heteroduplexes, and positive clones were further investigated with Western Blot against OGG1. Finally, the clones of interest were sequenced to make sure a frameshift had occurred.

The advantage of the CRISPR method in general is quick and precise editing, although the problem with off-target effects has been discussed (222, 223). Sequencing, the SURVEYOR assay and Western Blot altogether gave a robust verification of the knockout, although that did not exclude additional off-target effects of the CRISPR procedure or unknown effects of the handling of the cells and insertion of puromycin resistance to the system. It can be concluded that although CRISPR editing is very precise, the cells will be handled in a very “unnatural way”, which often is the case for *in vitro* culture, and can be a problem when studying delicate immune pathways.

Knocking out a protein completely rather than silencing it with small interfering RNA, is in general an advantage when studying the mechanism of action, but the sudden gene manipulation can both be toxic and cause sudden off-target effects. Another approach would

have been to use murine cells from OGG1 KO mice, as was the plan for the MTH1 studies, but we wanted to investigate human cells in the inflammation system. The limitation of using cells from animals with a complete KO is the risk of the animal developing compensatory mechanisms already during the development, which can cause a phenotype that is less relevant for the model.

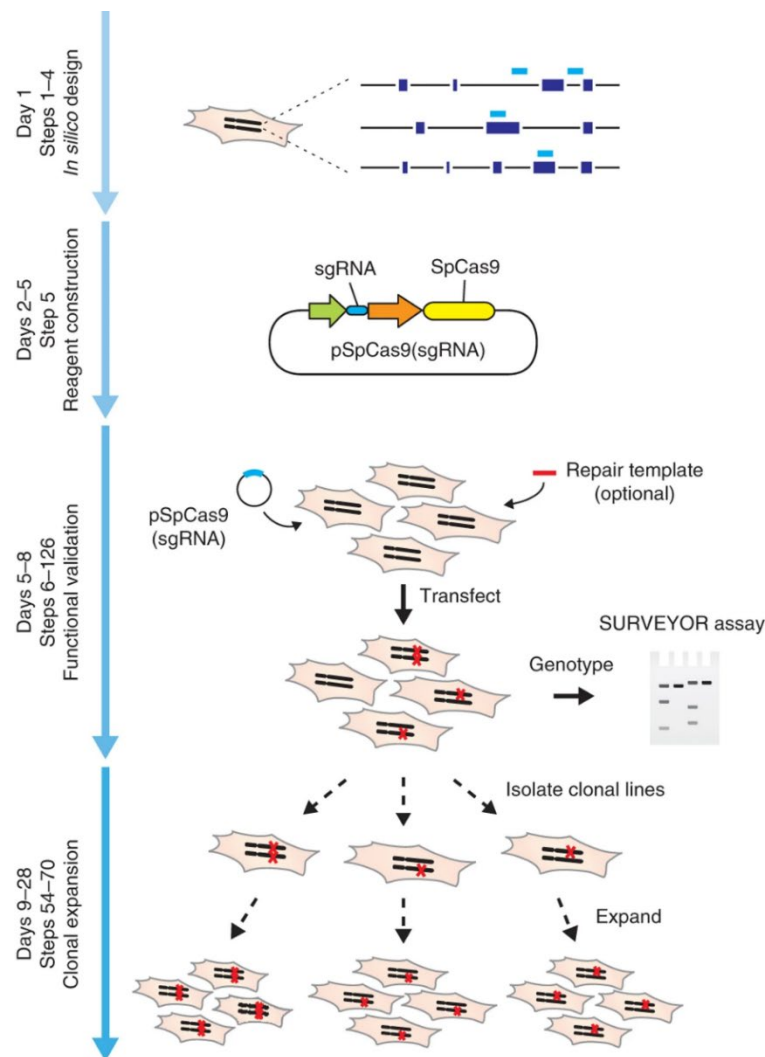


Figure 3.1 Schematic procedure of the CRISPR/Cas9 gene editing. sgRNAs were designed, created and inserted into a plasmid vector containing Cas9. Cells were transfected, selected for with puromycin and seeded out as single cells. Clones were screened for indel mutations with the SURVEYOR assay and Western Blot. In the clones of interest, a frame shift through random indel mutation was verified with sequencing. Reprinted with permission from Springer Nature, Nature Protocols, Ran et al 2013 (220).

3.2 ACUTE PNEUMONIA IN VIVO-MODEL

In the acute inflammatory models in **Paper I** and the preliminary data, mice were challenged with either TNF- α or LPS, to mimic bacterial infections and to some extent viral reactions. A

strong technical and economic advantage of the protocol is that there is no need to handle live microbes, like bacteria or viruses, as the reaction will be triggered by the microbial antigens. But that is also a limitation when studying immunological conditions, since the immune response has many branches – inhibiting them all would not only alleviate the symptoms, but possibly also benefit the bacteria or viruses, and thus have an adverse effect. The aspect of bacterial or viral overgrowth is not addressed in such a model. It is thus difficult to predict whether the subject would be able to clear out the pathogen, or if the bacteria and viruses would grow unhindered upon treatment with TH5487. However, we have seen promising results on survival also when using a model with live *Pseudomonas aeruginosa* (unpublished), and similar results have been observed by others in 2020 with other OGG1 inhibitors (97).

For the assessment of inflammation in the preliminary data, a flow cytometry panel for neutrophils and monocytes was developed. As it was a quite short stimulation period of 24 h, we did not assess lymphocytes or activation markers on macrophages. It could have been interesting to further investigate lymphocytes, although it could possibly require a longer time than 24 h, to observe a clear infiltration of T cells in animals not immunized before. In another publication assessing chronic inflammation and TH5487, a similar protocol was used, but in this case also looking at activated macrophages as they play a role in fibrosis (224), but that was not considered relevant for the 24 h protocol.

3.3 IN VIVO MODELS OF T CELL-DRIVEN DISEASES

For the T cell driven diseases in **Paper II** and **III**, psoriasis and EAE were induced. For psoriasis, the TLR7/8 ligand Imiquimod was administered locally, to induce inflammation and activate the IL-23/IL-17 axis (225). It is thus not a pure T cell disease model, as psoriasis is also not a pure T cell disease, although it engages T cells and chronic inflammation, and is to a large extent T cell driven (2-4) .

For the EAE model, mice were immunized with myelin oligodendrocyte glycoprotein (MOG), creating an immune reaction and antigen-specific lymphocytes towards the nervous system of the mice. EAE is not the only animal model for MS, but it is considered one of the most relevant ones to study MS (226).

In both models, overviewed in Fig. 3.2, a therapeutic effect was seen with MTH1 inhibition, possibly due to the T cell suppression, but potentially also via immunological effects on other cells not investigated further. Clinical scores were investigated for both **Paper II** and **III**. In **Paper II**, skin samples, splenocytes, lymph nodes and cytokines were also investigated, and in **Paper III** memory T cells were assessed. It would have been a strength to also study the histology in the EAE model and include more thorough splenocyte assessments in **Paper III**, but on the contrary the two studies complement each other well. In **Paper III**, splenocytes were stimulated after *in vivo* treatment to assess the function of the T cells, demonstrating an effect on memory T cells through *ex vivo* culture.

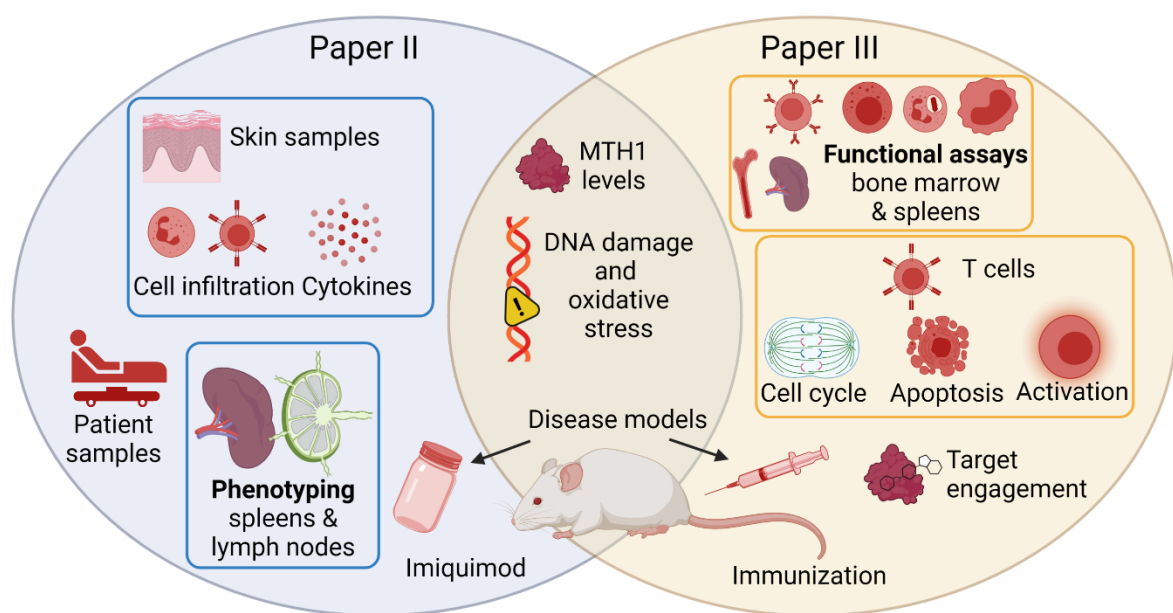


Figure 3.2. Overview of the methods covered in the two different papers, where Paper II assessed samples from patients, mouse skin, mouse spleens and mouse lymph nodes. In Paper III, more functional assays were performed, looking at the mechanism of action on T cells. Both papers assessed MTH1 levels, ROS and 8-oxoG in the samples, and both used mouse models. *Figure created with BioRender.com.*

3.4 FUNCTIONAL T CELL EXPERIMENTS WITH FLOW CYTOMETRY

As illustrated in Fig. 3.2, T cells were assessed in **Paper III** regarding cell cycle, proliferation, activation, apoptosis, and target engagement. Assays for cell cycle and DNA damage had already been extensively performed with MTH1 inhibitors from the Helleday lab in the context of cancer (86, 210, 213), but it was of importance to also verify the findings in normal human cells. *In vitro* experiments do not resemble the physiological state of cells, as they are for example kept in monocultures with bovine serum and normal air pressure, but by using freshly isolated primary cells instead of immortalized (cancer) cell lines, it could be considered one step closer to physiology.

The cells were isolated using negative selection for CD3, activated and when applicable treated consecutively or simultaneously, depending on the assay as described in Fig. 3.3. In this way, it was possible to study 1) untreated activated T cells, 2) the effect of the inhibitor on activated T cells and 3) the effect of the inhibitor on the activation itself, to elucidate if the inhibitors had any modulating capabilities.

Flow cytometry enabled investigation of the cells on high-throughput single cell level. The limitations of flow cytometry are that, as with many other assays but as opposed to imaging, the investigator is completely dependent on the fluorochrome signal, and it is not possible to know for sure if the events seen are cells or debris. Old tandem dyes can also give false signals, which can be difficult to recognize in the data. To overcome this, thorough gating strategies, including back-gating for verification, single-cell selection by both size and if applicable DNA, and proper controls were used. Controls consisted of both relevant biological positive and negative controls, and technical controls like fluorescence-minus-one (FMO) controls and single-stains for compensation. In addition to this, panels were designed according to brightness and density of the antigen, and so that colors that were close to each other in the spectrum were chosen for antigens not expected to be present on the same cell, like CD4 and CD8.

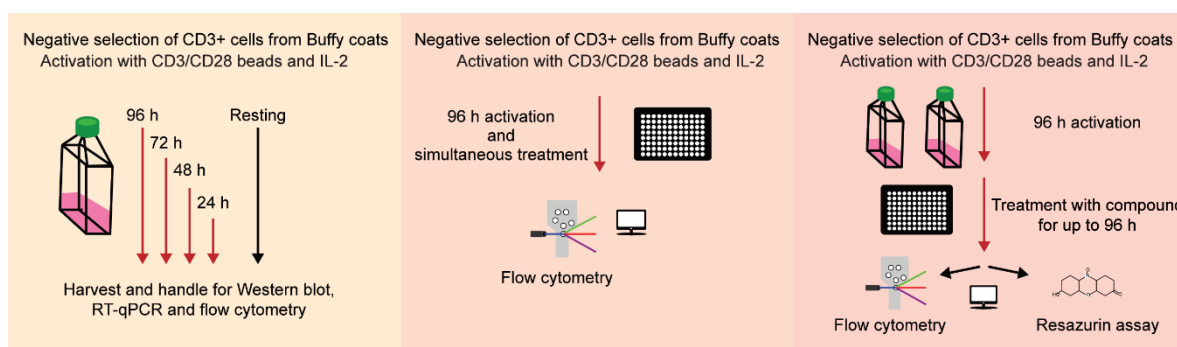


Figure 3.3. The different ways T cells were handled after separation. From left to right: Only activation; Activation and simultaneous treatment; First 96 h activation and then 96 h treatment. Figure modified from Paper III and reprinted with permission.

3.4.1 ROS and MTH1 assay

For the ROS assays, it was of importance to find a fixable dye, both due to the need for intracellular staining of MTH1 and due to later regulations regarding handling of blood products due to the Covid-19 pandemic. The CellROX Green probe by Thermo Fisher (#C10444) is fixable for up to 24 h and was thus suitable. An alternative would have been CellROX Deep Red (#C10422) but it was only fixable for a few hours. The Green probe localizes to the nucleus and mitochondria, whereas the Red probe remains in the cytoplasm according to the manufacturer.

The resting cells hardly gave any signal as compared to the FMO control, and for validation, Tert-butyl-hydroperoxide was used as a positive control, as shown in **Paper III**. Since both MTH1 and ROS are vastly up-regulated in activated cells, the use of other colors was minimized for that experiment to lower the spectral overlap and inter-channel leakage. It would have been interesting to add cell cycle, proliferation and fixable apoptosis markers into the same panel and assess ROS and MTH1 in the same experiment.

There were no suitable ready-conjugated MTH1 antibodies on the market, so the antibodies had to be added in two steps, first the MTH1-specific primary one, and then the secondary with fluorescence. This was challenging since the surface markers contained antibodies that could interact with the secondary. To minimize the risk of this, the primary and secondary antibodies for MTH1 were chosen to match different species (rabbit and donkey) than the mouse-anti-human ones for the surface markers. Since there can still be a slight inter-species interaction, MTH1 was stained for first, and after washing away any residual secondary antibody, the surface antibodies were added, despite it being considered best practice to add surface markers before fixation and permeabilization.

3.4.2 Cell cycle, proliferation, and apoptosis assay

To investigate cell cycle, the DNA stains DAPI and Propidium Iodide were used. 5-Ethynyl-2'-deoxyuridine (EdU) was added as an additional marker for cells in S-phase at the initiation of the treatment (Fig. 3.4). In that way it was possible to highlight only the cells in S-phase during the treatment initiation and investigate cell death, cell cycle and DNA damage. Treatment with EdU was a less harsh way of studying the cells in S phase as compared to synchronizing the cell cycle of all cells by starvation or treatment. However, as EdU is toxic in the long run, the experiment time points were only up to 24 h.

When defining the dead cells in the cell cycle assay, “SubG1” was defined as the cells below G1. It is a quite blunt way of defining dead cells as it cannot exclude the occurrence of dead cells above G1. The cells in SubG1 are already smaller and fragmented, which is a quite late stage of apoptosis. To complement the study, apoptosis assays with Annexin V and Sytox were also performed, although it had to be as a separate panel, as that method is based on live cell flow cytometry, whereas EdU-DAPI requires fixation and permeabilization. The lysed cells (“late apoptotic” and “necrotic”, Sytox positive) corresponded well to the SubG1 cells after 24 h, implying consistency between the two different experiments.

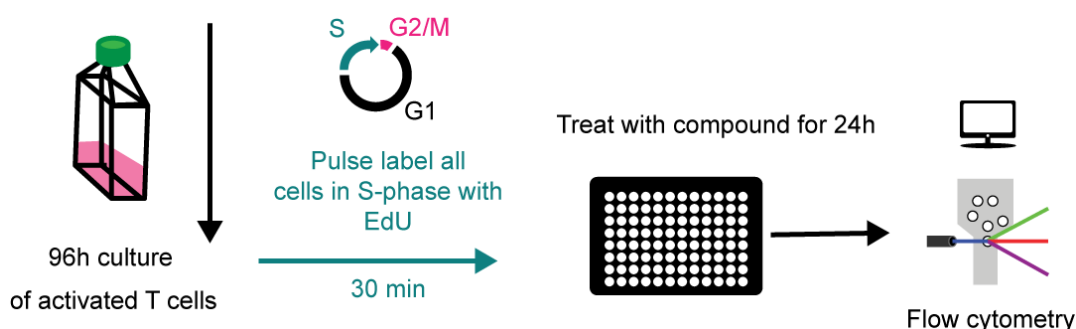


Figure 3.4. Schematic overview of the experimental procedure in the cell cycle experiments. Figure modified from Paper III and reprinted with permission.

3.4.3 Cytotoxic activity of T cells

In the preliminary data, the function of the surviving T cells after treatment was assessed. The protocol included co-culture with target cells for 10 h after an activation period of 96 h with

compound present. The cells were calculated after treatment and the numbers adjusted. All CD3 positive cells were in the culture, without calculating the CD8 cells separately. In **Paper III** we show no difference in sensitivity among CD4 or CD8 cells. We expected the CD4 cells to support the function of the CD8 cells and the procedure to be slightly more physiological than culturing only separated CD8 cells.

CD8 cells were investigated in the assay, but the experiment was not antigen dependent as that would have required more advanced techniques with immunized cells. Thus, the cytotoxicity of the CD8 cells is based on the alloreaction and potentially cancer antigens on the target cells recognized by the T cells. It cannot be 100 % concluded by this experiment that antigen recognition is not affected by the treatment. However, others have shown promising results with TH1579, where no effect was seen on T cell cytotoxicity *in vivo*, upon treatment with TH1579 (121).

For the MTH1 study, the Effector cell: Target cell (E:T) ratio was set to 10:1, where both CD4 and CD8 cells were among the effector cells, making the real E:T ratio somewhat smaller considering only CD8 cells. When the study was later repeated for the OGG1 preliminary data, only A3 cells were used as target cells, but instead different E:T ratios for quality control. As the efficacy of the T cells were quite high already in the lower E:T ratios, the assay could potentially be optimized even more, with a shorter co-culture period.

3.5 ETHICAL CONSIDERATIONS

The experiments included working with cell lines, primary cells from the blood bank, primary patient samples, and animal models in **Paper I-III**. For the animal models and patient samples, ethical permits were required and specified in the publications. The ethical permits consider the cost-benefit situation, i.e. cost for the animals in relation to the benefit of humans. The permits naturally only regulate the scientific practice to minimize the suffering of the experimental animals – the *in vivo* studies lead to disease and death of the animals nevertheless. As animal models are needed to fully understand potential human benefits, it is hard to exclude them from drug discovery.

The 3R's for animal research were followed – Replace (if possible, perform an alternative experiment without animals), Reduce (try to get as much information out of as few animals as possible) and Refine (perform well planned experiments to make sure the animals suffer as little as possible, taking into considering the procedures and housing of the animals).

For human samples, the need for an ethical permit arises when there is either an invasive intervention specifically for the study (like a blood sample that would not be taken otherwise) or if the sample can be traced back to the individual. For blood from the blood bank in **Paper III**, none of those requirements were fulfilled as the blood was donated for healthcare anyways and there was no way for the researcher to trace back the sample to the donor. The study with the skin patient samples in **Paper II** was approved by the Institutional Review Board at Linköping University (Sweden) and all participants had given their written informed consent.

There are many ethical considerations in all forms of life sciences, not only directly tied to the experimental subjects – academic research is to a large extent funded by tax money and experimental outcomes will work as a base for the decision to either continue or reject work that could lead to potential treatments of severe diseases. Poor planning and inadequate experiments will waste both tax funding and when applicable biological material from donors willing to donate material from their bodies to science. To our best abilities, all studies were planned thoroughly in this thesis.

The nature of academic research creates a constant balance between what is fundable, feasible and meaningful. What is interesting for the scientist, is not always interesting for the funder or the patient. Especially within the field of drug discovery like this thesis, commercial interests might be pronounced, and several conflicts of interest could affect the science in an ethical manner. All known conflicts of interests regarding the compounds and patents in this thesis are stated in the papers.

Moreover, it is easier to reach the clinic and market with novel drug candidates when providing the mechanism of action. In reality, it can often be enough to provide *a* mechanism of action, but it is difficult to assess if the mechanism demonstrated in a cell line or genetically modified animals is clinically relevant, despite making great efforts to do representative pre-clinical work. This urge to present a mechanism of action in the best available models of diseases that are not fully understood yet, might become an ethical dilemma when caregivers and patients participating in clinical trials are informed with solid data about the mechanism of action, without having a chance to assess if it is representative or not. On the other hand, it is almost impossible to fully know the mechanisms before testing the compounds in a patient, and the regulations require the best available models for validation. Having too strict regulations would instead make it even harder for functioning drugs to reach the patients.

As for TH1579 in **Paper III**, this thesis was not bound to the clinical trials, but we continued to assess new functions of the compound in the pre-clinical models. Any important adverse findings could have affected the clinical trials, which also opened a new dimension of ethical considerations for the thesis when planning experiments.

4 SUMMARY OF RESULTS

4.1 OGG1 INHIBITOR TH5487 SUPPRESSES INFLAMMATION

Paper I starts with the biochemical validation of the compound, and then moves on to one of the focuses of this thesis – the immunosuppressive effects of TH5487. The paper demonstrates anti-inflammatory effects on both human and murine cell lines, and proof of concept in a murine model of nonmicrobial acute pneumonia. The effect of the compound is compared to genetic KO of the OGG1 protein, and target engagement is also validated. Lastly, a model of the role of OGG1 for inflammation is presented, where OGG1 identifies 8-oxoG at guanine-rich pro-inflammatory gene promoter areas in DNA and attracts pro-inflammatory transcription factors. Treatment with TH5487 inhibits the binding of OGG1 to DNA as well as the recruitment and assembly of the transcriptional complex, thereby down-regulating the expression of proinflammatory mediators (Fig 4.1).

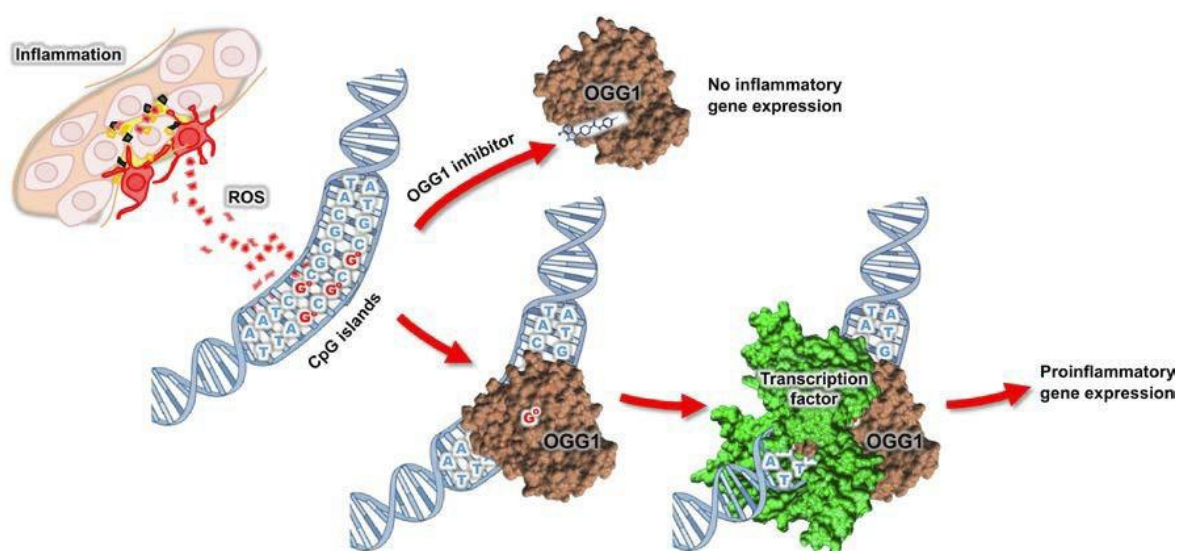


Figure 4.1. The suggested model of how inhibition of OGG1 suppresses inflammation. Inflammatory signaling involves induction of ROS and ROS induce inflammation. Oxidized guanine in promoter regions attracts OGG1, which further attracts transcription factors. Inhibition of OGG1 prevents OGG1 from binding to the DNA and thus the induction of pro-inflammatory gene expression is inhibited. Figure from Paper I and reprinted with permission.

4.2 THE IMMUNOSUPPRESSIVE EFFECT OF TH5487 IS COMPARABLE TO DEXAMETHASONE, BUT TH5487 DOES NOT SUPPRESS T CELLS

As a follow-up to **Paper I**, we investigated the effect of the inhibitor on T cells, and the effect on pneumonia was compared to dexamethasone. The IC_{50} value in T cells was about 20 μM (Fig. 4.2A), which was well above the efficient concentrations in the anti-inflammatory assays

in **Paper I** as well as previous works with cancer and fibrosis (224, 227). Cell count and apoptosis were affected at 25 μ M but not 2.5 μ M (Fig. 4.2 B-C).

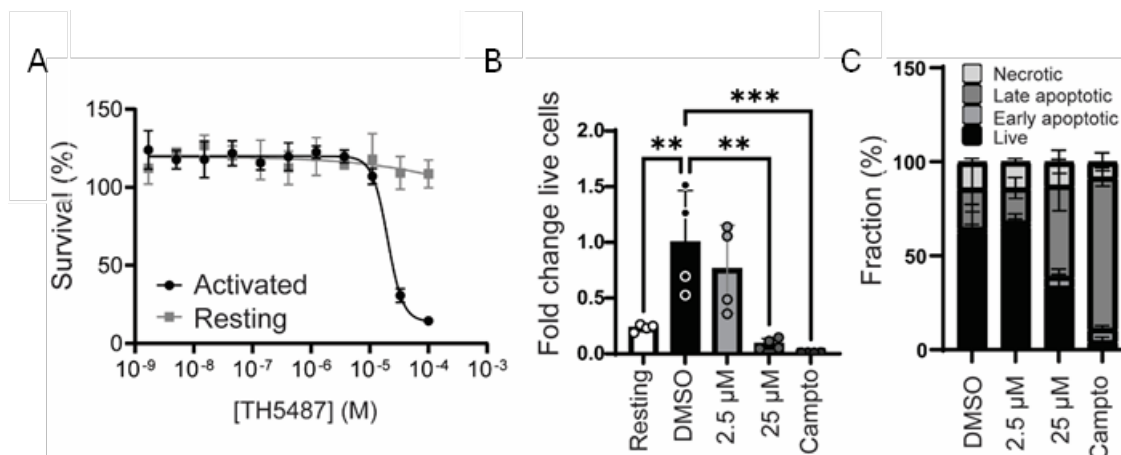


Figure 4.2. Survival of T cells upon treatment and simultaneous activation. A) Resazurin assay, showing the survival as compared to untreated cells (100%) of resting and activated T cells after 96 h of treatment and simultaneous activation (compound added 15 min before activation). B) Relative cell count (flow cytometry) as compared to the DMSO control after 96 h of treatment and activation. C) Apoptosis assay (flow cytometry) using Camptothecin (Campto) as a positive control. (*Unpublished*)

In concordance with the viability data in Fig 4.2, proliferation, activation, and cytotoxic function was also investigated, with effects at 25 μ M but not 2.5 μ M (Fig. 4.3). Cells were able to proliferate at 2.5 μ M after 96 h of stimulation (Fig.4.3A) and expressed normal amounts of CD71 at 2.5 μ M but a lower level at 25 μ M (Fig. 4.3B). When investigating the cytotoxic function of T cells, the T cells were able to kill off A3 cancer cells equally to untreated cells, but despite the compound itself being toxic to cancer cells at 25 μ M, it also inhibited the killing by the T cells (Fig. 4.3C).

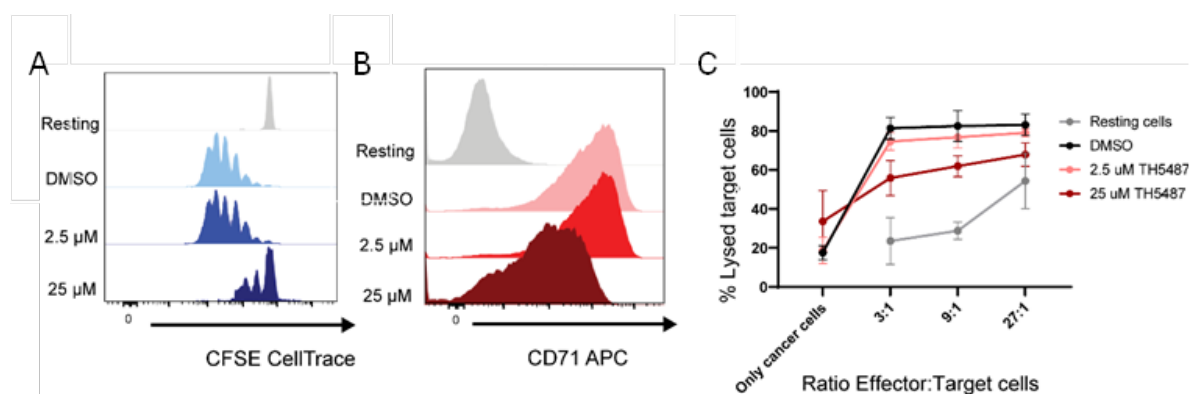


Figure 4.3. Activation and function of T cells upon treatment with TH5487 for 96 h. The compound was added 15 min before activation. A) Proliferation of T cells using CFSE CellTrace in flow cytometry. B) Expression of activation marker CD71 on the surface of cells, flow cytometry. C) Quantification of lysed target cells (A3 cancer cells) after a 10 h co-culture period with or without the compound, following the 96 h treatment and activation period. (*Unpublished*)

As it has been demonstrated that dexamethasone suppresses T cell activity (149, 151, 228), we wanted to test if the effect of TH5487 was comparable to dexamethasone. In an LPS *in vivo* model, like the one in **Paper I**, we could observe that the effect of TH5487 on the immunosuppression was comparable to dexamethasone (Fig. 4.4).

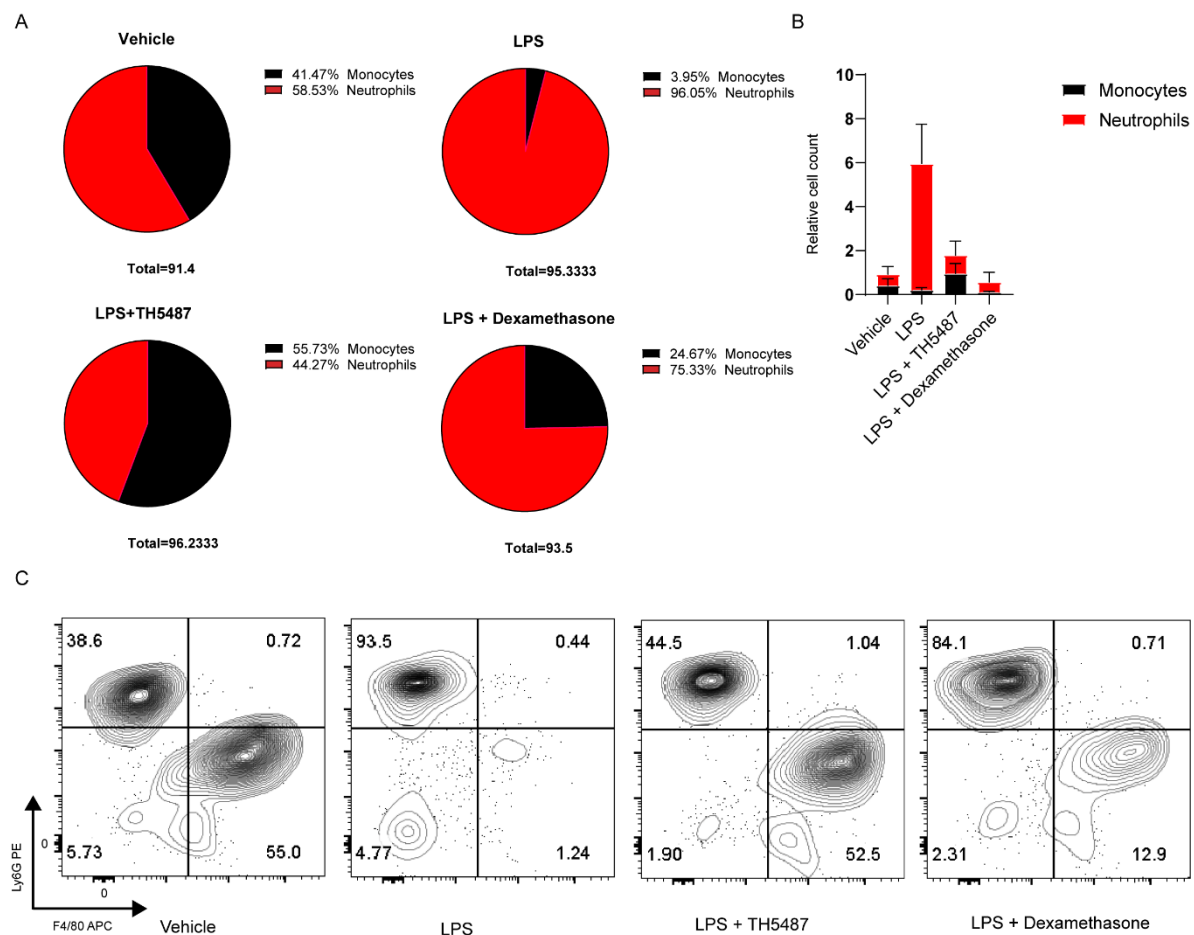


Figure 4.4. The effect of TH5487 is comparable to dexamethasone in an *in vivo* model of LPS-induced pneumonia. Fraction (A) and relative cell count (B) of neutrophils and alveolar macrophages from bronchoalveolar lavage fluids after treatment with either TH5487 or dexamethasone, and stimulation with LPS. C) Dot plots from the flow cytometry assay quantified in A-B. (*Unpublished*)

Conclusively, the results from **Paper I** and the unpublished data suggest that the small-molecule OGG1 inhibitor TH5487 suppresses inflammation in a novel and efficient way, potentially by inhibiting DNA-binding of NF- κ B like glucocorticoids (229), but via inhibiting DNA binding of OGG1. The minimal effect on T cells with the doses relevant for immunosuppression could be a great advantage from an infectious perspective, which is discussed further below.

4.3 MTH1 IS UP-REGULATED IN PRO-INFLAMMATORY TISSUE, AND INHIBITION SUPPRESSES T CELLS IN VITRO AND IN VIVO

Paper II shows that patients have increased MTH1 levels in their psoriatic tissue and in blood. In concordance with previous data, we also confirmed that activated T cells have higher MTH1 levels than resting, and that they can be selectively killed by MTH1 inhibition.

The data from **Paper II** and **III** conclude that T cell driven diseases such as psoriasis and MS can be ameliorated with MTH1 inhibition. The mice presented with better clinical scores upon treatment with MTH1, and several biological markers where also improved. The toxicity on other immune cells and the bone marrow was negligible with the concentrations needed for therapeutic effect.

The mechanism of action for T cell toxicity was also demonstrated, where TH1579 induced DNA damage and cell cycle arrest, and disrupted the mitotic spindle in **Paper III**. Proliferation was also inhibited, as well as expression of CD71. It was also shown that specifically IL-17 signaling was suppressed, and IL-17 producing $\gamma\delta$ T cells were fewer after MTH1 inhibition in mice in **Paper II**.

Investigating the cytotoxic effect of stimulated and treated T cells, no suppressing effect was observed. Instead, the ability to kill target cancer cells remained, in concordance with previous studies (121) (Fig. 4.5).

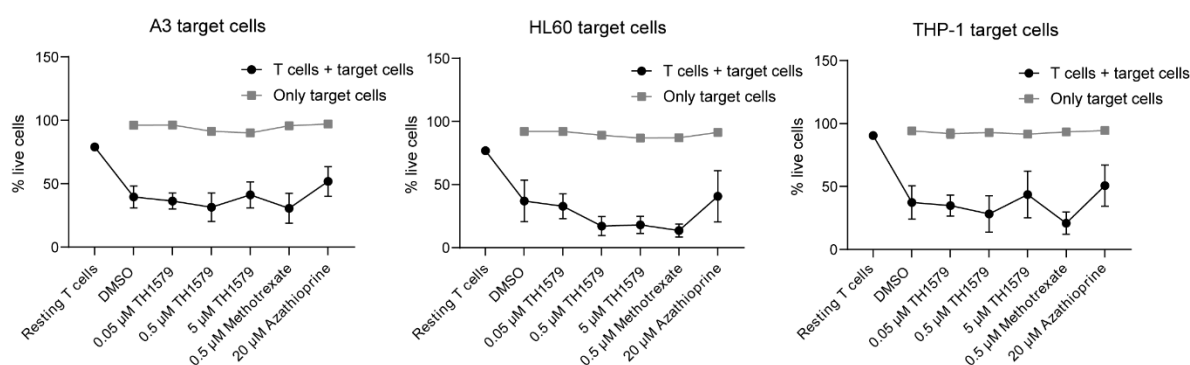


Figure 4.5. No effect on the cytotoxic activity of T cells. Separated CD3+ T cells were activated and treated with compound for 96 h, prior to a co-culture period of 10 h with an Effector:Target ratio of 10:1. Flow cytometry. Graphs show the viability of target cells. N = 4. (*Unpublished*)

4.4 THERE IS A HETEROGENEITY IN MTH1 AND ROS LEVELS, AND TH1579 DRIVES ACTIVATED T CELLS TOWARDS AN MTH1^{LOW}ROS^{LOW} PHENOTYPE

In concordance with what has been observed in cancer cells (58, 86), the ROS and MTH1 levels correlated with sensitivity to MTH1 inhibition in a dose-dependent manner in both keratinocytes and T cells. ROS induction increased MTH1 levels and made the cells more sensitive to MTH1 inhibition, both when caused by a glutathione synthesis inhibitor (**Paper II**) or TCR stimulation in T cells (**Paper III**).

Furthermore, it was demonstrated that MTH1 inhibition and simultaneous TCR stimulation drove the T cells towards an $\text{MTH1}^{\text{low}}\text{ROS}^{\text{low}}$ phenotype (Fig. 4.6). The heterogeneity in MTH1 levels among activated T cells was a novel finding on T cell biology.

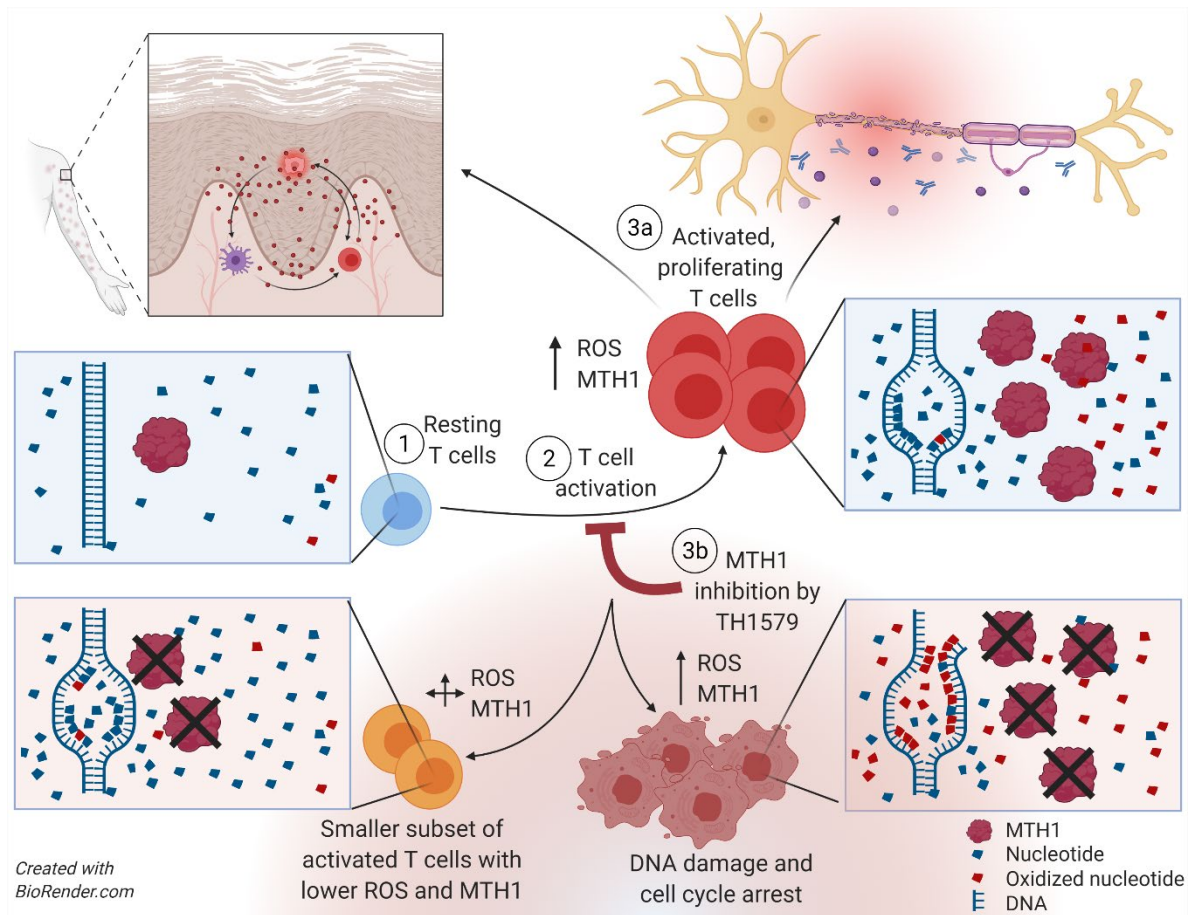


Figure 4.6. Illustration of the findings in Paper III. Activated pro-inflammatory T cells naturally have increased ROS and MTH1. Upon MTH1 inhibition, the $\text{MTH1}^{\text{high}}\text{ROS}^{\text{high}}$ cells die, and the remaining T cells are driven towards an $\text{MTH1}^{\text{low}}\text{ROS}^{\text{low}}$ phenotype, with less inflammatory activity. *Figure created with BioRender.com.*

5 DISCUSSION AND FUTURE PERSPECTIVES

The indications for the compounds assessed in this thesis cannot be fully known without clinical trials, but the studies support several potential areas of use. TH1579 is already in early clinical trials against cancer. Both TH5487 and the MTH1 inhibitors were originally developed as anti-cancer drugs, with focus on the mechanism on the cancer cells instead of immunomodulation.

5.1 OGG1 INHIBITION IN ACUTE INFECTIONS

For acute inflammation caused by infections, the treatment options are few, other than antibiotics, antivirals and antifungals, in combination with symptomatic and life-supporting care. TH5487 inhibits airway inflammation in the mouse models of **Paper I** and the preliminary data. This is in line with studies showing that OGG1 KO mice are more resistant to sepsis than WT mice (91) and that other OGG1 inhibitors work against bacterial sepsis (97). Thus, TH5487 could potentially be used for the treatment of sepsis and ARDS. The preliminary data suggest that there would be an advantage over corticosteroids, as TH5487 was not toxic to T cells (Fig. 5.1).

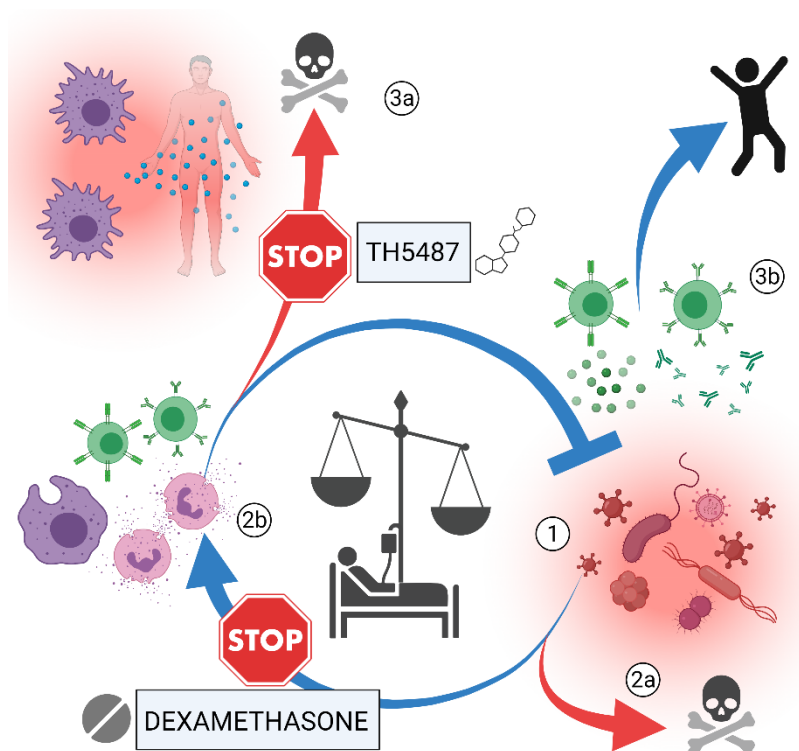


Figure 5.1. Proposed model for the advantage of TH5487 over dexamethasone. 1) Pathogens enter the body and can lead to death (2a) unless a proper immune response is induced. 2b) An immune response is induced to clear out the pathogen and recover (3b). In sepsis, the immune response is dysregulated and can thus lead to death (3a). Dexamethasone acts broadly and suppresses both the efficient and non-efficient branches of the immune reaction, whereas TH5487 would possibly only suppress the dysregulated hyperinflammation driven by cytokines, macrophages and neutrophil infiltration. Lymphocytes would still be able to clear the disease (3b). *Figure created with BioRender.com.*

In **Paper I**, several cytokines, like TNF- α , IL-6, CXCL2 and CCL2 are suppressed upon TH5487 treatment. They are central in the first-line innate response to inflammatory stimuli, and affect the tissue response further. Suppressing them would not necessarily result in an abolished ability to fight microbes and cancer, especially if the lymphocytes are unaffected. Cytokines like TNF- α and IL-6 might even promote tumor progression and up-regulation of IRs, so theoretically the cytokine suppression by TH5487 in **Paper I** could have anti-cancer effects (230). The effect is demonstrated in both human and mouse cell lines, but it would naturally have been an advantage to also assess the effect in primary human macrophages and endothelial cells, and ultimately to measure cytokines from the study animals.

In **Paper I** it is further demonstrated that the neutrophil infiltration is suppressed by TH5487. The effect is most prominent when treated prophylactically, but there is also an effect after disease onset. The latter is of particular interest, as the patients seek care when presenting symptoms of an ongoing infection. The compound could possibly suppress pro-inflammatory signaling from the cells that are already activated with 8-oxoG in their promoter regions attracting OGG1, and it would prevent additional inflammatory signaling from new oxidation and stimuli by the pathogens and damaged cells still present.

As it has been shown that OGG1 KO mice have more 8-oxoG than WT mice (231) and as we show in **Paper I** that TH5487 increases 8-oxoG, there is a risk that an inflammatory hyperreaction would take place after treatment withdrawal due to an excessive amount of 8-oxoG and free access to OGG1. This has not been studied specifically in this thesis, but such tendencies were seen when treating subjects with MTH1 inhibitors, also inducing 8-oxoG, in unpublished work related to **Paper II-III**. Both zebrafish and the recovery animals from the TDAR study in **Paper III** showed clinical signs of overstimulation in the form of zebrafish embryo death and rat hypomobility when stimulated with Poly(I:C) and KLH immunization respectively, after a treatment period with MTH1 inhibition (unpublished). Considering the explanation model where 8-oxoG attracts OGG1, which further drives inflammation, a hyperreaction due to elevated 8-oxoG without OGG1 inhibition is quite logical, and important to take into consideration from a safety perspective. Potentially the problem could be avoided by slowly phasing out the dosing of OGG1 inhibition when ending the treatment, instead of stopping abruptly. This could also be relevant when using the compounds in cancer settings. Furthermore, the mice in the studies are often euthanized and investigated quickly after the treatment and intervention, which could cause such complications to remain unknown, especially without a second immune stimulation and treatment withdrawal.

5.2 OGG1 INHIBITION BEYOND SEPSIS

TH5487 has also shown promising effects in cancer, as shown in Paper V (227). In addition to cancer and sepsis, many other diseases are also driven by acute inflammatory stimuli, like burns, trauma, pancreatitis and other tissue damaging causes. Myocardial infarcts and strokes belong to some of the most common diseases in the population. In both cases, ischemia is induced due to a clogged artery, and the treatment mainly consists of opening the obstruction or bypassing the flow. However, the actual damage leading to the sequelae are mainly caused

by the inflammation afterwards, which spans over a much larger area than the actual hypoxic area (232). In theory and based on **Paper I**, TH5487 could potentially protect against the inflammation and further tissue damage following the hypoxia and reperfusion in such conditions, like stroke.

As illustrated in Fig. 5.2, injuries and infections cause inflammatory signaling via DAMPs and PAMPs, causing immune cell infiltration and cytokine secretion. The response is initially driven by innate cells, where many of the cytokines in the panels of **Paper I** could be relevant, like CCL2 and IL-1. It is still unlikely that OGG1 inhibition would restore damaged cells that have been subject to hypoxia, viruses or trauma – it could even be more toxic to them or drive mutations instead of apoptosis – but it could lower the pro-inflammatory signaling and response of non-damaged cells that would drive the inflammation and sequelae further. Conclusively, it is difficult to predict the role of OGG1 in injuries and hypoxia-reperfusion contexts, and it would require advanced experimental models to study it in a representative way, but the data in **Paper I** is promising for many types of inflammation.

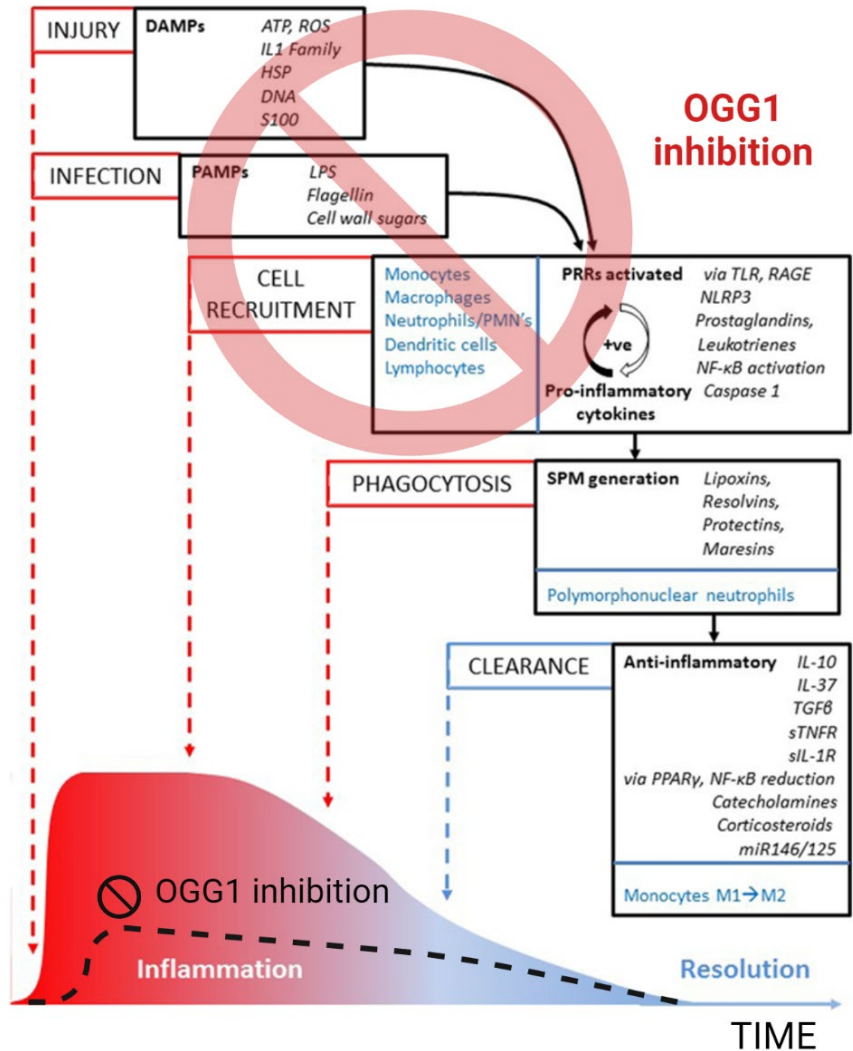


Figure 5.2 Overview of the immune reaction and OGG1 inhibition in different inflammatory scenarios. An injury or infection initiates an immune response via DAMPs and PAMPs, which could possibly be hindered by OGG1 inhibition. Inflammation is further driven by more cells and cytokines, which can cause more damage on the way towards clearance. Reprinted and carefully modified with permission from Frontiers, Frontiers in Immunology, Maeve Rea et al 2018 (17).

As it is shown in **Paper I** that TH5487 induces 8-oxoG, it is unclear if it would be suitable for chronic treatment. However, it has been suggested that TH5487 has anti-fibrotic properties (224), and as mentioned above, OGG1 KO mice live and grow old. Considering that some established chronic treatment options, like AZA (233), also induce mutations but still are suitable for chronic use, it is not yet known if TH5487 would be suitable or not.

As a final note on OGG1 inhibition, it is worth mentioning that the unpublished manuscript Paper VIII, not included in the thesis, presents OGG1 enhancers instead of inhibitors. Paradoxically, the enhancers would not necessarily induce more inflammation, as they are suggested to alter the dynamics of OGG1 and lower the time that OGG1 is bound to the site of the DNA (unpublished). There is thus a chance that also the enhancers would lower inflammation, but without increasing 8-oxoG as the inhibitors do. However, for acute inflammation with an increased amount of 8-oxoG in the genome, temporary treatment with an inhibitor could theoretically be less toxic than an activator, as an OGG1 activator could induce an excessive amount of DNA breaks in the presence of high levels of 8-oxoG, with the risk of becoming toxic. On the other hand, less 8-oxoG could also be less toxic.

It remains to be investigated in future studies, which approach that is more promising for the patients suffering from inflammation – inhibition or activation of OGG1.

5.3 MTH1 INHIBITION IN T CELL DRIVEN DISEASES

Finding new ways to alleviate T cell driven diseases is of great importance to offer efficient and safe treatment options for patients that suffer from severe symptoms despite treatment. In **Paper II** and **III**, we suggest a new approach to selectively suppress T cells, and in **Paper III** we compare it with the established drugs MTX and AZA. Potentially, TH1579 could be used for similar indications as MTX and AZA, but when the established drugs are not enough or cause too severe adverse effects.

In **Paper II** and **III**, the anti-inflammatory properties of TH1579 and single inhibition of MTH1 are demonstrated in human keratinocytes and T cells for the first time, in line with recent data on acute autoimmune hepatitis (Paper VI). The up-regulation of MTH1 in activated leukocytes has been described before (120), and it is close at hand to conclude that potentially MTH1 inhibition could be used for many other T cell driven diseases too (Fig. 5.3), like rheumatoid arthritis, inflammatory bowel disease, diabetes, graft-versus host disease (GvHD) and acute immunological disorders like Stevens-Johnson syndrome.

As TH1579 has been validated for the use against both solid and leukemic cancers, and the compound is under ongoing clinical trials for both cancer types, it is reasonable to consider TH1579 controversial in cancer treatment due to the T cell suppressing findings in **Paper III** from an oncoimmunologic perspective. However, as the preliminary data suggests, there is no evidence suggesting that the cytotoxic ability of the T cells would be altered, despite the decrease in cell number and shift towards MTH1^{low}ROS^{low}. This is also supported by other

studies (121), but it was interesting that MTX did not affect the cytotoxic activity either (but MTX killed almost all T cells, which required an over 10-fold adjustment to create the 10:1 E:T ratio of the remaining live cells). Instead, the selective effect on activated T cells could even be beneficial in contexts where both an anti-cancer effect and anti-inflammatory effect would be desirable, which is the case for many cancer patients.

Today, patients receiving allogeneic bone-marrow transplants initially receive CsA to suppress the donated T cells and thus avoids GvHD. But CsA has little effect on the cancer cells, except for some proven effect on small-cell lung cancer (126, 234). As TH1579 has been shown to have an effect on acute myeloic leukemic cells (235), it could potentially be a very good option for transplanted patients, to avoid both GvHD and cancer relapses. The graft-versus-cancer effect is suppressed by CsA whereas TH1579 would have an anti-cancer effect directly on the cancer cells in addition to the T cells suppressing effect.

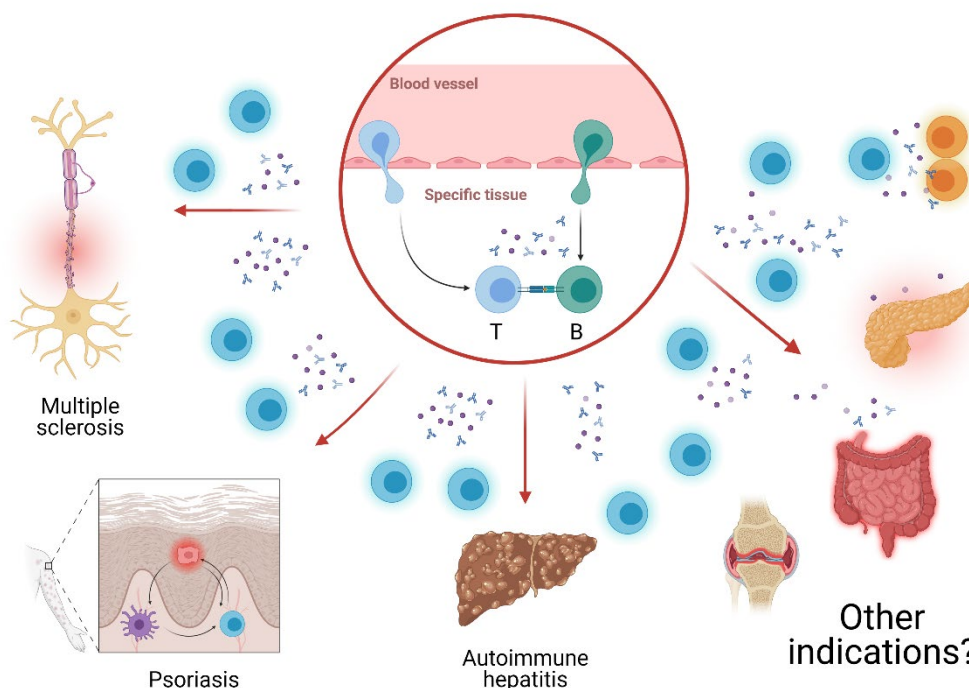


Figure 5.3 Potential inflammatory indications for MTH1 inhibition. Examples include MS, psoriasis, autoimmune hepatitis, rheumatoid arthritis, inflammatory bowel disease, diabetes, graft-versus-host disease. *Figure created with BioRender.com.*

To elucidate potential indications for TH1579 and MTH1 inhibition, further studies investigating T cell subtypes and cytokine signatures would be needed. If a specific subtype would turn out to be more sensitive than the other, such as Tregs or tissue-specific lymphoid cells, that could impact for what disease the treatment would be suitable. The role of T regs in inflammatory diseases is diverse and controversial (236, 237), and a stronger selectivity for Tregs over for example Th17 cells could result in adverse effects. However, the therapeutic effect seen in the different disease models in **Paper II, III** and VI makes it easy to assume that most likely pro-inflammatory cells are more sensitive than suppressive cells.

5.4 MTH1 HETEROGENEITY AMONG T CELLS

MTH1 levels had not been investigated thoroughly before in T cells, except for the study from 1997 where Oda et al. demonstrated higher levels in activated leukocytes, and even higher so in transformed blood cells (120). Previous studies suggest a model where MTH1 up-regulation is a consequence of high ROS levels (86), but MTH1 could also have immunomodulatory roles yet to be discovered.

In **Paper III**, interestingly the MTH1 increase was evident already after 24 h, whereas the ROS levels only showed slight tendencies to increase during the first 24 h, raising more after 48 h. If ROS induce up-regulation of MTH1, the opposite would be expected, unless the elevated ROS pressure is already present and sensed by the cells after 24 h, but compensated for during the first days. As described in section 1.3, TCR stimulation is tightly bound to ROS, but the question is whether the TCR associated ROS is enough for inducing MTH1 directly due to the ROS pressure. Another option is that MTH1 plays a direct part in the role of activating T cells and their differentiation upon TCR stimulation, independently of ROS.

To study this further, it would be of great interest to investigate the immune system of MTH1 KO mice, including TCR and T cell signaling, activation, function, and ROS status. Experiments on MTH1 KO cells was one of the aims of the thesis, where the results were planned to be in **Paper II** next to the psoriasis experiments with WT mice. However, it was later found out that the MTH1 KO mouse strain was a crossing of SKH hairless and C57/Bl6, and the SKH strain is described to be unsuitable for induction of psoriasis with Imiquimod (238). As the Imiquimod mouse model was the only inflammatory model we used for the KO mice, it was not representative to assess more inflammatory parameters of the mice, as was done for the WT mice in **Paper II**. Surprisingly, the T cells from the KO mice were equally capable to proliferate as compared to WT T cells when stimulated *in vitro* (unpublished). It would be intriguing to investigate this further, looking at T cells subsets, ROS status and metabolic profile of the KO cells, to see if they tend to have lower ROS like the MTH1^{low}ROS^{low} cells in **Paper III**, and to assess their ability to kill target cells.

Tissue-specific immunity is also an emerging field relevant for many diseases. Blood is readily available as opposed to tissue specific immune systems with $\gamma\delta$ T cells and innate lymphoid cells, and so the blood leukocytes are much more studied than tissue specific leukocytes. However, **Paper II** demonstrates that IL-17 producing $\gamma\delta$ T cells are selectively suppressed. It would be of great interest to study IL-17 producing $\gamma\delta$ T cells of the MTH1 KO cells, as well as cytokines associated to the IL-17 axis in the cancer patients from the clinical trials. As it has been shown that meningeal IL-17 producing $\gamma\delta$ T cells are of importance for brain development and affect the memory and synaptic plasticity (239), and as MTH1 is described to protect also the brain from 8-oxoG (209), it would be valuable to assess the cognitive function of MTH1 KO mice. However, in case the KO mice would lack meningeal $\gamma\delta$ T cells, TH1579 treatment would not necessarily be a problem, if the meningeal $\gamma\delta$ T cells are critical for memory functions mainly during development.

5.5 TARGETING DNA REPAIR ENZYMES AND ALTERING 8-OXOG FOR IMMUNOLOGICAL INDICATIONS

The work in this thesis focuses on suppressing the immune system. Targeting DNA repair and the immune system nonspecifically, especially T cells, could be associated with severe infections and even induce cancer in the long run, but that is a general issue for many established cancer- and immunomodulating drugs. In **Paper III** we show that TH1579 is selective for activated T cells, that other cells of the immune system are rather unaffected and that no significant general immunosuppressive effect was observed in the KLH study at the doses investigated. Only a transient leukopenia and a trend towards lower immunoglobulin responses in the highest doses was found. It was also shown that effector memory T cells are suppressed, but rats were still able to produce an antibody response with analogous doses. This could suggest that TH1579 does not deplete the ability to form immunologic memory, although the formation of specific T cell memory might be reversibly suppressed under ongoing treatment in mice, as well as antibody formation with high doses. As discussed above, there is also no indication that TH5487 would inhibit the antitumor response, despite its immunosuppressive effect demonstrated in **Paper I**. Taken together, both compounds could be suitable for both cancer treatment and immunomodulation, and the potential side effects might not be worse than the ones of drugs like AZA and MTX, possibly even less.

However, targeting a DNA repair enzyme is controversial, and even though MTH1 and OGG1 KO mice live and grow old, they develop tumors (94, 240, 241). Humans live longer than mice and might thus have even more tumors than the MTH1 KO mice, although the mice only have a slightly increased tumor rate. On the contrary, also AZA (73) and MTX (10) increase the cancer risk over time, both through their broad immunosuppressive effects and by inducing mutations. Equally the beneficial effects of MTH1 inhibition might still overcome the adverse effects. Furthermore, activated T cells are short-lived in relation to most other cells of the body, and so the window for potential mutations to originate is smaller than for established drugs that affect all dividing cells non-specifically, and still are suitable for chronic treatment.

Nevertheless, the relationship between MTH1 and tumor development remains unclear, as for example MTH1 KO mice have longer survival with crocidolite induced malignant melanoma when compared to OGG1 KO and MUTYH KO mice (242). Interestingly, it was shown in 2003 that MTH1 KO can reverse the tumor susceptibility of mice deficient in OGG1, since double KO of MTH1 and OGG1 are less prone to tumors than single OGG1 KO, despite the higher amount of 8-oxoG in the animals (231). The authors speculate that this could be due to an accumulation of oxidized ATP and dATP which would contribute to tumor suppression, since MTH1 hydrolyses all dNTPs and not only dGTPs (243), or that the generation of the KO mice resulted in co-transmission of some tumor suppression genes (231). However, that would not explain why the single MTH1/OGG1 KO mice are prone to tumors (240). Moreover, in 2021 it was concluded that MTH1 depleted cancer cells are *less* sensitive to OGG1 inhibition than WT cells (244), again shedding light on the controversial role of double KO or double inhibition.

Particularly controversial is also the fact that MTH1 and OGG1 double KO mice are more prone to develop Alzheimer's disease. The effect seems to be driven by inflammation through activation of microglia, possibly due to the increased 8-oxoG (245). This makes inhibition of OGG1 to treat inflammation like in **Paper I** more complicated, as well as chronic inhibition of MTH1 if the consequence is increased 8-oxoG.

The fact that both OGG1 and MTH1 as DNA repair enzymes are involved in inflammatory signaling might not be a coincidence as DNA damage is tightly involved in inflammation. Guanine is vulnerable to oxidation (73), and from an evolutionary perspective it is quite logical that the DNA-binding of the repair enzyme itself creates a quick although somewhat non-specific pro-inflammatory response to DNA damage. As an example, TNF- α induces ROS and DNA damage in the cells as a significant part of its mechanism of action (246). Moreover, if lower MTH1 induces 8-oxoG in the cells, the activity of OGG1 would be expected to increase upon MTH1 inhibition, which is also seen in many inflammatory diseases for OGG1, like MS and inflammatory bowel disease, whereas MTH1 expression remains somewhat controversial in both (87, 88).

In **Paper I** it is suggested that OGG1 binding to the promoter regions of pro-inflammatory genes is crucial for the function of OGG1 in inflammation. This has later been supported by others, where the catalytically dead OGG1 mutant K249Q induced more inflammation than WT OGG1 (247). However, that does not exclude other additional mechanisms of action. Studies also suggest that the excised free 8-oxoG could act as a second messenger (79, 99, 108). It would be fascinating to investigate this further by affecting the guanine pool through addition of 8-oxoG, inhibition of the guanine synthesis with compounds like Methotrexate and Mofetil, and over-expressing guanine deaminase to enhance its depletion. Assessing the immune response could then give a clue on the role of the guanine pool in the cell.

Altogether, this illustrates the controversial role of MTH1, OGG1 and 8-oxoG as factors involved in DNA repair, immunology and cancer, and there is still a lot to discover.

Furthermore, it remains to be discovered what role free dNMPs and dNTPs play for inflammatory signaling – if they could act as important regulators and if a skew in the oxidized dNTP/dNMP ratio could result in altered inflammatory signaling. Due to the vulnerability of guanine, dGTPs would be the most vulnerable with potentially the highest relative increase of oxidized dNTP, although oxidized dATP have the highest affinity to MTH1 of the nucleotides, and constitute the largest dNTP pool in the cells (84). As for the dNTP pool, there are many pathways and enzymes controlling it (248) to preserve genomic stability, but more studies on the direct effects of altered ratios in the dNTP pool, oxidized nucleotides, excised free oxidized bases and ATP- and GTPases are needed in non-cancerous cells, in order to elucidate their role in inflammation. Already today, several established drugs, like antifolates and nucleotide analogs, affect nucleoside and nucleotide metabolism with anti-inflammatory, anti-cancerous and anti-viral effects (249-253). Potentially many of the established anti-cancer drugs acting on DNA repair and cell cycle also affect the nucleotide metabolism in immune cells, through mechanisms yet to be discovered.

5.6 FROM BENCH TO BEDSIDE, OR THERE AND BACK AGAIN

All the **Papers I-III** included in the thesis, but also the additional IV-VIII, are about inhibitors originally developed for cancer treatment against the targets MTH1, OGG1 and MTHFD2, and in **Paper I-III** we elucidated the role of MTH1 and OGG1 inhibitors in inflammation. It could be considered a coincidence that the inhibitors have immunomodulatory effects in addition to the cancer effect. The altered ROS signaling of the immune cells and may cancer cells could explain the connection. On the other hand, the immunomodulating effects of many established drugs described to have other mechanisms of action are not always very thoroughly investigated, although the phenomenon has gained some attention over the years (254, 255).

As discussed briefly in the Ethical consideration-section, the road from pre-clinical trials to clinical trials can be significantly shortened when providing a mechanism of action. Despite this, as many as 97 % of drug-indication pairs tested in clinical oncology trials never advance to receive FDA approval, and off-target toxicity is a common mechanism of action of cancer drugs undergoing clinical trials (187, 188). One reason to the failure could be a lack of proper models where the whole organism, including the immune system, is accounted for. A large part of cancer research on drug-discovery focuses on cell lines, and even in the *in vivo* models, the cancer disease is typically represented by immunocompromised animals transplanted with cancer cell lines (256) (cf. Paper IV-V). Based on the effect on the cancer cell lines, the drug candidate then advances to the clinic, without much knowledge about the immunological effects, except for toxicology studies to exclude dangerous immunosuppression. These approaches with transplantations of cell lines in immunosuppressed animals are generally accepted as valid methods for proof of concept, despite the knowledge of the intra- and intertumoral heterogeneity, tumor microenvironment and stochastic nature of disease initiation (189, 190, 192). On the other hand, the lack of appropriate animal models is not unique for cancer research, and could also partly explain the struggles with creating new sepsis treatments over the past decades (140).

It was thus interesting to discover the immunomodulating roles of the inhibitors in **Paper I-III**, in addition to the earlier described effects on cancer cells. The immunological effect can have a great impact on the treated patients, despite not being determinant when entering clinical trials. Today great resources are put on sequencing the single cells of the tumor, but the translatable and clinical value has often been modest, and it is still difficult to predict prognosis and treatment compatibility despite the new techniques. By not assessing the immune system thoroughly, we might miss important findings, reject functional drug candidates, and not use established drugs in the most optimal ways.

In **Paper III**, the tubulin destabilizing agent Vincristine is used as a positive control. Interestingly, both Vincristine and the tubulin stabilizing agent Paclitaxel have been described to have immunomodulating properties beyond their cytotoxic effects on cancer (257, 258). Moreover, it has been suggested that part of the effect of TH1579 and its analogues is also via destabilization of tubulin (210, 213, 235). It would thus be interesting to study and compare the

immunomodulating effect of more established cancer drugs, also beyond anti-tubulin agents, and compare them further with MTH1 and OGG1 inhibitors.

Conclusively, the immune modulating effects of DNA damaging radio- and chemotherapy has gotten more and more attention over the past years. There is still a lot to discover to elucidate why some patients respond to therapy and others do not, when the answer cannot be not found in the tumor cells despite advanced molecular methods and proposed mechanisms of action. This was not the main focus of the thesis, but the work in **Paper I-III** are examples of significant immunomodulating roles of DNA damaging agents designed for cancer treatment, highlighting the role of DNA damage and immunophysiology and -pathology. It is possible that only the very tip of the iceberg has been discovered regarding immunological effects of conventional treatments such as anti-microtubule agents, alkylating agents, antimetabolites, topoisomerase inhibitors and radiotherapy, as well as how age, gender and co-morbidities affect clinical outcomes beyond what is known today.

6 CONCLUDING REMARKS

This doctoral thesis focuses on the inhibition of the DNA repair enzymes OGG1 and MTH1 in pre-clinical settings. OGG1 was well-known from before in the field of inflammation, whereas MTH1 was mainly assessed in cancer settings.

The **Papers I-III** and the preliminary data focus on small-molecule inhibitors from a strong drug-discovery perspective, where the inhibitors were assessed both from a toxicology- and efficacy perspective. However, we also provided new biology insights regarding OGG1 and MTH1 – in **Paper I** we support the DNA-binding theory as being the pro-inflammatory mechanism of OGG1, in **Paper II** we confirm that MTH1 is up-regulated in psoriatic patient samples, and in **Paper III** we suggest for the first time that activated T cells exhibit a diverseness in MTH1 levels, bound to ROS levels and activity.

The different therapeutic indications for the inhibitors are also discussed in both **Paper I-III** and the thesis. Potential adverse effects are also considered, but for now there is no indication that the inhibitors would cause more adverse effects than established DNA-damaging agents already approved for use. However, the true effects and side-effects of the treatment cannot be known for sure before challenging the compounds in clinical trials with an accurate patient group.

The work from this thesis thus provides some additional pieces of information to the complicated fight against immunological diseases, including cancers. Hopefully it can also inspire more immunologists to take place in the field of DNA repair, to ameliorate the understanding of physiological signaling of DDR beyond cancer, and ultimately provide new insights to treatment options, prophylaxis, and lifestyle characteristics of common diseases.

7 THE FELLOWSHIP OF THE THESIS

I would like to express my sincere gratitude to all who made this thesis possible, directly and indirectly. To quote my favorite author:

“I don't know half of you half as well as I should like; and I like less than half of you half as well as you deserve.”

To my supervisors:

Thank you, **Thomas**, my main supervisor, for hiring me without hesitation despite me originally contacting you with an e-mail that mistakenly didn't include a header. I had hardly any lab experience and still you gave me the opportunity to now, more than 8 years later, finish this PhD in your lab. Thank you for all the trust and freedom, and for being my only supervisor that didn't quit the Helleday lab before my graduation. Your energy and ability to push projects forward is inspiring, and I developed independence as well as both scientific and soft skills under your management.

Ulrika, my closest co-supervisor. I don't think I would have been able to finish the PhD as such without your support. You joined and took the lead in the supervisor team despite the somewhat unclear circumstances in this immunological project during the first half of my PhD. You helped me with everything from writing to planning experiments for the big picture, and you were always kind, caring and supportive. Thank you!

Thank you **Christina**, my co-supervisor, for stepping in as the OGG1 inflammation team leader and my supervisor in the middle of my PhD journey, and for all your support, discussions and swift answers, whether it was about a draft that needed revision or planning of projects.

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To my former supervisors:

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