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Tissue Damage Control in Autoimmune Diseases

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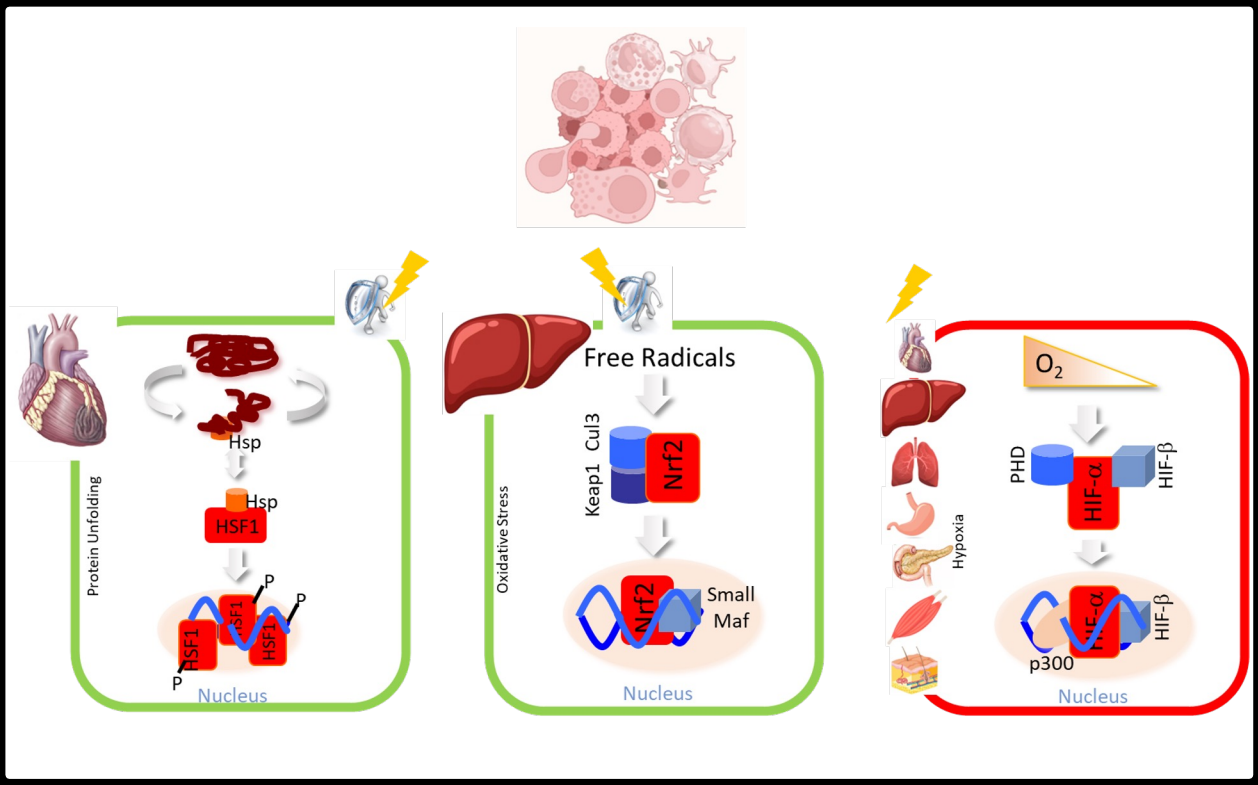

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Abbreviations used

AIRE	Autoimmune regulator
ARE	Antioxidant response elements
BCR	B cell antigen receptor
CAM	Cell adhesion molecules
CC	C-C Chemokine
CCL	C-C Motif Chemokine Ligand
CD	Cluster of differentiation or classification determinant
CSR	Class Switch recombination
CTL	Cytotoxic T cell
CTLA4	Cytotoxic T Lymphocyte Antigen 4
CXC	C-X-C chemokine
CXCL	C-X-C chemokine logand
DAMP	Damage-associated molecular-pattern molecules
DC	Dendritic cells
DDR	DNA damage response
DEREG	DEpletion of REGulatory T cells mouse model
DMOG	Dimethyl-oxaloylglycine
DSB	Double-strand breaks
DT	Dipththeria toxin
DTR	Dipththeria toxin receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic Reticulum
ERK	Extracellular signal-regulated kinase
ERT2	Estrogen receptor
FIH	Factor inhibiting HIF
FOXP3	Forkhead box P3 transcription factor
GLUT	Glucose transporter
GSH	Glutathione
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HRE	Hypoxia response elements
HSF	Heat Shock factor
HSP	Heat Shock protein
HSR	Heat Shock response
ICAM	Intercellular Adhesion molecule
ICOS	Inducible costimulator
IFN	Interferon
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleucin
ILC	Innate Lymphoid Cells
JAK	Janus Kinases
KEAP1	Kelch Like ECH Associated Protein 1
LFA	Leukocyte lymphocite antigen
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility complex
MRN	Mre11, Rad50 and Nbs1 complex
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB essential modulator
NF	Nuclear factor
NK	Natural killer cell
NKT	Natural killer T cell
NRF2	Nuclear factor erythroid 2-related factor 2
ODD	Oxygen-dependent degradation domain
OKD48	Keap1-dependent Oxidative stress Detector, No-48
PAMP	Pathogen-associated molecular patterns
PD1	Programmed cell death protein 1

PGK	Phosphoglycerate kinase
PHD	Prolyl hydroxylase domain
PI3K	Phosphatidylinositol 3-kinase
PRR	Pattern recognition receptors
RAG	Recombination-activating gene
RNA	Ribonucleic acid
ROR	Retineic-acid-receptor-related orphan nuclear recepto
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
SLE	Systemic Lupus Erythemathosus
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
T1D	Type 1 Diabetes
TAD	Terminal activation domain
TAM	Tamoxifen
TCR	T cell receptor
T_H	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{REG}	Regulatory T cell
VCAM	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

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Manuscripts

Vital Da Silva Domingues, Jocelyne Demengeot and Miguel Che Soares. "*Tissue Damage Control in Autoimmune Diseases*" – *in preparation*

Vital Da Silva Domingues, Jocelyne Demengeot and Miguel Che Soares. "*Hsf1 protects myocardium in immune mediated disease context*" – *in preparation*

Summary

Autoimmunity refers to when an immune response is directed against a self-antigen. Autoimmune diseases develop when autoimmune responses disrupt physiological functions and represent a considerable burden in western societies due to the young working-age group affected, chronicity, unpredictable flares and difficult clinical management.

Autoimmune diseases are tangled by common etiological factors that manifest by a diverse array of signs and symptoms according to the cell, tissue structure or organ targeted. The pathogenesis of autoimmune diseases is not fully understood which translates into the difficult clinical management of this group of diseases.

In this thesis, I will expand a concept whereby tissues can counter immune injury via coordinated responses that support cell survival and tissue function. This tissue damage control response is essential to counter different forms of stress and/or damage imposed by autoreactive antibodies and/or immune cells, suppressing the pathogenesis of autoimmune diseases.

The overarching hypothesis being tested is that genetic defects in specific stress and damage responses conferring tissue damage control can modulate the pathogenesis, progression and/or clinical outcome of autoimmune diseases. This hypothesis was tested in mice genetically ablated for Heat Shock Factor (HSF)1, a transcriptional master regulator that limits proteotoxicity, Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional master regulator that controls cellular responses to oxidative stress, Ataxia telangiectasia-mutated (ATM), a kinase that acts as a master regulator of DNA damage repair and Hypoxia-inducible factor (HIF)1α, a transcriptional master regulator controlling metabolic adaptation and tissue cytoprotection to hypoxia. The objective is to provide the "proof of principle" for the involvement of tissue damage control mechanisms in modulating the pathogenesis and/or progression of autoimmune diseases.

Using an inducible experimental model of systemic autoimmune disease triggered by the depletion Foxp3^{+} regulatory T (T_{REG}) cells, we found that mice lacking *Hsf1* in non-hematopoietic cells developed autoimmune cardiac damage upon depletion of regulatory T cells. Non-hematopoietic deletion of *Nrf2* increased susceptibility to autoimmune hepatitis and inducible deletion of *Atm* in non-hematopoietic cells was associated with severe autoimmune pancreatitis. These

findings support the notion that HSF1, NRF2 and ATM contribute to tissue damage control preventing autoimmune-driven deregulation in the heart, liver and pancreas function, respectively. Moreover, this thesis highlights a fundamental role of HIF-1α in promoting inflammation and the pathogenesis of autoimmune diseases, involving the pancreas, liver, stomach, skin and muscle, bringing forward the idea that HIF-1α promotes multi-organ systemic autoimmune disease.

In conclusion, an increasing awareness of autoimmune diseases is contributing to further research and understanding of the complexity underlying the pathogenesis of these diseases, from aetiology, triggers, clinical manifestation and organs targeted. We identified several master regulators of stress and damage responses that act specifically in non-hematopoietic tissues to fine-tune the pathogenesis and progression of autoimmune diseases. These findings should contribute to the development of novel therapeutic approaches limiting the clinical and social impact imposed by autoimmune diseases.

Resumo

Autoimunidade refere-se a uma resposta imune dirigida contra um auto-antigénio. As doenças autoimunes desenvolvem-se quando respostas autoimunes disrompem as funções fisiológicas, representando um fardo considerável nas sociedades ocidentais devido ao grupo etário de idade laboral afetado, pela sua cronicidade, crises imprevisíveis e difícil manejo clínico.

As doenças autoimunes estão associadas a fatores etiológicos comuns e manifestam-se por uma gama diversificada de sinais e sintomas de acordo com a célula, estrutura tecidual ou órgão visado. A patogénese das doenças autoimunes não é totalmente compreendida, o que se traduz na difícil gestão clínica deste grupo de doenças.

Nesta tese, expandirei um conceito pelo qual os tecidos podem combater a lesão imune por meio de respostas coordenadas que suportam a sobrevivência celular e a função do tecido. Essa resposta de controlo de dano tecidual é essencial para combater diferentes formas de stress e/ou dano imposto por anticorpos autorreativos e/ou células imunes, suprimindo a patogénese de doenças autoimunes.

A hipótese abrangente que está a ser testada é que defeitos genéticos em respostas específicas de stress e dano que conferem controle de dano tecidual podem modular a patogénese, progressão e/ou resultado clínico de doenças autoimunes. Esta hipótese foi testada em ratinhos para Heat Shock Factor (HSF)1, um factor de transcrição que limita a proteotoxicidade, fator nuclear eritróide 2 relacionado ao fator 2 (Nrf2), um fator de transcrição que controla as respostas celulares ao stress oxidativo, Ataxia telangiectasia- mutado (ATM), uma quinase que atua como um regulador da reparação de danos ao DNA e fator induzível por hipóxia (HIF)1α, um regulador de transcrição que controla a adaptação metabólica e a citoproteção tecidual à hipóxia. O objetivo é fornecer a "prova de princípio" para o envolvimento de mecanismos de controle de dano tecidual na modulação da patogénese e/ou progressão de doenças autoimunes.

Usando um modelo experimental induzível de doença autoimune sistémica desencadeada pela depleção de células T reguladoras Foxp3^{+} (T_{REG}), descobrimos que ratinhos sem *Hsf1* em células não hematopoiéticas desenvolveram dano cardíaco autoimune após a depleção de células T reguladoras. A deleção não hematopoiética de *Nrf2* aumentou a suscetibilidade à hepatite autoimune e a deleção induzível de

Atm em células não hematopoiéticas foi associada a pancreatite autoimune grave. Estes achados apoiam a noção de que HSF1, NRF2 e ATM contribuem para o controlo de dano nos tecidos, prevenindo a desregulação autoimune na função do coração, fígado e pâncreas, respectivamente. Além disso, esta tese destaca um papel fundamental do HIF-1α na promoção da inflamação e na patogénese de doenças autoimunes, envolvendo pâncreas, fígado, estômago, pele e músculo, trazendo à tona a ideia de que o HIF-1α promove doença autoimune sistémica de múltiplos órgãos.

Em conclusão, uma crescente conscientização sobre doenças autoimunes está a contribuir para mais pesquisas e compreensão da complexidade subjacente à patogénese destas doenças, desde a etiologia, desencadeantes, manifestação clínica e órgãos visados. Identificámos vários reguladores de respostas de stresse e danos que atuam especificamente em tecidos não hematopoiéticos para ajustar a patogénese e progressão de doenças autoimunes. Estes achados devem contribuir para o desenvolvimento de novas abordagens terapêuticas que limitem o impacto clínico e social imposto pelas doenças autoimunes.

Chapter 1

General Introduction

1.1. Immune System

Historically, immunity means protection from disease and, more specifically, from infectious diseases. Knowledge of the properties of the immune system underwent a seismic shift at the beginning of the twentieth century. Before that, immunity was considered only in the light of a beneficial defence strategy against infectious diseases. In 1901, Paul Ehrlich, one of the founders of immunology, propounded his dictum of *horror autotoxicous* to refer to the ability of the immune system to prevent self-harm. Erlich proposed that naturally produced molecules block "self-harming pathogenic molecules" [1]. A critical factor in the evolution of this idea was brought by McFarlane Burnet's theory of clonal selection in the 1950s, leading to the concept of self-non-self-discrimination and tolerance discussed in this introductory chapter [2].

The immune system is constituted by cells and soluble molecules that collectively and coordinately generate immune responses to potentially harmful biotic or abiotic "substances" [3]. The molecules that trigger an immune response are referred to as being immunogenic and include molecules expressed by microbes but also self-molecules released from damaged cells [3].

Innate and adaptive immune cells use germline-encoded receptors or receptors generated via genetic recombination, respectively, to recognise immunogenic molecules. Once activated, immune cells can circulate among different tissues, developing local and systemic responses regulated by positive and negative feedback loops that amplify or restrain immune responses, preventing pathological outcomes.

1.1.1. Innate immunity and inflammation

Innate immunity provides an immediate response to self or foreign molecules that can potentially disrupt homeostasis [4]. Typically, innate immune responses occur in a few hours or days upon sensing potentially damaging molecules and aim at the neutralisation and/or elimination of those molecules as means to maintain or restore homeostasis. The response itself is called generically an inflammatory response [4].

Innate immunity acts in most cases in conjunction with physical and chemical barriers provided by epithelia and secreted anti-microbial molecules, e.g. defensins, respectively. Innate immune cells, including neutrophils, macrophages, dendritic cells (DCs), mast cells, natural killer (NK cells) and innate lymphoid cells (ILCs), are activated as soon as microbes manage to trespass epithelial barriers. This first line of

defense relies as well on a variety of soluble molecules, such as those involved in the complement system or pentraxins, and other inflammatory mediators such as prostaglandins, acting locally and/or systemically [4].

Inflammatory responses are triggered by the recognition of pathogen-associated molecular patterns (PAMP)s expressed by viruses, bacteria, fungi or protozoan parasites. Alternatively, inflammatory responses are triggered upon recognition of alarmins, that is, self-molecules released or actively secreted from stressed or damaged cells [5]. Both PAMPs and alarmins are recognised by germline-encoded receptors/sensors, referred to as pattern recognition receptors (PRR)s. These also include soluble PRR, such as collectins, ficolins or pentraxins, which are essential to facilitate microbial clearance by innate immune cells [6].

Cellular PRRs, such as Toll-like receptors (TLR)s, elicit signal transduction pathways that converge at the activation of transcription factors regulating the expression of effector genes, supporting anti-microbial responses [6]. Among the transcription factors activated by PRR are the nuclear factor κB (NF-κB) family of transcription factors and the activation protein 1 (AP-1). These trigger the transcription/expression of inflammatory genes, including cytokines such as tumour necrosis factor (TNF) and interleukin 1 alpha and beta (IL-1α/β), chemokines, such as CC, CCL2 and CXCL8, as well as adhesion molecules such as E/P-selectin (cluster of differentiation (CD) CD62E/P), Intercellular adhesion molecule 1 (ICAM-1 or CD54) or Vascular cell adhesion molecule 1 (VCAM-1 or CD106) expressed on the vascular endothelium [7].

Phagocytes, including tissue-resident macrophages, express high levels of PRR, allowing for rapid and efficient detection of PAMPs or alarmins and acting as sentinels of microbial breaching at epithelial barriers and other tissues [8]. Circulating leukocytes, including monocytes and neutrophils, are recruited to "inflamed tissues" in response to signals generated by the sentinel cells, i.e. tissue-resident macrophages, as well as by invading microbes [8].

Inflammatory cytokines act locally or systemically, exerting unique or overlapping effects. As a general principle, cytokines can amplify inflammatory reactions by inducing the expression of other cytokines, setting a positive feedback loop that amplifies inflammation over time. The most prominent pro-inflammatory cytokines include Tumor necrosis factor (TNF), interleukin (IL) 1, IL6 and IL12 [9]. Among these, TNF and IL1 induce microvascular permeability to plasma proteins and

fluid while promoting the expression of chemokines by vascular endothelial cells, including CXCL8, and leukocyte adhesion molecules, such as P- or E-selectin [9]. Expression of chemokines and adhesion molecules by activated endothelial cells allows leukocytes to bind and transverse the vascular barrier through diapedesis. This process relies on the expression of integrins, such as Lymphocyte function-associated antigen 1 (LFA-1) and Very late antigen-4 (VLA-4) by leukocytes. These integrins bind specifically to cytokine-induced adhesion molecules of the Ig superfamily, such as ICAM-1 and VCAM-1, respectively [10]. Once recruited into non-lymphoid tissues, leukocytes sustain inflammatory responses until the initial stimulus is neutralised, destroyed or expelled.

Other tissue-resident phagocytic populations, including DCs, also secrete inflammatory cytokines that boost leukocyte recruitment into inflamed tissues. Still, the primary role of DC is to trigger the activation, proliferation and differentiation of naïve T cells [11]. The latter occurs upon migration of DC into specialised lymphoid organs, known as lymph nodes, maximising the chances of physical interaction with circulating antigen-specific naïve T cells, recognising the antigens captured, processed and displayed at the surface of DCs in association with major histocompatibility (MHC) molecules [11].

In summary, the innate system has different modes of recognition from diverse sensing branches by the immune system. These modes of recognition supply information to the adaptive immune system, guiding the activation of effector responses by the adaptive immune system [12].

1.1.2. Adaptive immunity

Adaptive immune responses rely on B and T lymphocytes, which express a highly diverse repertoire of antigen receptors, known as the B and T cell receptors, respectively [13]. These provide so-called humoral and cell-mediated immunity, respectively, targeting and eliminating different classes of invading microbes [13].

During haematopoiesis, T cell precursors seeding the thymus differentiate into conventional CD4 and CD8 T cells and T_{REG}, while B cell precursors seed in the bone marrow.

1.1.2.1. Properties of adaptive immunity

Humoral immunity is mediated by antibodies produced by B lymphocytes, as postulated by Paul Ehrlich in the 1890s [14]. Antibodies recognise discrete molecular patterns known as epitopes, which are part of larger structures known as antigens, capable of inducing an adaptive immune response. The binding of antibodies to microbial epitopes targets extracellular microbes or toxins for elimination by the complement system or phagocytes. Cellular immunity is mediated by T lymphocytes, which provide defense against intracellular microbes as initially proposed by Élie Metchnikoff [14]. In contrast to innate immunity, the cardinal features of adaptive immune responses mediated by either B or T cells are their specificity, diversity, memory and self-non-self-discrimination [15].

The specificity of adaptive immunity concerns the ability of antigen receptors to recognise discrete molecules within the antigen structure, known as epitopes. Each antigen receptor is expressed in a single B or T cell clone, adding to a total of 10^7 to 10^9 different clones in a non-immunised individual, known as the B and T cell repertoire. Once an antigen is recognised by an antigen receptor, B or T cell clones can expand and generate up to a 50000-fold/week increase in the number of cells specific for a given antigen, called clonal selection and expansion [15].

The diversity of adaptive immunity implies the ability of antigen receptors to recognise a vast number of molecules, i.e. epitopes, above 10^7 [15].This diversity is achieved by recombining different gene segments encoding the B and T cell receptor antigens. It is coordinated by the activity of several enzymes, including recombination-activating genes 1 and 2 (RAG1 and RAG2) [16,17]. These allow for the rearrangement of variable (V) region gene segments with diversity (D) and joining (J) gene segments called the V(D)J recombination, which produces a highly diverse repertoire of antigen receptors [16,17]. In B lymphocytes, activation-induced cytidine deaminase (AID) is another mechanism for added variability in the adaptive immune system. AID causes point mutations and double-stranded chromosomal breaks (DSBs), which contribute to high-affinity and high-specificity antibodies production by physiological processes of DNA gene alteration: somatic hypermutation (SHM), class-switch recombination (CSR) and functions by deaminating deoxycytidines (dC) to deoxyuridines (dU) [18].

Memory is another hallmark of adaptive immunity that refers to the ability of adaptive immune cells to generate, after the first exposure to a foreign antigen, long-

lived memory cells specific for that antigen. Upon secondary exposure to the same antigen, immune responses are typically quicker and more vigorous [15]. More recently, a new concept of trained immunity in innate immune cells has been brought forward [19]. Upon a second encounter, innate immune cells such as natural killers, macrophages and monocytes show enhanced responsiveness to pathogens. Epigenetic changes may be the main drive of trained immunity, a shorter-lived and less specific than classical memory but probably contributes to an improved immune response during many infections [19].

Finally, self-non-self-discrimination and the induction of self-tolerance are some of the most remarkable properties of the adaptive immune system. Self-tolerance means tolerance to self-antigens, in other words, the absence of harmful reaction to the individual's own (self) antigens. This feature is central to what concerns autoimmunity, which will be discussed later.

1.1.2.2. Innate licensing of adaptive immunity

Adaptive immune responses are initiated by antigen-presenting cells (APCs), such as DCs, that capture, digest and display microbial or other antigens to be presented in the context of MHC class I and II molecules to T lymphocytes or to B lymphocytes [20], which respond by proliferating and by differentiating. Activation of naïve T cells by APCs-antigen presentation requires the expression of costimulatory molecules, among which the best characterised are those from the B7:CD28 family and ICOS (Inducible COStimulator, CD278). The latter is particularly important in T follicular helper (Tfh) cell development in germinal centre reaction [21].

Antigen recognition by B lymphocytes, including other than proteins, polysaccharides, lipids or small molecules, triggers B cell differentiation into plasma cells that secrete different classes of antibodies with distinct effector functions [13]. Individual plasma cells, *i.e.,* clones, secrete antibodies that recognise the same antigen recognised by the surface antigen receptor expressed by the naïve B cell clone before activation and differentiation into a plasma cell. In the case of protein antigens, the response requires signals emanating from $CD4^+$ T helper (T_H) cells, which are essential to increased antibody affinity, a process called affinity maturation, improving the effector function of antibody response [13].

1.1.2.3. T cells

T cells play a central role in the adaptive immune system. They can be easily distinguished from other lymphocytes by the expression of the T cell receptor (TCRs) [13], which allows T lymphocytes to recognise peptides derived from antigens when presented in the context of MHC molecules [22], expressed by antigen-presenting cells, such as DCs. T cell proliferation will occur if the peptide carried by MHC interacts with a TCR that recognises an epitope [22].

Briefly, T lymphocytes are composed, in addition to T_H cells, of cytotoxic T cells (CTLs), gamma-delta ($\gamma\delta$) T cells and regulatory T cells (T_{REGs}) [13]. T_H cells express $CD4^+$ surface proteins essential to bind MHC class II molecules expressed by antigen-presenting cells such as DC. Upon activation, T_H cells secrete a variety of cytokines that contribute critically to recruit leukocytes and stimulate phagocytosis by macrophages while also exerting other effects on innate immune cells such as eosinophils in inflamed tissues. Moreover, activated T_H cells express costimulatory molecules such as CD40 ligand, which engage CD40 to induce B cell activation and class-switch recombination [13]. CTLs express $CD8^+$ coreceptors essential to bind MHC class I molecules expressed by infected cells, killing the targeted cells [13]. γδ T cells are T cells that promote the inflammatory responses of lymphoid and myeloid lineages and are especially vital to the initial inflammatory and immune responses. $CD4^+$ regulatory T cells are a fourth subset of T cells that develop under the control of the $Foxp3^+$ transcription factor and exert suppressive functions over the other T cell subsets [13].

1.1.2.3.1. T cell activation

Activation of naïve T cells requires recognition of antigen presented by APCs, while T cell proliferation and differentiation require signals via costimulatory molecules such as the B7:CD28 family and ICOS [23]. The outcome of T cell activation is inveigled by an equilibrium between activating and inhibitory receptors of the CD28 family. Cytotoxic T lymphocyte antigen 4 (CTLA-4) and Programmed death 1 (PD-1) are the inhibitory receptors of the CD28 family expressed following T cell activation and function to limit immune responses [23]. While CTLA-4 functions as a competitive inhibitor of CD28 by binding more strongly to B7 molecules, PD-1 delivers inhibitory signals that block signalling by the antigen receptor and CD28 [23]. It is still unknown if the various costimulatory and inhibitory B7-CD28 family members serve distinct roles

in different immune responses or at varying stages of a response. The T_H cell-dependent antibody responses rely on the CD28:B7 interaction, in an appropriate cytokine environment, which launch T cell responses through the activation of naïve T cells: ICOS:ICOS-ligand interactions [23]. While CTLA-4:B7 interactions inhibit activation of T cells in secondary lymphoid organs, PD1:PD-ligand interactions suppress activation of effector cells in peripheral tissues [23]. Other molecules, such as CD40-ligand expressed by T cells, may induce the expression of costimulatory molecules by APCs, including B7 molecules or cytokines such as IL12 [23].

Upon activation, T cells under a series of characteristic changes induce the expression of various surface molecules, these include: a) CD69, allowing the retention of activated T cells in lymphoid organs long enough to receive the signals, following a decrease that permits the egress from those organs; b) induction of CD25 (*i.e.,* IL2 receptor α) expression enabling a response to growth factor IL-2; c) induction of CD40 ligand expression enabling a positive feedback mechanism amplifying T cell responses, and d) expression of high levels of CD44 allowing activated T cells to bind hyaluronan and retaining effector T cells in the inflamed tissues [23].

Activated T cells also show altered patterns of molecules regulating cell migration, including selectins, e.g. $CD62L^{lo}$; integrins, i.e., LFA-1; and chemokine receptors, such as CCR4, CCR5, CCR10, CXCR3 or CXCR5 [23].

1.1.2.3.2. $CD4^+$ T cells subsets

Four major subsets of $CD4^+$ effector T cells, called T_H1, T_H2, T_H17, Tfh, function in the host defence against distinct pathogen classes and are involved in different types of tissue injury in immunologic diseases [24]. T_{REG} cells are a distinct $CD4^+$ T cell lineage whose function is to control immune system reactions. The differentiation of these different $CD4^+$ T cell subsets is driven mainly by cytokines.

T_H1 differentiation relies primarily on IL12, Interferon (IFN)γ and IL18 produced by activated DCs, macrophages and NK cells [25]. IL12 and IFNγ induce and activate signal transducer and activator of transcription (STAT) 1 and STAT4 and T-bet transcription factors. A positive loop further amplifies this signal through the mentioned transcription factors, enhancing the expression of IFNγ, the hallmark of the T_H1 responses underlying type 1 immunity. Moreover, IFNγ plays a critical role in the activation of innate immune cells such as macrophages while: i) inhibiting T_H2 and T_H17 development, ii) stimulating antigen presentation and further T cell activation (for

instance, by MHC or costimulatory proteins), iii) promoting B cell class switching to specific IgG subclasses (*i.e.,* IgG2a or IgG2c in mice) and iv) inhibiting B cell class switch to IL4-dependent isotypes, such as IgE. In addition to IFNγ, TNF and IL10 are also produced by T_H1 cells, promoting, respectively, activation and inhibition of this subset of cells [25].

T_H2 cells mediate type II immunity characterised by the phagocyte-independent defence, driven by eosinophils and mast cells [25]. Helminths and allergens induce type II immunity driven by the IL4 and IL-13 cytokines, which stimulate the activation of T_H2 via the transcription factor STAT6, that, together with TCR signals, induce expression of the GATA-3 transcription factor. This transcription factor stimulates the characteristic T_H2 cytokine expressing genes *IL4, IL5 and IL13*. These act as a positive feedback loop to enhance T_H2 differentiation, B cell activation and IgG and IgE production, eosinophil activation, epithelial mucous secretion and the activation of macrophages towards a tissue repair function [25].

Another subset of IL-4-secreting T helper cells is T follicular helper (Tfh) cell. Localised in germinal centres, Tfh derive from naïve T cells by IL6 signalling induction of Bcl6, in addition to inducible costimulatory (ICOS) ligand, IL2 and TCR signal strength. Ultimately this subset of cells located in secondary lymphoid organs contributes to the formation of germinal centres and induces B cell activation and differentiation into antibody-secreting plasma cells and long-lasting antibody production type of B cells – memory B cells [26].

T_H17 cells are primarily involved in recruiting neutrophils and, to a lesser extent, monocytes to sites of inflammation. This occurs via the secretion of pro-inflammatory cytokines, such as IL6, IL1 and IL23, in response to invading bacteria and fungi. Surprisingly, transforming growth factor (TGF)β, an anti-inflammatory cytokine produced by many cell types, also promotes the development of T_H17 cells in the presence of other mediators such as IL1 or IL6 [25]. These cytokines work cooperatively to induce the activation of the transcription factors Retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγ)t and STAT3 to drive the T_H17 responses in mucosal/epithelial tissues, suggesting a tissue specific role, including epithelial production of inflammatory chemokines and integrity maintenance, and proliferation of effector $CD8^+$ T and NK cells by IL17, IL22 and IL21 generation, respectively [25].

T_{REG} cells are a suppressive subset of T cells that generally act by secreting anti-inflammatory cytokines, expressing co-inhibitory molecules, such as lymphocyte

activation gene 3 protein (LAG3) and cytotoxic T lymphocyte antigen 4 (CTLA4), killing effector cells through cytotoxicity and modulating antigen-presenting cells activity [25]. Other mechanisms of T_{REG} cell action involve growth factor depletion 'starving' effectors cells, amino acid consumption and effector cell metabolic disruption [25].

1.1.2.3.2.1. Regulatory T cells

Peter Medawar and Ray Owen's discovery in the second half of the 20th century of immune tolerance executed the vision of Paul Ehrlich's immune tolerance [27]. Miller and Cooper subsequently clarified the adaptive immunity dogma proposing the existence of regulatory interactions in the thymus function as the generative organ of T cells and the cooperativity of B and T_H cells [28–30].

In the 1970s, the so-called suppressor T cells became one of the prevailing subjects in immunology research. More than 20 years later, the idea had a rebirth, with the application of better approaches to define, purify and analyse populations of T lymphocytes that inhibit immune responses, the T_{REG} cells. Briefly, T_{REG} cells are produced from two sources: i) self-antigen recognition in the thymus and ii) recognition of foreign and self-antigens in peripheral lymphoid organs.

Generation of T_{REG} cells requires the cytokine transforming growth factor (TGF)-β, which induces the expression of Foxp3, and the cytokine IL-2 that sustains the survival and functional competence of T_{REG} cells [31,32]. IL-2, provided by conventional T cells in response to self or foreign antigens, activates STAT5 in T_{REG} cells. This transcription factor further enhances the expression of Foxp3 and other genes involved in T_{REG} cell function [31,32]. The T_{REG} cells then act at multiple levels to repress T and B cell activation in lymphoid tissues and effector development of these responses in other tissues while also inhibiting innate immune responses, for example, by repressing the proliferation and differentiation of natural killer (NK) cells. All these actions are managed through numerous mechanisms from which the best studied are: i) the production of immunosuppressive cytokines (e.g. IL-10 and TGF-β), ii) reduced ability of antigen-presenting cells to stimulate T cells through molecules such as CTLA-4 or consumption of IL-2, iii) depriving other cell populations of IL2-dependent proliferation and differentiation [31,32].

1.1.2.3.2.1.1. Roles of Regulatory T cells in Self-tolerance and Autoimmunity

Sakaguchi and colleagues originally described T_{REG} cells as a functionally distinct T cell subgroup [33]. The proof that this subgroup was relevant in sustaining self-tolerance and homeostasis in the immune system was provided by IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome genetic basis characterisation and Scurfy mice mutations in the Foxp3 gene causing a multisystemic autoimmune disease [34,35]. Hori and colleagues later targeted the Foxp3 gene by homologous recombination and exposed an inflammatory disease from the resulting knockout mice identical to what was observed in scurfy mice [36,37]. Moreover, the lack of suppression activity was ascertained by Khattri and Fontenot using an *in vitro* suppression assay comparing the anti-proliferative activity of $CD25^+$ activated T cells (*i.e.,* T_{REG} cells) from wild type mice versus mice carrying the scurfy mutation or carrying a functional deletion of the *Foxp3* gene [35,36]. Meanwhile, Hori and Fontenot provided a role for *Foxp3* in mediating suppressor function using retroviral transfer of the *Foxp3* gene to activated effector $CD4^+$ T cells [35,36]. Khattri and colleagues went on to show that suppressor activity of T_{REG} cells can be exerted over $CD8^+$ T cells [35].

Due to the importance of T_{REG}s in maintaining self-tolerance and immune homeostasis, autoimmune diseases such as type 1 diabetes, inflammatory bowel disease, multiple sclerosis and allergic disorders have been widely scrutinised to identify defects in the development and function of T_{REG} cells [38]. Defects in T_{REG} cells or resistance of effector cells to suppression by T_{REG} cells may contribute to the pathogenesis of autoimmune diseases. The finding that Foxp3 is the decisive developmental transcription factor orchestrating T_{REG} cell differentiation and function was a milestone for the fundamental understanding of the immune system's functionality and unveiled new therapeutic options for orphan diseases. Beyond transplantation of healthy donor hematopoietic stem cells, these include a variety of clinical conditions where T_{REG} cells expansion has been safely used [39], arguing that immune balance restoration by a living therapeutic drug is setting the ground for a new chapter in treating chronic inflammatory and autoimmune diseases.

In addition to controlling autoimmunity, T_{REG} cells have many other critical roles in maintaining homeostasis. Tissue residents T_{REG} cells have unique transcription signatures and perform functions tailored to different tissues, as perhaps best

illustrated by the role of adipose tissue T_{REG} cells in the control of fat metabolism [40]. Moreover, skin, muscle or lung resident T_{REG} cells promote tissue repair via a mechanism favouring stem cell proliferation and differentiation and contributing critically to restore tissue integrity after inflammatory reactions resolve. Additionally, T_{REG} cells are also critical for maintaining foetal tolerance and preventing the rejection of foetuses [40]. Another critical role of T_{REG} cells is the control of immune reactivity against commensal microbes, as provided by research from Round et al. [41] and others [42,43] where intestinal bacterial colonisation induces mutualistic regulatory T cell responses and prevents gut inflammation.

1.1.2.3.3. $CD8^+$ T cells

The activation of $CD8^+$ T cells is initiated by antigens presented by DC and may require the participation of $CD4^+$ helper cells depending on the type of antigen being presented[44]. For instance, IL12 and type I interferon (IFN), also involved in $CD4^+$ T cell differentiation into T_H1, induce the differentiation of naïve $CD8^+$ T cells into effector CTLs [44], killing infected cells via the expression of cytotoxic proteins, including granzyme B and perforin. In addition to direct cell killing, $CD8^+$ T cells secrete IFNγ and, in some cases, IL17, thus contributing to classical macrophage activation and inflammation in host defence and hypersensitivity reactions [44].

1.1.2.4. B cells and humoral immunity cell effector functions: humoral immune response

In contrast to cell-mediated immunity, B cells activation results in differentiation into antibody-secreting plasma and memory cells underlying humoral immune responses. Both protein and nonprotein antigens are initially recognised by B cells in secondary lymphoid organs [13], with protein derived antigens requiring Th cells to fully activate B cells. The B cell antigen receptor (BCR) complex expressed at the surface of mature B cells serves two critical roles. First, it provides the means for the recognition of non-processed antigens, and second, it internalises the bound antigens to be processed into peptides that will be presented to helper T cells in the context of class II MHC molecules in the T-B cell interaction zone in lymphoid follicles. Complement receptor type 2 and Toll-like receptors, which recognise complement fragments and PAMPs, respectively, also enhance B cell activation by a T-independent response [13].

Activated T follicular helper (Tfh) cells expressing high levels of CXCR5 chemokine receptor may also interact with B cells migrating to the edge of a follicle drawn by increasing levels of CXCR5 ligand [45]. These have critical roles in germinal centre formation and function [45]. In addition to CXCR5, Tfh cells express ICOS, PD1 and IL21 and Bcl6 essential for the germinal centre reaction and differentiation of activated B cells [45].

In germinal centres created within lymphoid follicles in response to different types of antigens and regulated by T_H cells, B cells undergo class switching by VDJ recombination of the heavy chain, generating antibodies with heavy chain isotypes, other than the initial IgM. Class switching is controlled by the cytokines milieu as exemplified by class switching towards IgA driven by TGFβ as well as by the TNF family members – BAFF and APRIL [13]. Signalling via CD40, upon recognition of CD40L expressed by activated Tfh cells, is also critical to induce isotype switching and somatic hypermutation via activation-induced deaminase (AID).

Besides class switching, humoral responses are also characterised by an increase in the binding affinity of antibodies imposed by point mutations in Ig variable (V) genes, known as somatic hypermutation. Through this process, only B cells with high-affinity antigen receptors are selected and survive. Differentiation towards antibody-secreting plasma cells requires a further structural Ig alteration imposed via BCR and IL21 receptor signalling, which leads to the secretion of antibodies into the circulation. Antibody secretion will be regulated [13] by antigen-antibody complexes that bind to antigen receptors and Fcγ receptors on antigen-specific B cells, repressing further antibody secretion. In addition to plasma cells, another type of cell is generated during the germinal centre reaction, the memory B cells. These express high levels of antiapoptotic protein Bcl2 and are responsible for producing high-affinity isotype-switched antibodies upon secondary exposures to antigens [13].

1.1.2.5. Immune tolerance

Immune tolerance is defined as immunological "unresponsiveness to an antigen-induced by previous exposure to that antigen" [26]. One antigen may be an inducer of both immune response and tolerance on certain exposure conditions and concomitant stimuli like costimulators [26].

As previously mentioned, antigen receptor gene segments in lymphocytes recombine randomly without previous distinction about self from non-self, thus,

contributing to a large and diverse repertoire. Consequently, recognising self-atigens expressed by individual cells and tissues may occur, and lymphocytes may react against those cells, leading to autoimmune disease. A failure of tolerance to self-antigens results in immune activation against self (*i.e.,* autologous) antigens, prompting autoimmunity [46]. This may occur as a failure of central or peripheral tolerance mechanisms acting in immature or mature self-reactive lymphocytes, respectively [46].

1.1.2.5.1. Central tolerance and Peripheral T cell tolerance

In central tolerance, lymphocytes engage cell death or replacement of the self-reactive antigen receptor by a non-self-reactive once upon recognition of self-antigens. In peripheral tolerance, mature lymphocytes recognise self-antigens but fail to become activated or to proliferate by lack of costimulation, undergoing programmed cell death by apoptosis or becoming anergic when re-exposed to that antigen [46]. Peripheral tolerance is maintained by T_{REG} cells along with these mechanisms. Other mechanisms underlying the establishment of peripheral tolerance to self-antigens rely on antigen sequestration from the immune system, for instance, in the testis and eyes, whereas others antigens are inexplicably ignored, failing to elicit an immune response [46].

Central tolerance occurs during T cell maturation in the cortex and thymic medulla, whereby immature double-positive ($CD4^+CD8^+$) T cells that recognise antigens with high antigen affinity undergo apoptosis (*i.e.,* negative selection) [46]. Negative selection occurs when antigens are presented in the thymus, by local expression or upon delivery by blood, to thymocytes expressing TCRs that recognising that antigen with high affinity [46].

Thymic antigens are representative of many circulating and cell-associated proteins distributed in tissues. Autoimmune regulator (AIRE) protein allows medullary thymic epithelial cells to produce peripheral tissue antigens to select the T repertoire [46]. Interestingly, T_{REG} cells arise from this pool of self-reactive T cells (*i.e.,* recognising self-antigens in the thymus) [46]. Factors that promote T_{REG} cell thymic development include the local availability of particular cytokines, the affinity of antigen recognition by the TCR and the types of APCs presenting the antigen [46]. In contrast to $CD4^+$ and $CD8^+$ cell development, the costimulatory receptor CD28 is required to generate T_{REG} in the thymus [47]. These so-called natural T_{REG} cells that emerge from thymus inhibit

immune responses against self-antigens in the periphery, one of the peripheral tolerance mechanisms.

Not all autoantigens are expressed in the thymus, allowing autoreactive clones to become potentially activated and proliferate in the periphery. Peripheral tolerance mechanisms include, besides the above-mentioned ignorance mechanism, clonal deletion, anergy and immune regulation [48].

Peripheral deletion of autoreactive lymphocytes is also achieved by activation-induced cell death. This apoptotic cell death consists in coordinated and cooperative mechanisms in eliminating chronically stimulated T lymphocytes by self-antigens through Fas-mediated apoptosis via the caspase pathway and Bim-dependent triggering of a Bcl2 and Bcl-xL-regulated mitochondrial death pathway [49].

In peripheral tolerance, regulatory T cells are key cells for immune response suppression [50]. T_{REG} may do so through cell-cell interaction or by production of anti-inflammatory cytokines such as TGFβ, IL10 and IL35 [51]. In the first instance, T_{REG} cell surface expression of high levels of interleukin-2 (IL-2) receptor α chain (CD25) deplete local IL2 conduces to deprivation-mediated effector T cell apoptosis. Also, TGFβ associated with latency peptide on T_{REG} cell surface can suppress the proliferation of activated T cells [51]. TGFβ, IL10 and IL35 participate by inhibiting the differentiation, proliferation, and activation of effector T cells, suppressing cytokine production by effector T cells and promoting the conversion of activated T conventional cells to cells with an immunosuppressive phenotype [52].

Along with suppression by T_{REG}, another mechanism of peripheral tolerance is anergy (*i.e.,* functional unresponsiveness) [50]. Two signals are required for immune activation, which in the case of T cells occurs via antigen recognition by TCR (signal 1) and costimulation recognition of B7-1 and B7-2 by CD28 expressed in T cells (signal 2). As observed in central tolerance, prolonged signal 1 may lead to anergy, other mechanisms such as blocked TCR-induced signal transduction, activation of cellular ubiquitin ligases by self-antigen recognition leading to proteolytic degradation of TCR-associated proteins, and interconnect inhibitory receptors of the CD28 family which function is to terminate T cell responses [53]. Among these inhibitory receptors, CTLA-4 (*i.e.,* cytotoxic T lymphocyte antigen-4) and PD-1 (*i.e.,* programmed death-1) have the best-established physiologic role in self-tolerance [54].

In summary, immune tolerance is the indifference of lymphocytes upon exposure to an antigen to which they are specific. Several mechanisms account for

this strength at the organismal level through layers of central and peripheral tolerance. In case self-tolerance mechanisms fail, this leads to autoimmunity, on which regulatory T cells have a pivotal role in preserving it.

1.2. Autoimmunity and Immune disease

Autoimmunity emerges when self-antigens trigger an immune response. Autoimmune disease develops when autoimmunity disturbs a physiological function or leads to tissue damage. This distinction between autoimmunity and autoimmune disease is crucial, as autoimmune responses may arise without the development of an associated disease similar to the dissociation between infection and infectious disease [53]. Inflammation is a major driving force in the pathogenesis of autoimmune diseases, similar to other immune-mediated inflammatory diseases.

Autoimmunity is believed to arise from genetic, epigenetic, hormonal and environmental factors (e.g., infections, microbiota, drugs, physical agents). The development of autoimmune disease involves two other phases: i) propagation and ii) resolution [53]. The burden of autoimmune diseases is considerable in western society [55] afflicting up to 5% of the population, mainly affecting working-age people [48]. Almost all autoimmune diseases are more common in women *vs.* men, with exceptions such as ankylosing spondylitis [48]. Northern latitudes and westernised, industrialised societies tend to have a higher prevalence of autoimmunity [48]. Nowadays, it remains to be clarified if geographic and socioeconomic variation in autoimmune disease indicates variations in exposure to pathogens, nutrition or other factors [48]. Although there is evidence of family clustering, unveiling a genetic aetiology.

Depending on the distribution of the autoantigens recognised, autoimmune diseases may be organ-specific or systemic. Typically, organ-specific autoimmune disease results from the production of autoantibodies or the activation of self-reactive T cells against self-antigens expressed in a tissue specific manner. Examples include multiple sclerosis (MS), myasthenia gravis, type 1 diabetes (T1D), in which immune cells recognize antigens in myelin, acetylcholine receptor or β cells (gluramic acid decarboxylase (GAD) 65, insulin or tyrosyl phosphatase (IA-2)) [56–58].

Systemic autoimmune disease develops when the immune response extends further to different tissues, such as kidney, skin and lung due for example to polyclonal cell activation or, in the case of autoimmune regulator (AIRE) involvement, diseases

such as Addison disease, vitiligo or parathyroiditis, where immune cells recognise antigens of these structures [59,60]

Moreover, systemic autoimmune disease may also develop when there is the formation of circulating immune complexes composed of self-antigens and antibodies recognising those self-antigens, as in the case of systemic lupus erythematosus (SLE).

Immune complexes, circulating autoantibodies and autoreactive T lymphocytes are among the dominant immune responses responsible for tissue injury [53]. According to the nature of the dominant autoimmune response, the disease may express particular clinical and pathological features.

Autoimmune diseases tend to be chronic, progressive and self-perpetuating, due to the persistence of the self-antigen and amplification mechanisms sustaining autoimmune responses [53]. Importantly, once tissues are injured through an immune-mediated response, they release alarmins as well as self-antigens which may sustain immune activation and autoimmune disease [53].

1.2.1. Pathogenesis of autoimmune disease

The lighting match of autoimmunity is currently held under three main pillars: defective regulation, genetic predisposition and environmental triggers [53]. Koch's postulate application to explain the pathogenesis of autoimmunity has been one time-honoured approach [61]. The major criteria included:

- "autoantibodies or autoreactive T cells with specificity for the affected organ are found reliably in the disease" (criteria met in endocrine autoimmune diseases);
- "autoantibodies and/or T cells are found at the site of damage" (for instance, in some glomerulonephritis);
- "the levels of autoantibody or T-cell response reflect disease activity" (e.g., systemic lupus erythematosus);
- "reduction of the autoimmune response leads to improvement" (as observed by the use of immunosuppression in these diseases);
- "transfer of antibody or T cells to a second host leads to the development of autoimmune disease in the recipient" (easily demonstrated in animal models or cases of recipients of bone marrow transplants from diseased donors);

- "immunisation with autoantigen and consequent induction of an autoimmune response causes disease" (as described in animal models, many self-proteins induce autoimmune response with an appropriate adjuvant)" [61].

Even though Koch's criteria contributed to the definition of autoimmunity, its pathogenesis remains completely uncovered due to the multiple players that contribute to its development.

1.2.1.1. Immunologic abnormalities leading to autoimmunity

While autoimmune responses are essentially not different from other types of immune responses against non-self-antigen, the development of autoimmunity implies a failure of immunoregulatory mechanisms, including [53]:

i) Abnormal self-tolerance due to defective elimination or regulation of self-reactive B or T cell or

ii) Defective display of self-antigens due to lack of self-antigen clearance or structural changes in antigens due to enzymatic modifications, injury or cellular stress leading to new antigenic epitopes;

iii) Pathology associated with cell injury or infection overcoming regulatory mechanisms controlling unfettered activation of B and T cells [53].

Regarding immunologic abnormalities, experimental studies have provided hints about features of proteins that determine whether these protein antigens will promote T cell activation or tolerance, namely, persistence, portal of entry/location, presence of adjuvants and properties of APCs, as in the case of dendritic cells [62].

Self-antigens are expressed in primary (*i.e.,* generative) lymphoid organs, engaging B and T cell antigen receptors for prolonged periods without concomitant inflammation. Dendritic cells in tissues usually are in a resting (immature) state, expressing low levels of costimulators; some of them may traffic at a low level at a steady-state [63]. These APCs present self-antigens without co-stimulation, driving T cells that recognise these specific antigens to anergy or differentiation into regulatory T cells [64]. In contrast, if DCs engage PRR upon recognition of PAMP expressed by microbes, they go on to express costimulatory molecules and can breakdown T cell self-tolerance, which conduces to autoimmune reactions against self-antigens [11,62,65,66].

1.3. Inflammation and the breakdown of homeostasis

Inflammation is a fundamental component of immune responses that is essential to survival in response to injury and/or infection and upholds collateral tissue homeostasis to various harmful conditions. Tissue homeostasis refers to systemic processes that maintain vital variables within an upper and lower threshold sustaining optimal tissue function. These variables include tissue architecture, cell number and composition as well as nutrient and metabolite concentration but also O_2 tension, temperature, pH or osmolarity [67]. Mechanical stress, DNA damage, heat, cold and hypoxia are some insults that can result in cellular and tissue damage. Injured, distressed, damaged or necrotic cells can liberate intracellular molecules known as alarmins that engage PRR to activate APC [68]. Cells and tissues evolved several mechanisms to sense different stressors, which will trigger an appropriate adaptive response [69]. In the case of cellular homeostasis, alterations such as protein folding, levels of reactive oxygen species (ROS) and nutrient availability trigger signalling proteins that function as sensors [67]. For instance, heat shock, hypoxia, high levels of reactive oxygen species and DNA damage are sensed by HSF (heat shock factor)-1, HIF (Hypoxia inducible factor) 1-α, NRF (Nuclear factor erythroid-derived 2-like) 2 and ATM (Ataxia-telangectasia mutated), respectively, in alterations in cellular physiology that allow adaptation to abnormal circumstances [67].

If homeostasis is the aim because of a scant stress response, an inflammatory response is persuaded. At this moment, both mechanisms, stress response and inflammatory response, engage in ousting the stressor. Furthermore, adaptation strategies include recovery of homeostasis by restoring regulated variables to homeostatic set points or switching these set points to values better suited to deal with persistent abnormal conditions [69]

1.3.1. Tissue Damage Control and Disease Tolerance

Upon an inflammatory host response to pathogens or physical injury, immunopathology of host tissues may ensue. Host tissues have a functional physiological reserve that warrants parenchymal tissue function and maintains tolerance to internal and external precipitants without concerning the etiological cause. Initially designated disease tolerance by plant ecologists, more recently, this concept was adapted in the animal kingdom [70–74].

As defined by others, disease tolerance is more than mere resistance to the injury imposed because it protects the host by promoting host health while having a neutral to a positive effect on pathogen fitness [71,74,75]. Nevertheless, the term tolerance may mislead to immunological tolerance, which involves the elimination of self-reactive cells. In certain circumstances, immunological tolerance may be considered a disease tolerance mechanism [76].

In the context of autoimmunity, the concept of disease tolerance is interpreted as a decrease of host susceptibility to tissue damage or other fitness costs caused by the immune system [74]. The capacity to tolerate varies between different physiological processes and tissues. For instance, the function of intrinsic damage susceptibility, functional autonomy repair capacity, and damage sequelae are factors that define the tolerance level [74]. As an example, cardiomyocytes have a low tolerance to hypoxia (i.e., lower than physiologic O_2) because they rely primarily on oxidative metabolism to produce energy. On the other hand, apoptosis induced by DNA-damaging agents, such as epithelial and hematopoietic cell types, which have a higher regenerative capacity and therefore a high intrinsic tissue damage control and "tissue" tolerance. In the case of hepatocytes, the loss of individual cells may be overcome by the remaining cells performing the same function due to their functional autonomy [74]. Moreover, depending on the damage degree or malfunction, tolerance capacity may vary [74].

Each tissue has a basal level of tolerance, an intrinsic ability to tolerate some degree of stress, damage, or malfunction. The basal tolerance is granted by constitutively active cytoprotective mechanisms, differing between tissues and organs. These tolerance mechanisms are inducible, even though cytoprotective genes may be constitutively expressed at low levels. Moreover, some can only operate once the damage has taken place when compared to tissue renewal that operates under basal conditions [74].

Tissue damage in autoimmune diseases is mainly mediated by antibodies (types II and III hypersensitivity) or by $CD4^+$ T_H-cell activation of macrophages or cytotoxic T cells (type IV hypersensitivity). Although many autoimmune diseases involve a predominance of one or another form of hypersensitivity, there is often considerable overlap between antibody and T-cell mediated damage. In this context, tissue damage control consists of cellular adaptive responses that protect the targeted tissues from stress, dysfunction, and/or damage, required to limit the pathogenic effect associated with immunopathology [75].

Tissue damage control can be achieved via diverse mechanisms that restrict the damaging effects of host cellular and/or tissue and confine the immune effector burden – the harmful side-effects of stress and damage imposed by the host autoreactive immune system. Breakdown of these preventive measures leads to exacerbation of stress, tissue dysfunction and/or damage and for that reason, we perceive another factor of susceptibility for autoimmune diseases [75].

1.3.1.1. Stress responses underlying Tissue Damage Control

Adverse conditions such as hypoxia, ROS, exposure to noxious xenobiotics or endoplasmic reticulum (ER) overload induce cellular stress sensed by particular stress response pathways. These pathways trigger a transcriptional master regulator, setting off a battery of stress response genes. These genes function to help the in-need cells to survive the adverse condition, at the cost of normal cell or tissue function. For instance, heat shock activates the transcription factor HSF-1, triggering induction of genes that prevent proteotoxicity, controlling refolding or degradation of misfolded proteins [77]. Hypoxia induces the activation of HIF-1α transcription factor acting in a non-cell autonomous manner to increase O_2 delivery while providing cellular metabolic adaptation to hypoxia [78]. Oxidative stress activates the transcription factor Nrf2 which regulates the expression of several genes that scavenge free radicals, clearance of damaged proteins, metabolise oxidised membrane lipids, and damaged DNA repair [79]. Later in chapters 4, 5 and 6, we will further expand these examples of stress response and explore their role in the context of autoimmunity.

1.3.1.2. Damage responses underlying Tissue Damage Control

Damage to macromolecules such as DNA, proteins or lipids and organelles in the context of cellular stress is sensed to trigger damage responses. Contrary to stress, damage sensors and ensuing damage responses aim at repairing macromolecular and/or organelle damage, as the means to maintain cellular function [75]. In the case of DNA-damage response the aim is to rapidly prevent mutations and genomic instability for which ATM acts as a regulator [75].

ATM, a Ser/Thr protein kinase responsible for DNA damage repair, has been shown to protect against severe sepsis, acting predominantly at the lung epithelium and eliciting tissue damage control [80]. In case of maladaptive response or failure to enforce tissue damage control, cells will undergo programmed cell death, irreversible

tissue damage, organic dysfunction and disease [75]. Later in chapter 7, we will further expand ATM role in the context of autoimmunity.

In summary, the abovementioned stress and damage responses are part of inflammatory responses, adjusting host immunity to the type of danger and aiming at a harmony status of homeostasis [67]. These systemic adaptive and cellular responses are part of tissue damage control and coordinately act as shield of protection of the host parenchymal tissues against stress, dysfunction and/or damage [75].

1.4. Unmet needs in autoimmunity

A full understanding of autoimmunity is yet to be achieved. Nowadays it is still a research field of interest partly because the questions about which predispositions and how they influence autoimmune diseases development remain to be answered.

Over the last decades, there were significant breakthroughs in understanding the mechanism that leads to disease, which gave rise to substantial therapeutic advances to manage the symptoms of these diseases. Though, a clear understanding of the mechanisms that underlie them is still lacking, which stalls the development of treatments for these diseases. We are missing several milestones of the disease, namely perceive its beginning, engage to its clinical appearance, control and prevention of its reappearance as flares and collateral side effects of systemic and untargeted treatment. We refer to new molecular biomarkers that may aid in stratifying and treating autoimmune diseases, withstanding the autoreactivity of the immune system yet protecting the tissues from damage.

1.5. Aims of the thesis

Efforts to unveil and blunt tissue damage caused by sterile inflammation have primarily focused on tuning immune responses and restraining the immune activation response. Nevertheless, they have not always been enough to defend from the immunopathology caused by damaged self-induced by the immune. Furthermore, the immune effector burden may endure; yet, the target tissues' capacity to stand this immune effector burden may be decisive for disease severity. In this thesis, I tested the hypothesis that defects in specific stress and damage responses conferring tissue damage control can act in non-hematopoietic tissues to modulate the pathogenesis of autoimmune diseases. I tested this hypothesis using genetic loss of function

approaches targeting HSF-1 (*i.e.,* preventing damage responses to proteotoxicity), HIF-1α (*i.e.,* preventing metabolic adaptation and tissue cytoprotection in response to hypoxia), NR2 (*i.e.,* preventing cellular resistance to oxidants) and ATM (*i.e.,* preventing DNA repair). The overall Aim of this thesis was to provide the "proof of principle" that tissue damage control can act in tissues targeted by autoimmunity to modulate the pathogenesis and progression of autoimmune diseases.

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Chapter 2

Material and Methods

2. Material and Methods

Material and methods are shared by all the chapters, for this reason an independent chapter was created to prevent multiple entries of the same subjects.

Mice. Mice were bred and maintained under specific pathogen-free conditions at the Instituto Gulbenkian de Ciência. Experimental protocols were approved by the Ethics Committee of the IGC (A003.2017, A008.2010 and A009.2011) and the Portuguese National Entity (Direcção Geral de Alimentação e Veterinária; 008959 and 018071). Experimental procedures were performed according to the Portuguese (Decreto-Lei 113/ 2013) and European (Directive 2010/63/EU) legislations.

Nrf2$^{-/-}$ mice (RIKEN Bioresource research center; B6.129P2-Nfe2l2<tm1Mym>/MymRbrc;# RBRC01390) were crossed with a *Rag2*$^{-/-}$ mouse (Taconic; B6.129S6-*Rag2*^{tm1Fwa}N12; # RAGN12) (*Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$) used in Chapter 4.

Hif1α$^{lox/lox}$ (*Hif1α*$^{fl/fl}$) mice (Jackson laboratory; B6.129-Hif1α^{tm3Rsjo}/J) were crossed with *Rosa26*$^{CreERT2/CreERT2}$ (*Hif1α*$^{R26fl/fl}$) (Jackson Laboratory; B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J; #008463) and after crossed in a *Rag2*$^{-/-}$ background (Taconic©; B6.129S6-*Rag2*^{tm1Fwa}N12; # RAGN12) (*Rag2*$^{-/-}$ *Hif1α*$^{R26fl/fl}$) used in Chapter 5.

Hsf1$^{lox/lox}$ (*Hsf1*$^{fl/fl}$) mice were provided originally by Elisabeth Christians (Toulouse, France)[1], crossed with *Rosa26*$^{CreERT2/CreERT2}$ (*Hsf1*$^{R26fl/fl}$) (Jackson Laboratory©; B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J; #008463) and thereafter with *Rag2*$^{-/-}$ mice (Taconic©; B6.129S6-*Rag2*^{tm1Fwa}N12; # RAGN12) at Instituto Gulbenkian de Ciência to generate *Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$ mice used in Chapter 6.

Atm$^{lox/lox}$ (*Atm*$^{fl/fl}$) mice (Jackson laboratory; B6. 129-*Atm*$^{tm2.1Fwa}$/J ;#021444) [2] were crossed with *Rosa26*$^{CreERT2/CreERT2}$ (*Atm*$^{R26fl/fl}$) (Jackson Laboratory; B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J; #008463) and afterwards crossed in a *Rag2*$^{-/-}$ background (Taconic; B6.129S6-*Rag2*^{tm1Fwa}N12; # RAGN12) (*Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$) used in Chapter 7.

Foxp3^{DTR} mice (B6.129(Cg)-*Foxp3*$^{tm3(DTR/GFP)Ayr}$/J; #016958) were purchased from Jackson Laboratories. C57BL/6 mice (*Foxp3*^{WT}) were bred in Instituto Gulbenkian de Ciência. Ultimately, *Nrf2*$^{-/-}$ and *Foxp3*^{DTR} mice were crossed and bred at Instituto Gulbenkian de Ciência.

OKD48^{luc} (RIKEN Bioresource research center; B6.C57BL/6 OKD48, RBRC05704) were crossed with *Foxp3^{DTR}* mice (B6.129(Cg)-*Foxp3$^{tm3(DTR/GFP)Ayr}$*/J; #016958) used in Chapter 4.

ODD^{luc} mice (Jackson laboratory; *Gt(ROSA)26Sor$^{tm2(HIF1A/luc)Kael}$*, #006206) were crossed with *Foxp3^{DTR}* mice (B6.129(Cg)-*Foxp3$^{tm3(DTR/GFP)Ayr}$*/J; #016958) used in Chapter 5.

HSF reporter (hsp-lucF/hsp-mPlum) mice were provided by Franck Couillaud (Bordeaux, France)[3] and crossed with *Foxp3^{DTR}* mice (B6.129(Cg)-*Foxp3$^{tm3(DTR/GFP)Ayr}$*/J; #016958) used in Chapter 6.

Genotyping was conducted by PCR from genomic DNA using Taq DNA polymerase, dNTPack (Roche life sciences©), High Fidelity PCR Enzyme Mix (Thermo scientific©) and the following primers.

Table 1: Pcr primers for mice genotyping

Primer	Sequence
*Hsf1*lox Forward	5'-CAC CAC TTC TCA CTC CCC TGC TGG-3'
*Hsf1*lox Reverse	5'-GTT GTG GTC AGC TCC TGT C-3'
*Hif1α*lox Forward	5'-CGT GTG AGA AAA CTT CTG GAT G-3'
*Hif1α*lox Reverse	5'-AAA AGT ATT GTG TTG GGG CAG T-3'
*Atm*lox Forward	5'-CAT CCT TTA ATG TGC CTC CCT TCG CC-3'
*Atm*lox Reverse	5'-GCC CAT CCC GTC CAC AAT ATC TCT GC-3'
Nrf2-5'	5'-TGG ACG GGA CTA TTG AAG GCT G-3'
Lacz	5'-GCG GAT TGA CCG TAA TGG GAT AGG-3'
Nrf2-AS	5'-GCC GCC TTT TCA GTA GAT GGA GG-3'
*Rosa26*Cre Mutant Reverse	5'-CGG TTA TTC AAC TTG CAC CA-3'
*Rosa26*Cre Common	5'-AAG GGA GCT GCA GTG GAG TA-3'
*Rosa26*Cre Wild type Reverse	5'-CCG AAA ATC TGT GGG AAG TC-3'
Rag2 Wild type Forward	5'-CTG ACT GCC TAC CCC ATG TT-3'
Rag2 Common	5'CCA TGT TGC TTC CAA ACC AT-3'
Rag2 Mutant Forward	5'-AAT TCG CCA ATG ACA AGA CG-3'
Foxp3^{DTR} Mutant Forward	5'-GGG ACC ATG AAG CTG CTG CCG-3'
Foxp3^{DTR} Mutant Reverse	5'-TCA GTG GGA ATT AGT CAT GCC-3'
Foxp3^{DTR} WT Forward	5'-CCC TAT CTA GCT GCC CTC CT-3'
Foxp3^{DTR} WT Reverse	5'-GGG GGA TGT AAT TGT GAA GGT-3'
LucF 12D	5'-TCC ATT CCA TCA CGG TTT TGG-3'

LucF 13R	5'-GCT ATG TCT CCA GAA TGT AGC-3'
mPlum 3D	5'-AGG GCG AGG AGG TCA TCA AG-3'
mPlum 5R	5'-GAT GTC GGT CTT GTA GGC GC-3'
OKD48 WT Forward	5'-ATC ACC AGA ACA CTC AGT GG-3'
OKD48 WT Reverse	5'-TAG CGC TTC ATA GCT TCT GC-3'
oIMR0042 OKD48 IC Forward	5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'
oIMR0043 OKD48 IC Reverse	5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'
oIMR6326 Mutant Forward	5'-CGG TAT CGT AGA GTC GAG GCC-3'
oIMR6327 Mutant Reverse	5'-GGT AGT GGT GGC ATT AGC AGT AG-3'
oIMR9020 WT Forward	5'-AAG GGA GCT GCA GTG GAG TA-3'
oIMR9021 WT Reverse	5'-CCG AAA ATC TGT GGG AAG TC-3'

Once identified breeding pairs were kept in final format (*Rag2*$^{-/-}$ *Hsf1*$^{R26fl/fl}$, *Rag2*$^{-/-}$ *Hif1α*$^{R26fl/fl}$, *Rag2*$^{-/-}$ *Atm*$^{R26fl/fl}$, *Rag2*$^{-/-}$ *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$ *Foxp3*^{DTR}, *Rag2*$^{-/-}$ *Hsf1*$^{fl/fl}$, *Rag2*$^{-/-}$ *Hif1α*$^{fl/fl}$, *Rag2*$^{-/-}$ *Atm*$^{fl/fl}$, *Rag2*$^{-/-}$, *Nrf2*$^{-/-}$) and experimental groups weaned together. Breeding pairs and progeny were maintained under RM3 diet (SDS©) at Instituto Gulbenkian de Ciência. Mice were used at 8 to 12 weeks of age.

Cre recombinase gene deletion. Cre-mediated recombination was induced by tamoxifen (TAM) via activation of estrogen receptor (CreERT2). Tamoxifen ((Z)-1-(p-Dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine ≥99%; Sigma-Aldrich® T5648) was dissolved in 5% EtOH/Corn Oil (Corn Oil; Sigma-Aldrich® C8267-500ML) and gavaged (225 mg/kg body weight in 100 µL; 3 times every other day).

For the activation of CreERT2 recombinase in *Rag2*$^{-/-}$ *Atm*$^{R26fl/fl}$ animals were fed initially for one week on low phytoestrogen content, soybean and alfalfa products free, gamma-irradiated complete food (Ssniff Spezialdiaten GmbH; #V1154-703). Afterwards, mice were fed low phytoestrogen content, soybean and alfalfa products free, gamma-irradiated complete food with tamoxifen citrate 360mg/kg (TAM 240mg/Kg), flavoured and supplemented in sucrose for four weeks long (Ssniff Spezialdiaten GmbH; #A115T70360).

Deletion of the *LoxP sites flanked Hsf1*$^{fl/fl}$, *Hif1α*$^{fl/fl}$, *Atm*$^{fl/fl}$ alleles by the Cre recombinase was assessed by qRT-PCR in *Rag2*$^{-/-}$ *Hsf1*$^{R26\Delta/\Delta}$, *Rag2*$^{-/-}$ *Hif1α*$^{R26fl/fl}$ or

Rag2$^{-/-}$*Atm*$^{R26fl/fl}$ relative to its respective control (*Rag2*$^{-/-}$ *Hsf1*$^{fl/fl}$, *Rag2*$^{-/-}$ *Hif1α*$^{fl/fl}$, *Rag2*$^{-/-}$*Atm*$^{fl/fl}$) mice using the following set of primers:

Table 2: Primers for Deletion check of the LoxP

Primer	Sequence
Hsf1 exon1 Forward	5'-GTA GTC CAC ATT GAG CAG GGT-3'
Hsf1 exon1 Reverse	5'- TCC TGT CCA CGC AAG AAA CA-3'
Hif1α Forward	5'-TCA TCAG TTG CCA CTT CCC CAC-3'
Hif1α Reverse	5'-CCG TCA TCT GTT AGC ACC ATC AC-5'
Atm Forward	5'-GAA GTG CAG AAG AAG TCT-3'
Atm Reverse	5'-TGT AAC CGA CGA TAG AAG-3'
Arbp0	5'-CTTTGGGCATCACCACGAA-3'
Arbp0	5'-GCTGGCTCCCACCTTGTCT-3'

RNA isolation and qRT-PCR. For mRNA extraction and deletion check, organs from individual mice were harvested, snap-frozen in liquid nitrogen, grinded and resuspended in TRIzol (Invitrogen®). RNA was extracted with chloroform and purified with the Macherey-Nagel™ NucleoSpin™ RNA Columns Kit (#12373368). cDNA synthesis was performed from 2 µg of RNA using random hexamer primers (0.3 µg/reaction; Invitrogen®), dNTPs (0.5 mM/reaction; Invitrogen®)(5 min., 65°C). 5x First Strand buffer (Invitrogen®) was added in the presence of DTT (10 mM/reaction; Invitrogen®) and RNAse Out recombinant ribonuclease inhibitor (40 U/reaction; Invitrogen®) (2 min., 42°C). SuperScriptII reverse transcriptase (200 U/reaction; Invitrogen®) was added, completing a final volume of 20 µl (50 min., 42°C; 15 min., 70°C). cDNA (1 µl) was used for PCR reactions (10 µl) using the Power SYBRGreen PCR master mix (Applied Biosystems®) and optimal primer concentrations (previously determined for each transcript). PCR products were detected by qRT-PCR (ABI-7900HT; Applied Biosystems®) (2 min., 50°C, 10 min., 95°C, and 40 cycles of 15 sec at 95°C, 1 min., 60°C). Primers used to amplify mouse mRNA transcripts were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Steve Rozen and Helen Skaletsky) according to the specifications of the ABI-7900HT equipment (Applied Biosystems) and are listed in the previous table. Transcript number was calculated from the Ct of each gene using a $2^{-\Delta\Delta CT}$ method (relative number) and normalizing results to Arbp0.

Bone marrow chimera: Bone marrow from tibia and femur of *Foxp3*^{DTR} and C57BL/6 (*Foxp3*^{WT}) mice were washed in HBSS (Hank's Balanced Salt Solution, Gibco®, #14025-050) on ice, counted, resuspended in PBS 1x (Phosphate-buffered saline 10x, Gibco®, #70011-036) in a volume 2.5x10^{7} cells/mL and then transferred (5x10^{6}/200µL/mouse) retro-orbitally into 8-12 week-old gender-matched, 10h after sub-lethal irradiation (500rad unless otherwise mentioned; Gammacell© 2000; Mølsgaard Medical). Bone marrow reconstitution check was performed 6-8 weeks after transfer by flow cytometry analysis in peripheral blood (description below).

Systemic Autoimmune Disease. Lyophilized diphtheria toxin from Corynebacterium diphtheriae (Sigma-Aldrich D0564-1MG) was reconstituted to 0.5 mL with sterile distilled water and then diluted in PBS1x for a final concentration of 15µg/kg/mouse (intraperitoneal). Mice were injected with diphtheria toxin for regulatory T cell depletion (15µg/kg/mouse, every three days for two weeks – 5 times). Clinical and vital signs were evaluated every three days for 26 days.

Vital Signs Monitoring. Total body weight and rectal temperature were measured every three days for the length of the experiment using the Ohaus® CS200 CS Compact Portable Scale and BIOSEB® thermometer BIO-TK8851, respectively. Glucose blood levels were measured on a tail blood drop by Roche AccuCheck Performa® glucose strips every three days for 26 days.

Histology. Mice were sacrificed and perfused with PBS. Organs were harvested and submerged in 10% formalin on the day of assessment. Heart, lung, pancreas, liver, stomach, kidney, small intestine, large intestine, skin, muscle and salivary glands sections were embedded in paraffin, sectioned (3µm) and stained with Hematoxylin & Eosin or with Luxol fast blue stains. The severity of autoimmunity affecting the different organs was scored across the specimen in a blinded fashion according to the following definitions in tables 3, 4, 5, 6 and 7.

Table 3: Severity of Autoimmunity Scores for Liver and Lung [4]

	Organs	Liver			Lung
	Areas	*Portal inflammation*	*Lobular inflammation*	*Necrosis*	
Score	**0**	no inflammatory infiltrate	no inflammatory infiltrate	none	normal
	1	low level of inflammatory cell infiltration	low level of inflammatory cell infiltration	small necroses	minor perivascular inflammation
	2	moderate level of inflammatory cell infiltration	moderate level of inflammatory cell infiltration	large necrotic areas	increases perivascular and peribronchial inflammation
	3	severe inflammation	severe inflammation (>50% parenchyma)	bridging necroses	severe perivascular, peribronchial and interstitial inflammation

Table 4: Severity of Autoimmunity Scores for Salivary glands, Heart, Muscle, Kidney [5] and Pancreas [4]

	Organs	Salivary glands, Heart, Muscle and Kidney	Pancreas
Score	**0**	no inflammation	no inflammation
	1	Minimal leukocyte infiltration	minor perivascular inflammation
	2	Mild leukocyte infiltration	increased perivascular and intralobular inflammation
	3	Moderate leukocyte infiltration	severe inflammation

Table 5: Severity of Autoimmunity Scores for Skin [4]

	Organ	Skin
Score	**0**	no inflammation
	1	minimal: few scattered lymphocytes, plasma cells multifocally in the pinnae
	2	mild: increased numbers of lymphocytes, plasma cells and neutrophils multifocally in the pinnae
	3	moderate: diffuse infiltration of lymphocytes, plasma cells and neutrophils associated with multifocal abrasion or ulcerations in the pinnae
	4	moderate: diffuse infiltration of lymphocytes, plasma cells and neutrophils associated with multifocal abrasion or ulcerations in the pinnae

Table 6: Severity of Autoimmunity Scores for Stomach [6]

	Organ	**Stomach**			
	Parameter	*Thickening*	*Forestomach*	*Corpus*	*Antrum*
Score	**0**	absent	absence of granulocytes, mononuclear cells and hyperkeratosis	absence of granulocytes, rare mononuclear cells, no parietal cell loss	absence or rare granulocytes and rare mononuclear cells
	1	present	presence of granulocytes; focal presence of mononuclear cells 1–5 cells in a 40x field; hyperkeratosis	focal presence of granulocytes, 2–5 cells in a 40x field; focal presence of mononuclear cells; irregular staining, and morphology of the parietal cells	focal presence of granulocytes, 2–5 cells in a x40 field and focal presence of mononuclear cells
	2		aggregates of mononuclear cells	granulocytes present in more than 1 field with 5 cells, diffuse mononuclear infiltration with more than 30 cells in a 40x field; moderate loss of parietal cell mass	granulocytes present in more than 1 field with 5 cell and diffuse mononuclear infiltration
	3			luminal presence of granulocytes, aggregates of mononuclear cells and marked loss of parietal cell mass	luminal presence of granulocytes and aggregates of mononuclear cells

Each region of the stomach (forestomach, corpus, and antrum) was assessed individually for several parameters and the result was considered the sum of each individual score.

Table 7: Severity of Autoimmunity Scores for Small Intestine and Colon [6]

	Organ	**Small Intestine and Colon**						
	Para-meter	*Extent of inflammation*	*Degree of inflammation*	*Abnormal crypt morphology*	*Neutrophil Infiltration*	*Globet cell loss*	*Mucosal erosions or ulceration*	*Crypt Abcess*
Score	**0**	Nome	None	None	None	None	None	None
	1	Lamina propria	Mild	Mild	Mild	Moderate	Lesion present	Lesion present
	2	Submucosal	Moderate	Moderate	Moderate	Severe		
	3	Transmural	Severe	Severe	Moderate-severe			
	4				Severe			

Each region of the small intestine and colon was assessed individually for several parameters and the result was considered the sum of each individual score within the organ. The maximum score was 17 [6].

Serology. Blood was withdrawn on day 26 of analysis and outsourced (DNTAtech©) ELISA measurements were obtained of the following biochemical parameters: urea, creatinine, troponin, total proteins, total bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase.

Blood Pressure. The CODA® mouse rat tail-cuff system was used to measure blood pressure through Volume Pressure Recording sensor technology. Each mouse was placed inside a mouse restrainer and blood tail pressure was measured continuously every other minute for 15 min.

Physiological Signs Measurement. Mice were anaesthetized with inhaled isoflurane (IsoFlo®100% p/p, Zoetis®) mixed with oxygen (medical liquid oxygen 21%, Linde®) for moderate sedation (unaffected airway, adequate spontaneous ventilation and maintained cardiovascular function). Non-invasive real-time neck pulse oximeter for rodents (MouseOx® Plus; Harvard Apparatus®) measured continuous arterial O_2 saturation, heart rate (bpm: beats per minute), breath rate (bpm: breaths per minute), pulse distention and breath distention. Pulse distention is simply a measurement of the change in the effective path length of the light that passes through only the arterial or pulsating blood, and it has precise linear distance units of μm. Breath distention is an overlaying pulsatile signal on the blood pressure at the artery caused by changes in thoracic cavity pressure due to breathing. Data were analysed by MouseOx® Plus software.

Total IgG quantification. Total IgG was detected by Enzyme-linked immunosorbent assay (ELISA) using the following antibodies: Goat anti-mouse IgG total antibody (SBA-1030-01, 2μL/mL), mouse IgG total standard antibody (SBA-0107-01, 0.5μg/mL) and goat anti-mouse IgG total – HRP antibody (SBA-1030-05), 1:4000). Flat bottom polyvinyl chloride plates (Corning Incorporated-Life Sciences©, MA, USA) were coated with 50 μl of goat anti-mouse IgG (0.2 μg/ml of sodium carbonate buffer, pH 9.6) and incubated overnight at 4°C. The antigen solution was then poured off and the plates were blocked with 150 μl of 1% Gelatin (Sigma-Aldrich©, #G1393-100) PBS 1x for 1 hour at 37°C. The plates were washed three times with PBS-Tween 0.1% (Polyoxyethylenesorbitan monolaurate, Tween® 20, Sigma-Aldrich©, #P7949-100). Plasma samples serially diluted from 1:600–1:4050 in 1% gelatin PBS1x were added to the plates and incubated for 1 hour at 37°C. The plates were washed three times with PBS-Tween 0.1% and 50 μl of 1:1000 dilution of Goat anti-Human IgG peroxidase conjugate 1% gelatin PBS was added to the plates, which were then incubated for 1 hour at 37ºC. The plates were then finally washed three times with PBS-Tween 0.1%. 50 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate Reagent Set (BD

Pharmingen™; #555214) was added to the plates and the colour was allowed to develop at 37°C. The absorbance was then read at 650nm with a microplate reader (Molecular Devices, Menlo Park, CA, USA) after stopping the reaction with sulfuric acid 0.1M (H2SO4).

Isolation of splenocytes. Spleens were harvested and homogenized into a single-cell suspension in PBS 2% FBS (Fetal bovine serum, Gibco®, #10500-064). Splenocytes were subjected to erythrocyte lysis in 3 ml of a hypotonic red blood cell lysis buffer (155 mM NH4Cl, 10 mM NaHCO3, 0.1 mM EDTA, Sigma-Aldrich®) while centrifuging (375g, 5 min., 4ºC). Cells were washed twice in 15 ml PBS 2% FBS (375 g, 5 min., 4ºC). Subsequently, using a Neubauer hemocytometer, cells were counted in suspension (10µL of diluted sample in 5mL), 1:9 dilution in trypan blue. $2x10^6$ cells were then used for staining.

Cell stimulation for cytokines. For intracellular cytokine staining, cells were cultured in supplemented RPMI 1640 (Biowest®, #L0495-500) containing 50 ng/ml PMA (Phorbol 12-myristate 13-acetate, Sigma-Aldrich®, #P8139), 500 ng/ml ionomycin (Sigma-Aldrich®, #I3909-1),10 µg/ml brefeldin A (Biolegend®, #420601) and monensin (BD GolgiStop™, BD biosciences®, #554724) for 4h at 37°C (5% CO2, 95% humidity). Afterwards, cells were stained for surface markers as detailed below, adding brefaldin A and monesin solutions. For intracellular staining, the protocol was followed as described.

Flow cytometry and antibodies. The following table displays the monoclonal antibodies used for flow cytometry staining.

Table 8: Flow cytometry monoclonal antibodies

Marker	Fluorochrome	Clone
CD11b	PerCpCY5.5 or PE-e610	M1/70
CD19	PE or BV711	1D3
CD25	PE or A647	PC61
CD3	BV711	17A2
CD4	PE-Cy7 or PE-Cy5	GK1.5
CD44	e450	IM7
CD45.1	A647	A20
CD62	PE-Cy7 or PE-Cy5	Mel14
CD69	PE	H1.2F3
CD8	BV605 or PE-Cy5.5	A-53-6.7
CXCR5	PE	L138D7
F4/80	PE	BM8
FcgIII/II receptor	-	2.4G2

Fixable Viability Dye	eFluor 780	-
Foxp3	APC or PE or e450	FJK-16s
GR1	PE-Cy5.5	RB6-8C5
ICOS	PE-Cy7	7E.17G9
IgD	e45	11-26c
IgM	PE-Cy7	II/41
IL10	A700	JES5-16E3
IL17A	e660	17B7
IL2	PE-Cy7	JES6-5H4
IL4	PerCP-eFluor 710	11B11
INFg	Bv650	XMG1.2
Ly6C	PerCP-Cy5.5	HK1.4
Ly6G	PE-Cy7	Poly4053
Ly77	FITC	GL7
NK1.1.	A647	PK136
PD1	PE	RMP1-30
TCRß	Bv510 or Bv605	H57-597
TNFa	PE	MP6-XT22
TNFa	e660	TN3-19

For surface staining (flow cytometry), cells were incubated with anti-FcγIII/II receptor antibody in PBS 2% FBS (20 min., 4°C). Surface staining was performed in PBS 2% FBS (20 min., 4°C). After washing and centrifugation (666g, 2 min., 4°C), cells were incubated with an antibody mix (25 µL, 1:100) for 20 minutes (4°C). Dead cells were excluded for flow cytometry analysis of surface staining using fixable viability dye® (eBiosience™). For intracellular cytokine and intracellular Foxp3 stainings, fixation and permeabilization were achieved by eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (#00-5523-00). Cells were fixed by adding 150µL of IC fixation buffer and incubated for 45 minutes (4ºC). After washing with 100µL of permeabilization buffer and centrifuge (666g, 2 min., 4°C), the supernatant was discarded. Cells were then re-suspended in the antibody mix (25 µL, 1:100) constituted by the combination of the above-listed antibodies and diluted in permeabilization buffer (45min., 4ºC). Finally, after washing with 100µL of permeabilization buffer, centrifugation (666g, 2 min., 4°C) and supernatant discard, stained cells were re-suspended in 200µL of PBS 2% FBS before sample analysis.

Cell numbers were assessed by flow cytometry, using a fixed number of latex beads (Beckman Coulter®) co-acquired with a pre-established volume of the cellular suspensions. Flow cytometry analyses was performed in the BD LSRFortessa™ X-20 cell analyzer, using the FACS DIVA™ software (BD Biosciences®) for acquisition. Post-acquisition analysis was performed with FloJo® software (Treestar®).

Adoptive transfer of splenocytes. Previously described harvested splenocytes were counted by flow cytometry, using a fixed number of latex beads (Beckman Coulter®) co-acquired with a pre-established volume of the cellular suspensions. Cells were then transferred ($5x10^6$/200µL/mouse) retro-orbitally into 8-10 weeks-old gender-matched *Rag2*$^{-/-}$ and followed for survival.

Bioluminescence monitoring. Three days before imaging setup, the area of analysis was shaved while mice were sedated with isoflurane (2% in medical liquid oxygen, Linde®). For in vivo heating, we used a large water bath with automatic temperature regulation, preventing fluctuations in temperature during the experiment. Water temperature was measured with a calibrated thermometer (Luxtron®, California, USA). For applying the heat-shock (45°C, 8 min), animals were sedated with ketamine (150mg/Kg; DOMTOR© 1 mg/ml, Orion®) and xylazine (12mg/Kg; Seton© 20 mg/ml, Calier®) and their left leg was introduced in the water [3].

For hypoxia induction, mice were placed in hypoxia chamber (INVIVO2 from Biotrace, Bridgend, U.K.) containing 21% O_2. The O_2 levels were decreased 2% every 10 min., until 8% O_2, mixing with CO at a concentration of 1% (10,000 ppm) in compressed air. CO concentrations were controlled by varying the flow rates of CO in a mixing cylinder before delivering it to the chamber. A CO analyzer (Interscan Corp., Chatsworth, CA) was used to measure CO levels in the chamber. The mice were left at 8% O2 for 4 h before imaging [7,8].
For oxidative stress induction, sodium arsenite solution (ASN; NaAs, Sigma-Aldrich® #1062771000-1L, 12.5 mg/kg) was intraperitoneally injected. As a control for ASN, PBS was injected. Mice were analysed 6 hours after injection [9,10].

For bioluminescence measure, mice were injected intraperitoneally with D-luciferin (2.9 mg in 100 µL PBS, Promega®, Madison, Wisconsin, USA) and sedated 7 min later with ketamine (150mg/Kg) and Xylazine (12mg/Kg). Luciferase activity was visualized by whole-mice imaging using an electron-multiplying charge-coupled device (EM-CCD) photon-counting camera (ImagEM®; Hamamatsu®). Quantification and analysis were done using ImageJ/Fiji® software.

Psoriasis-like dermatitis. *Hsf1*$^{R26\Delta/\Delta}$ mice and controls at 8 to 11 weeks of age received a daily topical dose for 5 consecutive days of 62.5 mg of commercially available Imiquimod cream (5%) (Aldara®; 3M Pharmaceuticals®) on the 5-days previously shaved back and the right ear, translating in a daily dose of 3.125 mg of the active compound. Back and right ear skin were harvested, submerged in 10% formalin and histologically scored on day 9.

Statistics. Statistical analyses were performed on Prism 7 (GraphPad®) using the Nonparametric Mann-Whitney U test, when the sample size was smaller than 5, or did not follow a normal distribution, and when it did, Unpaired Student's t-test for unequal variances was used. Comparisons between groups were performed using unpaired, two-sided t-test for normally distributed data or Mann-Whitney otherwise. For grouped comparisons, bootstrapping resampling method was applied, unless otherwise mentioned, one-way or two-way ANOVA followed by Tukey's multiple comparisons analysis were used when appropriate. Normal distributions were assessed using the Kolmogorov-Smirnov test. $p<0.05$ was considered statistically significant.

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Chapter 3

Regulatory T cell depletion: An autoimmune disease model

Abstract

In this chapter we describe an experimental model whereby autoimmune disease develops in Foxp3^{DTR} mice in response to depletion of Foxp3^{+} T_{REG} cells [1]. To test whether specific stress and damage responses conferring tissue damage control modulate the pathogenesis of autoimmune diseases that develops when peripheral self-tolerance is compromised, we used a genetic loss-of-function approach to impair specific stress and damage responses conferring tissue damage control in parenchymal tissues. To this aim we used bone marrow chimeras in which hematopoietic and non-hematopoietic cells are genetically disentangled, such that wild type hematopoietic cells are confronted to non-hematopoietic cells lacking or not the ability to respond to different forms of stress and/or damage. The data described in this chapter shows that ablation of Foxp3^{+} T_{REG} cells leads to the development of disease. This is associated with the development of autoimmunity and with varying levels of damage to different organs, including the liver, stomach, pancreas and skin. Disease development was associated with lymphoid organ enlargement and a progressive increase in the number of activated CD4^{+}CD44^{hi}CD62^{lo} T_H cells and CD8^{+}CD44^{hi}CD62^{lo} T_c as well as activated B cells. This experimental model was then adapted and applied to bone marrow chimeric mice with similar results, allowing to disentangle the immune system from non-hematopoietic cells in a controlled manner. In conclusion, T_{REG} cell ablation in Foxp3^{DTR} mice leads to the development of autoimmune disease affecting different organs with different levels of severity.

3.1. Introduction

3.1.1. Strategies for the induction of experimental autoimmune diseases

The first experimental evidence of a human autoimmune disease was documented in 1888 when patients undergoing rabies vaccination occasionally developed paralysis [2]. This evidence that immune cells can attack the brain was later recognised by Rivers et al. and led to the first breakthrough in autoimmunity [3], acknowledging that complete Freund's adjuvant was the cause [4].

Experimental models of human disease using different organismal models are widely accepted research tools used to investigate the pathogenesis and pursuit of novel therapeutics against human diseases [5]. Experimental animal models of autoimmune diseases may be divided into spontaneous and induced. In the former, disease develops spontaneously with or without genetic modifications, while in the latter, disease is artificially induced [6]. Different strategies have been used to induce autoimmune diseases in animals. These can be grouped into three categories: 1) immunisation with autoantigens, 2) transfer of autoimmunity and 3) induction by environmental factors, such as infection, drugs or adjuvants [7].

We developed an experimental animal model of autoimmune disease whereby autoimmune cells can cause stress and/or damage to parenchyma cells that can deploy or not different stress and damage responses conferring tissue damage control and disease tolerance. To this aim we decided on an experimental model developed by Rudensky[1] whereby Foxp3^{DTR} T_{REG} cells are ablated upon administration diphtheria toxin leading to the development of an aggressive form of disease in adults which involves different organs, including the liver, lungs and the skin [1]. This model was chosen against other experimental models whereby T_{REG} cell function is impaired using anti-IL-2 neutralizing antibodies [8] or T_{REG} cells are transiently depleted due to an expression of diptheria toxin receptor under the control of Foxp3 promoter on a bacterial artificial chromosome – DEpletion of REGulatory T cells (DEREG) mouse model [9]. In comparison to DEREG mice, depletion of Foxp3^{DTR} T_{REG} cells by the administration diphtheria toxin was not associated with a marked “rebound” of T_{REG} cells and presented with a more aggressive disease [1], similar to human autoimmune diseases. This experimental model allows autoreactive cells to develop progressively

upon deletion of Foxp3^{DTR} T_{REG} cells, imposing over time increasing levels of stress and damage to different organs which, from a clinical standpoint, may resemble patients where autoimmunity develops leading or not to overt autoimmune disease.

3.1.2. Strategy to disentangle hematopoietic from non-hematopoietic cells

Testing experimentally whether stress and damage responses operate specifically in non-hematopoietic cells to prevent the pathogenesis of autoimmune disease required the development of an experimental model whereby stress and damage responses are regulated specifically in parenchyma (*i.e.,* non-hematopoietic) cells. To this aim we performed bone marrow chimeras using bone marrows from Foxp3^{DTR} mice to reconstitute Rag2-deficient mice lacking adaptive immune cells. The recipient *Rag2*-deficient mice were genetically engineered (see Chapter 2. Methods) to delete specific master regulators of stress and damage responses specifically in non-hematopoietic cells. These *Rag2*-deficient mice which are unable to produce adaptive immune cells will be grafted with a donor complete immune system. In turn, each donor bone marrow will also carry, in Foxp3^{+} cells, a DTR marked with green fluorescent protein (GFP), which grants their recognition. Once the mouse chimera is created, the hematopoietic system, particularly the adaptive immune system, will be from the donors and the non-hematopoietic cells from the host, allowing the study of distinct features of these systems.

3.2. Results

To break immune tolerance, we used Foxp3^{DTR} mouse model that allows specific elimination of T_{REG} cells *in vivo*. Foxp3^{DTR} mice express the human diphtheria toxin receptor (DTR) fused to a green fluorescent protein (GFP) after an internal ribosome entry site (IRES), knocked in into the 3' untranslated region of *Foxp3* (Fig.3.1A). Expression of the DTR was monitored by PCR (Fig. 3.1B).

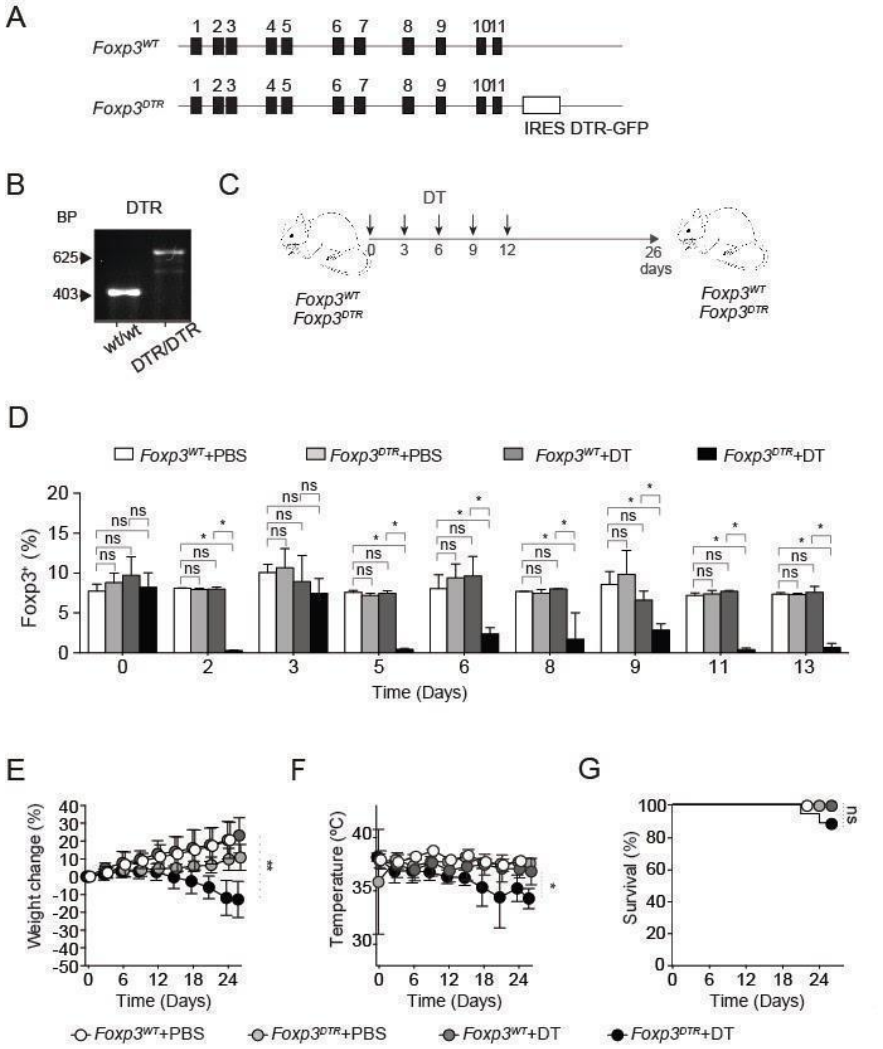

Figure 3.1. Disease development upon depletion of T_{REG} cells in Foxp3^{DTR} mice

(A) Schematic diagram of the Foxp3^{DTR} allele insertion of a human diphtheria toxin receptor (DTR) fused to sequences encoding a green fluorescent protein (GFP) and equipped with an internal ribosome entry site (IRES) into the 3′ untranslated region of Foxp3 locus. **(B)** Electrophoresis of PCR products amplified from genomic DNA of mice, generated as depicted in A) and carrying DTR insertion. **(C)** 8-12 weeks-old mice bearing a diphtheria toxin receptor on regulatory t cells (Foxp3^{DTR}) or from wt mice (Foxp3^{WT}) were treated with diphtheria toxin (DT) every three days for two weeks and followed up to 26 days. **(D)** Peripheral blood depletion of T_{REG} in mice treated as depicted in (C) (Foxp3^{WT}+PBS n=3, Foxp3^{DTR}+PBS n=3, Foxp3^{WT}+DT n=5, Foxp3^{DTR}+DT n=5). **(E-G)** Weight change (E), rectal temperature (F) and survival (G) during the 26 days follow-up time. (Foxp3^{WT}+PBS n=8, Foxp3^{DTR}+PBS n=16, Foxp3^{WT}+DT n=12, Foxp3^{DTR}+DT n=20). *p < 0.05, as determined by log-rank (Mantel-Cox) test for survival comparison and ANOVA test with Tukey's multiple comparisons for the remainder. Standard bars represent mean ± SD. ns, not significant. Results from 3 independent experiments.

To delete Foxp3$^+$ T_{REG} cells and unleash autoreactive cells, we administered diphtheria toxin (DT) at different dosage every three days for sixty days, adapting the originally proposed protocol [1]. We first compared the effect of DT administration on the survival, weight change and organ damage in WT mice (Fig.3.2A-D).

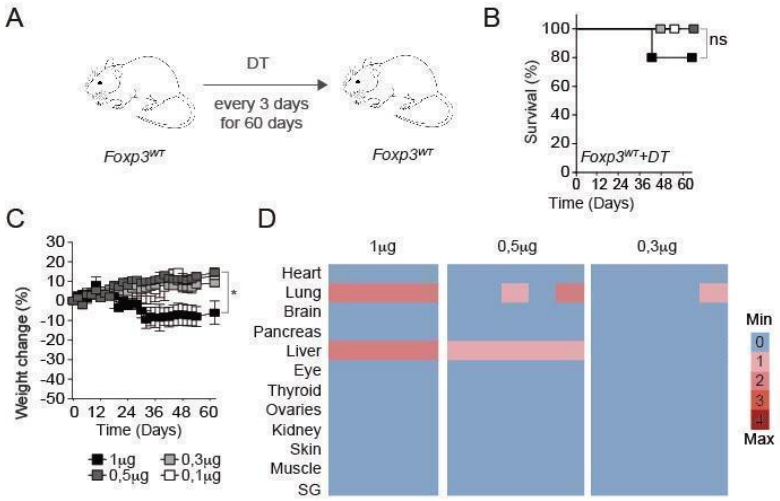

Figure 3.2. Diphtheria toxin toxic effect is dose-dependent

(A) Illustration of diphtheria toxin (DT) administration every three days to wild type animals (Foxp3^{WT}). **(B-C)** Survival (B) and weight change (C) of Foxp3^{WT} treated with different DT concentrations as described in (A). Representative data of one experiment. **(D)** Array plot analysis of DT toxin concentration-dependent toxicity. Each line represents an organ, and each column represents an individual mouse. SG, salivary glands. *p < 0.05, as determined by log-rank (Mantel-Cox) test for survival comparison and two-way ANOVA test with Tukey's multiple comparison for the remainder. Standard bars represent mean ± SD. ns, not significant.

We found that DT was partially lethal (Fig.3.2B) and associated with weight loss (Fig.3.2C) as well as damage to the lungs and liver (Fig.3.2D) when administered to C57BL/6 mice at a dosage above 0.5 µg per Kg of body weight every third day. Therefore, DT doses >0.5 µg/Kg are highly toxic irrespective of T_{REG} depletion which was not observed when DT was administered at a dosage 0,3µg per Kg of body weight every third day (Fig.3.2B-D). This DT dosage and schedule was sufficient however to induce the depletion of Foxp3$^+$ T_{REG} cells. Briefly, there was a 98% depletion of Foxp3$^+$ cells 48h after DT administration to *Foxp3^{DTR}* mice *vs.* control *Foxp3^{DTR}* mice receiving PBS. Moreover, there was noticeable depletion of Foxp3$^+$ cells 48h after DT administration when compared to control *Foxp3^{WT}* mice receiving PBS (Fig.3.1D). There was a rebound of Foxp3$^+$ T_{REG} cells 3 days after the first DT administration to

Foxp3^{DTR} mice (Fig.3.1D). This Foxp3^{+} T_{REG} cells rebound was not yet observed after 11 days of the scheduled DT, rendered by a low percentage of peripheral Foxp3^{+} cells from that day on (Fig.3.1D).

Depletion of Foxp3^{+} T_{REG} cells in *Foxp3*^{DTR} mice was associated, 15 days after receiving DT, with the development of sickness behaviour as well as with a decrease in weight (Fig.3.1E) and temperature (Fig.3.1F), without impacting on survival (Fig.3.1G). This was not observed in control *Foxp3*^{WT} mice receiving DT or in control *Foxp3*^{DTR} mice receiving PBS, which gained weight and maintained body temperature over the same period.

We conclude that the dose and posology of DT administration of 0,3ug per Kg BW, every 3d day for two weeks, efficiently depletes regulatory T_{REG}s in *Foxp3*^{DTR} mice. Moreover, this experimental model is associated with the development of disease along the follow-up time.

To determine if depletion of T_{REG} cells is associated with the development of autoimmune disease, we analysed different organs 26 days after the first DT administration. While we found that weight and temperature loss, two hallmarks of disease following T_{REG} cell depletion in *Foxp3*^{DTR} mice, became apparent, organs such as liver, stomach, pancreas and skin were moderately affected when compared to control groups with physiological levels of circulating T_{REG} cells (Fig.3.3).

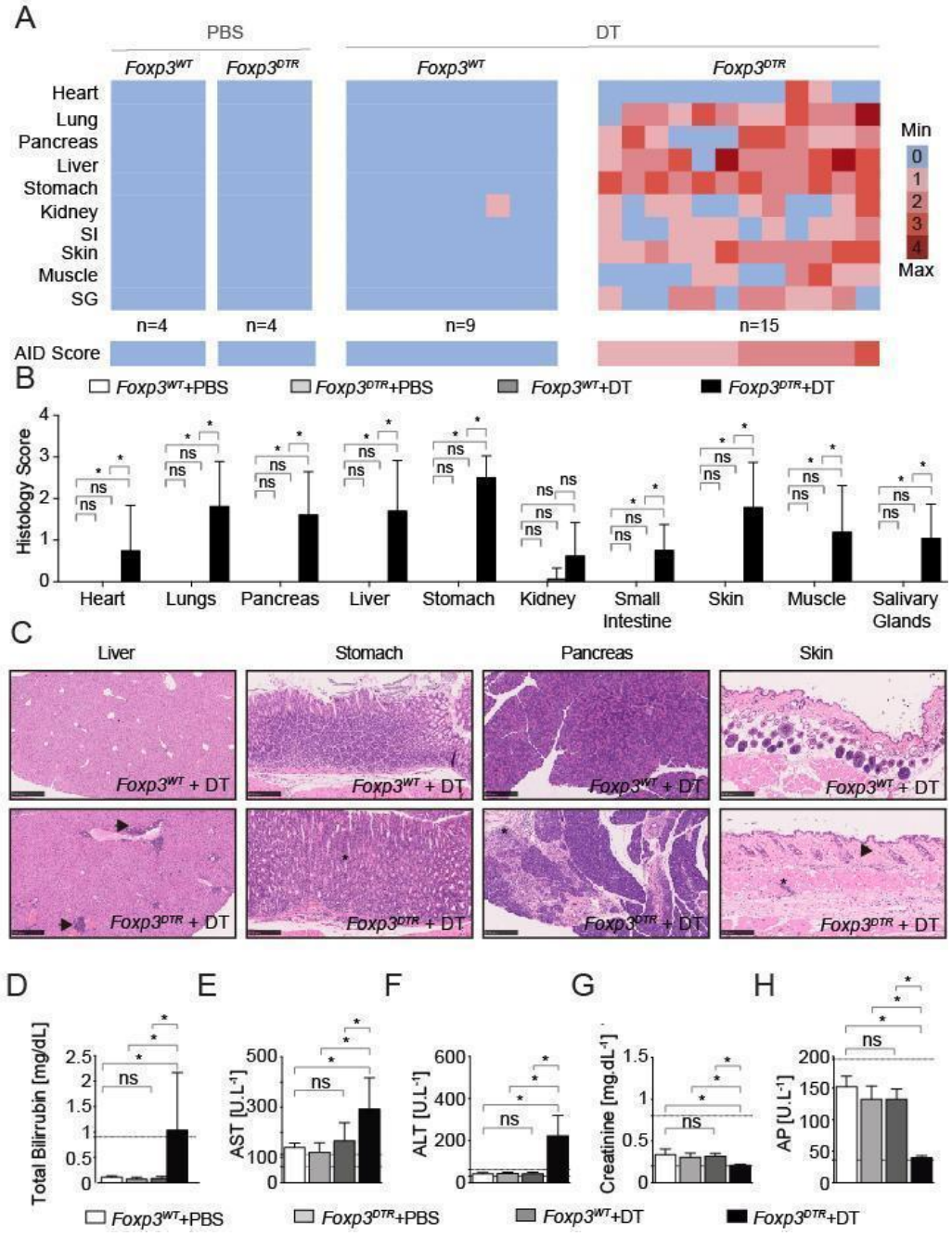

Figure 3.3. T_{REG} cell elimination triggers the multiorgan autoimmune disease

(A) Autoimmune disease (AID) score of organs from mice as described in (3.1.C), retrieved from histological analyses on day 26 after starting diphtheria toxin (DT). Each column represents an individual mouse. Mean AID scores are pictured in the bottom horizontal line. **(B)** Bar plot of histological pathology score per organ as in (A). Standard bars represent mean ± SD. *p < 0.05, as determined by bootstrapping resampling method. **(C)** Hematoxylin and eosin representative staining of liver, stomach, pancreas and skin from $Foxp3^{DRT}$ and $Foxp3^{WT}$ mice that received DT. T_{REG} depleted mice present liver periportal and perivascular infiltration (arrows), gastric submucosa widening with mononuclear and granulocyte invasion (*), pancreatic destruction of exocrine acini (*) and dermal thickening with a decrease of hair follicles (arrow) and focal immune cells invasion (*). **(D-H)** Liver serology (total bilirubin (C), AST: alanine transaminase (D), ALT: aspartate transaminase (E)), creatinine (F) and alkaline phosphatase (AP) n=3 per group. *p < 0.05, as determined by ANOVA and Tukey's multiple comparisons test. ns, not significant. Results from 3 independent experiments.

Organ damage presented histologically by extravascular mononuclear cell infiltrates into parenchyma and more or less extensive foci of disruption of tissue architecture (Fig3.3C). This organ damage was observed by periportal and perivascular cellular infiltration in the liver, by gastric submucosa widening with mononuclear and granulocyte invasion in the stomach, by the destruction of exocrine acini in the pancreas and by dermal thickening with a decrease in the relative number of hair follicles and focal immune cells invasion in the skin (Fig3.3C).

Organ damage was confirmed by the accumulation of organ-damage-specific biomarkers in serum. This was the case for liver damage by the accumulation of bilirubin, aspartate aminotransferase and alanine aminotransferase in the serum of *Foxp3^{DTR}* mice lacking T_{REG} cells in response to DT administration, compared to control *Foxp3^{DT}* or *Foxp3^{WT}* mice receiving PBS as well as control *Foxp3^{WT}* mice receiving DT (Fig3.3D-H). There was a concomitant decrease in creatinine (*i.e.,* a serological marker of muscle and renal damage) and also a decrease of alkaline phosphatase (*i.e.,* a serological marker of bone and liver damage) in *Foxp3^{DTR}* mice lacking T_{REG} cells in response to DT administration, compared to controls.

To establish whether T_{REG} cell depletion affected cardiovascular function, we measured pulse distention, as a proxy for blood pressure and heart rate. Comparison of *Foxp3^{DTR}* mice lacking T_{REG} cells (*i.e., Foxp3^{DTR}*+DT) *vs.* control mice (*i.e., Foxp3^{DTR}+PBS, Foxp3^{WT}+DT, Foxp3^{WT}+PBS)*, showed a decrease in pulse distention (Fig3.4A) without affecting heart rate (Fig3.4B). This suggests that T_{REG} cell depletion impairs peripheral vascular resistance and/or heart stroke, without an expected heart rate compensation to maintain the mean blood pressure.

We conclude from these observations that T_{REG} cell depletion affects a wide range of organs, which are probably targeted by autoreactive cells. Presumably this leads to organ damage and dysfunction overtime without impacting on survival (Fig.3.1G).

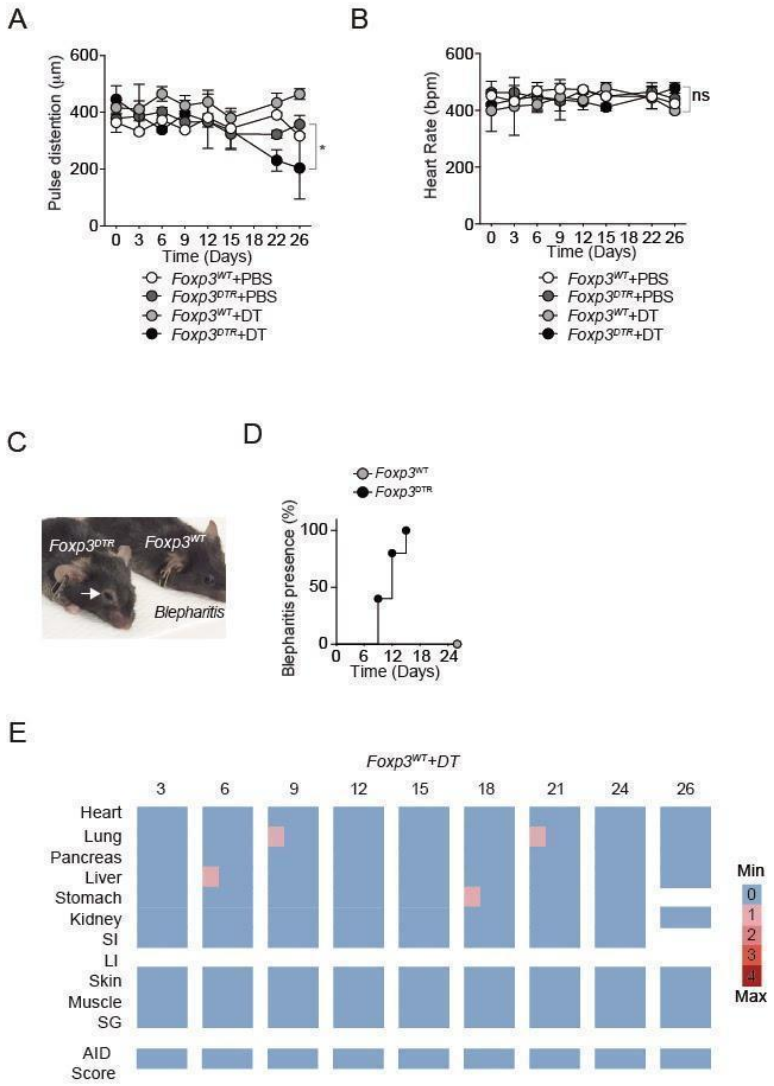

Figure 3.4. Arterial volume flow reduction and macroscopic autoimmunity signs of autoimmunity during autoimmune disease pathogenesis

(A-B) Mice bearing a diphtheria toxin receptor (*Foxp3^{DTR}*) and wild type animals (*Foxp3^{WT}*) treated or not with diphtheria toxin were analysed for pulse distention (A) (variation between pulsatile and non-pulsatile arterial blood volume) and heart rate (B). n=6, Results from 2 independent experiments. **(C-D)** Same mice analysed in (A-B) display signs of blepharitis (C), whose incidence is represented in (D). **(E)** Organ histological score every three days of wild type (*Foxp3^{WT}*) mice receiving diphtheria toxin as described in (3.1.C). (n=3 per time point) *p < 0.05, as determined by two-way ANOVA and Tukey's multiple comparisons tests. Standard bars represent mean ± SD. ns, not significant.

To further understand whether T_{REG} cell depletion was associated with unfettered immune activation, we analysed the lymphoid organs (Fig.3.5A-E) and tissues (Fig.3.5F;3.6). We found that T_{REG} cell depletion in *Foxp3^{DTR}* mice receiving DT led to an enlargement of the spleen and lymph nodes, associated with a progressive increase in the number of activated $CD44^+CD62L^-CD4^+$ T_H cells and $CD44^+CD62L^-CD8^+$ T_C cells (Fig3.5B-C), compared to control *Foxp3^{WT}* mice receiving DT. This increase in $CD4^+$ and $CD8^+$ activated T cells was associated with a decrease in the percentage of $CD19^+$ B cells, which did not reflect the overall increase in the numbers

of CD19^{+} B cells (Fig3.3D). This was associated with an increase in circulating IgG (Fig3.3E). These observations confirm that T_{REG} cell depletion is associated with unfettered T and B cell activation and the production of circulating IgG natural antibodies.

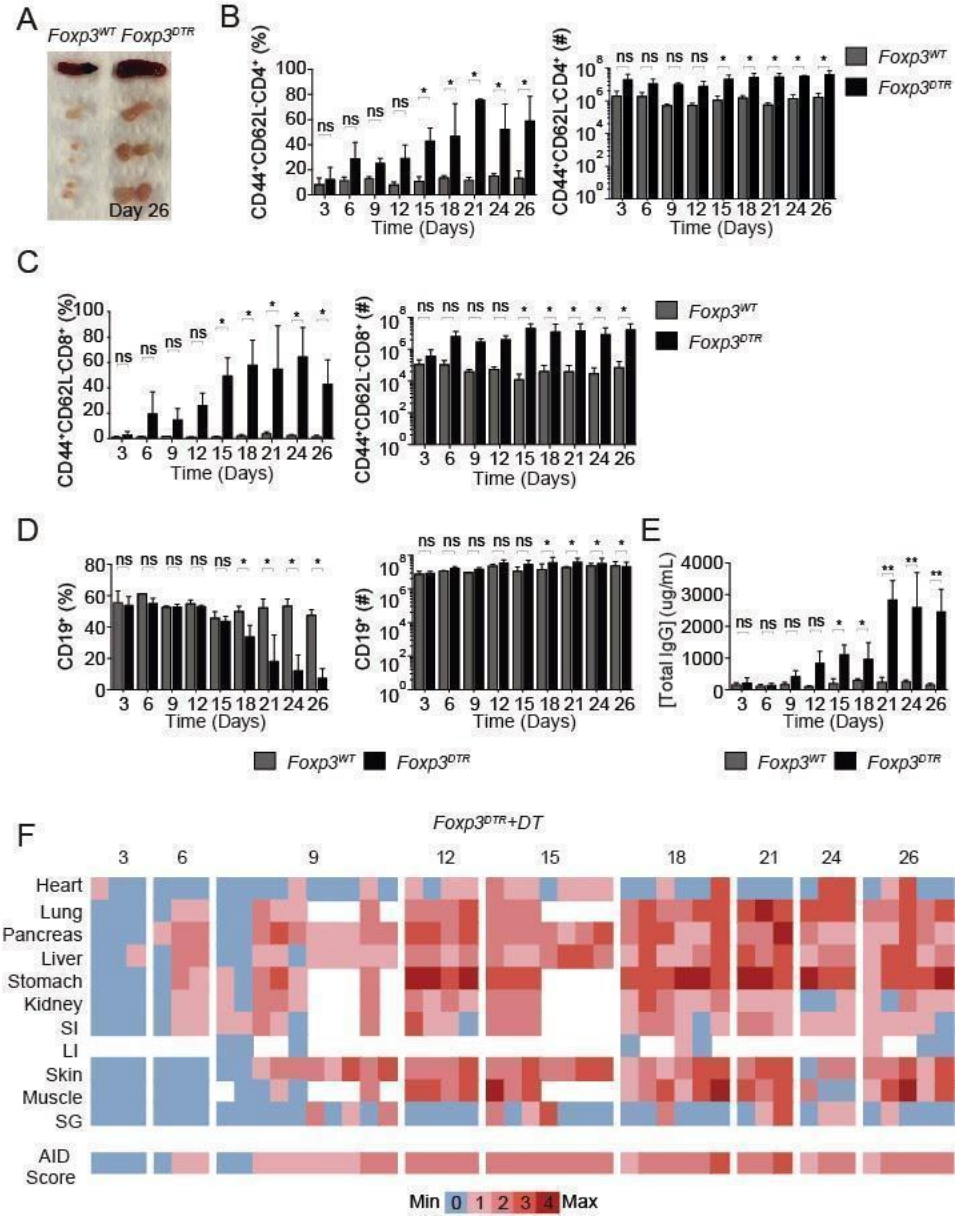

Figure 3.5. Kinetics of adaptive immune system activation in response to acute elimination of T_{REG} cells and consequently multiorgan autoimmune disease

(A) Regulatory T cell (T_{REG}) depletion promotes lymphadenopathy and splenomegaly of 8-12-month-old Foxp3^{DTR} and Foxp3^{WT} mice injected with diphtheria toxin (DT) every three days. Splenic effector memory (CD62L^{low} and CD44^{high}) CD4^{+} **(B)** and CD8^{+} **(C)** T cells in mice upon DT administration and analysed by flow cytometry every 3 days (n=6 per time point). **(D)** Splenic B (CD19^{+}) cells in the same mice as (A-C). **(E)** Total serum concentration of immunoglobulin G of the same mice as (A-D; n=3). **(F)** Array plot of histological Autoimmunity Disease (AID) score in organs from T_{REG} depleted (Foxp3^{DTR}+DT) mice obtained every three days after the first bolus of DT administration. Each horizontal line represents an organ and each column an individual mouse. Mean AID scores are illustrated for each genotype in the bottom horizontal lines. SI: small intestine, LI: large intestine, SG: salivary glands. *p < 0.05, as determined by two-way ANOVA and Sidak's multiple comparisons tests. Standard bars represent mean ± SD. ns, not significant.

Time-lapse of histological organs analysis after T_{REG} cell depletion disclosed a mild to severe inflammatory damage in 8 out of the 11 organs analysed (Fig3.5F), namely in the lung, pancreas, liver, stomach, skin, kidney, small intestine and muscle (Fig3.6; Fig3.5F). The lungs, pancreas, liver and stomach showed signs of immunopathology from day 6 after the first DT administration onwards. At the end of the follow-up (*i.e.,* day 26), the most affected organs were lungs, liver, pancreas, stomach and skin (Fig.3.5F). Mild signs of DT-driven toxicity were rarely detected in the lung, liver and stomach of control $Foxp3^{WT}$ mice receiving DT, at the same dosage and schedule (Fig. 3.4E). Externally, blepharitis was a sign previously reported of autoimmunity in this model (Fig.3.4C) and its prevalence increased over time, beginning on day 9 and with widespread prevalence around day 15 (Fig.3.4D). The histological and external signs of disease allow to conclude that this range of organ targeting and severity is ideal for testing different experimental settings in an autoimmune context.

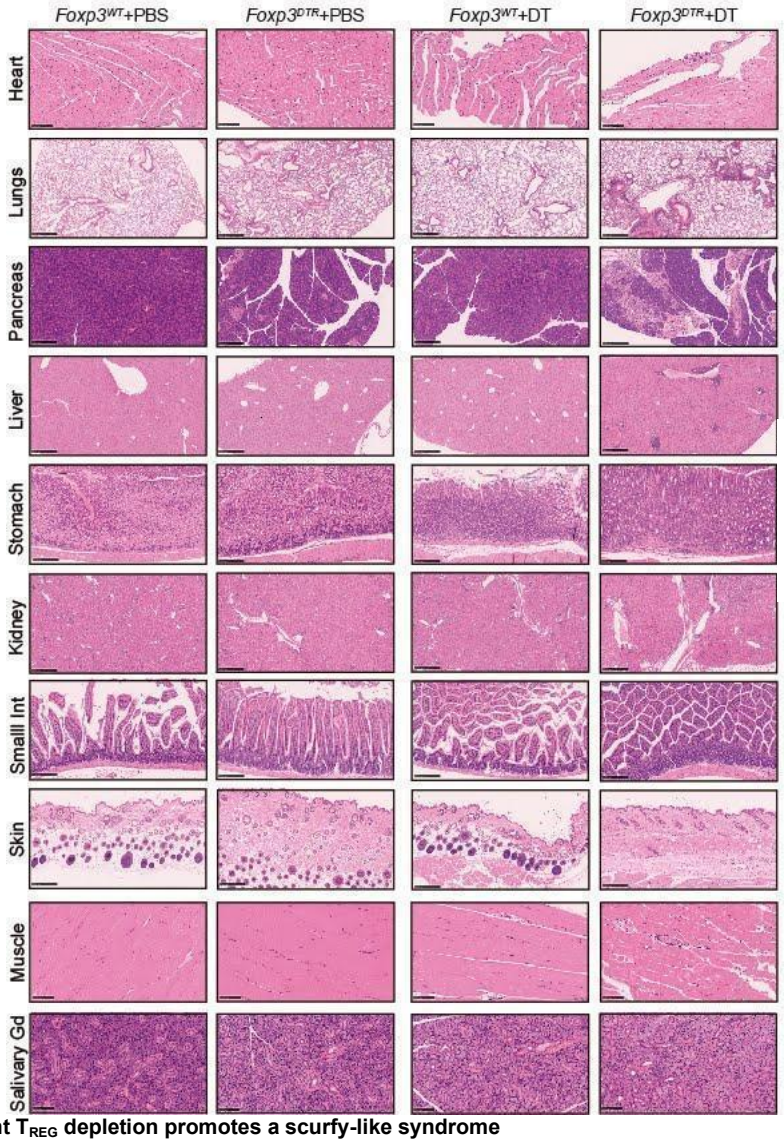

Figure 3.6. Transient T_{REG} depletion promotes a scurfy-like syndrome

Hematoxylin and eosin pictures representative of different organs from *Foxp3*^{WT} and *Foxp3*^{DTR} mice, on day 26 after begin PBS or diphtheria toxin (DT) administration (*Foxp3*^{WT}+PBS n=4, *Foxp3*^{DTR}+PBS n=4, *Foxp3*^{WT}+DT n=9, *Foxp3*^{DTR}+DT n=15). Scale bar = 500μm. Mice treated with vehicle (PBS) do not present signs of inflammatory infiltrates in any organ. Once upon diphtheria toxin administration, *Foxp3*^{WT} present a similar phenotype across the organs except for the stomach, where scattered inflammatory cells are observed in the lamina propria of the gastric mucosa. On the other hand, *Foxp3*^{DTR} DT treated mice present inflammatory infiltrates in all organs. In the heart, focal minimal infiltrates in the myocardium are present. The lungs display mild perivascular, and peribronchial inflammation (arrow) and the pancreas show mild inflammation with focal acinar atrophy (arrow). In the liver, a mild portal to lobular inflammation is seen (arrow), and the gastric mucosa shows moderate granulocytic and mononuclear infiltrates. The kidney displays minimal interstitial infiltrates (arrow), and the small intestine presents minimal inflammatory infiltrates in the lamina propria. The skin of regulatory T cell-depleted animals (*Foxp3*^{DTR}+DT) exhibits mild, multifocal inflammation with increased numbers of lymphocytes, plasma cells and neutrophils (arrow). Finally, muscle and salivary glands also show minimal inflammatory infiltrates (arrows).

To establish whether age affects the extent of organ damage imposed by T_{REG} cell depletion, the same protocol of T_{REG} depletion was used in 18 weeks-old mice (Fig3.7A). T_{REG} depleted *Foxp3^{DTR}* mice lost weight and temperature, when compared with age-matched *Foxp3^{WT}* mice receiving DT (Fig3.7B-C). Autoimmune organ damage was also observed after T_{REG} cell depletion in 18-month-old *Foxp3^{DTR}* mice receiving DT, with a range of severity varying from mild to severe in the large intestine, skin, liver, pancreas, salivary glands, muscle, lungs, kidney, small intestine and stomach, respectively (Fig3.7E). Of note, glycemia levels suffer a fluctuation along the 26 days of follow-up, being lower, but within a normal range, when T_{REG} cells were depleted *vs.* controls. These observations grant that this protocol efficiently depleted T_{REG} and induced disease in mice with 18 weeks-old.

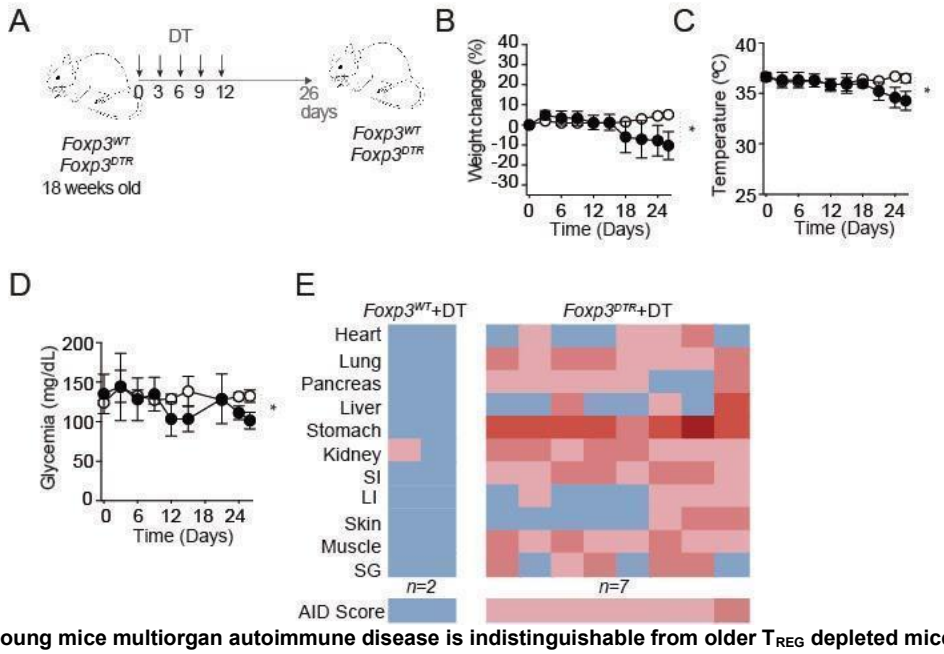

Figure 3.7. Young mice multiorgan autoimmune disease is indistinguishable from older T_{REG} depleted mice

(A) 18 weeks-old mice bearing a diphtheria toxin receptor on regulatory t cells (*Foxp3^{DTR}*) or from WT mice (*Foxp3^{WT}*) were treated with diphtheria toxin (DT) every three days for two weeks and followed up to 26 days. **(B-D)** Weight change (B), rectal temperature (C) and glycemia (D) during the 26 days follow-up time (*Foxp3^{DTR}* n=7, *Foxp3^{WT}* n=2). *p < 0.05, as determined by two-way ANOVA test with Sidak's multiple comparison for the remainder. Standard bars represent mean ± SD. ns, not significant. **(E)** Array plot of histological Autoimmunity Disease (AID) score in organs from T_{REG} depleted (*Foxp3^{DTR}*+DT) mice obtained on day 26 after the first bolus of DT administration. Each horizontal line represents an organ and each column an individual mouse. Mean AID scores are illustrated for each genotype in the bottom horizontal lines. SI: small intestine, LI: large intestine, SG: salivary glands.

Testing whether stress and damage responses operate specifically in non-hematopoietic cells to prevent the pathogenesis of autoimmune disease requires an experimental model whereby stress and damage responses are regulated specifically in parenchyma (*i.e.,* non-hematopoietic) cells. To this aim we asked whether autoimmune disease can be triggered upon T_{REG} cell depletion in bone marrow chimeric *Rag2*-deficient mice, carrying hematopoietic cells derived from the bone marrow cells of Foxp3^{DTR} *vs. Foxp3^{WT}* mice (Fig3.8A).

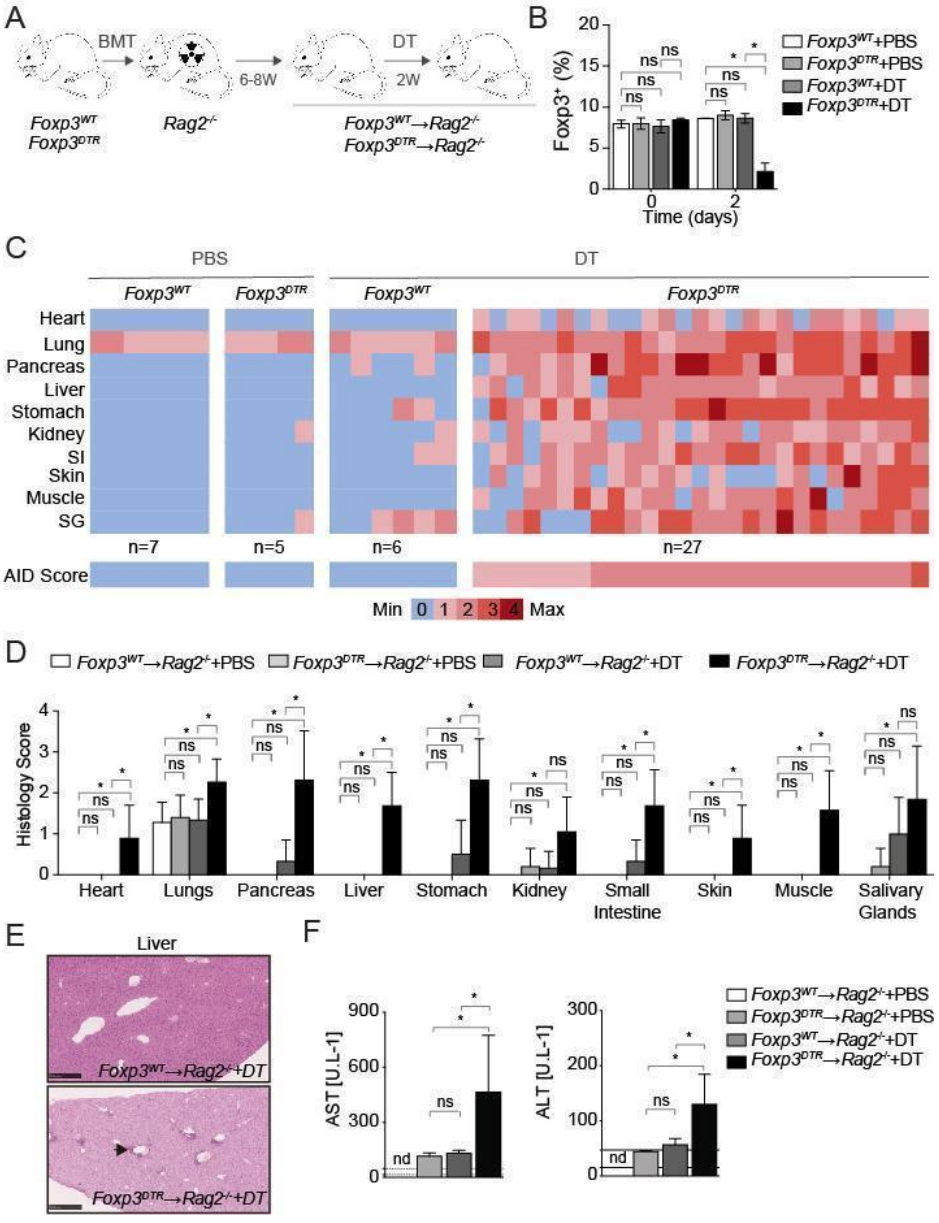

Figure 3.8. T_{REG} depletion in bone marrow chimeric mice reproduces multiorgan autoimmune disease

(A) Schematic representation of the strategy used to generate chimeric mice in which hematopoietic tissues are genotypically different from the non-hematopoietic ones. Non-lethally irradiated *Rag2* deficient mice lacking adaptive immune system were grafted with bone marrow (BTM) from mice harbouring a diphtheria toxin receptor on regulatory T cells (*Foxp3*^{DTR}) or from WT mice (*Foxp3*^{WT}). Reconstitution lasted for 6-8 weeks when diphtheria toxin (DT) was administered to induce depletion of regulatory T cells (T_{REG}) or vehicle as control (PBS) for two weeks. Finally, mice were analysed on day 26 after DT started, similar to the

protocol represented in (Figure 3.1C). **(B)** *Foxp3*$^{+}$ cells depletion in *Rag2* chimeric mice (n=3), as described in (A). **(C)** Autoimmune disease (AID) histological pathology score in organs of mice described in (A). Each column represents a separate mouse, and each line represents an organ. Mean Autoimmunity Disease score is depicted in the bottom horizontal line. SI: small intestine, LI: large intestine, SG: salivary glands. **(D)** Quantification of AID scores *per* organ *per* genotype of the mice represented in (C) and shown mean ± SD. *p < 0.05, as determined by bootstrapping resampling method. **(E)** Representative H&E staining of liver from chimeric mice developed as described in (A). Scale bar=500μm. **(F)** Liver enzymes concentration on day 26 after DT treatment beginning from the mice defined in (A). AST: alanine transaminase, ALT: aspartate transaminase. *Foxp3*^{WT}→*RAG2*$^{-/-}$ +DT n= 4, *Foxp3*^{DTR}→*RAG2*$^{-/-}$+PBS n=4, *Foxp3*^{DTR}→*RAG2*$^{-/-}$+DT n=13; Results from 2 independent experiments. *p < 0.05, as determined by ANOVA test and Tukey's multiple comparisons test. ns, not significant. nd, not determined.

Bone marrows from *Foxp3*^{DTR} or *Foxp3*^{WT} mice were adoptively transferred into non-lethally irradiated *Rag2*$^{-/-}$ mice to produce chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ and *Foxp3*^{WT}→*RAG2*$^{-/-}$ mice, respectively (Fig.3.8A). Chimeric mice *Foxp3*^{DTR}→*RAG2*$^{-/-}$ receiving bone marrow cells from *Foxp3*^{DTR} mice should allow for specific depletion of T_{REG} cells upon DT administration while control chimeric *Foxp3*^{WT}→*RAG2*$^{-/-}$ mice receiving bone marrows from *Foxp3*^{WT} mice should express physiologic numbers of T_{REG} cells upon DT administration.

Initially, we asked if T_{REG} cell depletion could be achieved using a protocol similar to the one depleting T_{REG} cells non-chimeric *Foxp3*^{DTR} mice (Fig.3.1D). There was a 50% reduction in the number of T_{REG} cells, two days after DT administration to chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice, as compared to control chimeric *Foxp3*^{WT}→*RAG2*$^{-/-}$ receiving DT or control chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice receiving PBS (Fig.3.8B).

We then asked whether this chimeric model would also reproduce the systemic autoimmunity observed upon T_{REG} cell depletion in non-chimeric *Foxp3*^{DTR} mice (Fig3.3A). T_{REG} cell depletion in chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice was associated with multiorgan autoimmune damage, as illustrated histologically in the heart, lungs, pancreas, liver, stomach, small intestine, skin and muscle, 26 days after the first DT administration (Fig.3.8C-D;3.9). This was not the case for control chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice receiving PBS or *Foxp3*^{WT}→*RAG2*$^{-/-}$ receiving DT or PBS (Fig.3.8C-D; 3.9).

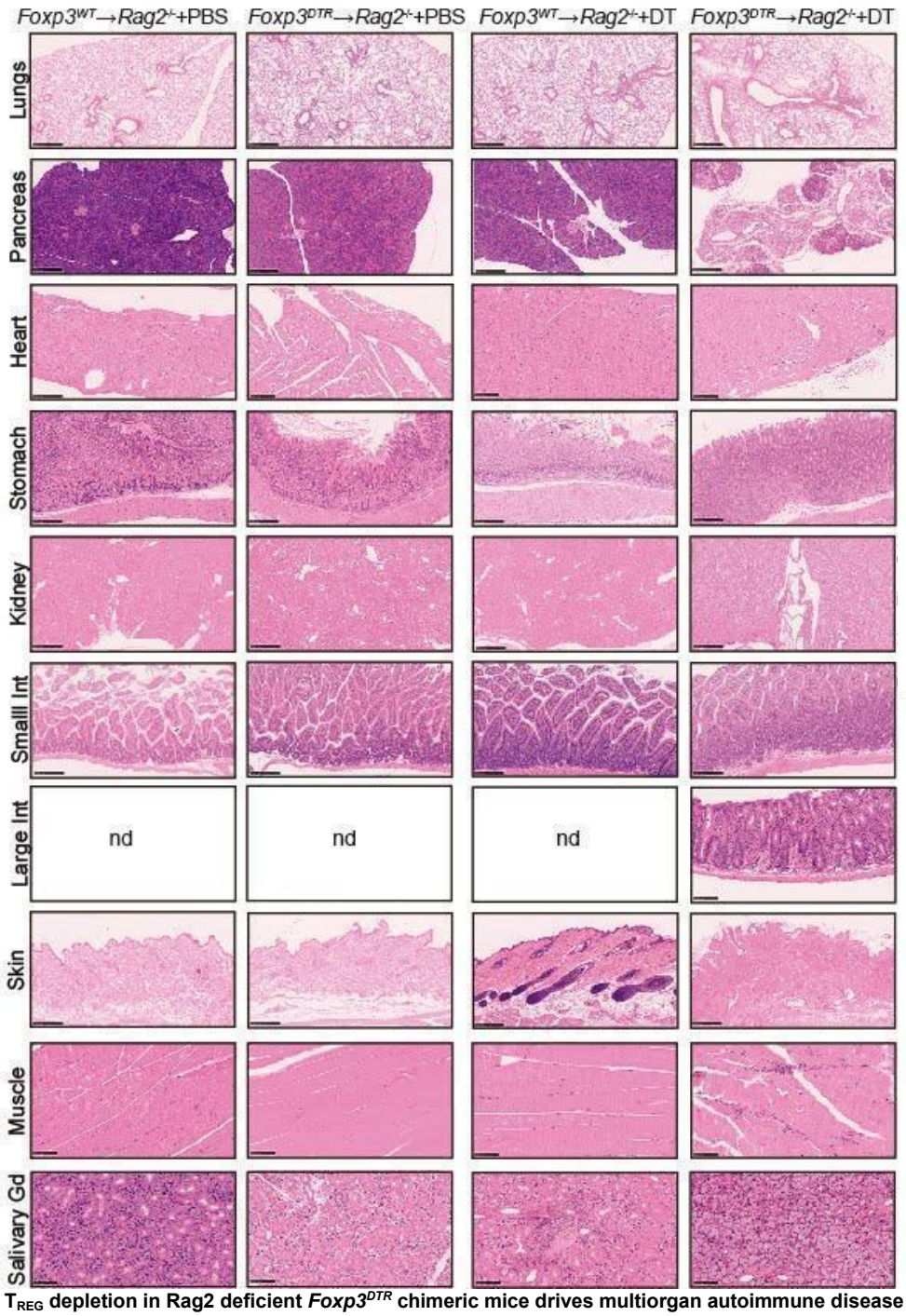

Figure 3.9. T_{REG} depletion in Rag2 deficient *Foxp3^{DTR}* chimeric mice drives multiorgan autoimmune disease targeting

H&E pictures representative of different organs from each chimeric group of mice on day 26 after diphtheria toxin administration began. (*Foxp3^{WT}→Rag2$^{-/-}$*+PBS n= 7, *Foxp3^{DTR}→Rag2$^{-/-}$*+PBS n=5, *Foxp3^{WT}→Rag2$^{-/-}$*+DT n= 6, *Foxp3^{DTR}→Rag2$^{-/-}$*+DT n=27) Scale bar = 500μM. Same as Figure 3.6. Lungs from chimeric mice were very mild to mild infiltrated by mononuclear and polynuclear cells around the peribronchial (arrow) and perivascular areas. The pancreas reveals an altered architecture with the destruction of exocrine cells (*) and preservation of islet cells. The forestomach mucosa, corpus and antrum are mildly inflamed with aggregates of mononuclear cells (*) in *Foxp3^{DTR}* chimeras compared with normal mucosa in *Foxp3^{WT}* chimeras. Similar results are shown for the heart, kidney, small intestine, muscle (arrow) and salivary glands (*) with scattered lymphocytes multifocally in T_{REG} depleted chimeras. The skin of the same chimeric mice depicts a mild inflammation with increased numbers of mononuclear and granulocytes multifocally dispersed. nd, not done.

Histological analyses of the liver showed periportal mono and polymorphonuclear cell infiltration upon T_{REG} depletion in chimeric *Foxp3^{DTR}→RAG2$^{-/-}$* mice, compared to normal hepatic structure control chimeric *Foxp3^{WT}→RAG2$^{-/-}$* mice receiving DT at the same dosage and schedule (Fig3.8E). Hepatic lesions were associated with accumulation of liver enzymes in plasma, confirming the hepatic damage observed histologically (Fig3.8F). Levels of total bilirubin, total proteins and alkaline phosphatase were similar in all experimental groups (Fig.3.10A-E).

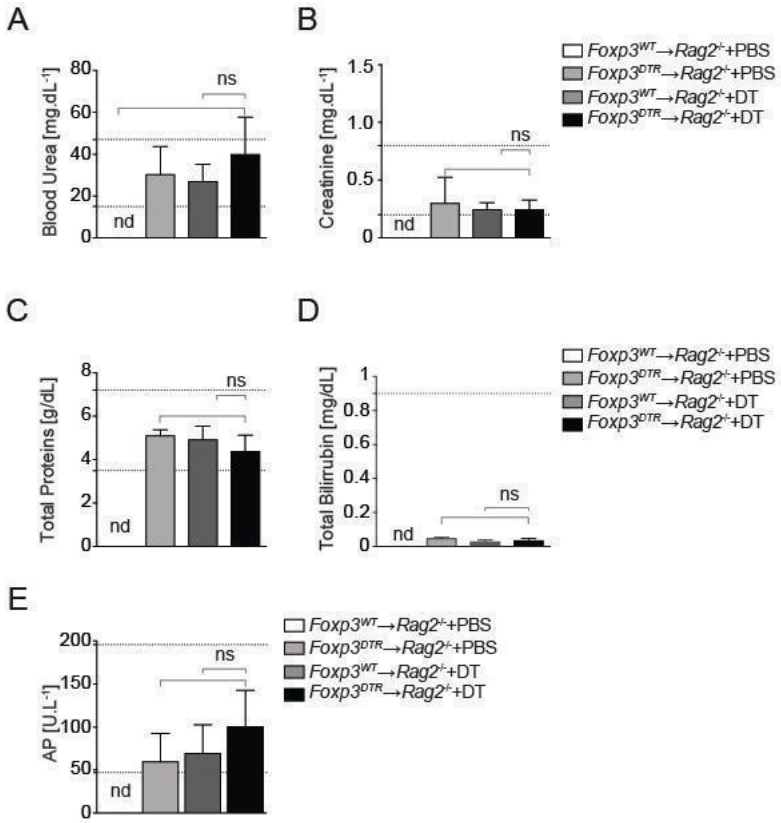

Figure 3.10. Upon T_{REG} depletion, kidney and liver function remains unaffected

(A-G) Kidney and liver function evaluation in blood analysis of urea (A), creatinine (B), total proteins (C), total bilirubin (D) and alkaline phosphatase (AP) **(E)** concentrations on day 26 after DT administration beginning from *Foxp3^{WT}→Rag2$^{-/-}$*+DT n= 4, *Foxp3^{DTR}→Rag2$^{-/-}$*+PBS n=4, *Foxp3^{DTR}→Rag2$^{-/-}$*+DT n=13. *p < 0.05, as determined by ANOVA test and Tukey's multiple comparisons test. ns, not significant. nd, not determined. Results from 3 independent experiments.

Of note, mild levels of immunopathology were detected in the lungs of control chimeric *Foxp3^{DTR}→RAG2$^{-/-}$* mice receiving PBS and in *Foxp3^{WT}→RAG2$^{-/-}$* receiving DT as well as in *Foxp3^{WT}→RAG2$^{-/-}$* mice receiving PBS, probably ensuing from a radiation effect (Fig.3.8C-D;3.9). Mild immunopathology was also observed in the stomach, salivary glands, pancreas and kidney of control chimeric *Foxp3^{WT}→RAG2$^{-/-}$* mice receiving DT, probably ensuing DT administration (Fig.3.8C-D; 3.9). These

observations argue that radiation and DT administration probable exert a combined effect that favours tissue damage, which was not observed in non-chimeric mice (Fig.3.2). Nevertheless, this intricate system provides similar levels of autoimmune organ injury upon disruption of peripheral self-tolerance imposed by T_{REG} cell deletion in non-chimeric mice (Fig.3.3). These similarities extended to survival, weight and temperature loss following T_{REG} cell depletion in chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice, compared to control chimeric *Foxp3*^{WT}→*RAG2*$^{-/-}$ receiving DT (Fig.3.11.B-D).

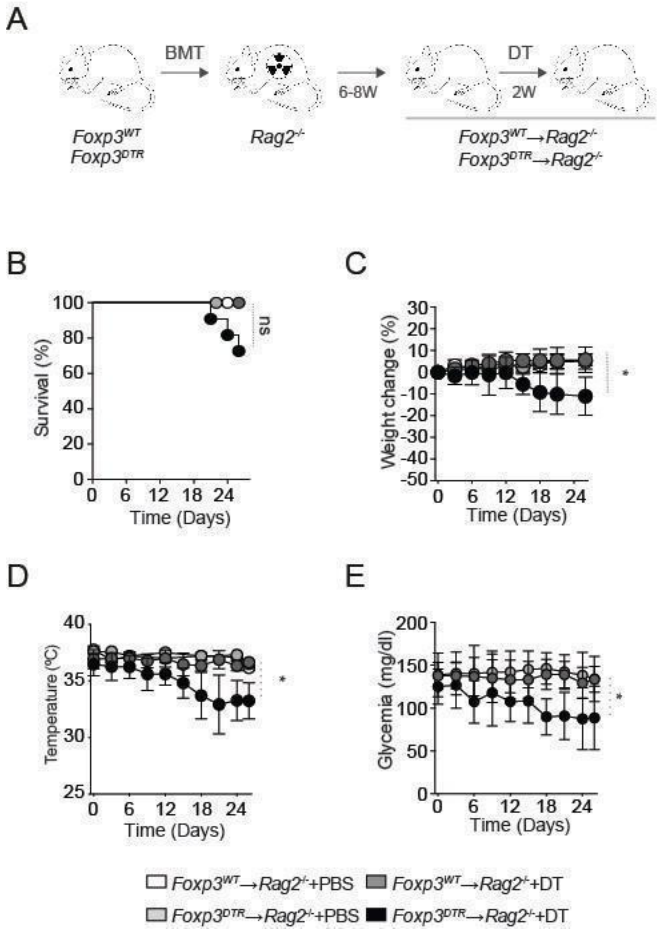

Figure 3.11. Rag2 T_{REG} depleted chimeric mice display signs of sickness behaviour

(A) Schematic representation of the strategy used to generate chimeric mice in which hematopoietic tissues are genotypically different from the non-hematopoietic ones. Non-lethally irradiated *Rag2* deficient mice lacking adaptive immune system were grafted with bone marrow (BTM) from mice harbouring a diphtheria toxin receptor on regulatory t cells (*Foxp3*^{DTR}) or from WT mice (*Foxp3*^{WT}). Reconstitution lasted for 6-8 weeks when diphtheria toxin (DT) was administered to induce depletion of regulatory T cells (T_{REG}) or vehicle as control (PBS) for 2 weeks. Finally, mice were analysed on day 26 after DT started. **(B-E)** Survival (B), weight change (C) according to initial day of T_{REG} depletion, rectal temperature (D) and glycemia (E) throughout the disease development: *Foxp3*^{WT}→*RAG2*$^{-/-}$+PBS n= 4, *Foxp3*^{DTR}→*RAG2*$^{-/-}$+PBS n=5, *Foxp3*^{WT}→*RAG2*$^{-/-}$+DT n=12, *Foxp3*^{DTR}→*RAG2*$^{-/-}$+DT n=24). *$p < 0.05$, as determined by one-way ANOVA test and Tukey's multiple comparisons test. ns, not significant. Results from 3 independent experiments.

We also asked whether T_{REG} depletion impacted on glycemia. Although within a normal range, there was a reduction of glycemia following T_{REG} cell depletion in chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice, compared to control chimeric *Foxp3*^{WT}→*RAG2*$^{-/-}$ mice receiving DT (Fig. 3.11E). This is consistent with the histological analyses of the pancreas showing lesions to exocrine glands but mostly preserved islet cells (Fig.3.9), leading to a malabsorptive syndrome and consequently associated weight loss (Fig.3.7C).

We confirmed that T_{REG} cell depletion in chimeric *Foxp3*^{DTR}→*RAG2*$^{-/-}$ mice was associated with unfettered immune activation, as assessed in the spleen 26 days after DT administration (Fig.3.12A-G). This was characterized by an increase in the number and percentage of activated $CD4^+CD44^+CD62L^-$ T cells and $CD8^+$ $CD44^+CD62L^-$ T cells, as compared to control chimeric *Foxp3*^{WT}→*RAG2*$^{-/-}$ mice receiving DT (Fig3.12A-D).

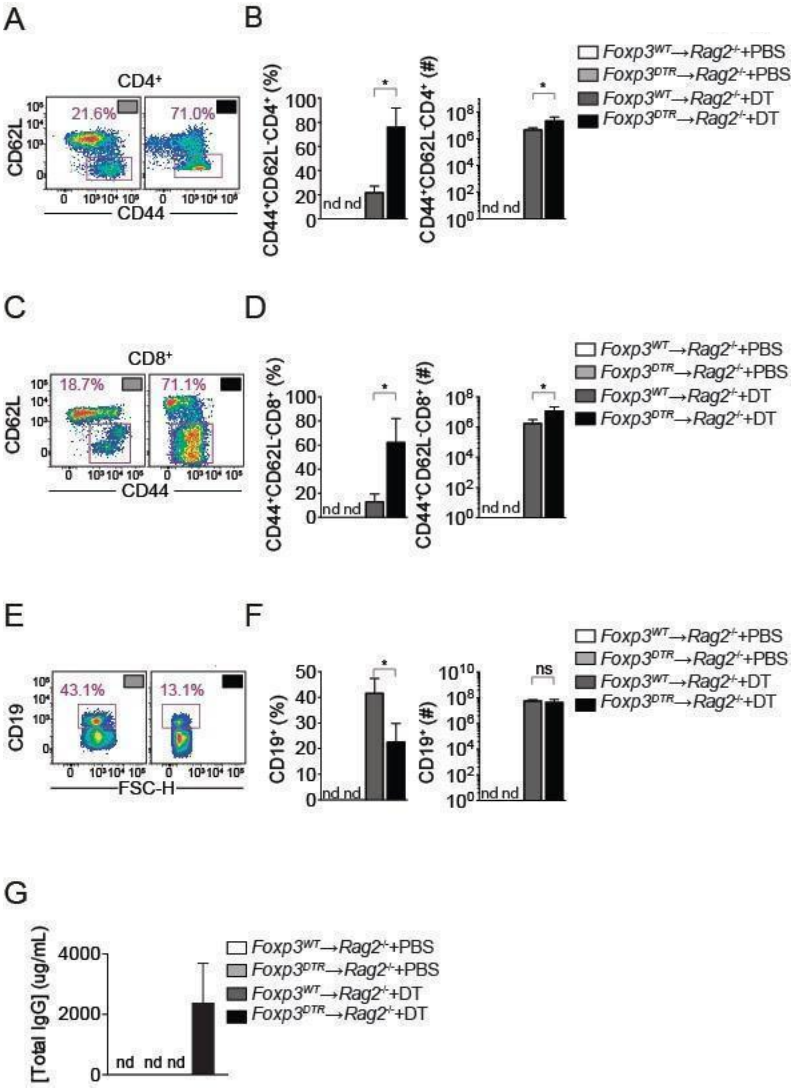

Figure 3.12. T_{REG} depletion triggers a systemic immune activation

Splenic effector memory (CD62L^{low} and CD44^{high}) CD4$^+$ **(A,B)** and CD8$^+$ **(C,D)** T cells from chimeric mice as outlined in Figure 3.8A were analysed by flow cytometry. **(E,F)** In the same mice as (A-D), splenic B cells (CD19$^+$) were quantified. **(G)** Serum total immunoglobulin G concentration in the same mice as (A-F; n=3). *Foxp3*^{WT}→*Rag2*$^{-/-}$+DT n= 5, *Foxp3*^{DTR}→*Rag2*$^{-/-}$+DT n=7. *p < 0.05, as determined by unpaired T-student test. ns, not significant. Standard bars represent mean ± SD. nd, not determined. Results from 3 independent experiments.

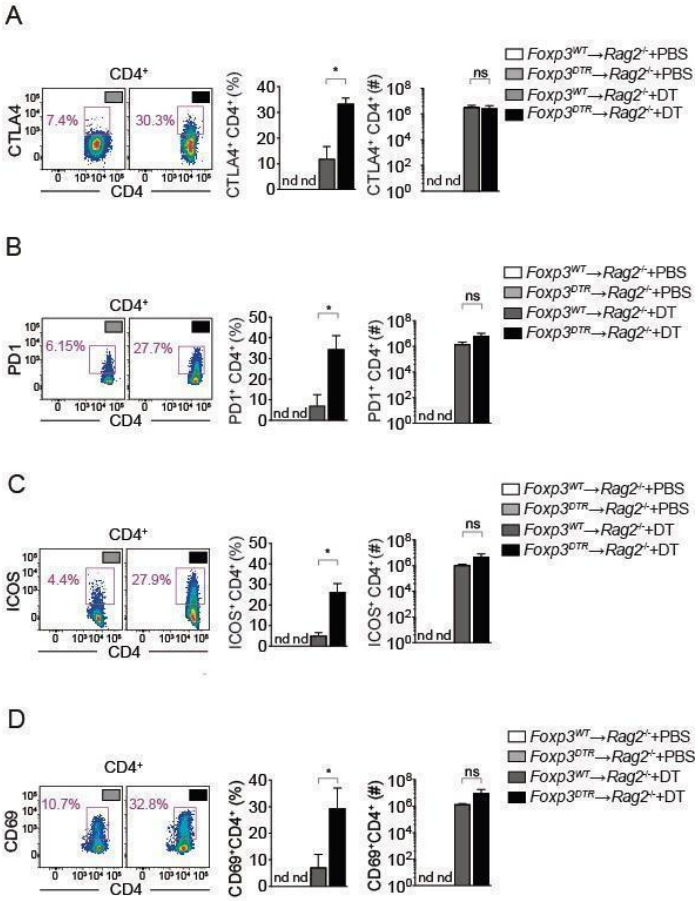

Figure 3.13. Transient T_{REG} depletion in *Foxp3^{DTR} Rag2* chimeric mice activates the systemic adaptive immune response
Splenic activated CTLA4$^+$ **(A)**, PD1$^+$ **(B)**, ICOS$^+$ **(C)** and CD69$^+$ **(D)** in CD4$^+$ T cells analysis by flow cytometry, from the same mice as depicted in Figure 3.8, on day 26: *Foxp3*^{WT}→*Rag2*$^{-/-}$+DT n= 5, *Foxp3*^{DTR}→*Rag2*$^{-/-}$+DT n=7. *p < 0.05, as determined by unpaired *T-student* test. ns, not significant. Standard bars represent mean ± SD. nd, not determined. Results from 3 independent experiments.

We also determined other markers of T cell activation by the levels of ICOS and CD69 which were also increased in regulatory T cell-depleted chimeric mice compared to WT bone marrow chimeras (Fig.3.13C-D). Moreover, we questioned if these mice faced a defective re-balance of immune tolerance by ascertaining the levels of expression of known immune checkpoints molecules (PD1 and CTLA4). We found that immune equilibrium is being attempted by downregulating the immune system and promoting self-tolerance through an increased percentage of PD1$^+$ and CTLA4$^+$ cells (Fig.3.13A-B) in T_{REG} depleted chimeric mice in comparison with chimeras presenting physiological T_{REG} cells.

T cell activation was, in addition, associated with a decrease in the percentage of CD19$^+$ B cells (Fig.3.12E), along with an increase in total IgG concentration in plasma (Fig3.12G), without difference in numbers of CD19$^+$ B cells (Fig3.12F). There was no overt effect of T_{REG} cell depletion on splenic neutrophils and macrophages, on day 26 after DT administration (Fig.3.14A-B). We conclude that this bone marrow chimeric model recapitulates the development of autoimmune disease elicited upon T_{REG} cell deletion in non-chimeric mice and is a potential tool for assessing effects on hematopoietic and non-hematopoietic systems.

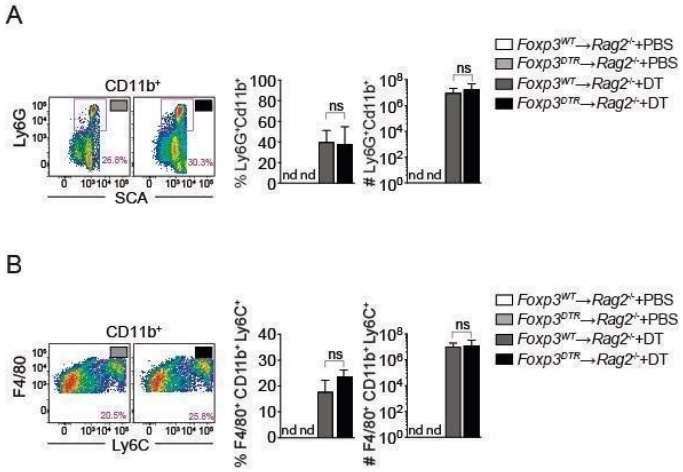

Figure 3.14.After transient T_{REG} depletion in Foxp3^{DTR} Rag2 chimeric mice, the innate immune system remains unaffected (A,B) Splenic neutrophils (Ly6G$^+$ CD11b$^+$) (A) and macrophages (F4/80$^+$ CD11$^+$) (B) assessed by flow cytometry on day 26 after DT started, from the same mice as depicted in Figure 3.8: *Foxp3*^{WT}→*Rag2*$^{-/-}$+DT n= 5, *Foxp3*^{DTR}→*Rag2*$^{-/-}$+DT n=7. *p < 0.05, as determined by unpaired *T-student* test. ns, not significant. Standard bars represent mean ± SD. nd, not determined. Results from 3 independent experiments.

3.3. Discussion

T_{REG} cells have a vital function in regulating the extent of innate and adaptive immune responses to prevent their deleterious effects and concomitant immunopathology. Moreover, T_{REG} cell dysfunction has been proposed to be at the core of the pathogenesis of autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, multiple sclerosis and inflammatory bowel disease [1,10–18]. Possible causes for this impairment of peripheral self-tolerance include: i) Inadequate numbers of T_{REG} cells, ii) T_{REG} cell-intrinsic functional defects and iii) T_{REG} cells extrinsic functional defects that allow immune cells to escape T_{REG} cell suppression, such as those imposed by pro-inflammatory cytokines and antigen-presenting cells under inflammatory conditions [19].

In this chapter, we tested and developed several mouse models that allow inducible unleash of autoreactive cells by acutely ablating T_{REG} cells. These experimental models target T_{REG} cells for depletion in response to DT and assume that mice are resistant to DT due to intrinsic differences in their DTR which recognises preferentially the non-toxic DT fragment B instead of the toxic fragment A recognized by the human DTR [20]. As such, mice bearing human DTR specifically in T_{REG} cells can be used to deplete T_{REG} cells in response to DT administration [21]. While not widely reported [22], we found that DT was toxic and eventually lethal to control mice that did not express the human DTR in T_{REG} cells, when administered at a dosage higher than 0,5µg per Kg body weight every third day. For this reason, we scaled down the DT administration regimen reducing its toxicity, even when administered for a prolonged period of time.

As described by Kim et al., ablation of $Foxp3^+$ T_{REG} cells in *Foxp3^{DTR}* mice was highly efficient reaching around 98% depletion 48h after DT administration [1]. We noticed however, a rebound of T_{REG} cells, consistent with the known dynamics of T_{REG} cells in other experimental models [9]. For example, this rebound was also observed in the DEpletion of REGulatory T cells (DEREG) mice expressing a DTR-eGFP transgene under the control of a Foxp3 promoter expressed in a bacterial artificial chromosome (BAC), depleting T_{REG} cells carrying the BAC transgene. Upon DT administration to DEREG mice, T_{REG} cell depletion is followed by a rapid return of circulating T_{REG} cells that do not carry the BAC transgene and originate from the

thymus. This does not occur in the *Foxp3^{DTR}* mice, which grant a sustained ablation of T_{REG} cells over time.

As expected, depletion of T_{REG} cells led to the development of autoimmunity and ensuing immunopathology in the *Foxp3^{DTR}* mice. Since T_{REG} cells have a heterogeneous TCR repertoire [23,24], multiple organs were targeted for damage upon T_{REG} cell depletion, confirming previously published results [1]. Tissue pathology was manifested essentially by extravascular lymphocytic and mononuclear infiltrates, in the liver, stomach, pancreas and skin, in keeping with previously published results [1]. A time-lapse histological analysis showed that the pancreas, lungs, liver and stomach were the first to be damaged and were also the most severely affected organs upon T_{REG} cell depletion in *Foxp3^{DTR}* mice. Our findings are consistent with the C57BL/6 genetic background of *Foxp3^{DTR}* mice, known to be relatively resistant to autoimmune disease [25], in which autoimmune damage to the lung, liver and stomach was expected [26–30]. Moreover, there was a reduction of creatinine and alkaline phosphatase levels in plasma suggesting that T_{REG} cell depletion causes muscle wasting.

We also confirmed that T_{REG} cell ablation caused a vigorous activation of the immune system in *Foxp3^{DTR}* mice, as reported by others [1]. T_{REG} cell depletion in adult C57BL/6 *Foxp3^{DTR}* mice led to a more aggressive development of autoimmune disease, compared to neonatal T_{REG} cell depletion [1], probably due larger repertoire of self-reactive B and T cells at adulthood. However, older *Foxp3^{DTR}* mice showed no further differences in autoimmune disease progression upon T_{REG} cell depletion, suggesting that *Foxp3^{DTR}* mice can be tested with older ages without interfering with the outcome of autoimmune disease.

To test whether stress and damage responses operate in parenchyma cells to confer tissue damage control and modulate the pathogenesis of autoimmune diseases we adapted the experimental model of induction of autoimmunity upon T_{REG} depletion to bone marrow chimeric mice. This approach is essential to disentangle the genetic background of hematopoietic *vs.* non-hematopoietic cells such that wild type T and B cells are confronted to parenchyma (*i.e.,* non-hematopoietic) cells lacking or not the ability to respond to different forms of stress and/or damage. We used recipient mice in the Rag2-deficient genetic background to prevent a putative effect of tissue resident T_{REG} cells in the control of autoimmune disease in chimeric mice.

We confirmed that autoimmune disease can be elicited upon T_{REG} cells depletion in chimeric mice receiving bone marrows from *Foxp3^{DTR}* mice. Autoimmune disease 26 days after T_{REG} cell deletion was comparable to the autoimmune disease in non-chimeric Foxp3^{DTR} mice. Our findings also suggest that host irradiation might elicit a non-pathogenic inflammatory response that presumably increase the susceptibility to autoimmune disease in the chimeric mice [31,32]. This is consistent with previous findings by the group of Sakaguchi and colleagues showing that ionising radiation can promote autoimmune disease [33]. Of note however, this report used high dose (42.5 Gy) and fractionated ionising radiation (2.5Gy 17 times) on the lymphoid organs/tissues by affecting the T cell immune system, instead of the targeted self-antigens, supposedly by altering T cell-dependent control of self-reactive T cells [1].

In our chimeric model, a second hit seems more evident on salivary glands and stomach, in which the combination of radiation and DT administration leads to a mild immune infiltration of those organs. No further characterisation was pursued, although we speculate that due to a high cellular turnover these epithelial organs may be more prone to mutations imposed by radiation and inducing inflammation. Despite these effects, this chimeric experimental system provides us similar results of organ targeting upon disruption of immune tolerance by T_{REG} depletion compared to non-chimeric Foxp3^{DTR} mice. Extension of these results to survival, weight change and temperature drop confirm the reproducibility of the T_{REG} depletion model in bone marrow chimeras.

In conclusion, T_{REG} cells play a critical role in preventing autoimmunity by inhibiting T cell proliferation. The ablation of this specialised population of T cells in Foxp3^{DTR} mice leads to the development of autoimmune disease in a multiorgan range of severity. Moreover, this model can be applied in a chimeric bone marrow model, reproducing similar results, which allow us to disentangle the immune system from non-hematopoietic cells in a controlled manner.

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Chapter 4

Contribution of the Oxidative Stress Response To Tissue Damage Control in Autoimmune Diseases

Abstract

Cellular accumulation of reactive oxygen and nitrogen species activates the Nuclear factor-E2-related factor 2 (*Nrf2*), a transcriptional master regulator of oxidative stress responses that is essential to re-establish redox homeostasis and cytoprotection. Once activated, Nrf2 binds to antioxidant response elements (ARE) in the promoter of Nrf2-regulated genes that detoxify xenobiotics, re-establish redox cycling reactions and maintain cellular reducing equivalents, to counteract the cytotoxic effects of reactive oxygen and nitrogen species. In this Chapter we tested the hypothesis that activation of *Nrf2* in parenchyma cells regulates tissue damage control and modulates the pathogenesis and/or outcome of systemic autoimmune disease. To this aim, we generated bone marrow chimeric mice carrying a deletion of the *Nrf2* allele in non-hematopoietic (*i.e.,* parenchyma) cells. We found that the development of autoimmune disease, induced upon T_{REG} cell depletion, was more severe in mice lacking *Nrf2* expression in parenchyma cells. This was most apparent in the liver, where *Nrf2*-deletion exacerbated leukocyte infiltration and was associated with the accumulation of liver enzymes in plasma, corroborating an increase in liver damage. While expression of *Nrf2* in parenchyma cells did not affect immune activation, a more severe form of autoimmune disease developed when immune cells from chimeric mice lacking *Nrf2* in parenchyma cells were adoptively transferred to naïve *Rag2*$^{-/-}$ mice. In conclusion, expression of *Nrf2* in parenchyma cells contributes to organ protection, namely the liver, upon immune deregulation which may impact in the development of autoimmune disease in humans carrying polymorphic *Nrf2* alleles.

4.1. Introduction

4.1.1. Nuclear factor erythroid 2-related factor 2: An adaptive response to oxidative stress

Cellular processes occurring in peroxisomes, mitochondria and endoplasmic reticulum generate by-products that can be toxic under physiological and pathological conditions. Among those by-products are reactive oxygen species (ROS) [1], including superoxide anion radical ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), perhydroxyl radical ($HO_2^{\cdot}$) and hydroxyl radical ($^{\cdot}OH$). These highly reactive ROS are short-lived, as they react with proteins, carbohydrates, lipids and nucleic acids [2].

ROS production during cellular metabolism can be increased in response to exogenous stimuli associated with an increase in metabolic demand, such as associated with inflammatory reactions [1]. These reactive oxygen species can activate redox-sensitive phosphatases, kinases and transcription factors early in inflammatory processes [3,4]. Oxidative cellular stress happens when ROS concentration levels surpasses the anti-oxidant cellular capacity, *Nrf2*, a member of the cap-n-collar (CNCfamily of basic region-leucine-zipper (bZIP) transcription factors[2], acts as a master regulator in defense mechanism towards redox homeostasis and cytoprotection. The family of *Nrf2* transcription factor also includes *Nrf1*, *Nrf3* and NF-E2 p45 subunit [2].

4.1.2. Nrf2: Regulation of activity

The Kelch-like ECH-associated protein 1 *(Keap1)* is a negative regulator of *Nrf2* that functions as an E3 ubiquitin ligase substrate-recognition. Under homeostatic conditions, *Nrf2* is bound at Nrf2-ECH homology domain 2 (Neh2) of Keap1, which acts as an adaptor protein for Cul3 E3 ubiquitin ligase, responsible for *Nrf2* ubiquitylation and degradation. Due to *Nrf2* short half-life (*i.e.,* 10-30 minutes), the high turnover of *Nrf2* imposed by *Keap1* makes that the basal levels of Nrf2 protein expression remain at low levels. Upon oxidative or electrophilic stress, oxidation of *Keap1* leads to its inactivation, Nrf2 stabilisation and translocation into the nucleus. Once in the nucleus, *Nrf2* heterodimerises with sMaf proteins (Maft, MafG and MafK), to bind the anti-oxidant response element (ARE; 5'-TGACXXXGC-3') in the promoter region of *Nrf2*-target genes, including *Nrf2* itself. This allows for the induction of *Nrf2* synthesis that flood the cytoplasm and translocate into the nucleus [5], without

interfering with the levels of Keap1 and Cul3 protein expression [5]. Oxidative/electrophilic stress can activate protein kinase C, mitogen-activated protein kinases (MAPKs) or phosphatidylinositol 3-kinase (PI3K), which, in turn, phosphorylate Nrf2, facilitating Nrf2 release from Keap1 and allowing its translocation into the nucleus [6]. As *Nrf2* also regulates Nrf2 transcription and protein expression, polymorphisms in the promoter region of *Nrf2* regulate overall Nrf2 activity [7].

Nrf2 regulates the expression of several members of the glutathione (*Gsh*) and thioredoxin (*Txn*) anti-oxidant system, as well as enzymes involved in ROS and xenobiotic detoxification, NADPH regeneration and heme metabolism [2], including Heme oxygenase (Ho)-1, NAD(P)H Quinone Dehydrogenase (Nqo)1, Glutamate-Cysteine Ligase Catalytic Subunit (Gclc) and Glutamate-cysteine ligase modifier subunit (Gclm) 8.

4.1.2.1. Cross-talk between Nrf2 and inflammatory signaling

Pro-inflammatory cytokines can promote ROS production and oxidative stress contributing to the pro-inflammatory responses via activation of redox-sensitive transcription factors such as NF-κB. While under basal redox conditions, IκB kinase (IKK) is targeted by Keap1 for ubiquitination and proteasomal degradation [9], oxidative stress promotes phosphorylation of the NF-κB inhibitor IκB by IKK, releasing NF-κB and promoting its nuclear translocation [8], therefore fostering inflammation. ROS can also regulate other kinases, including the MAPKs p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) as well as PI3K [8], contributing to pro-inflammatory responses.

4.1.2.2. Nrf2 in immune and nonimmune cells: tissue damage control effect of Nrf2

Nrf2 is expressed ubiquitously although at different levels across different cells and tissues [10]. The immunoregulatory effect of Nrf2 are thought to be exerted in a cell autonomous manner via the expression of *Nrf2* innate and adaptive immune cells. Its activation in the innate players suppresses or eliminates several bacterial and viral pathogens [11]. Genetic loss of function approaches have shown that Nrf2 regulates phagocytosis, Fc receptor effector function, complement activation and immune globulin regulation [12]. They have also demonstrated the anti-inflammatory effects of

Nrf2. For example, Nrf2-deficient mice have decreased survival in experimental models of sepsis [13], harsher pathology in experimental autoimmune encephalomyelitis[14], enhanced production of inflammatory mediators in LPS-induced neuroinflammation[15] and worsened damage in T cell-mediated hepatitis model [16].

Nrf2 activation in $CD4^+$ T cells leads to decreased production of IL-2 and expression of CD25 and CD69, early activation markers [17]. Nrf2 also induces an anti-inflammatory phenotype that modulates the functions of $CD8^+$ T cells [18] and macrophages and microglia [19,20] due to increased levels of cysteine and glutathione (GSH). Surprisingly, Klemm et al. have shown that Nrf2 is a negative regulator of T_{REG} function and Foxp3 specific activation of Nrf2 results in a loss of immune tolerance and spontaneous accumulation of IFN-γ producing effector T cells and inflammation[21]. Furthermore, Suzuki et al. showed, in the context of autoimmunity, that Nrf2 activation at multiple cell lineages appears to be required for an anti-inflammatory effect and that Nrf2 inhibits effector T cell activities independently of T_{REGs} [22]. Another group showed that wild-type bone marrow transplantation into $Nrf2^{-/-}$ mice slowed lung inflammation development and correlated with the presence of macrophages expressing Nrf2-regulated anti-oxidant enzymes and anti-proteases [23]. Recently, Tsai et al. reported that the absence of Nrf2 in donor T cells enhanced the persistence of T_{REG} and reduced systemic inflammation in graft-versus-host disease [24]. Altogether, these data hints at a role for Nrf2 and impaired anti-oxidant activity in determining susceptibility to autoimmune disease.

Nrf2 deficiency has been shown to exacerbate rheumatoid arthritis [25] and systemic lupus erythematosus [26]. Yet, whether expression of Nrf2 in parenchyma cells expression modulates the pathogenesis of autoimmune diseases is still to be established. In this chapter we tested the hypothesis that Nrf2 acts in parenchyma cells to provide tissue damage control and establish disease tolerance to autoimmune diseases.

4.2. Results

To study whether *Nrf2* is activated upon unleash of autoreactive immune cells, we generated an *Nrf2* bioluminescent mouse reporter (*OKD48*) in which T_{REG} cells carry a diphtheria toxin receptor (*Foxp3*^{DTR}) and confirmed the genotype by PCR (Fig.4.1A). T_{REG} depletion triggers an immune-mediated disease, as previously shown (Chapter 3). Induction of *Nrf2* was initially tested in *OKD48 Foxp3*^{DTR} mice by an *Nrf2* inducer – Arsinite (Ars). Six hours after administration of Ars to an OKD48 mouse, independently of DTR expression, bioluminescence intensity increases 7 folds compared to an OKD48 mouse receiving vehicle (Fig.4.1B), providing a model to assess *Nrf2* expression during autoimmunity development. Once tested, we followed OKD48 reporter mice for at least 26 days every three days after starting depletion of T_{REG} cells to induce autoimmune disease. As expected, OKD48 *Foxp3*^{DTR} reporter mice lost weight (Fig.4.1E), however, during the following up time every three days, we did not find differences in bioluminescence intensity between depleted (*Foxp3*^{DTR}) or non-depleted (*Foxp3*^{WT}) mice (Fig.4.1C-D), remaining open a question about activation of *Nrf2* in these mice.

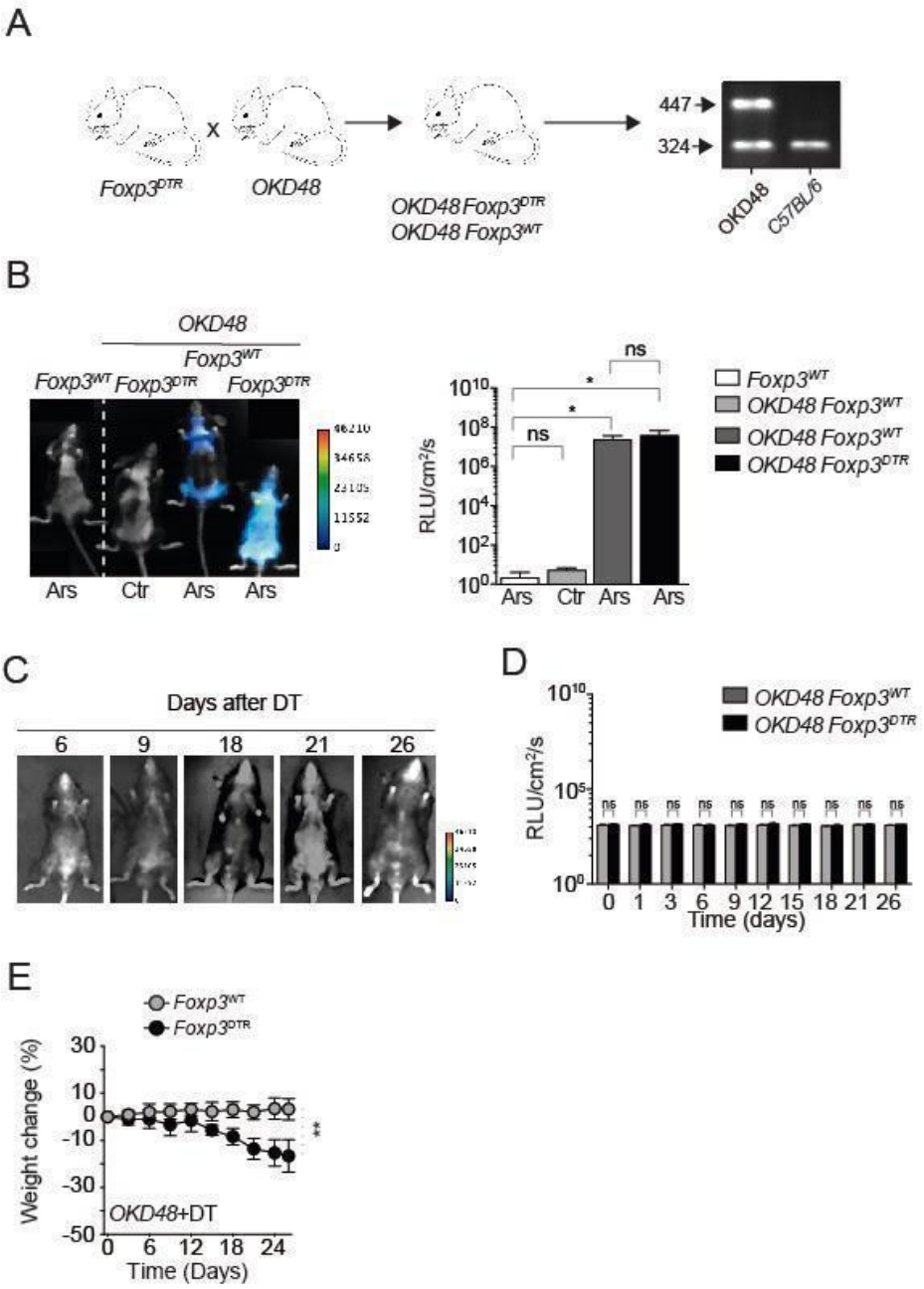

Figure 4.1. OKD48 reporter mice do not report expression of Nrf2 during autoimmunity devolvement

(A) Generation of a bioluminescent and immunofluorescent *Nrf2* reporter (*OKD48*) mouse with depletable regulatory T cells (*Foxp3*^{DTR}). Amid *Nrf2* activation, the *OKD48* mouse, which has a promoter with 3 antioxidant responsive elements and a flag-tagged luciferase, emits luminescence when luciferin is administered. Electrophoresis of PCR products amplified from genomic DNA of mice carrying a *OKD48* allele. **(B)** Bioluminescence activity 6 hours after arsenite (Ars) and PBS (Ctr) administration in mice carrying WT or a heterozygous *OKD48* allele (exposure 8s; n=4). **(C-E)** *OKD48 Foxp3*^{DTR} reporter mice bioluminescence pictures (C), quantification (D) and weight change (E) for a 26 days period with every 3 days follow-up (exposure 8s; *OKD48 Foxp3*^{WT} n=5 and *OKD48 Foxp3*^{DTR} n=5 per time point). *p < 0.05 determined by ANOVA and Tukey multicomparison test. ns, not significant (p>0.05). Standard bars represent mean ± SD. Results from 2 independent experiments.

We generated *Rag2*$^{-/-}$*Nrf2*$^{-/-}$ mice whereby the *Nrf2* allele is constitutively deleted in mice lacking adaptive immunity (Fig.4.2A). The *Rag2*$^{-/-}$*Nrf2*$^{-/-}$ and control *Rag2*$^{-/-}$*Nrf2*$^{+/+}$ genotypes were identified by PCR (Fig.4.2B-C). Similar to Chapters 3, 5 and 6, we also generated bone marrow chimeric mice whereby the *Nrf2* allele is deleted specifically in non-hematopoietic cells (Fig.4.2A).

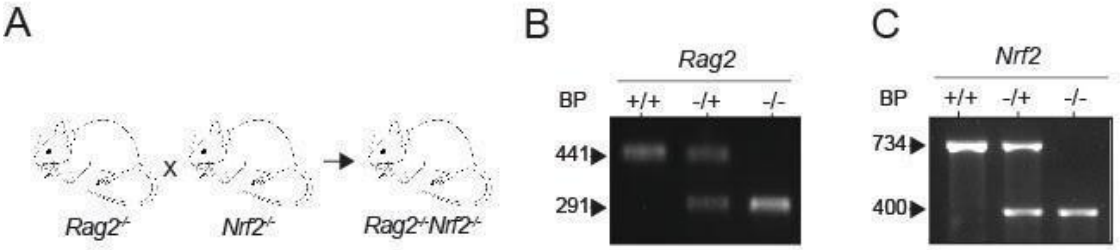

Figure 4.2. Generation of *Nrf2* and *Rag2* double knockout mice

(A) Schematic representation of the breeding strategy used to generate Rag2 deficient (*Rag2*$^{-/-}$) mice, in which the transcription factor nuclear factor erythroid 2-related factor 2 (*Nrf2*) is invariably deleted. **(B,C)** Electrophoresis of PCR products amplified from genomic DNA of mice carrying: B) wild type (+/+), heterozygous (+/-) or homozygous (-/-) *Rag2* alleles and C) wild type (wt/wt), heterozygous (wt/-) or homozygous (-/-) *Nrf2* alleles.

Bone cells from *Foxp3*^{DTR} mice were adoptively transferred into non-lethally irradiated (400 Rad) *Rag2*$^{-/-}$*Nrf2*$^{-/-}$ mice to generate *Foxp3*^{DTR}→*Rag2*$^{-/-}$*Nrf2*$^{-/-}$ mice and control *Foxp3*^{DTR}→*Rag2*$^{-/-}$*Nrf2*$^{+/+}$ mice (Fig.4.3A). In addition, Foxp3^{WT} donors were also used as controls to generate control *Foxp3*^{WT}→ *Rag2*$^{-/-}$*Nrf2*$^{+/+}$ and *Foxp3*^{WT}→*Rag2*$^{-/-}$*Nrf2*$^{-/-}$ mice, expressing physiologic numbers of Treg cells. This experimental system allows to assess, for the first time, the impact of *Nrf2* expression in non-hematopoietic cells on the pathogenesis and outcome of experimental autoimmune diseases, as triggered upon Treg cell depletion.

Similar to chapters 3, 5 and 6, we compared the pathogenesis and outcome of systemic autoimmune disease triggered upon T_{REG} cell depletion in chimeric mice (Fig.4.3B, 4.4, 4.5). We found that deletion of *Nrf2* in non-hematopoietic cells from chimeric *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ mice was associated with more pronounced hepatic leukocyte infiltration and liver damage, compared to control *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ mice, as assessed 26 days after DT administration (Fig.4.3B-D). As anticipated control *Foxp3*^{WT}→*Nrf2*$^{+/+}$ or *Foxp3*^{WT}→*Nrf2*$^{-/-}$ mice, did not present liver infiltration or damage. Taken together, this shows that *Nrf2* deletion in parenchyma cells is not sufficient per se to induce liver damage (Fig.4.3B-C).

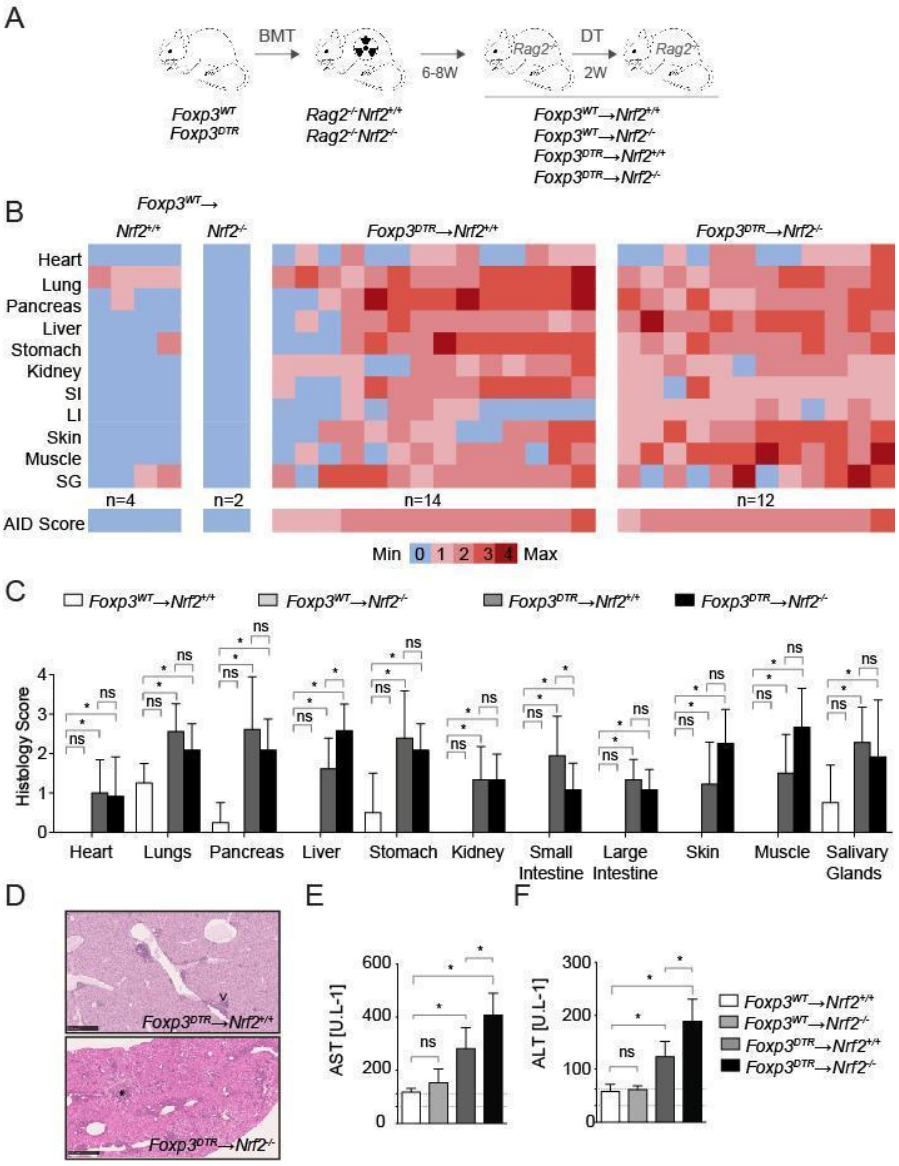

Figure 4.3. *Nrf2* deletion in non-hematopoietic tissues promotes autoimmune hepatitis

(**A**) Schematic representation of the strategy used to generate bone marrow chimeric mice in which the *Nrf2* allele is knockout specifically in parenchyma and connective (non-hematopoietic) tissues, but not in hematopoietic cells. Briefly, non-lethally irradiated *Rag2*$^{-/-}$*Nrf2*$^{+/+}$ or *Rag2*$^{-/-}$*Nrf2*$^{-/-}$ mice received bone marrow transplants (BMT) from wt (*Foxp3*^{WT}) mice or *Foxp3*^{DTR} mice bearing a diphtheria toxin receptor (DTR) specifically on regulatory T cells. After 6-8 weeks (W), chimeric (BMT→Recipients) mice received intra-peritoneally diphtheria toxin (DT) for two weeks (2W) to achieve T_{REG} cell depletion and were analysed on day 26 after DT started. (**B**) Array plot of histological Autoimmunity Disease (AID) score in organs from the mice previously described (A), obtained 26 days after the first bolus of DT administration. Each horizontal line represents an organ and each column an individual mouse. Mean AID scores are illustrated for each genotype in the bottom horizontal lines. SI: small intestine, LI: large intestine, SG: salivary glands. **(C)** Relative quantification of AID scores *per* organ *per* genotype, obtained as indicated in (B) and represented as mean ± SD. P values determined by bootstrapping resampling method. *p < 0.05. **(D)** H&E staining of representative livers from chimeric mice, generated as described in (A). Scale bar=500 µm. Interface hepatitis (*) is depicted in *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ chimeras**,** while *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ present periportal infiltration (V). **(E-F)** Liver enzymes (AST: aspartate aminotransferase (E), ALT: alanine aminotransferase (F)) concentration in the serum of chimeric mice, generated as described in (A). Data is represented as mean ± SD for the following chimeric mice: *Foxp3*^{WT}→*Nrf2*$^{+/+}$ (n=2), *Foxp3*^{WT}→*Nrf2*$^{-/-}$(n=3), *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ (n=5), *Foxp3*^{DTR}→*Nrf2*$^{-/-}$(n=10). *p < 0.05 determined by ANOVA test and Tukey's multiple comparison test. ns, not significant (p>0.05). Results from 3 independent experiments.

Liver damage in chimeric *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ mice was associated with the accumulation of ALT and AST in plasma, compared to control *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ mice, as assessed 26 days after DT administration (Fig.4.3E,F). As anticipated control *Foxp3*^{WT}→*Nrf2*$^{+/+}$ or *Foxp3*^{WT}→*Nrf2*$^{-/-}$ mice, did not accumulate AST and ALT in plasma (Fig.4.3E,F).

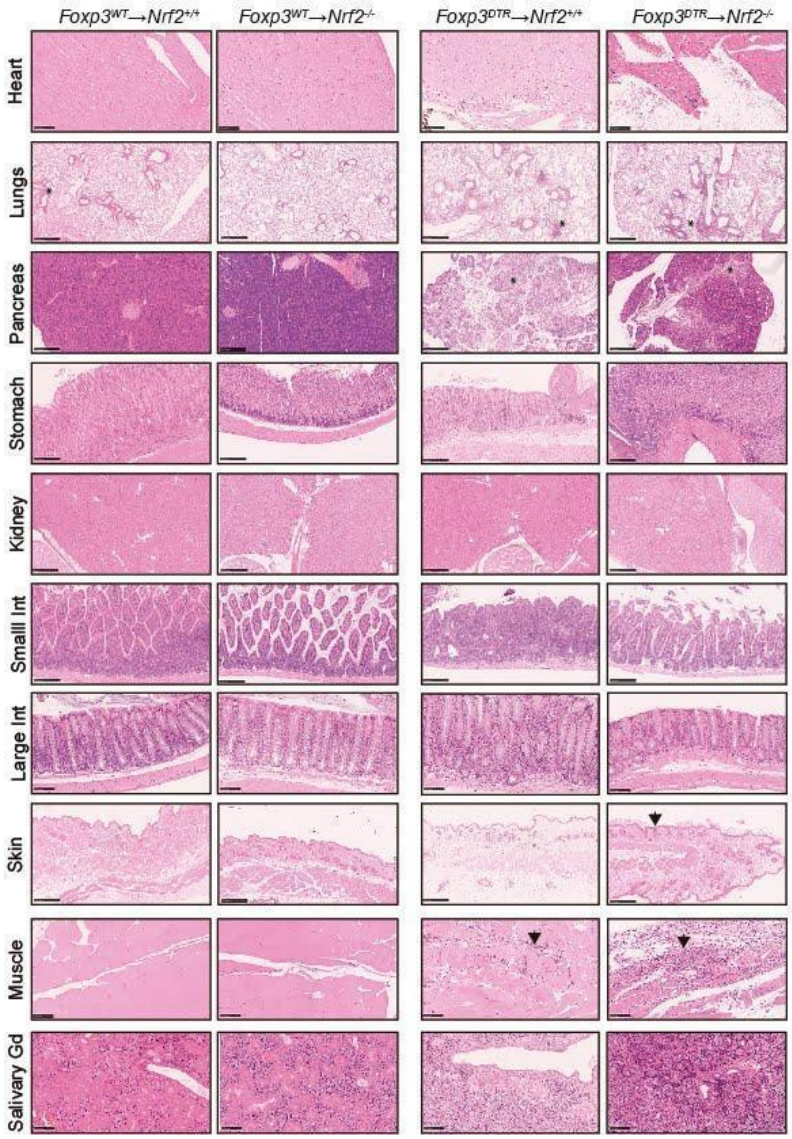

Figure 4.4. *Nrf2* absence impacts on the severity of organ targeting

H&E pictures representative of different organs from each chimeric group of mice on day 26 after begin diphtheria toxin administration: *Foxp3*^{WT}→*Nrf2*$^{+/+}$ n=4, *Foxp3*^{WT}→*Nrf2*$^{-/-}$n=2, *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ n=14 and *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ n=12. Scale bar = 500μM. In the absence of *Nrf2,* the heart, lungs, stomach, kidney, small and large intestines and salivary glands are not differently infiltrated by mono and polymorphonuclear cells when compared to controls carrying a functional *Nrf2* gene. Of notice, the lungs in *Foxp3*^{WT} mice present a peribronquial infiltration of polymorphonuclear cells. Pancreatic acini area is less atrophic in *Foxp3*^{DTR} chimeric mice without non-hematopoietic *Nrf2* when compared to having the allele. Chimeras lacking *Nrf2* and T_{REG} unveil moderate dermal inflammation with diffuse infiltration of lymphocytes, plasma cells and neutrophils covered by a hyperkeratotic epithelium (arrow), which isn't found in controls (*Foxp3*^{DTR}→*Nrf2*$^{+/+}$). Muscle in T_{REG} depleted chimeras is infiltrated distinctly. In the absence of *Nrf2*, fibres disruption and moderate infiltration (arrow) are observed, whilst in the presence of the gene, mild infiltration (arrow) is detected.

Chimeric *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ mice also presented a higher histopathological score of leukocyte infiltration and tissue damage in skin and muscle, when compared to control chimeric *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ mice as well as to control *Foxp3*^{WT}→*Nrf2*$^{+/+}$ or *Foxp3*^{WT}→*Nrf2*$^{-/-}$ mice (Fig.4.3B,C;4.4). These results show that failure to activate a stress-responsive transcriptional program orchestrated by Nrf2 in parenchyma cells exacerbates the autoimmune disease elicited upon T_{REG} depletion.

When compared chimeric mice upon depletion of regulatory T_{REG}, no differences were observed on survival, weight loss, temperature or glycemia (Fig. 4.5).

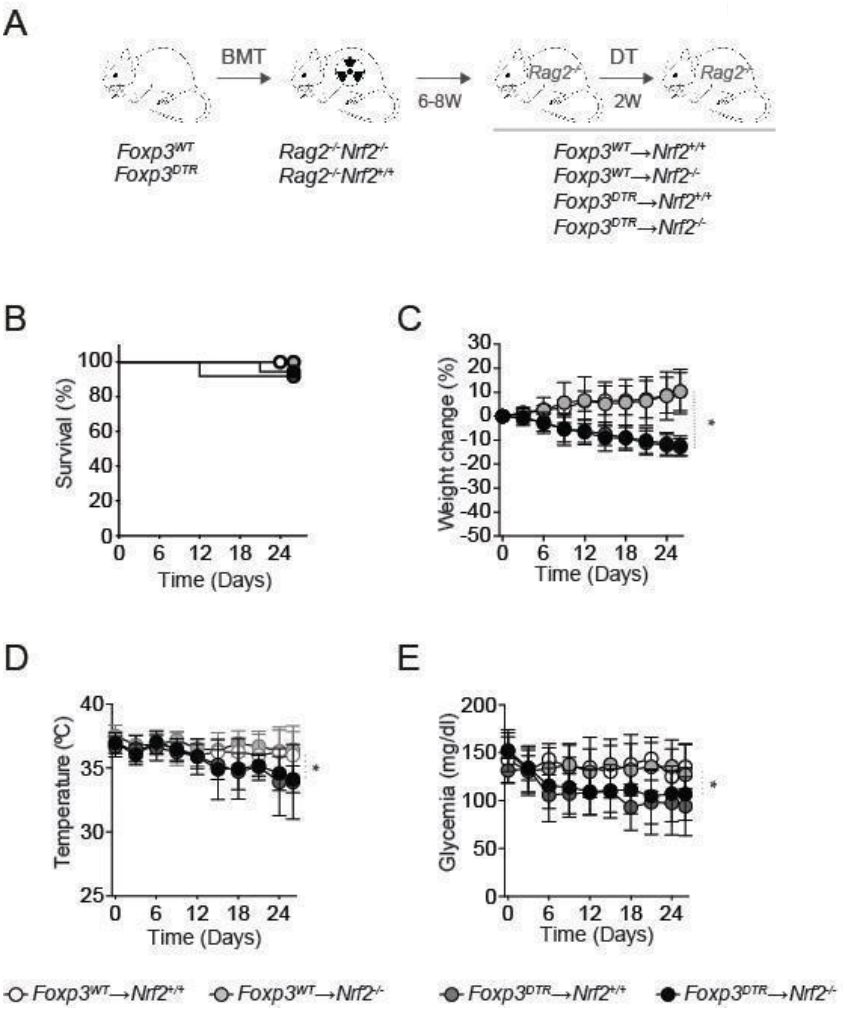

Figure 4.5. Upon regulatory T cell depletion, Nrf2 does not interfere in vital parameters outcome

(**A**) Schematic representation of the strategy used to generate bone marrow chimeric mice in which the *Nrf2* allele is knockout specifically in parenchyma and connective (non-hematopoietic) tissues, but not in hematopoietic cells. Briefly, non-lethally irradiated *Rag2*$^{-/-}$*Nrf2*$^{+/+}$ or *Rag2*$^{-/-}$*Nrf2*$^{-/-}$ mice received bone marrow transplants (BMT) from wt (*Foxp3*^{WT}) mice or from *Foxp3*^{DTR} mice bearing a diphtheria toxin receptor (DTR) specifically on regulatory T cells. After 6-8 weeks (W) chimeric (BMT→Recipients) mice received intra-peritoneally diphtheria toxin (DT) for 2 weeks (2W) to achieve T_{REG} cell depletion and were analysed on day 26 after DT started. (**B-E)** Survival (A), Weight change (B), rectal temperature (D) and glycemia of chimeric mice as described in (A) during autoimmune disease development: *Foxp3*^{WT}→*Nrf2*$^{+/+}$ n=4, *Foxp3*^{WT}→*Nrf2*$^{-/-}$ n=10, *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ n=14, *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ n=18. *p < 0.05 determined by ANOVA test and Tukey's multiple comparison test. ns, not significant.

We then asked whether non-hematopoietic *Nrf2* expression would tune systemic (auto) immune activation driven by T_{REG} cell depletion. We found no differences in the expression of $CD4^+$ and $CD8^+$ T cell activation markers (*i.e.,* $CD44^{hi}CD62^{lo}$) nor the proportion of $CD19^+$ B cells in the spleen of chimeric *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ mice, when compared to control chimeric *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ or *Foxp3*^{WT}→*Nrf2*$^{-/-}$ mice (Fig.4.6A-F).

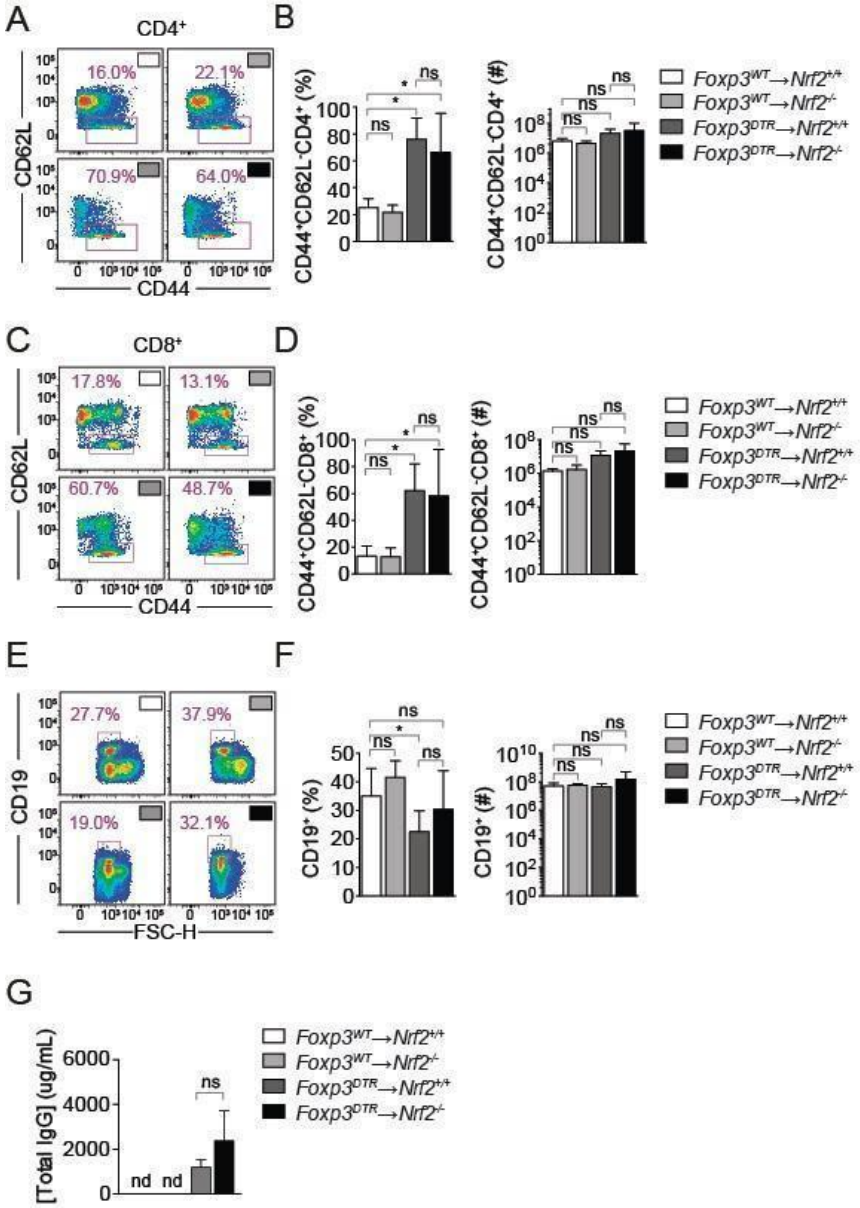

Figure 4.6. *Nrf2* non-hematopoietic knockout does not interfere with immune activation upon regulatory T cell depletion
Flow cytometry analyses of splenic effector memory ($CD62L^{low}$ and $CD44^{high}$) $CD4^+$ (**A, B**) and $CD8^+$ (**C, D**) T cells in chimeric mice*, generated as described in Figure 5.2A. (**E-F**) Splenic B ($CD19^+$) cells in the same mice as (A-D). (**G**) Concentration of circulating IgG of the same mice as (A-D; n=3). *p < 0.05 determined by ANOVA test and Tukey's multiple comparison test. ns, not significant (p>0.05). Standard bars represent mean ± SD. *(*Foxp3*^{WT}→*Nrf2*$^{+/+}$ n=5, *Foxp3*^{WT}→*Nrf2*$^{-/-}$ n=5, *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ n=7, *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ n=10).

IgG circulating levels were also similar between *Foxp3*^{DTR} chimeric mice in the presence and absence of *Nrf2* (Fig.4.6G). Accordingly, follicular splenic T cells, responsible for B cell response, and the activation marker ICOS were also increased in regulatory T cell-depleted mice, regardless of *Nrf2* expression (Fig.4.7C). Furthermore, we examined if immune tolerance was affected in these mice by verifying PD1 and CTLA4 positive cells. We determined that only PD1^{+} cells were increased, as expected upon T_{REG} depletion, while CTLA4^{+} cells present similar levels, independently of *Nrf2* expression or T_{REG} depletion (Fig.4.7A-B). On day 26 after depletion, CD4^{+} cells in T_{REG} depleted chimeras were still activated, as shown by the increased percentage of the positive early activation marker CD69 (Fig.4.7D).

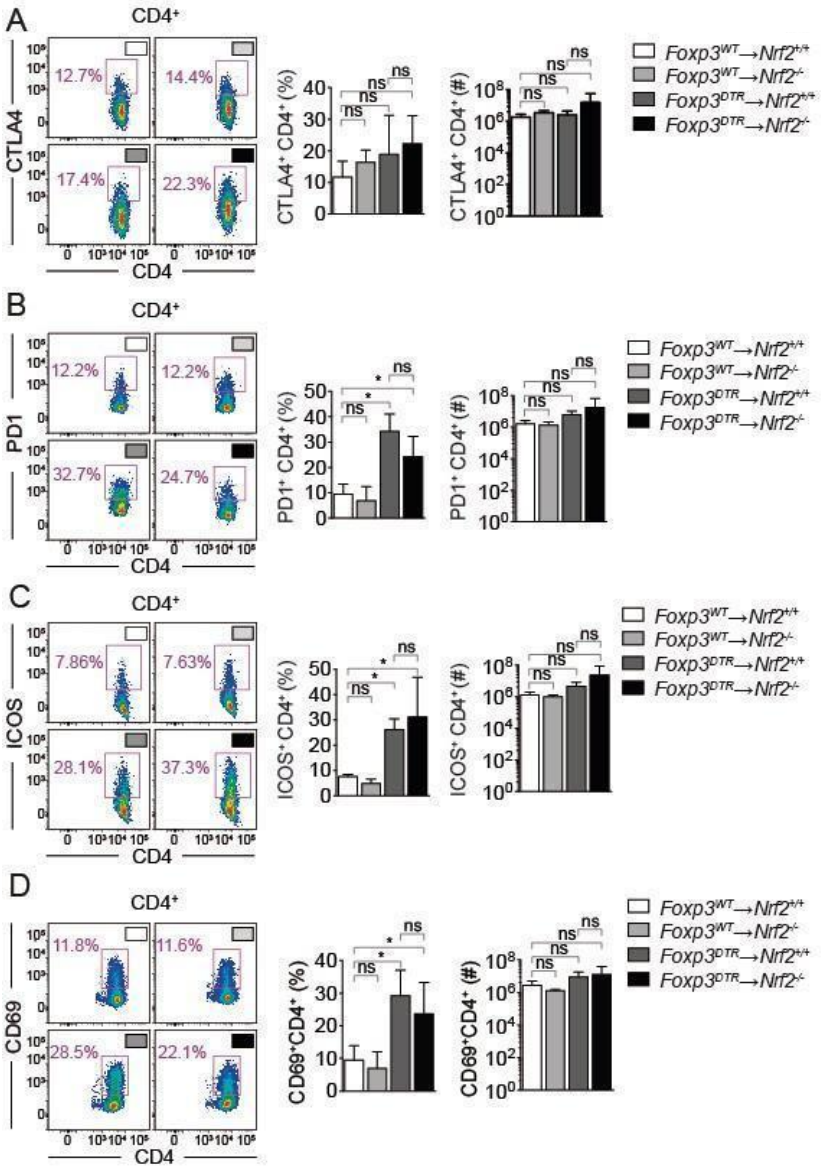

Figure 4.7. During autoimmunity pathogenesis, non-hematopoietic *Nrf2* does not modulate the activation status of immune

Flow cytometry analyses of splenic CTLA4$^+$ CD4$^+$ **(A)**, PD1$^+$ CD4$^+$ **(B)**, ICOS$^+$ CD4$^+$ **(C)** and CD69$^+$ CD4$^+$ **(D)** cells from mice as describe in Figure S5.2A: *Foxp3*^{WT}→*Nrf2*$^{+/+}$ n=5, *Foxp3*^{WT}→*Nrf2*$^{-/-}$ n=5, *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ n=7, *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ n=10. *p < 0.05 determined by ANOVA and Tukey multiple comparison tests. ns, not significant (p>0.05). Standard bars represent mean ± SD.

Within the innate immune system, more specifically neutrophils and macrophages, no differences among chimeric groups were observed on day 26 after depletion (Fig.4.8A-B). We may therefore surmise that non-hematopoietic *Nrf2* deletion does not hamper adaptive and innate immune activation.

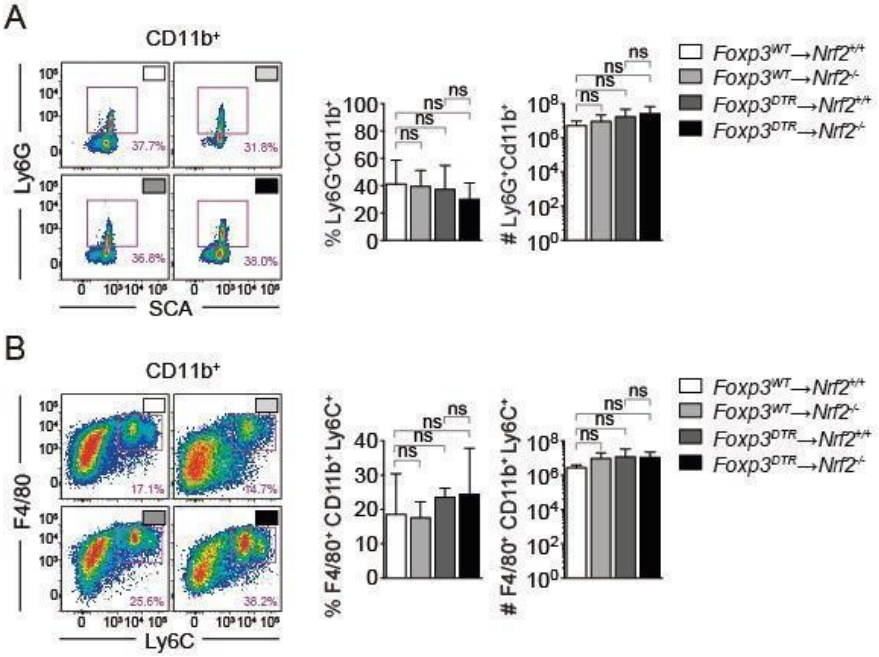

Figure 4.8. *Nrf2* non-hematopoietic knockout does not interfere on innate immune activation upon regulatory T cell depletion

(A,B) Splenic neutrophils (Ly6G$^+$CD11b$^+$) (A) and macrophages (F4/80$^+$CD11$^+$Ly6C$^+$) (B) of chimeric mice as described in Figure S5.2A: *Foxp3*^{WT}→*Nrf2*$^{+/+}$ n=5, *Foxp3*^{WT}→*Nrf2*$^{-/-}$ n=5, *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ n=7, *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ n=10. *p < 0.05 determined by ANOVA and Tukey multiple comparison tests. ns, not significant (p>0.05). Standard bars represent mean ± SD.

We then analysed the capacity of immune cells from *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ compared to control chimeric mice to elicit autoimmunity upon adoptive transfer to *Rag2*$^{-/-}$ mice (Fig.4.9A). We observed that *Rag2*$^{-/-}$ mice grafted from T_{REG} depleted and *Nrf2* non-hematopoietic deleted donors (*Foxp3*^{DTR}→*Nrf2*$^{-/-}$) died earlier and more of autoimmune disease (Fig.4.9B) when compared to controls also T_{REG} depleted but expressing physiological levels of Nrf2 (Foxp3^{DTR}→*Nrf2*$^{+/+}$). This observation leads us to conclude that the previously activated immune system remains functional, autoimmune disease is interchangeable and empty mice receiving non-hematopoietic *Nrf2* deleted Foxp3^{DTR} bone marrow chimeras immune system are less likely to survive.

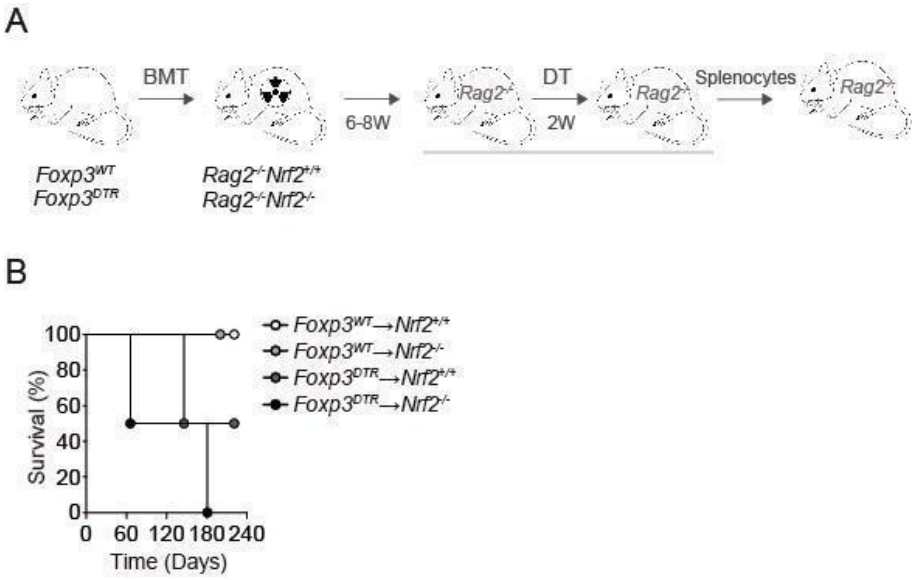

Figure 4.9. *Nrf2* non-hematopoietic knockout does not interfere with immune function upon regulatory T cell depletion
(**A**) Schematic representation of the adoptive transfer of splenocytes from mice described in (5.2.A) into naïve Rag2 deficient recipients. Note that donor chimeric mice received Diphtheria toxin (DT) for two weeks (2W) to deplete T_{REG} cells before the adoptive transfer of splenic cells. (**B**) Survival of *Rag2*$^{-/-}$ after splenocytes adoptive transfer, as previously described; *Foxp3*^{WT}→*Nrf2*$^{+/+}$ n=1, *Foxp3*^{WT}→*Nrf2*$^{-/-}$ n=1, *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ n=2, *Foxp3*^{DTR}→*Nrf2*$^{-/-}$ n=2.

Following the previous premisses, to understand whether Nrf2 acts in parenchyma cells to provide tissue damage control and establish disease tolerance to autoimmune diseases, we used an alternative model in which all Foxp3^{+} cells, including tissue resident Fox3^{+} T cells, bear a DTR, allowing us to deplete all T_{REG} cells including tissue-resident cells.

We bred Foxp3^{DTR} *Nrf2*$^{-/-}$ (F.*Nrf2*$^{-/-}$) in which *Nrf2* was fully body knockout and T_{REG} cells bore a DT receptor (Fig.4.10A). Then, we created bone marrow chimeras in which F.*Nrf2*$^{-/-}$ mice were non-lethally irradiated and grafted with bone marrow from *Foxp3*^{DTR} or *Foxp3*^{WT} mice that express physiological *Nrf2* levels, *Foxp3*^{DTR}→*F.Nrf2*$^{-/-}$ and *Foxp3*^{WT}→*F.Nrf2*$^{-/-}$, respectively (Fig.4.10A). Previous studies showed that T_{REG} cells depletion in chimeric grafted *Foxp3*^{WT} with *Foxp3*^{DTR} bone marrows were not a long stand success due to *Foxp3*^{WT} expansion (data not shown).

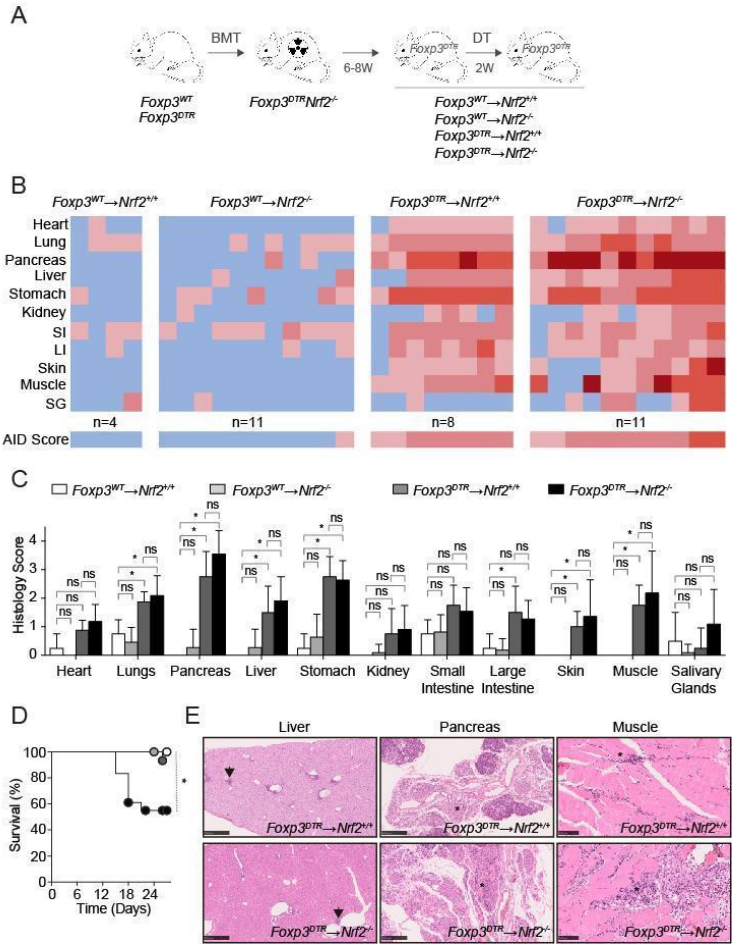

Figure 4.10. Non-hematopoietic *Nrf2* and resident regulatory T cells are relevant for survival in autoimmunity

(**A**) Schematic representation of the strategy used to generate bone marrow chimeric mice in which the *Nrf2* allele is knockout specifically in parenchyma and connective (non-hematopoietic) tissues, but not in hematopoietic cells. Briefly, non-lethally irradiated (700rad) *Foxp3*^{DTR}*Nrf2*$^{+/+}$ or *Foxp3*^{DTR}*Nrf2*$^{-/-}$ mice received bone marrow transplants (BMT) from wt (*Foxp3*^{WT}) mice or *Foxp3*^{DTR} mice bearing a diphtheria toxin receptor (DTR) specifically on regulatory T cells. After 6-8 weeks (W), chimeric (BMT→Recipients) mice received intra-peritoneally diphtheria toxin (DT) for two weeks (2W) to achieve T_{REG} cell depletion and were analyzed on day 26 after DT started. (**B**) Autoimmunity Disease (AID) score in organs from the mice described in (A), obtained 26 days after the first bolus of DT administration. Each horizontal line represents an organ and each column an individual mouse. Mean AID scores are illustrated for each genotype in the bottom horizontal lines. SI: small intestine, LI: large intestine, SG: salivary glands. **(C)** AID scores relative quantification of individual organs *per genotype*, obtained as indicated in (B) and represented as mean ± SD. P values determined by bootstrapping resampling method. *p < 0.05. **(D)** Survival curve of chimeric mice represented in (A). *Foxp3*^{WT}→*Nrf2*$^{+/+}$ (n=8), *Foxp3*^{WT}→*Nrf2*$^{-/-}$(n=12), *Foxp3*^{DTR}→*Nrf2*$^{+/+}$ (n=15), *Foxp3*^{DTR}→*Nrf2*$^{-/-}$(n=18). *p < 0.05 determined by Log-rank (Mantel-Cox) test. **(E)** H&E staining of representative liver, pancreas and muscle from chimeric mice, generated as described in (A). Liver presents mild infiltrations of granulocytes and lymphocytic cells (arrows). Pancreas is destroyed especially exocrine acini (*), configuring general atrophy of the organ. Lymphocytes invade muscles, and fibres are damaged (*).

Upon DT depletion of T_{REG} cells, we assessed similar immunopathology scores among groups on day 26 after DT started (Fig.4.10B). We found that deletion of *Nrf2* in non-hematopoietic cells from chimeric Foxp3^{DTR}→*F.Nrf2*$^{-/-}$ mice was not differently associated with leukocyte infiltration or damage, when in contrast to what was

observed in control *Foxp3*^{DTR}→*F.Nrf2*$^{+/+}$ mice, expressing *Nrf2* physiologic levels (Fig.4.3B,C). As foreseen, the pancreas, liver, stomach, muscle and skin were the most common target organs, independently of *Nrf2* expression (Fig.4.3E). Curiously, in mice not depleted of T_{REG}, lacking (*Foxp3*^{WT}→*F.Nrf2*$^{-/-}$) or not *Nrf2* (*Foxp3*^{WT}→*F.Nrf2*$^{+/+}$), we found mild levels of infiltration, which might suggest an irradiation effect (Fig.4.3B,C).

Noteworthy and not observed in the previous models, chimeric mice that lack non-hematopoietic *Nrf2,* once T_{REG} are depleted (*Foxp3*^{DTR}→*F.Nrf2*$^{-/-}$), survive less when compared to controls with depleted but expressing physiological *Nrf2* levels (*Foxp3*^{DTR}→*F.Nrf2*$^{+/+}$) (Fig.4.10D). This observation rises at potential role of *Nrf2* in non-hematopoietic tissues because no survival difference was observed when *Nrf2* was expressed (Fig.4.5B).

4.3. Discussion

Redox homeostasis is a fundamental function and *Nrf2* has been considered an orchestrator of this process. In our work, we found that when expressed in parenchyma cells the transcription factor Nrf2 protects the liver from autoimmune-mediated injury.

Compared to previous work in which systemic activation of *Nrf2* was described to be protective under an autoimmune context [31], we narrow down its cytoprotective role to non-hematopoietic cells in the same context. Consistent with the protective effect of Nrf2 against autoimmune disease is the observation that aged *Nrf2*-deficient female mice develop a lupus-like syndrome characterized by splenocyte apoptosis, increased lipid peroxidation and DNA oxidation, anti-double-strand DNA and anti-Smith antibodies, along with tissue damage (myocarditis, vasculitis, glomerulonephritis and hepatitis), and, in some cases, death [27,28]. More recently, systemic activation of *Nrf2* (*i.e., Keap1* deletion) in Scurfy (Sf) mice, which present T_{REG} deficiency and develop severe multiorgan inflammation and lethality, was shown to present reduce tissue inflammation and fatality. This was not owed however to *Nrf2* activation in T cells, myeloid cells or dendritic cells [22], consistent with our finding that *Nrf2* activation outside of the immune system exerts anti-inflammatory effects that are protective against autoimmune disease triggered in the absence of T_{REG} cells. Other experimental models have also identified *Nrf2* as part of a protection mechanism against autoimmune disease, as illustrated by Uruno et al. in an experimental model of diabetes [29] or by Wu et al. in an experimental model of skin sclerosis where keratinocyte-specific *Nrf2* deletion promoted disease progression [30].

Systemic deficiency of *Nrf2* impacts also in the immune response, namely with an increased proliferative response of $CD4^+$ T cells, altered $CD4^+/CD8^+$ ratio and promotes pro-inflammatory T_H17 [31,32]. Suzuki et al., using scurfy mice overexpressing *Nrf2*, observed a dampen of $CD4^+$ T cell activation, with a mitigated activation of $CD25^+$, $CD44^+$ and $CD69^+$ populations [22]. Moreover, Sf mice exhibit T_H1-, T_H2-, and T_H17-type effector $CD4^+$ T cells increases, followed by increased production of interferon-gamma production (IFN- γ), interleukin-4 (IL-4), and IL-17A, respectively. These cytokine-producing T cells decreased more than 80% when compared to mice overexpressing Nrf2 (Sf::$Keap1^{-/-}$ mice) [22].

Under a stressful insult, Nrf2 initiates the transcription of a diverse array of cytoprotective molecules and enzymes that detoxify and eliminates ROS, xenobiotics and damaged proteins and organelles, namely through the expression of NQO1 and GSTs. One possible explanation for our findings is that the cytoprotective effect of Nrf2 in parenchyma cells prevents the release of inflammatory alarmins, which engage immune cells to sustain inflammatory reactions in systemic autoinflammatory diseases associated with oxidative stress [33–35]. Preventing the release of alarmins has been widely discussed as a possible mechanism underlying how *Nrf2* promotes tissue damage control and a point of further research through NF-κB pathway and inhibition of pro-inflammatory cytokines [36].

Another possible explanation for the cytoprotective effect of Nrf2 expression in parenchyma cells is to sustain regenerative responses [37] that promote tissue damage control and limit the pathogenetic effects of autoimmune mediated tissue injury. Our results point out to a protective role of *Nrf2* in the liver, this means in case of this cytoprotective loss, this organ becomes severely affected which relates to the immune infiltration and increase of liver enzymes (AST and ALT). *Nrf2* protective role has also been confirmed in another model of chimerism, where both donors and host expressed *Foxp3*^{DTR} while *Nrf2* is only expressed in donors. Yet, no statistical differences in organ targeting were observed due to a survival decrease in *Nrf2* deficient mice following T_{REG} ablation, which further substantiates how crucial *Nrf2* is to overcome the tissue the damage imposed by the immune attack, aligned with a possible regenerative response. Not surprisingly, the liver is indeed a vital organ that has the capacity of regeneration however its dysfunction conduces to death. The relevance of *Nrf2* in liver function was also reported in several disease contexts, such as cadmium-induced acute liver injury [38], hepatic ischemia-reperfusion injury [39], alcohol-induced liver steatosis [40], toxin-induced liver injury and fibrosis [41], where *Nrf2* activation halts oxidative stress, liver damage and promotes detoxification through modulation of anti-oxidant defence-associated genes [42].

The skeletal muscle is the most abundant muscle in the body that allows movement and many other physiological processes, namely metabolism, thermogenesis and production of peptides that have autocrine, paracrine or endocrine effects [43]. Our results evidence a tendency for muscle targeting in *Nrf2*-deficient mice, which may imply *Nrf2* role also in this organ. In addition, Yamamoto's group proved that *Nrf2* upregulation improved glucose tolerance by increases in glycogen branching

enzymes (GBE) and phosphorylase b kinase α unit (PhKα) expression in a skeletal muscle (SkM)-specific Keap1knockout (Keap1MuKO) [44]. *Nrf2* role in the skeletal muscle goes beyond glucose control. Wruck et al. provided new data regarding muscle regeneration [45], which we foresee as a possible explanation for the tendency that we observed. By comparing *Nrf2*$^{-/-}$ and WT mice, Wruck et al. showed impaired muscle regeneration after hindlimb ischemia/reperfusion injury by disturbing myogenic regulatory factors (MyoD and myogenin) in *Nrf2*-deficient mice [45].

The regeneration and repair function of *Nrf2* was also shown beyond of the muscle [46]. In the skin, keratinocyte growth factor (KGF) expression, a key player in epithelial repair processes [47], activates *Nrf2* upregulation for the repair process of skin wounds. In the absence of *Nrf2*, dermal infiltration of macrophages persists, sustaining the inflammation [48], which may explain an increased susceptibility to the skin targeting in our model.

Remarkably, Nrf2 expression in parenchyma cells is deleterious against autoimmune damage to the small intestine (Fig.4.3C). The reason for this is not entirely clear. Previous studies have shown that *Nrf2*$^{-/-}$ mice presented an increased intestinal permeability and plasma endotoxin level in a traumatic brain injury-induced intestinal mucosa damage model [49]. This would suggest that Nrf2 is cytoprotective in intestine epithelial cells. However, these studies used constitutive *Nrf2* deletion in immune and parenchyma cells, suggesting that there may be a particular role for *Nrf2* in the local immune milieu that we do not observe in our chimeric model in which the immune system expresses physiological levels of Nrf2.

Nrf2 has pivotal roles in inflammation [8]. Under a physiologic state, reactive oxidative species are produced but quickly neutralized to prevent cellular damage [50]. Nrf2 takes part in this neutralizing process, allowing a buffering of oxidants species, thus preventing its deleterious inflammatory effects [50,51]. In line with these premisses, our results demonstrate that non-hematopoietic Nrf2 loss increases disease susceptibility. We know that Nrf2 is a relevant player in balancing the oxidative status [8], including the proinflammatory mediators, including IL-1β, IL-6, TNF-α, iNOS and COX2, as determined in dextran sulfate sodium colitis [52] and LPS-induced pulmonary sepsis [13] models of *Nrf2*-deficient. Oppositely, there is an inhibition of pro-inflammatory cytokines and chemokines overproduction and activation of NF-κB when *Nrf2* is overexpressed in endothelial cells [13,53].

Nrf2 anti-inflammatory effect expands further by influencing chemoattraction and transmigration of immune cells. Cell adhesion molecules (CAMs), such as ICAM-1 and VCAM-1 [54], are also influenced by Nrf2 expression. Nrf2 represses the promotor of VCAM1 [13], mediated by the expression of Hemoxigenase 1 (HO-1) [55–57], while in *Nrf2*-deficient mice there is an increased expression [58]. Along with inflammatory cytokines, chemokines, such as CCL-3, CCL-4, CCL-5 and CXCL-10, facilitate the recruitment of immune cells into inflamed tissues. Wu et al. observed an increase expression of chemoattractants such as Mcp-1, Ccl-3 and Cxcl-10 in a *Nrf2*-deficient skin fibrosis mouse model [30]. Accordingly, the fact that *Nrf2,* in an inflammatory context, decreases chemoattraction and transmigration of the immune cells by chemokines and CAMs, provides another possible explanation for our results of diverse organ targeting (Fig. 4.3).

By the comparison of the two chimeric experimental models, we observed a crucial role of *Nrf2* in non-hematopoietic tissues that hinders mice survival (Fig.4.10D, Fig.6.5B). Such survival difference observed in chimeric mice expressing *Foxp3*^{DTR} in donors and host can be justified by the more widely depletion of T_{REGs}, including tissue-T_{REGs} that also express Foxp3, and may present different functions according to the organs that they inhabit [59,60]. Accordingly, due to a decrease in survival we do not observe similar organ targeting between the two models.

In conclusion the pathogenesis of autoimmune diseases, resulting from failure to sustain peripheral self-tolerance, is associated with oxidative stress and therefore with the induction of protective-stress responsive mechanisms. These are orchestrated via the activation of Nrf2 in parenchyma cells, which contributes critically to provide protection against autoimmune injury, namely in the liver. If these findings in mice translate into human autoimmune disease, they suggest that, polymorphic *Nrf2* tissue expression may result in differently organ targeting during autoimmunity.

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Chapter 5

Contribution of the Hypoxia Stress Response To Tissue Damage Control in Autoimmune Diseases

Abstract

Hypoxia occurs when oxygen (O_2) supply falls below the metabolic requirements of cells, tissues or organs. In response, cells in different organs activate hypoxia-inducible factors (HIFs), which are responsible for a transcriptional program towards a protective/adaptive molecular response to hypoxia [1]. In this chapter we tested whether modulating cellular adaptation to hypoxia regulates tissue damage and modulates the pathogenesis of autoimmune diseases. To this aim, we generated bone marrow chimeric carrying a deletion of the *Hif1α*$^{fl/fl}$ allele in non-hematopoietic (*i.e.,* parenchyma) cells. We found that the development of autoimmune disease upon T_{REG} cell deletion was more severe when *Hif1α* was expressed at physiologic levels in parenchyma cells. Autoimmune pathology was exacerbated especially in the pancreas, liver, stomach, skin and muscle. These findings bring forward *Hif1α* as a susceptibility factor in the development of multi-organ systemic autoimmunity. These findings highlight a critical role of HIF-1α in promoting inflammation in autoimmune diseases. Additional studies will be required to address a role for *Hif1α* tissue specificity in autoimmunity disease pathogenesis.

5.1. Introduction

5.1.1. Hypoxia: when tissues lack oxygen

Oxygen (O_2) is a crucial component of inorganic compounds and a fundamental component of all major biomolecules. Eukaryotic cells adopted specialised systems to enhance O_2 uptake and distribution in order to function efficiently. At the organismal level, if O_2 supply is disrupted and the intracellular O_2 concentration decreases, hypoxia develops. Hypoxia refers to when tissues are deprived of oxygen to meet their metabolic requirements; hence, it will be sensed differently across several tissues and cells. Notably, hypoxia may be a physiological stress related to processes that lead to adaptation, for instance, to high altitude or human diseases (e.g., cancer) [1]. The overall impact of hypoxia on tissues is also determined by the degree and the temporal and spatial pattern of activation of these hypoxia-related processes to elicit a transcriptional response [2]. Inside tissues, such as the renal medulla, intestinal mucosa, placenta, bone marrow or germinal centres, oxygen gradients regulate pathways critical to maintaining physiologic homeostasis.

Moreover, physiological hypoxia is vital in maintaining adaptive and innate immune cell homeostasis and the control of tissue features such as antibody production [2]. However, oxygen role in oxidative metabolism and maintenance of cellular ATP levels at a micro-environment level may potentially threaten cellular life, the so-called pathological hypoxia, an unstructured and chaotic cellular exposure to oxygen gradients (e.g., myocardial infarction, atherosclerosis and stroke) [3]. Pathological hypoxia was found in inflammatory disorders such as inflammatory bowel disease, rheumatoid arthritis and chronic infection, as a combination of disrupted blood flow, increased metabolic activity of inflamed resident and infiltrating cells and oxygen consumption in the case of bacterial infection [4–6]. This pathological hypoxia may provoke more inflammation and cell death, contributing to disease development [7–9]. As autoimmunity imposes stress and damage in the target tissue, the ability of the host parenchyma to accommodate stress and damage should influence the development and extent of the pathology. Acknowledging these facts, we aimed at understanding whether modulating hypoxia cellular adaptation which confers tissue damage control, would modulate the pathogenesis of T cell mediated tissue injury.

5.1.2. Hypoxia-inducible factors: an adaptive response to hypoxia

O_2 deprivation elicits profound changes in gene transcription, inducing an adaptive transcriptional response [10] and hypoxia-inducible factors (HIFs) are transcriptional master regulators of this adaptive response [2]. The first member of this family, HIF-1α was discovered almost 30 years ago by G. Semenza et al. through studies of erythropoietin gene expression [11]. HIFs form heterodimers composed of two constitutively expressed subunits, an O_2-sensitive alpha unit (*i.e.,* HIF1α, HIF2α or HIF3α) and an O_2-insensitive beta subunit Hif1β/ARNT[12]. HIF-1α and HIF-2α have critical cellular functions as transcription factors with common targets [3]. Even though HIF-2α protein shares similarities and functional overlap with HIF-1α, its distribution is restricted to specific cell types, and in some cases, it mediates distinct biological functions [12].

5.1.2.1. HIF1α: molecular mechanism of stability under hypoxia and normoxic conditions

Under normoxic conditions, HIF1α is synthesised but rapidly targeted, via a process involving the enzymatic hydroxylation of two conserved prolyl residues at oxygen dependent domain (ODD) (Pro-402 and Pro-564), for degradation by the ElonginC-ElongiB-Cullin2-E3-ubiquitin-ligase complex recruited by von Hipple–Lindau disease tumour suppressor (pVHL) [13]. This process is dependent upon the availability of non-mitochondrial O_2 [2] and involves a family of O_2-dependent dioxygenases termed prolyl hydroxylase domain (PHD) enzymes and an asparagine hydroxylase termed factor inhibiting HIF (FIH) [14]. These hydroxylases repress HIF-1α in normoxia through a combination of targeted degradation and transcriptional repression. Until now, three PHDs proved to hydroxylate HIF1α: PHDs 1, 2 and 3. PHD2 has the highest affinity for HIF-1α, whereas PHD1 and PHD3 for HIF2α [15]. These PHDs require iron (Fe^{2+}) and α-ketoglutarate (α-KG) as co-factors for their catalytic activity plus O_2 as a co-substrate, repressing their activity when O_2 availability is reduced, *i.e.,* hypoxia [16]. FIH serves as a second O_2 sensor and prevents HIF-1α interaction with coactivators by hydroxylation at a specific asparaginyl residue (Asn-803) in terminal activation domain (TAD)-C [13], repressing chromatin remodelling by recruitment of histone deacetylases. Like the PHDs, FIH is a Fe^{2+} and 2-oxoglutarate dependent dioxygenase enzyme, requiring vitamin C to retain the ferrous state of the iron [17]. Therefore, nutrient

conditions may regulate HIF stability independent of oxygen tension. Other mechanisms regulating HIF function independent of O_2 are by binding pVHL independently and recruiting histone deacetylase that interferes with the TAD function [17], or by regulating E3 ubiquitin ligase activity through receptor-activated protein kinase C (RACK1) and carboxyl terminus of Hsp70-interacting protein (CHIP) [17,18]. Thus, post-transcriptional phosphorylation, acetylation and S-nitrosylation may regulate HIF stability or activity irrespectively of O_2.

PHDs activity is repressed under hypoxia, due to the lack of O_2, inhibiting the hydroxylases responsible for targeted degradation of HIF1α. Therefore, the inhibition of PHDs increases HIF1α stability and availability to form a heterodimer with HIF-β in the nucleus. This hydroxylation-dependent switch also regulates the ability of HIF1α TAD-C to interact with coactivators such as CBP/p300 [17,18], steroid receptor coactivator-1 family activators [19], nuclear redox regulator Ref-1 [19] or molecular chaperon heat shock protein 90 [20]. Only then, HIF heterodimer binds the consensus cis-acting hypoxia response element (HRE) nucleotide sequence 5'-RCGTG-3' in HIF target genes [21,22]. The coactivators own histone acetyltransferase activity and can remodel the chromatin structure to activate gene transcription [19].

HIFs are self-regulated by several negative feedback loops, such as upregulation of the PHD2 and PHD3 hydroxylases and by microRNAs, which control HIF expression [23]. HIF hydroxylases sense the metabolic status of a cell, besides acting as O_2 sensors. PHDs are able to a broader metabolic sense [24,25]. Since amino acids control intracellular levels of α-KG, oscillations in α-KG, a substrate for PHD reaction, allow PHD to sense amino acid availability [24,25], as described by 2-oxoglutarate, 2-hydroxyglutarate, succinate and reactive oxygen species (ROS) [26,27]. Other factors, such as mechanical stress [28,29], heat [30], pH [31], cytokines [32], hormones [33], growth factors [34], have also been demonstrated to induce HIF independently of hypoxia. More recently, a lysosomal degradation pathway in an O_2-independent manner has also been found to control HIF stability [35]. Although the mechanisms behind it are not yet settled, a common cellular pathway may be responsible for triggering hypoxia-responsive targets [13].

Regulation of HIF1α protein synthesis may be another way to overcome the O_2 sensor mediated degradation under normoxic conditions. Growth factors induce HIF1α protein translation irrespectively of hypoxia, via activation of the phosphatidylinositol

3-kinase (PI3K), upstream of mitogen-activated protein kinase (MAPK) [36,37]., enhancing the transcriptional activity of HIF [13].

Under inflammatory conditions, the NF-κB, a key regulator of inflammation and immunity, regulates *Hif1α* promoter positively and, inducing HIF1α synthesis and stabilisation in chronically inflamed context [38,39]. Curiously, NF-κB is also hypoxia-induces through hydroxylation of key upstream regulators of the pathways, namely IKKβ and ankyrin repeat domain-containing proteins for instance p105 (also known as NFKB1) and its inhibitor IκBα by PHDs [40].

There is a crosstalk between NF-κB and HIF1α pathways, by activation of the canonical NF-kB pathway, such as driven by cytokines or bacterial remains, which promotes HIF1α transcription, expression and activity under conditions of chronic inflammation[2], suggesting that HIF1α may be recruited as an inflammatory mediator in tissue inflammation.

5.1.2.2. HIF1α transcriptional activity

HIF heterodimers regulate a broad range of cellular processes through the expression of 100 target genes [3], controlling different aspects of cellular metabolism, for example promoting glycolysis and repressing mitochondrial OXPHOS while controlling amino acid, nucleotide and extracellular matrix metabolism. HIFs also control cell proliferation, programmed cell death via apoptosis, cellular motility and cytoskeletal structure as well as cell adhesion. At organismal level HIFs regulate erythropoiesis and promote vascular endothelial growth factor (VEGF)-dependent angiogenesis while also controlling iron metabolism, inflammation, pH, vascular tone, epithelial barriers and fibrinogenesis [2]. This occurs through the cognate binding of the transcription factor to DNA hypoxia responsive elements (HRE) in the promoter or enhancer of target genes. More recently, HIF signalling has been demonstrated to regulate Notch [41] and p53 [42] signalling pathways, relevant in cell-cell communication such angiogenesis sprouting [43,44].

5.1.2.3. HIF1α in immune and nonimmune cells: tissue damage control

Different tissues face different levels of O_2 pressure, relying on HIF1a activation to orchestrate gene expression profiles compatible with cell function under sustained gradients of O_2 typical of physiological hypoxia. Intermittent O_2 gradients tend to be more common in chronically inflamed tissue as seen in inflammatory bowel disease or

in a growing tumour [2,45]. The degree of hypoxia, presence of cytokines, inflammatory mediators[46], immune metabolites, such as succinate, 2-oxoglutarate and 2-hydroxyglutarate [26,47], oxidative stress [48,49] or gasotransmitters such as nitric oxide, carbon monoxide or hydrogen sulfide regulate HIF cellular activity and shape inflammatory responses [2]. Tissue hypoxia can be both pro-inflammatory or anti-inflammatory depending on cell type and context and, thus, the degree of HIF activation in specific cell subtypes.

Both HIF1α and HIF2α are expressed in the kidney, where interstitial renal fibroblasts produce erythropoietin (EPO) in a HIF2α-dependent manner. Endothelial HIF2α, but not HIF1α, is protective against renal ischemia-reperfusion injury by reducing the expression of adhesion molecules such as VCAM1 [50]. HIF1α activation in other epithelial cells, such as imposed by *Phd2* deletion, accelerates wound healing and keratinocyte migration in skin epithelium, through the induction of β3 integrin [51].

HIFs regulate macrophage motility and bactericidal activity shaping macrophage polarization and inflammatory responses [2,52]. HIFs also regulate neutrophil lifespan, invasiveness, antimicrobial response and cell death [53,54]. Myeloid-specific *Phd2* deletion (*i.e.,* targeting macrophage and neutrophil) or PHD2 pharmacological inhibition to promote HIF1α activation, exacerbates neutrophilic inflammation and lung injury in response to infection [55]. This is explained in part by the neutrophil release of antimicrobial factors that are highly toxic to pulmonary epithelial cells, promoting lung epithelium permeability [56]. In contrast, induction of HIF1α and downstream target genes in the intestinal epithelium is protective and depends on the migration of neutrophils and O_2 consumption to fuel neutrophil-derived NADPH oxidase [57]. HIF also promotes NF-κB activation in neutrophils and keratinocytes, sustaining an inflammatory response [58,59].

HIFs also act in T cells to regulate T_H cell differentiation towards T_H17 *vs.* T_{REG} cells, whereby HIF1α acts as a RORγt coactivator to promote the pro-inflammatory response of T_H17 cells while repressing the transcription factor FOXP3 underlying T_{REG} cell function and differentiation [60]. While HIF1α deletion in T cells enhances T_{REG} cell differentiation [60,61], perhaps intriguingly, sustained *Hif1α* activation promotes T_{REG} cell transdifferentiation towards inflammatory T_H1 cells, due to loss of FOXP3 and sustained IFNγ expression [62]. This is consistent with *Hif1α* knockout mice in response to T cell receptor (TCR) activation increasing pro-inflammatory cytokine production [63].

HIF1α upregulation is also associated with improved viral and tumour cell clearance as well as with increased immunopathology and mortality during chronic viral infections due to persistent $CD8^+$ T cell activation [64]. Accordingly, HIF1β-deficient T cells show an altered expression of molecules involved in T cell migratory capacity and diminished expression of perforin and granzymes [65]. Moreover, HIF1β-deficient $CD8^+$ T cells display increased T cell homing to secondary lymphoid organs and sustained CD62L expression, likely due to upregulation of chemokine receptors [65]. Given the above, it is apparent that HIFs integrate TCR and cytokine receptor-mediated signals involved in T_H and $CD8^+$ T, irrespectively of O_2 availability.

HIF also regulates B cell development survival and antibody production [66], with B cell-specific *Hif1α* deletion reducing IL-10 production and exacerbating collagen-induced arthritis as well as experimental autoimmune encephalomyelitis (EAE) [67]. On the other hand, constitutive HIF1α stabilisation in B cells led reduces class-switching, antigen-specific germinal centre formation, preventing the generation of early memory B cells [66].

HIF has been found crucial to the normal cell functions which depend on distinct isoforms. For instance, in intestinal epithelial cells, HIF1α modulates ion transport, antimicrobial peptide production and barrier function while HIF2α has been reported to be associated with the promotion of inflammation [68]. In the kidney, both HIF1α and HIF2α are expressed, however interstitial renal fibroblasts produce erythropoietin (EPO) in a HIF2α-dependent manner, while endothelial HIF2α, but not endothelial HIF1α, protects from renal ischemia-reperfusion injury by reducing the expression of VCAM1 [50].

Interestingly, Chi et al analysed the HIF transcriptional response in several cell types and found that the degree of induction of HIF targets was higher in epithelial cells than in mesenchymal cells, plus the number of genes induced was significantly higher [69]. Renal proximal tube epithelial cells had a clear specific set of genes induced, mostly involved in immune regulation, solute transport and genomic integrity [69]. This unique case may be due to a specific function in hypoxic response. In contrast, increased HIF protein abundance is unlikely to cause cell type-specific differences as their regulation depends also on chromatin status, partner transcription factors or coactivators [70]. In fibroblasts, HIF activates the expression of collagen and lysyl hydroxylases, both required fibrogenesis, collagen deposition and extracellular matrix stiffening [71]. Myofibroblasts, that result from differentiation of fibroblasts, are also a

result of hypoxia and HIF1α upregulation of transforming growth factor β [72]. Similar effects occur in pulmonary fibrosis, in which lactate accumulation by HIF-mediated upregulated glycolytic metabolism results in TGFβ activation. In the skin, HIF1-dependent induction of β3 integrin accelerates wound healing and keratinocyte migration, on a PHD2 keratinocyte specific-deletion model [51]. Deletion of HIF-1α in endothelial cells interrupts an autocrine loop necessary for the hypoxic induction of VEGFR-2 expression [69]. In contrast, models of liver fibrosis with hepatocyte-specific HIF-1α deletion show a reduced fibrosis through downregulation of profibrotic proteins such as type I collagen and α smooth muscle actin (αSMA) [73]. Alveolar epithelial cells are at a unique region, exposed to sterile and non-sterile inflammation, where HIF1α plays a determinant role. Resolution and repair in the alveolus after inflammatory stimuli rely on the proliferation and transdifferentiation of alveolar cells, for which HIF1α-dependent VEGF expression is crucial [74]. In acute lung injury, consistent with mechanical ventilation, normoxic induction of HIF was observed to be protective while its alveolar epithelial loss is associated with mortality [75]. However, in a different context, namely influenza-induced alveolar epithelial loss, activation of HIF-1α may be associated dysfunctional alveolar remodelling [76]. In this study, mice lacking epithelial cell HIF1α recovered faster, with improved expansion of the type II alveolar cells and restoration of normal epithelium [76].

5.1.2.4. HIF1α in inflammatory and non-inflammatory diseases

In inflammatory microenvironments, one can easily foresee a combination of O_2 consumption by local cell proliferation, migrating inflammatory cells, and through activation of oxidases, monooxygenases and dioxygenases expressed in multiple cell types [8]. Combined inflammation and hypoxia have been identified in several diseases, including rheumatoid arthritis, psoriasis, systemic sclerosis, lupus nephritis, inflammatory bowel disease or cancer [77–81].

In this chapter we tested whether modulating cellular adaptation to hypoxia regulates tissue damage and modulates the pathogenesis of autoimmune diseases.

5.2. Results

To explore whether *Hif1α* is implicated in the pathogenesis, progression and/or outcome of systemic autoimmune disease, we started by generating *Rag2*$^{-/-}$*Hif1α*$^{R26fl/fl}$ mice (Fig.5.1A), in which Tamoxifen-inducible expression of the Cre^{ERT2} recombinase under the control of the *Rosa 26* (*R26*) promoter removes *LoxP* site-flaking exon 2, from the *Hif1α*$^{fl/fl}$ allele (Fig.5.1B). *Rag2*$^{-/-}$*Hif1α*$^{R26fl/fl}$ and control *Rag2*$^{-/-}$ *Hif1α*$^{fl/fl}$ were identified by PCR (Fig.5.1C-E) and deletion of *Hif1α*$^{fl/fl}$ allele in response to tamoxifen was confirmed by qRT-PCR in several organs from *Rag2*$^{-/-}$ Hif1α$^{R26Δ/Δ}$ *vs.* control *Rag2*$^{-/-}$*Hif1α*$^{fl/fl}$ mice expressing physiologic levels of *Hif1α* (Fig.5.1F).

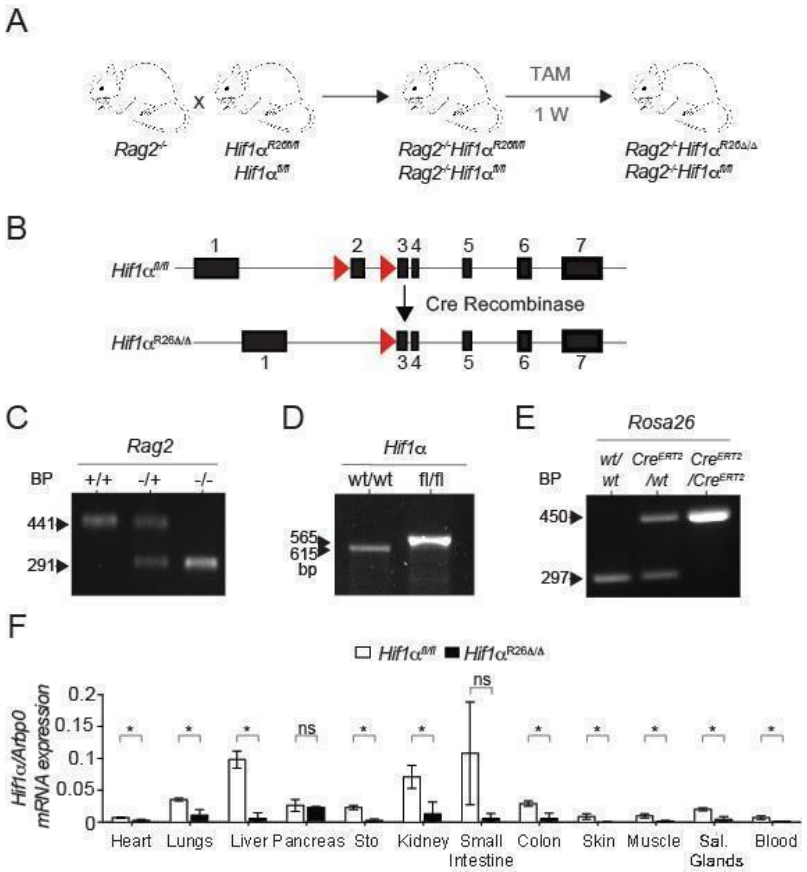

Figure 5.1. *Hif1α* is widely deleted in Rag2 deficient mice

(**A)** Graphical depiction of the strategy used to generate *Rag2* deficient (*Rag2*$^{-/-}$) mice in which the transcription factor hypoxia-inducible factor 1 α (*Hif1α*) is globally deleted, under the control of the *rosa26* (R26) promoter, one week (W) after Tamoxifen (TAM) administration, as described in *Methods*. **(B)** Schematic representation of *Hif1α* locus containing two *LoxP* sites (red triangles), at the introns between exon 2 (black rectangles) [82]. Deletion of exon 2 in *the Hif1α*$^{fl/fl}$ allele is achieved by TAM-driven induction of CreERT2 recombinase activity. **(C-E)** Electrophoresis of PCR products amplified from genomic DNA of mice, generated as depicted in A) and carrying: C) wild type (+/+), heterozygous (+/-) or homozygous *Rag2* alleles, D) wild type (wt/wt) and homozygous (fl/fl) *Hif1α* alleles and E) wild type (wt/wt), heterozygous (Cre^{ERT2}) or homozygous (*Cre*^{ERT2}/*Cre*^{ERT2}) Cre^{ERT2} allele inserted in the *Rosa26* locus. **(F)** Quantification of *Hif1α* mRNA expression by qRT-PCR in different organs from mice receiving TAM, as indicated in (A). Data is represented as mean ± SD (n=3/group) in a single experiment. P value was determined by unpaired Student's t-test. * p< 0.05. ns, not significant. BP: base pairs.

We then generated bone marrow chimeric mice to delete the *Hif1α*$^{fl/fl}$ allele specifically in non-hematopoietic cells (Fig.5.2A). To this aim, bone marrows from *Foxp3*^{DTR} mice were adoptively transferred into non-lethally irradiated *Rag2*$^{-/-}$ *Hif1α*$^{R26fl/fl}$ (*Foxp3*^{DTR}→*Rag2*$^{-/-}$*Hif1α*$^{R26fl/fl}$) and control *Rag2*$^{-/-}$*Hif1α*$^{fl/fl}$ (*Foxp3*^{DTR}→*Rag2*$^{-/-}$*Hif1α*$^{fl/fl}$) mice (Fig.5.2A). Tamoxifen was used to delete the *Hif1α*$^{fl/fl}$ allele specifically in non-hematopoietic cells of the chimeric mice. DT administration was used to deplete T_{REG} cells depletion from *Foxp3*^{DTR}→*Rag2*$^{-/-}$*Hif1α*$^{R26fl/fl}$ mice as in Chapter 3. Control *Foxp3*^{WT}→*Rag2*$^{-/-}$*Hif1α*$^{R26fl/fl}$ and *Foxp3*^{WT}→*Rag2*$^{-/-}$*Hif1α*$^{R26fl/fl}$ mice express physiologic numbers of T_{REG} cells. This experimental system allows to investigate whether *Hif1α* expression specifically in non-hematopoietic cells regulates the pathogenesis, progression and/or outcome of systemic autoimmune disease.

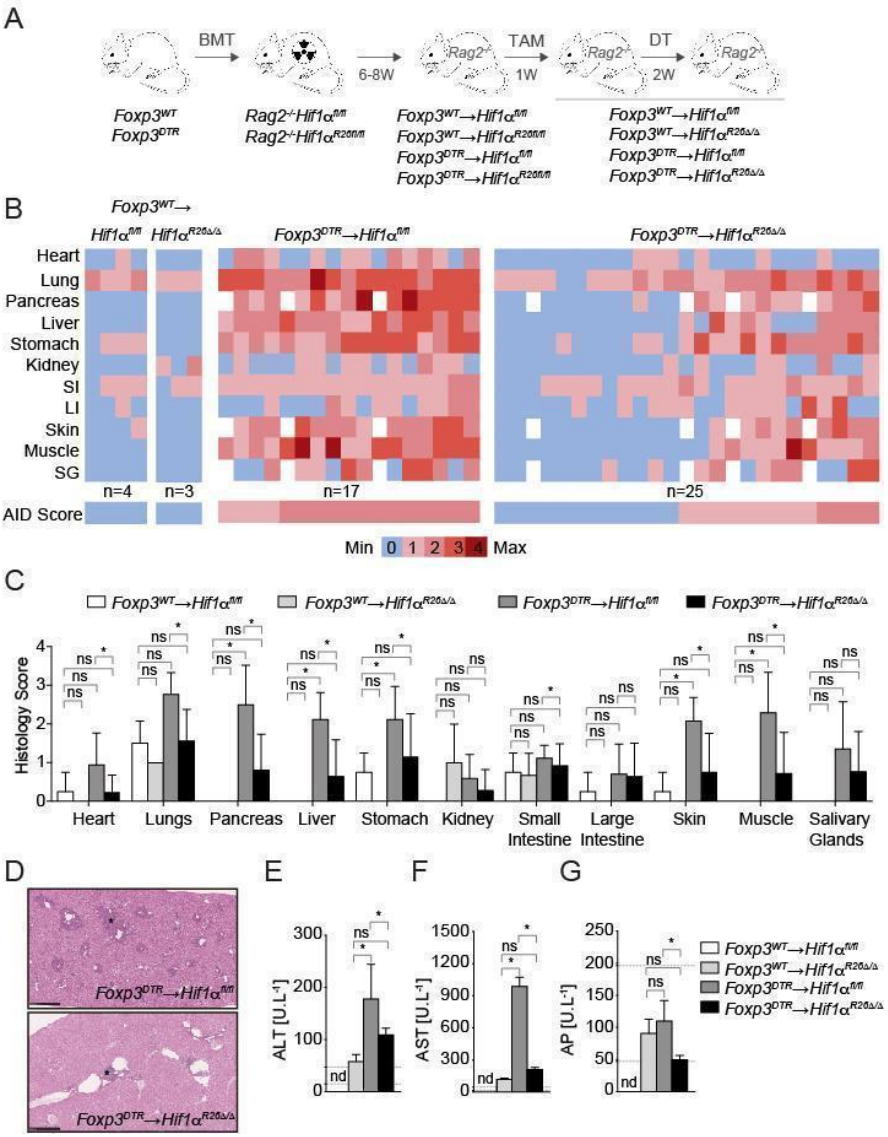

Figure 5.2. *Hif1* deletion in non-hematopoietic tissues protects from multi-organ targeting

(A) Simplified diagram of the strategy used to generate chimeric mice in which the transcription factor hypoxia-inducible factor 1*α* (*Hif1*) is globally deleted except in the hematopoietic compartment. Bone marrow transplants (BMT) from mice bearing a diphtheria toxin receptor on regulatory T cells (*Foxp3*^{DTR}) or from wt mice (*Foxp3*^{WT}) were transferred into non-lethally irradiated *Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ or *Rag2*$^{-/-}$*Hsf1*$^{R26f/lf}$. After 6-8 weeks (W), chimeric mice (BMT→recipients) were given tamoxifen (TAM) for one week to delete *Hif1α* in non-hematopoietic tissues by cre recombinase activation (R26fl/fl→R26Δ/Δ). Thereafter, diphtheria toxin (DT) was administrated for two weeks to induce depletion of regulatory T cells, and finally, mice were analysed on day 26 after DT started. **(B)** Systematized plot of histological pathology score in the same mice as (A) on day 26 after starting diphtheria toxin. Each column represents a single mouse within a given experimental group. AID score is a mean of all the organ scores of a given mouse. **(C)** Bar plot of histological pathology score per organ as B). Standard bars represent mean ± SD. *p < 0.05, as determined by bootstrapping resampling method. **(D)** Representative H&E staining of liver from regulatory T cells depleted mice (*Foxp3*^{DTR}) *Hif1α* globally deleted (*Hif1α*$^{R26Δ/Δ}$) or not (*Hif1α*$^{fl/fl}$). Scale bar=500mm. **(E-G)** Serum liver enzymes concentration on day 26 after starting regulatory T cells depletion. ALT, aspartate transaminase. AST, alanine transaminase. AP, alkaline phosphatase. nd, not determined, *p < 0.05, as determined by ANOVA and Tukey's multiple comparisons test. Data from 4 independent experiments.

As expected (read Chapter 3), T_{REG} cell depletion in response to DT administration was associated with the development of systemic autoimmune disease in all chimeric mice (Fig.5.3). Namely, *Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$ mice developed multi-organ leukocyte infiltration, while this was not the case in control chimeric mice *Foxp3*^{WT}→*Hif1α*$^{fl/fl}$ in which T_{REG} cells were not depleted and *Hif1α* was expressed at physiologic levels (Fig.5.2.B,C; Fig.5.3; Fig.5.4). Notwithstanding, the severity of autoimmune disease was lower in *Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$ mice, in which *Hif1α* was deleted in parenchyma cells, as compared to control *Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$ mice expressing physiologic levels of *Hif1α* in parenchyma cells (Fig.5.2B-D). This decrease in immunopathology was especially notable in the liver, pancreas, stomach, skin and muscle. This was associated with lower accumulation of liver injury biomarkers (*i.e.,* alanine and aspartate aminotransferases; ALT and AST), as assessed 26 days after T_{REG} cell depletion (Fig.5.2E,F). Of note, *Hif1α* deletion in non-hematopoietic cells did not *per se* cause tissue damage in control *Foxp3*^{WT}→*Hif1α*$^{R26\Delta/\Delta}$ mice (Fig.5.2B,C). These findings bring forward the idea that activation of *Hif1α* in parenchyma cells increases susceptibility to autoimmune diseases.

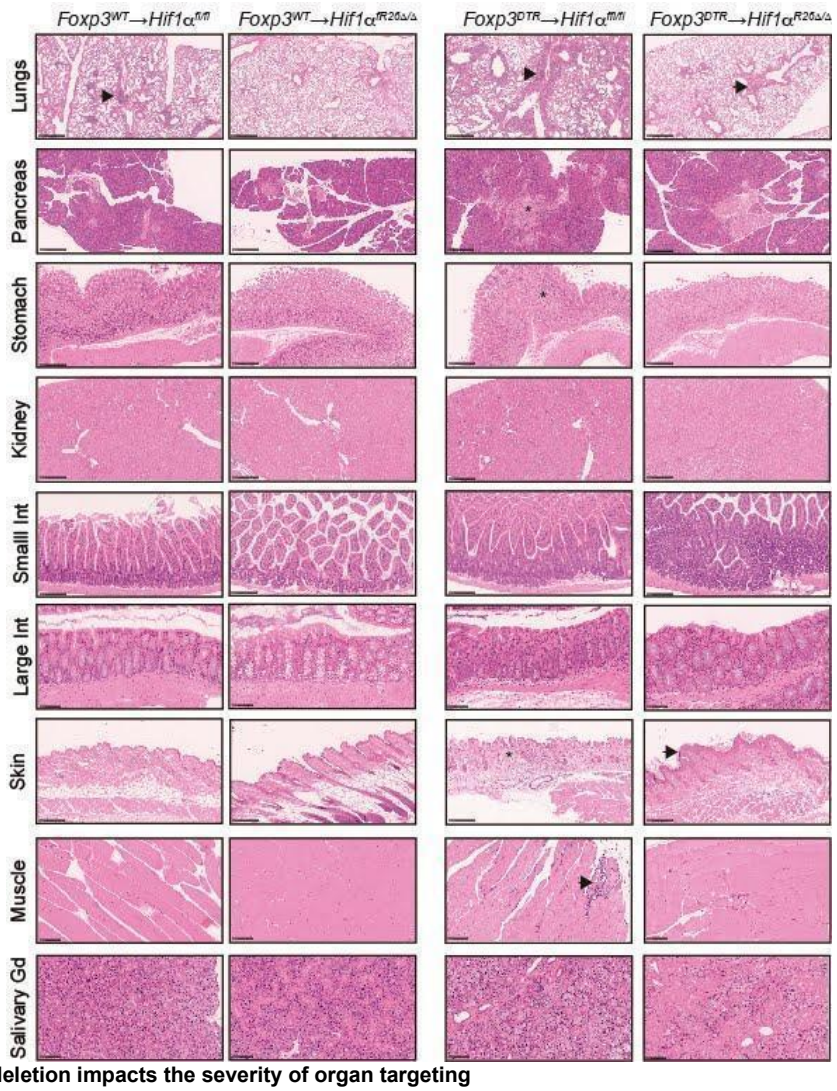

Figure 5.3. *Hif1α* deletion impacts the severity of organ targeting

H&E pictures representative of different organs from each chimeric group of mice on day 26 after beginning diphtheria toxin administration: *Foxp3*^{WT}→*Hif1α*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hif1α*$^{R26\Delta/\Delta}$ n=3; *Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$ n=17; *Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$ n=25. Lungs from chimeric mice present a very mild to moderate perivascular and peribronchial inflammation (arrow). The pancreatic structure is moderately infiltrated by mononuclear and polynuclear cells (*) in the presence of *Hif1α;* in its absence (*Hif1α*$^{R26\Delta/\Delta}$), the pancreatic structure is preserved. Gastric mucosa has mild immune cells infiltrations (*) in *Foxp3*^{DTR} chimeric mice presenting *Hif1α*, compared to a very mild infiltration when *Hif1α* is missing (*Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$). The kidney is not different between chimeric mice. The small and large intestine (Int) present a mild inflammation in the mucosa and submucosa. Chimeras lacking *Hif1α* and T_{REG} unveil very mild dermal inflammation with hyperkeratosis (arrow), while in the presence of *Hifα (Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$*)*, there are diffuse infiltration of lymphocytes, plasma cells and neutrophils (*). Muscle and salivary glands (Gd) are mildly infiltrated by inflammatory cells (arrows) in T_{REG} depleted chimeras presenting *Hif1α*$^{fl/fl}$, however, in *Hif1α*$^{fl/fl}$ deleted chimeras, the pathology induced is very mild.

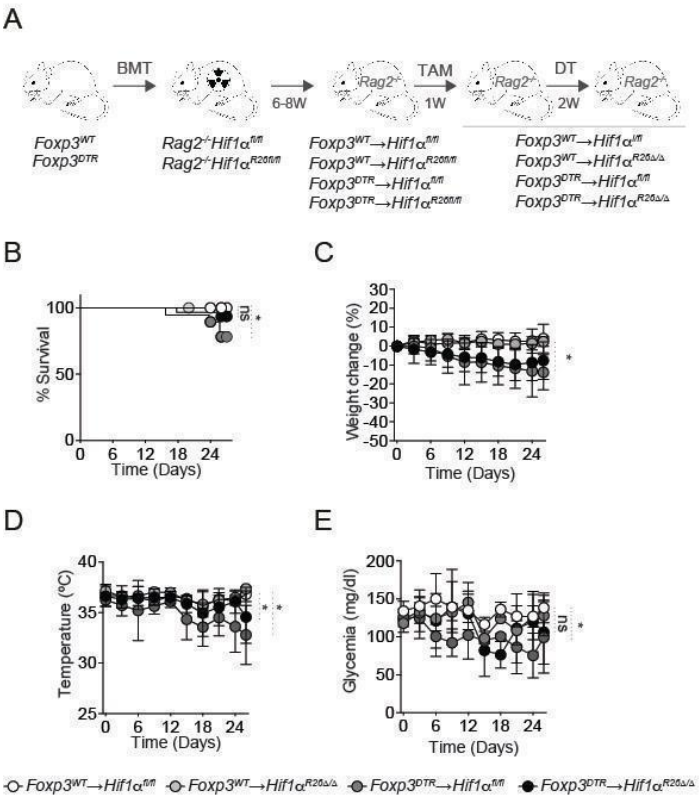

Figure 5.4. Loss of *Hif1α* does not influence mice vital parameters upon regulatory t cell depletion

(A) Simplified diagram of the strategy used to generate chimeric mice in which the transcription factor hypoxia-inducible factor 1 (*Hif1*)*α* is globally deleted except in the immune compartment. Bone marrow transplants (BMT) from mice bearing a diphtheria toxin receptor on regulatory t cells (*Foxp3*^{DTR}) or from wt mice (*Foxp3*^{WT}) were transferred into irradiated *Rag2* deficient mice carrying a homozygous *Hif1α* floxed allele and *Rosa26* locus with Cre^{ERT2} homozygous allele (*Rag2*$^{-/-}$ *Hif1α*$^{R26fl/fl}$) or wt allele (*Rag2*$^{-/-}$ *Hif1α*$^{fl/fl}$). After 6-8 weeks, chimeric mice (BMT→recipients) were given tamoxifen (TAM) for one week to delete *Hif1α* by cre recombinase activation (*R26*fl/fl→ *R26*Δ/Δ). Thereafter, diphtheria toxin (DT) was administrated for two weeks to induce depletion of regulatory T cells, and finally, mice were analysed on day 26 after DT started. **(B)** Survival of chimeric mice during autoimmunity development. **(C-E)** Weight change according to initial day of disease induction (C). Rectal temperature (D) and blood glycemia (E) throughout the disease development. (*Foxp3*^{WT}→*Hif1α1*$^{fl/fl}$ n=6; *Foxp3*^{WT}→*Hif1α1*$^{R26Δ/Δ}$ n=5; *Foxp3*^{DTR}→*Hif1α1*$^{fl/fl}$ n=18; *Foxp3*^{DTR}→*Hif1α1*$^{R26Δ/Δ}$ n=25). *p < 0.05, as determined by log-rank (Mantel-Cox) test for survival comparison and ANOVA test with Tukey's multiple comparisons for the remaining. Standard bars represent mean ± SD. ns, not significant. Data from 4 independent experiments.

When compared chimeric mice upon depletion of regulatory T_{REG}, no differences were observed on survival, weight loss, temperature or glycemia (Fig. 5.4), regardless of *Hif1α* expression.

We then asked whether *Hif1α* expression by non-hematopoietic cells modulates the systemic autoimmune activation observed in response to T_{REG} cell depletion. We found no apparent differences in the number of activated splenic $CD4^+$ and $CD8^+$ T cells according to the expression of CD44 and CD62 nor on the number of splenic $CD19^+$ B cells (Fig.5.5A-F) comparing the different chimerix groups. Similar

results were observed for regulators of T cell activation, including PD1, CTLA4 and ICOS (Fig.5.6).

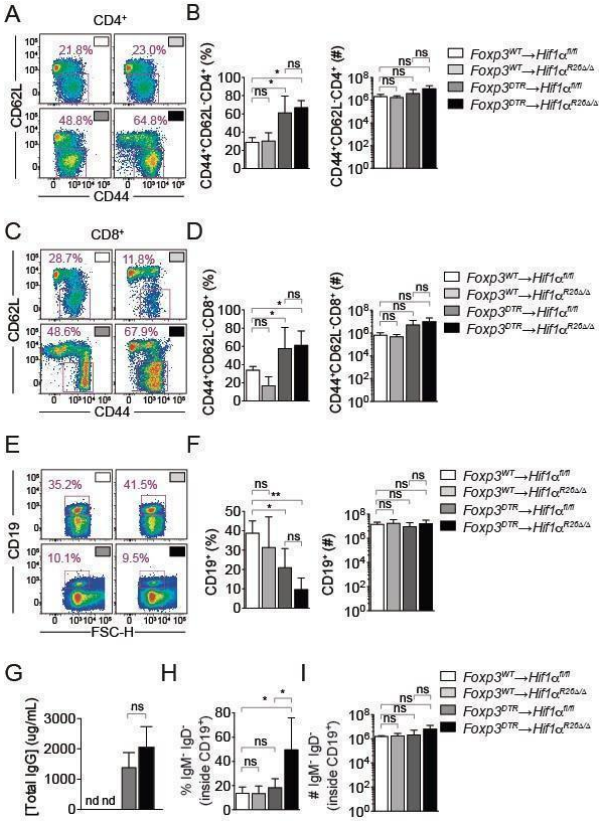

Figure 5.5. Impact of non-hematopoietic *Hif1α* tissue deletion on immune system activation

Flow cytometry analysis of splenic effector memory (CD62L^{low} and CD44^{high}) CD4$^+$ **(A, B)** and CD8$^+$ **(C, D)** T cells in chimeric mice♦, generated as described in Figure 5, 26 days after DT administration (Treg cell depletion). **(E-F)** Splenic B (CD19$^+$) cells in the same mice as (A-D). **(G)** Total serum concentration of immunoglobin G of the same mice as (A-D; n=3). **(H-I)** B cell class switching (IgM-IgD-CD19+ cells) analysis by flow cytometry on the same mice as (A-D). *p < 0.05, as determined by unpaired Student's t-test. Standard bars represent mean ± SD. ♦(Foxp3^{WT}→Hif1α$^{fl/fl}$ n=4; Foxp3^{WT}→Hif1α$^{R26Δ/Δ}$ n=2; Foxp3^{DTR}→Hif1α$^{fl/fl}$ n=5; Foxp3^{DTR}→Hif1α$^{R26Δ/Δ}$ n=7). Data from 2 independent experiments.

The levels of circulating IgG were also similar, irrespectively of *Hif1α* expression in non-hematopoietic cells (Fig.5.5G). We also found no differences in the number of splenic F4/80$^+$Ly6C$^+$ macrophages and CD11b$^+$Ly6G$^+$ neutrophils (Fig.5.7). Of notice, the number of splenic IgM$^-$IgD$^-$CD19$^+$ B cells was increased in *Foxp3^{DTR}→Hif1α$^{R26Δ/Δ}$* mice, in which *Hif1α* was deleted in parenchyma cells, compared to control *Foxp3^{DTR}→Hif1α$^{fl/fl}$* mice expressing physiologic levels of *Hif1α* in parenchyma cells (Fig.5.5H), suggesting for a role for parenchyma tissue *Hif1α* on the B cell activation.

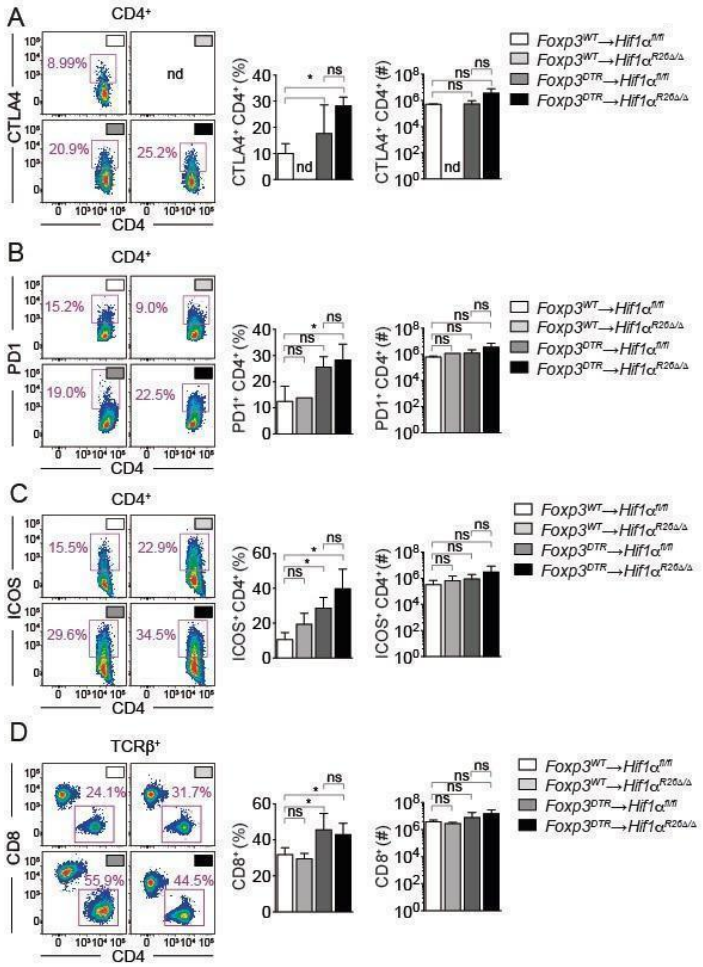

Figure 5.6. Non-hematopoietic *Hif1α* does not modulate the activation status of the immune system during autoimmunity development

Flow cytometry analysis of splenic CTLA4$^+$ CD4$^+$ **(A)**, PD1$^+$ CD4$^+$ **(B)**, ICOS$^+$ CD4$^+$ **(C)** and CD8$^+$ **(D)** cells on day 26, with corresponding graphical analysis of percentage and cell numbers of four chimeric groups$^\blacklozenge$. *$p < 0.05$, as determined by ANOVA test and Tukey's multiple comparison test. Standard bars represent mean ± SD. $^\blacklozenge$(*Foxp3*^{WT}→*Hif1α*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hif1α*$^{R26Δ/Δ}$ n=2; *Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$ n=7; *Foxp3*^{DTR}→*Hif1α*$^{R26Δ/Δ}$ n=7). Data from 2 independent experiments.

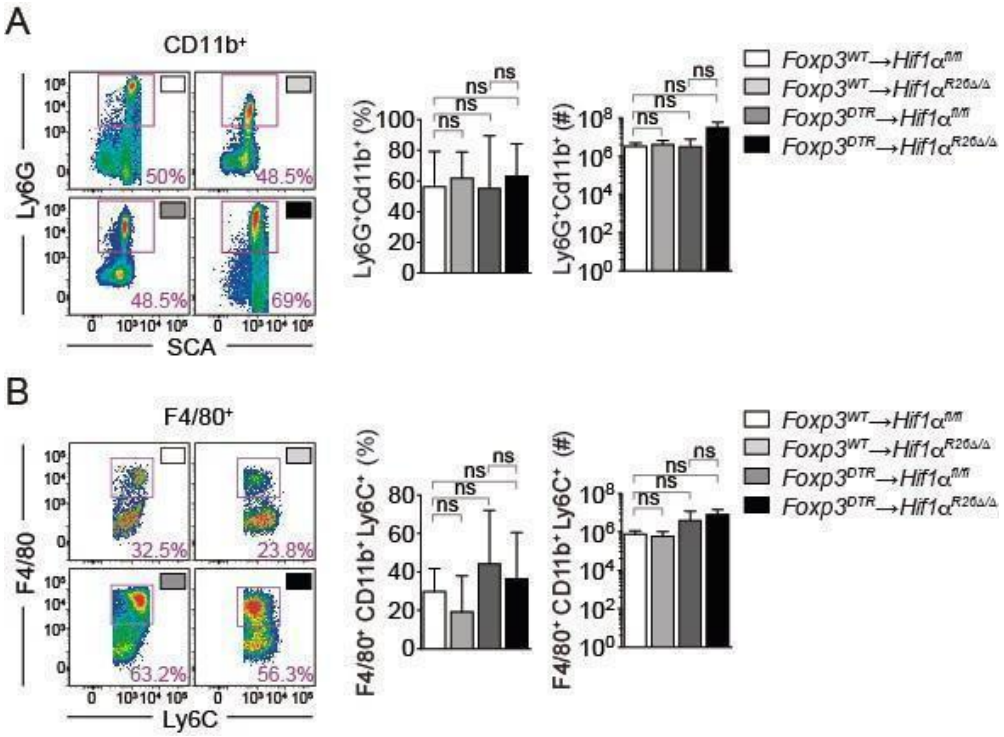

Figure 5.7. Non-hematopoietic *Hif1α* does not modulate the activation status of the innate immune system during autoimmunity development

Representative flow cytometry plots and graphical analysis of neutrophils (Ly6G$^+$ CD11b$^+$) **(A)** and macrophages (F4/80$^+$ CD11$^+$ Ly6C$^+$) **(B)** of four chimeric groups$^\blacklozenge$ on day 26. *p < 0.05, as determined by ANOVA test and Tukey's multiple comparison test. Standard bars represent mean ± SD. ns, not significant. $^\blacklozenge$(*Foxp3*^{WT}→*Hif1α*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hif1α*$^{R26\Delta/\Delta}$ n=2; *Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$ n=7; *Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$ n=7). Data from 2 independent experiments.

To test functionally the effect exerted by parenchyma *Hif1α* expression on the pathogenic effect of autoimmune cells, we transferred splenocytes from different chimeric mice into naïve *Rag2*$^{-/-}$ mice (Fig.5.8A). Transfer of splenocytes from chimeric *Foxp3*^{DTR}→ *Hif1α*$^{fl/fl}$ mice was lethal, in contrast to transfer of splenocytes from chimeric *Foxp3*^{DTR}→ *Hif1α*$^{R26\Delta/\Delta}$ mice, that was not lethal to *Rag2*$^{-/-}$ mice (Fig.5.8A). Transfer of splenocytes from chimeric *Foxp3*^{WT}→*Hif1α*$^{R26\Delta/\Delta}$ or *Foxp3*^{WT}→*Hif1α*$^{fl/fl}$ mice was not lethal to *Rag2*$^{-/-}$ mice (Fig.5.8A). This suggests that the expression of *Hif1α* in parenchyma cells promotes the pathogenesis and progression of autoimmune disease, via a mechanism that somehow amplifies functionally the pathogenic effect of immune cells.

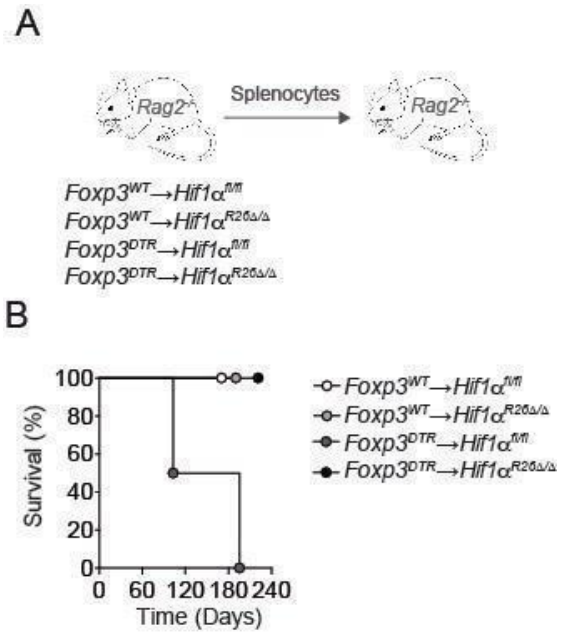

Figure 5.8. Impact of non-hematopoietic Hif1α tissue deletion on immune system function

(A) Schematic representation of the adoptive transfer of splenocytes from mice described in (4.2.A) into naïve *Rag2* deficient recipients. Note that donor chimeric mice received Diphtheria toxin (DT) for two weeks (2W) to deplete T_{REG} cells before the adoptive transfer of splenic cells on day 26. (**B**) Survival of *Rag2*$^{-/-}$ after splenocytes adoptive transfer, as previously described; (*Foxp3*^{WT}→*Hif1α*$^{fl/fl}$ n=1; *Foxp3*^{WT}→*Hif1α*$^{R26\Delta/\Delta}$ n=1; *Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$ n=2; *Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$ n=3).

5.3. Discussion

Calor (heat), *dolour* (pain), *rubor* (redness), *tumour* (swelling) and *functio laesa* (loss of function) are the five hallmarks of inflammation. Three of these are associated with enhanced blood flow and vascular permeability, likely related to O_2 distribution in inflamed tissues. This suggests that hypoxia and inflammation coexist and cross-regulate each other, such as observed during the inflammatory reactions underlying autoimmune diseases [83]. This idea is supported at a molecular level by the overlap in target genes controlled by the two key transcription factors regulating hypoxia and inflammation, namely, HIF1α and NF-κB, respectively [84].

Inflammation-induced hypoxia creates a microenvironment that affects immune, endothelial and parenchymal cells in different tissues. As discussed above (see 5.1.2.3.), HIF1α acts in a cell autonomous manner in immune cells to shape innate and adaptive immunity, while its activation in parenchymal cells and endothelial cells triggers the release of alarmins that can act in a non-cell autonomous manner to modulate immune cell activation [85,86]. This occurs via a number of mechanisms including for example the induction of β2 integrin expression by leukocytes, which

promotes leukocyte adhesion to vascular endothelial cells and extravasation [85]. In response to hypoxia, endothelial cells release prostaglandins and induce the expression of cell adhesion molecules such as P-selectin, while releasing platelet-activating factor (PAF) that promotes platelet aggregation [86]. Moreover, hypoxia disrupts the anticoagulant function of endothelial cells through a decrease of thrombomodulin expression, eventually comprising endothelial barrier integrity and heightened expression of pro-inflammatory cytokines, while promoting angiogenesis[86].

Our findings highlight a fundamental role of HIF1α expression by non-immune parenchyma cells, including most likely endothelial cells, in the pathogenesis and outcome of autoimmune diseases. Presumably HIF1α acts together with other transcription factors, such as NF-κB, to regulate the expression of chemokines, selectins and integrins as well as matrix metalloproteinases and cathepsins, according to the anatomic context and growth factor milieu generated by tissue injury.

In other words, activation of HIF1α in non-hematopoietic cells may confer a predisposal for autoimmune damage. In support of this notion, deletion of *Hif1a* in the non-hematopoietic cell compartment of chimeric mice was associated with a reduction of leukocyte interstitial infiltration. Presumably HIF-1α regulates immune cell trafficking, justifying differences in immune infiltrates and tissue damage. In keeping with this interpretation, HIF1α regulates the expression of integrins, selectins and chemokines, involved in immune-vascular cell adhesion [85,87,88] as it occurs in inflammatory processes. These function of HIF1α in parenchyma cells, including in vascular endothelial cells, may justify the differences observed, where chimeric mice that lack *Hif1α* show less immune cell interstitial infiltration, probably due to an impaired recruitment function in the endothelium lacking *Hif1α*. In keeping with our findings, other laboratories have also shown that tissue specific differences in vascular endothelial cell function might impact on the dynamics and profile of vascular interaction with leukocytes, likely explaining the organ-specific increase in susceptibility exerted by *Hif1α* expression in parenchyma cells. When considering that Hif1α might act in parenchyma cells to regulate leukocyte transmigration across the vascular endothelium, these effects may be overviewed in a broader context to also explain organ-specific immunopathology in response to infections.

The organ-specific differences in immunopathology may also be explained by differences in the extent of *Hif1α* deletion in chimeric *Foxp3*^{DTR}→*Hif1*$^{\alpha R26\Delta/\Delta}$ *vs.*

Foxp3^{DTR}→*Hif1α*$^{fl/fl}$ mice, as illustrated in the pancreas. As an alternative explanation *Hif2α* might compensate for *Hif1α* deletion in a manner that would be organ specific[89]. If that is the case, we may find these disparities of organ damage by virtue of an intrinsic activation of these two HIF isoforms that may act coincidently or in different time-frame over the time course of the disease [90].

Perhaps not surprisingly, we did not find overt differences in immune activation when *Hif1α* was deleted in parenchyma cells. Notwithstanding, there was a higher number of splenic IgM$^-$IgD$^-$CD19$^+$ B cells, presumably corresponding to B cells that underwent class switch, when *Hif1α* was deleted in parenchyma cells, without interfering, however, with antibody production. This contrasts with the finding that *Hif1α* expression by B cells reduces class-switching [66]. While our findings suggest that there is a cross talk between non-hematopoietic cells and B cells regulating B cell class switching, this should be confirmed in other experimental models.

The observation that *Rag2*$^{-/-}$ hosts receiving splenocytes from chimeric *Foxp3*^{DTR}→*Hif1α*$^{R26\Delta/\Delta}$ donors survive while those receiving splenocytes from chimeric *Foxp3*^{DTR}→*Hif1α*$^{fl/fl}$ donors succumbed suggests that the non-hematopoietic expression of *Hif1α*, probably by tissue resident leukocytes, might account for this effect [87,91,92]. Nevertheless, given the small number of mice analysed, this remains to be confirmed.

In this chapter we tested whether modulating cellular adaptation to hypoxia regulates tissue damage and modulates the pathogenesis of autoimmune diseases. We found that the development of autoimmune disease upon T_{REG} cell deletion was more severe when *Hif1α* was expressed at physiologic levels in parenchyma cells. Autoimmune pathology was exacerbated especially in the pancreas, liver, stomach, skin and muscle. In conclusion, *Hif1α* promotes cell survival skills, conferring adaptive mechanisms that rescue cells from stress-induced by hypoxia or other stimuli. Its involvement in autoimmune diseases has long been in question, and recent findings narrowed our curiosity by implying a role in the immune system. We went beyond and found that this factor, by its pro-inflammatory role, also modulates systemic autoimmunity. Additional studies are still lacking to address a role for *Hif1α* tissue specificity in autoimmunity disease pathogenesis.

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Chapter 6

Contribution of the Heat Shock Response To Tissue Damage Control in Autoimmune Disease

Abstract

The heat shock factor 1 (HSF1) is the transcriptional master regulator of the heat shock response (HSR)[1], a damage response that limits the deleterious effects and sustains cellular homeostasis in response to proteotoxicity [2]. In this Chapter we asked whether the transcriptional regulation of the HSR by HSF1 in parenchyma cells impacts on the pathogenesis and/or progression of autoimmune disease. We found that *Hsf1* deletion in non-hematopoietic cells promoted a more severe form of autoimmune cardiac damage elicited upon T_{REG} cell depletion. The expression of *Hsf1* in parenchyma cells did not interfere directly with autoimmune activation nor did it interfere with the ability of autoreactive B and/or T cells to prompt autoimmune disease, when adoptively transferred to *Rag2*$^{-/-}$ mouse. These findings reveal that regulation of the HSR in parenchyma cells confers tissue damage control and contributes to establish disease tolerance to autoimmunity.

6.1. Introduction

6.1.1. Heat Shock Response: When the heat hits the cell function

Under acute and chronic challenges, cells, tissues, organs and organisms ensure the conformational integrity and function of their proteome which is crucial for cellular and organismal homeostasis. Cells rely on proteostasis for protein quality control and maintain proteome homeostasis via a network of integrated sensors of protein structure-function relationships, regulating protein translation, chaperone assisted folding and degradation [3].

Different forms of stress can be associated with varying levels of protein misfolding and consequently with the formation and accumulation of aggregates made of misfolded proteins [4]. This is countered by protein chaperones that connect with, sustain or help other proteins to (re)acquire their functional active conformation, while restraining themselves from the final structure. These molecular chaperones can also promote protein translocation to cellular organelles and target damaged proteins for degradation by the proteasome or autophagy apparatus.

Heat-shock proteins (HSPs) are amongst the best-described molecular chaperones, upregulated under stress conditions associated with misfolding of mature proteins, as originally described upon exposure of cells to heat. These protein chaperones were classified originally according to their molecular weight as HSP40, 60, 70, 90, 100 and smaller HSPs [5].

Under homeostatic conditions, HSPs assist folding of nascent polypeptide chains and prevent their aggregation. The expression of HSP is induced in response to different forms of abiotic stress (*i.e.,* heat, ultraviolet radiation or heavy metals), physiologic stress (*i.e.,* growth factors or cell differentiation) and biotic stress (*i.e.,* infection). This is essential to support various proteome-maintenance functions, such as *de novo* protein folding, refolding of stress-denatured proteins, oligomeric assembly, trafficking and assistance in proteolytic degradation [5].

6.1.2. Heat shock factors: More than an adaptation to heat

The HSR encompasses a wide range of functions. This response is mediated at the transcription level by simultaneous binding of multiple heat shock factors (HSF) to inverted pentameric nGAAn - heat shock elements (HSE) in the regulatory region of HSP genes [6].

HSF are a family of transcription regulators, which consist of seven proteins: HSF1, 2, 3, 4, 5, HSFX and HSFY. These have distinct but overlapping functions, tissue-specific expression patterns and multiple posttranslational modifications. All have been described in humans, except for HSF3 that was only identified in mice [1].

6.1.2.1. HSF1: molecular mechanism under heat shock and normal conditions

HSF1 is the transcriptional master regulator responsible for the induction of genes encoding HSP and maintaining cellular integrity in response to heat as well as different forms of stress, as demonstrated using different model organisms [2,7]. In the absence of stress, HSF1 is constitutively expressed but kept inactive by its association with HSPs. Under stress conditions, HSF1 reshapes its 3D structure to form a trimer, coordinately regulated through multiple post-translational modifications. This conversion of HSF1 from an inactive monomeric to a high HSE affinity trimmer is the first step in the activation of the HSR shared by most, if not all, eukaryotes[1]. Briefly, monomeric HSF1 dissociates from Hsp90 to produce the trimeric form of HSF1, which can remain inactive when its regulatory domain persists bound to a chaperone complex formed by Hsp90, co-chaperone 23 and immunophilin FK506-binding protein[1]. HSF1 activity is also inhibited by Hsp70 and Hsp40, which prevent HSF1 trimerization [2]. Other regulatory chaperonins such as the cytosolic chaperonin TCP1 ring complex, valosin-containing protein and histone deacetylase 6 were more recently thought to support the formation of restraining heterocomplexes that control HSF1 activity [1]. This negative feedback enforced by the end products of HSF1-dependent transcription grants an essential regulatory step in adapting the intensity and duration of the HSR [8]. This activation-attenuation cycle also depends on posttranslational modifications of HSF1, including acetylation, phosphorylation and sumoylation. The last two occur rapidly in response to heat shock, whereas the kinetics of acetylation are delayed and go along with the attenuation phase of the HSF1 activation cycle [9].

6.1.2.2. Heat shock proteins and tissue specificity

While ubiquitously expressed, HSPs exhibit some level of tissue specificity. For instance, small HSPs are particularly abundant in the heart, exerting important roles in cardiac protein homeostasis and function [10]. As part of the HRS pathway, HSPs play a crucial role in normal conditions and an important protective role during

pathological processes. Induction of the HSR in the myocardium is cardioprotective [11] with HSP70 promoting post-ischemic myocardial recovery and reducing infarct size [4].

Growth and differentiation of the eye lens requires *Hsf*4 in combination with *Hsf*1 for maintenance of sensory organs [4].

Furthermore, Tanguay et al. examined several tissues of young and adult mice and found, while ubiquitously expressed, stress-inducible HSP such as HSP71 and HSP25 were in higher levels in tissues exposed to toxic environmental or metabolic products such as stomach, intestine, colon and bladder [12]. Altogether, HSPs tissue-restricted expression suggests potential specialized properties of specific members of these proteins.

6.1.2.3. Heat shock proteins can act as alarmins

HSPs can be released extracellularly in response to different forms of stress associated with infection and/or cellular damage. Extracellular HSPs are sensed by PRR, such Toll-like receptor (TLR)-4, -2 but also other PRR such as CD14, CD91, CD94, Lectin-type oxidise low lipoprotein receptor (LOX)-1, SR-A [5]. Sensing of extracellular HSP via PRR is thought to contribute to inform the immune system on the occurrence of cellular damage, similar to other alarmins [5,13].

6.1.2.4. Heat shock proteins regulate adaptive immunity

Upon proteasomal degradation of proteins, the generated peptides can be loaded into MHC I molecules in antigen presenting cells and be cross presented to the adaptive immune system. HSP90 has been associated with the proteasome and take over generated peptides [14]. Afterwards, it associates with HSP70 and takes the antigen to the endoplasmic reticulum through protein transporter associated with antigen processing (TAP) reproducing the standard trafficking into MHCI complex loading [14]. Moreover, HSP90 cellular distribution and trafficking to the extracellular milieu as a signal for the activation of dendritic cells contributes to the regulation of the adaptive immunity by triggering adaptive immunity against pathogens as well autoantigens [15].

We are discussing about proteins that evolved from prokaryotic to eukaryotic organisms and which are acknowledged as self by our defences. How can such broad and conserved proteins be distinguished by the adaptive immune system? Conservation of sequences prompts the conservation of immunogenic epitopes,

recognized by T cells and triggering immune response. Presumably, this justifies why patients with autoimmune diseases such as ankylosing spondylitis have antibodies that recognize a *Drosophila* antigen or why rheumatoid arthritis patients have higher titers of IgG antibodies against mycobacterial HSPs [16]. Cross-reactivity may arise from these commonalities between host and pathogen HSPs and precipitate autoimmune disease.

In animal models of arthritis or non-obese diabetic models, immunization against mycobacterial HSP60 is protective against autoimmune disease [16]. While self-HSPs can exert immunoregulatory effects by inhibiting TNF-α through the activation of type 2 immunity (*i.e.,* IL-4 and IL-10), this regulatory effect was not observed in response to non-self HSPs isolated from *Mycobacterium tuberculosis* [17]. This suggests that in an acute stress context HSPs prime the adaptive immune responses, acting as a danger signal to prepare against a subsequent challenge.

6.1.2.5. Heat shock factors gene expression

HSF1 regulates a broad array of genes in stressed cells and leads flexible physiological processes also in non-stressed settings. Along with some of the previously mentioned functions, HSF1 drives a transcription program which includes genes involved in translation (mitochondrial ribosomal protein *Mrpl18* and fragile X mental retardation *Fxr1*), cell cycle and signalling (CDC28 protein kinase *Cks1B* and cell division cycle *Cdc6*), protein folding and stress response (*Serpin1* and *Hsp90*), energy metabolism (phosphoglycerate kinase *Pgk1*), adhesion and extracellular matrix (integrin beta *Itgb3*), immune process (signal transducer and activator of transcription *Stat6 and* interleukin Il7) or DNA repair and chromatin remodelling (chromobox *Cbx3,* chromodomain helicase DNA binding protein *Chd3 and Rad51* recombinase) [28].

While the expression of several HSP is increased in autoimmune diseases [20] whether HSP regulate the pathogenesis and/or outcome of autoimmune diseases is not established. In this Chapter we tested the hypothesis that the master regulator of the HSP network, HSF1, acts in parenchyma cells to provide tissue damage control and establish disease tolerance to autoimmune diseases.

6.2. Results

To determine whether *Hsf1* regulates the pathogenesis, progression and/or outcome of systemic autoimmune disease, we generated *Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$ mice, allowing for tissue specific and inducible *Hsf1* loss of function in mice lacking adaptive immunity (Fig.6.1A). Briefly, Tamoxifen-inducible expression of the Cre^{ERT2} recombinase, under the control of the *Rosa 26* (*R26*) promoter, removes *LoxP* site-flaked exons 2-4, from the *Hsf1*$^{fl/fl}$ allele (Fig.6.1B). The resulting *Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$ and control *Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ genotypes were identified by PCR (Fig.6.1C-E). Tamoxifen-induced deletion of the *Hsf1*$^{fl/fl}$ allele *in Rag2*$^{-/-}$*Hsf1*$^{R26\Delta/\Delta}$ was confirmed by qRT-PCR in several organs, as compared to control *Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ mice expressing physiologic levels of Hsf1 (Fig.6.1F).

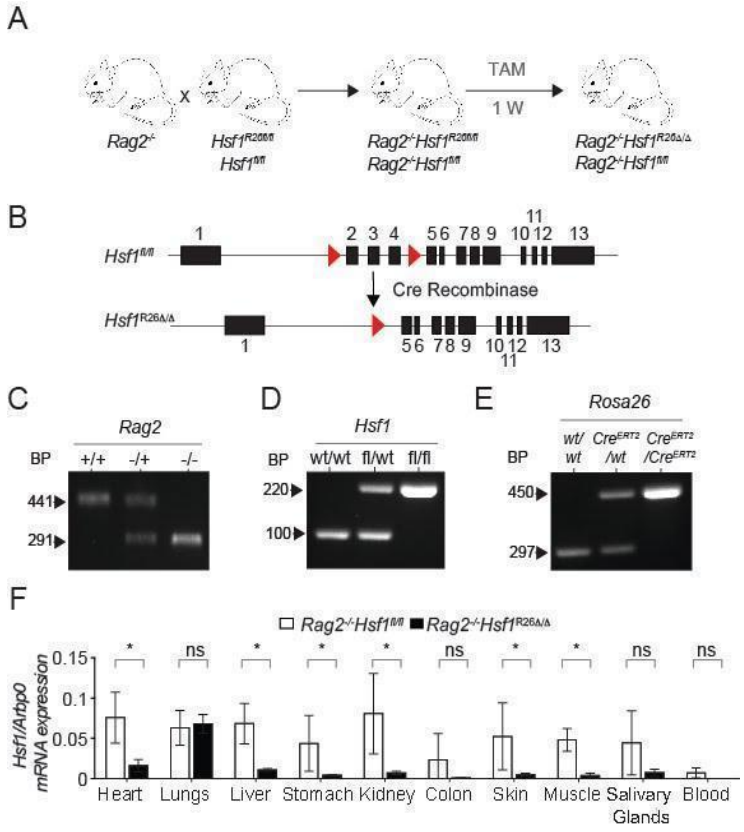

Figure 6.1. *Hsf1* deletion in Rag2 deficient mice

(A) Schematic representation of the breeding strategy used to generate Rag2 deficient (*Rag2*$^{-/-}$) mice, in which the transcription factor heat shock factor 1 (*Hsf1*) is deleted in an inducible manner, under the control of the Rosa26 (R26) promoter. Deletion of the *Hsf1*$^{fl/fl}$ allele is achieved by Tamoxifen (TAM) administration during one week (W), as described in Methods. **(B)** Schematic representation of the two LoxP sites (red triangles), flanking exons 2 and 4 (black rectangles) in the *Hsf1*$^{fl/fl}$ allele [15]. Deletion of exons 2-4 in the *Hsf1*$^{fl/fl}$ allele is achieved by TAM-driven induction of CreERT2 recombinase activity. **(C-E)** Electrophoresis of PCR products amplified from genomic DNA of mice carrying: C) wild type (+/+), heterozygous (+/-) or homozygous (-/-) Rag2 alleles, D) wild type (wt/wt), heterozygous (wt/fl) or homozygous (fl/fl) Hsf1 alleles and E) wild type (wt/wt), heterozygous (CreERT2) or homozygous (CreERT2/CreERT2) allele inserted in the Rosa26 locus. **(F)** Quantification of *Hsf1* mRNA expression by qRT-PCR in different organs from mice receiving TAM, as indicated in (A). Data represented as mean ± SD (n=3/group) in one experiment. P values determined by unpaired Student's t test. * p< 0.05. ns, not significant (p>0.05). BP: base pairs.

Initially, we attempted to reveal an *Hsf1* activation exploiting an *in vivo Hsf1* downstream gene reporter (Fig.6.2). Amid the time of follow-up, we did not detect differences between sick animals and controls (Fig.6.2D-E) which may be as a result of a limited activation time not caught or a non-activation at all. Nonetheless, we cannot conclude that *Hsf1* isn't recruited in the context of an immune mediated inflammation caused by an imbalance in regulatory:effector T cells.

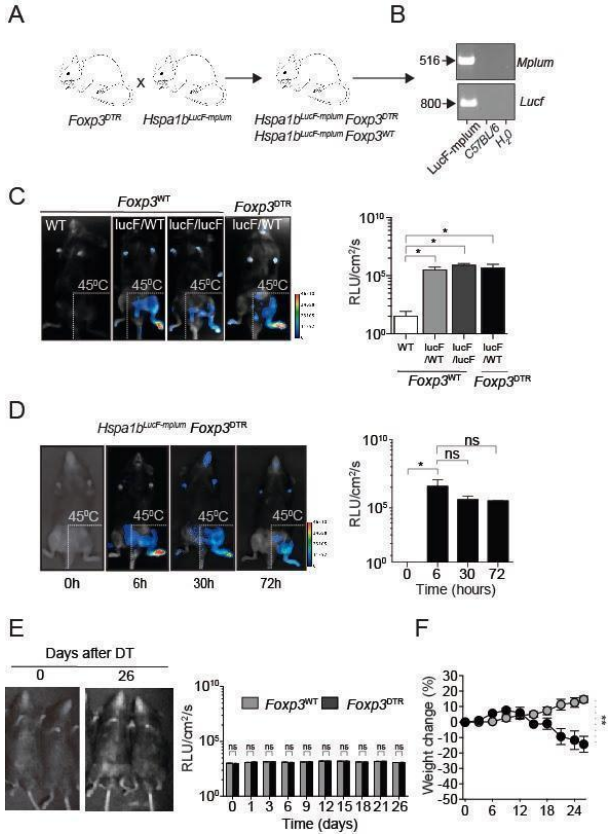

Figure 6.2. Luciferase *Hsf1* reporter mice do not report expression of Hsf1 during autoimmunity devolvement

(A) Generation of a bioluminescent and immunofluorescent *Hsf1* reporter (*Hspa1*$^{\text{lucF-mplum}}$) mouse with depletable regulatory T cells (*Foxp3*$^{\text{DTR}}$). **(B)** Electrophoresis of PCR products amplified from genomic DNA of mice, generated as depicted in (A) and carrying a *mplum* and *lucF* allele. **(C)** Bioluminescence representative pictures and quantification of luciferase activity after 45ºC-water left lower limb bathing for 8 minutes (exposure 8s; n=4) in mice carrying a *lucF* homozygous (*Hsf1*$^{\text{lucF/lucF}}$) and heterozygous (*Hspa1b*$^{\text{lucF/WT}}$) allele. **(D)** *Hsf1 Foxp3*$^{\text{DTR}}$ mice reporter (Hspa1$^{\text{lucF-mplum}}$Foxp3$^{\text{DTR}}$) representative pictures of bioluminescence kinetic and quantification after 45ºC-water left lower limb bathing for 8 minutes (exposure 8s; n=4) at 0h, 6h, 30h and 72h. **(E-F)** *Hsf1* reporter mice weight change (F), bioluminescence pictures and quantification (E) after regulatory T cell depletion upon diphtheria toxin administration (DT) every three days for 2 weeks (exposure 8s; *Hsf1*$^{\text{luc/WT}}$ *Foxp3*$^{\text{WT}}$ n=3 and *Hsf1*$^{\text{luc/WT}}$ *Foxp3*$^{\text{DTR}}$ n=4 per time point). *p < 0.05 determined by ANOVA test and unpaired Student's t test for (E). ns, not significant (p>0.05). Standard bars represent mean ± SD. Data from 2 independent experiments.

To delete the *Hsf1*$^{fl/fl}$ allele specifically in non-hematopoietic cells, we generated bone marrow chimeric mice (Fig.6.3A). Briefly, bone marrows from *Foxp3*^{DTR} mice were adoptively transferred into non-lethally irradiated *Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$

(*Foxp3*^{DTR}→*Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$) or control *Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ (*Foxp3*^{DTR}→*Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$) mice (Fig.6.3A). Upon tamoxifen administration, the *Hsf1*$^{fl/fl}$ allele is deleted specifically in the non-hematopoietic cell compartment of the chimeric mice. Using *Foxp3*^{DTR} mice as bone marrow donors allows for specific depletion of T_{REG} cells upon DT administration. Chimeric mice receiving bone marrows from *Foxp3*^{WT} and expressing physiologic numbers of T_{REG} cells were used as controls (*i.e.,* *Foxp3*^{WT}→*Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ and *Foxp3*^{WT}→*Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$ mice). This complex experimental system is required to test experimentally the putative protective effect exerted by *Hsf1*, specifically in parenchyma cells, and assess in this manner its impact in the pathogenesis, progression and/or outcome of systemic autoimmune disease, as triggered upon T_{REG} cell depletion. Similar results, as previously mention in Chapter 3, regarding weight loss, temperature drop, survival decrease and hypoglycemia were observed in these chimeric groups, regardless of *Hsf1* expression (Fig.6.3B-E).

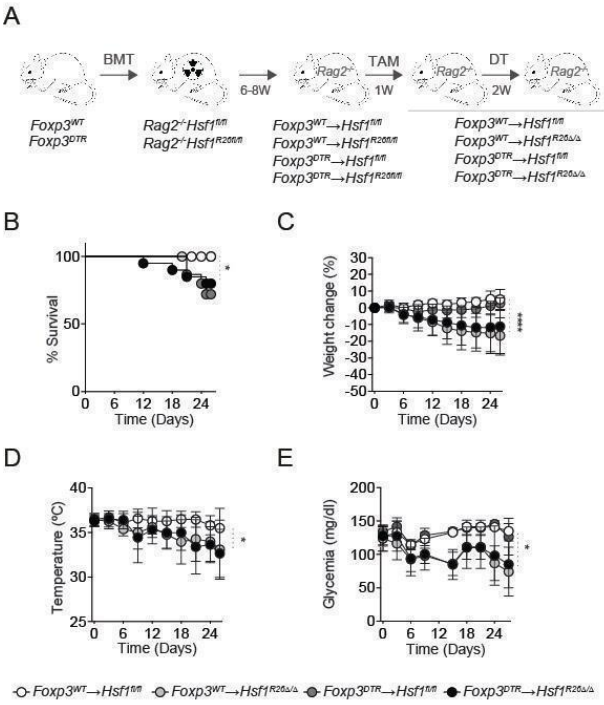

Figure 6.3. Upon regulatory T cell depletion, *Hsf1* deletion does not interfere in vital parameters outcome

(**A**) Schematic representation of the strategy used to generate bone marrow chimeric mice in which the *Hsf1*$^{fl/fl}$ allele is deleted specifically in parenchyma and connective (non-hematopoietic) tissues, but not in hematopoietic cells. Briefly, non-lethally irradiated *Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ or *Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$ mice received bone marrow transplants (BMT) from wt (*Foxp3*^{WT}) mice or from *Foxp3*^{DTR} mice bearing a diphtheria toxin receptor (DTR) specifically on regulatory T cells. After 6-8 weeks (W) chimeric **CONTINUED Fig. 6.3.** (BMT→Recipients) mice received Tamoxifen (TAM) for one week (1W) to delete the *Hsf1*$^{fl/fl}$ allele, specifically in non-hematopoietic tissues (*R26*Δ/Δ). Diphtheria toxin (DT) was administered for 2 weeks (2W) to achieve T_{REG} cell depletion. (**B-E)** Survival (A), Weight change (B), rectal temperature (D) and glycemia of chimeric mice as described in (A) during autoimmune disease development: *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ n=11; *Foxp3*^{WT}→*Hsf1*$^{R26Δ/Δ}$ n=7; *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ n=18;

Foxp3^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ n=23. *p < 0.05 determined by unpaired Student's t test. ns, not significant (p>0.05). Standard bars represent mean ± SD. Data from 4 independent experiments.

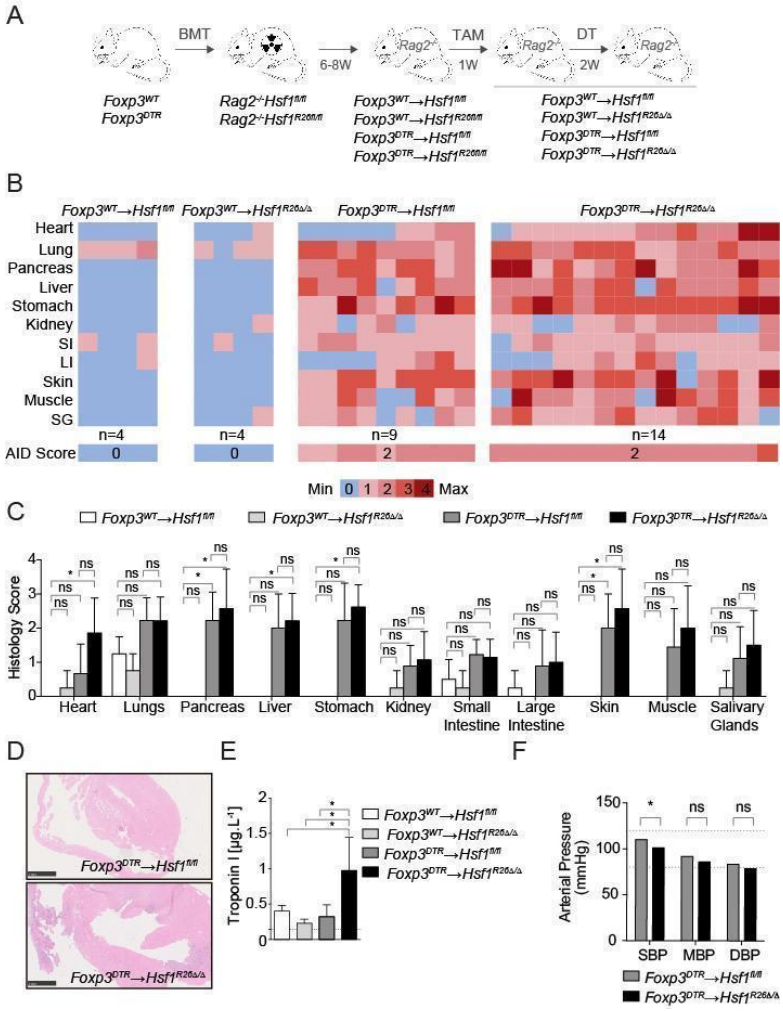

Figure 6.4. *Hsf1* deletion in non-hematopoietic tissues promotes autoimmune cardiac damage

(**A**) Schematic representation of the strategy used to generate bone marrow chimeric mice in which the *Hsf1*$^{fl/f}$ – allele is deleted specifically in parenchyma and connective (non-hematopoietic) tissues, but not in hematopoietic cells. Briefly, non-lethally irradiated *Rag2*$^{-/-}$*Hsf1*$^{fl/fl}$ or *Rag2*$^{-/-}$*Hsf1*$^{R26fl/fl}$ mice received bone marrow transplants (BMT) from wt (*Foxp3*^{WT}) mice or from *Foxp3*^{DTR} mice bearing a diphtheria toxin receptor (DTR) specifically on regulatory T cells. After 6-8 weeks (W) chimeric (BMT→Recipients) mice received Tamoxifen (TAM) for one week (1W) to delete the *Hsf1*$^{fl/fl}$ allele, specifically in non-hematopoietic tissues (R26Δ/Δ). Diphtheria toxin (DT) was administered for 2 weeks (2W) to achieve Treg cell depletion. **(B)** Autoimmune Disease (AID) score in organs from the mice described in (A), obtained from histological analyses, 26 days after the first bolus of DT administration. Each horizontal line represents an organ and each column an individual mouse. Mean AID scores are illustrated for each genotype in the bottom horizontal lines. SI: small intestine, LI: large intestine, SG: salivary glands. **(C)** Relative quantification of AID scores *per* organ *per* genotype, obtained as indicated in (B) and represented as mean ± SD. p values determined by bootstrapping resampling method. *p < 0.05. **(D)** Representative H&E staining of the heart from chimeric mice, generated as described in (A). Scale bar=1mm. **(E)** Troponin concentration in the serum of chimeric mice, generated as described in (A). Data is represented as mean ± SD for the following chimeric mice: *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ (n=2), *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ (n=2), *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ (n=3), *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ (n=3). *p < 0.05 determined by unpaired Student's t test. **(F)** Arterial pressure represented as mean ± SD in the same mice as (E): *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ (n=5) and *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ (n=5) mice. Systolic blood pressure (SBP), Mean blood pressure (MBP), Diastolic blood pressure (DBP) *p<0.05 determined by unpaired Student's t test. ns, not significant (p>0.05). Data from 4 independent experiments.

Immunopathology scores were compared in different chimeric mice 26 days after T_{REG} cells depletion (Fig.6.3B,6.5). We found that deletion of *Hsf1* in non-hematopoietic cells from chimeric *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ mice was associated with marked increase in cardiac leukocyte infiltration and myocardial damage, not observed in control *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ mice in which T_{REG} were not deleted and *Hsf1* was expressed at physiologic levels (Fig.6.4B,D). Moreover, leukocyte infiltration and myocardial damage were also not observed when T_{REG} cells were depleted in *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ mice expressing physiologic levels of Hsf1 in parenchyma cells, when compared to control *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ mice (Fig.6.4.B). As expected *Hsf1* deletion in parenchyma tissues of *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ mice, was not sufficient *per se* to elicit cardiac leukocyte infiltration and damage (Fig.6.4B,C). This suggests that autoimmune cardiac damage occurs only when two events concur, namely, impaired peripheral immune tolerance (*i.e.* T_{REG} cells depletion) and failure to activate a stress responsive transcriptional program in parenchyma cells of the heart (*Hsf1*$^{R26\Delta/\Delta}$).

We then asked whether cardiac muscle tissue injury induced by leukocyte infiltration was associated with the accumulation of a well-established highly specific and sensitive biomarker of cardiac damage – cardiac troponin I[16]. To this aim we measured the levels of cardiac troponin I in plasma 26 days after the first administration of DT (Fig.6.4E). We found that chimeric *Foxp3*^{DTR}→ *Hsf1*$^{R26\Delta/\Delta}$ mice had higher levels of cardiac troponin I in blood when compared to chimeric *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ or *Foxp3*^{WT}→ *Hsf1*$^{R26\Delta/\Delta}$ controls. This suggests that upon T_{REG} cell depletion the development of autoimmune cardiac injury occurs only when *Hsf1* is deleted in parenchyma cells.

Furthermore, we asked whether the development of cardiac damage was associated with cardiac dysfunction, as assessed by measuring peripheral blood pressure. We found that systolic blood pressure (SBP) was compromised in chimeric *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ mice, when compared to chimeric *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ controls. This was not associated with a reduction of heart rate. Systolic blood pressure (SBP) is the result of the heart contraction, heart rate and peripheral vessel resistance, suggesting that the reduction of SBP in *Foxp3*^{DTR}→ *Hsf1*$^{R26\Delta/\Delta}$ mice is not due to a defect in signal conduction for heart contraction but at the heart muscle itself, translating in an impaired contraction and consequently a lower SBP (Fig.6.4F). Both cardiac troponin release and lower SBP results allude to a role of *Hsf1* in autoimmune cardiac damage, conducting a normal function upon T_{REG} imbalance.

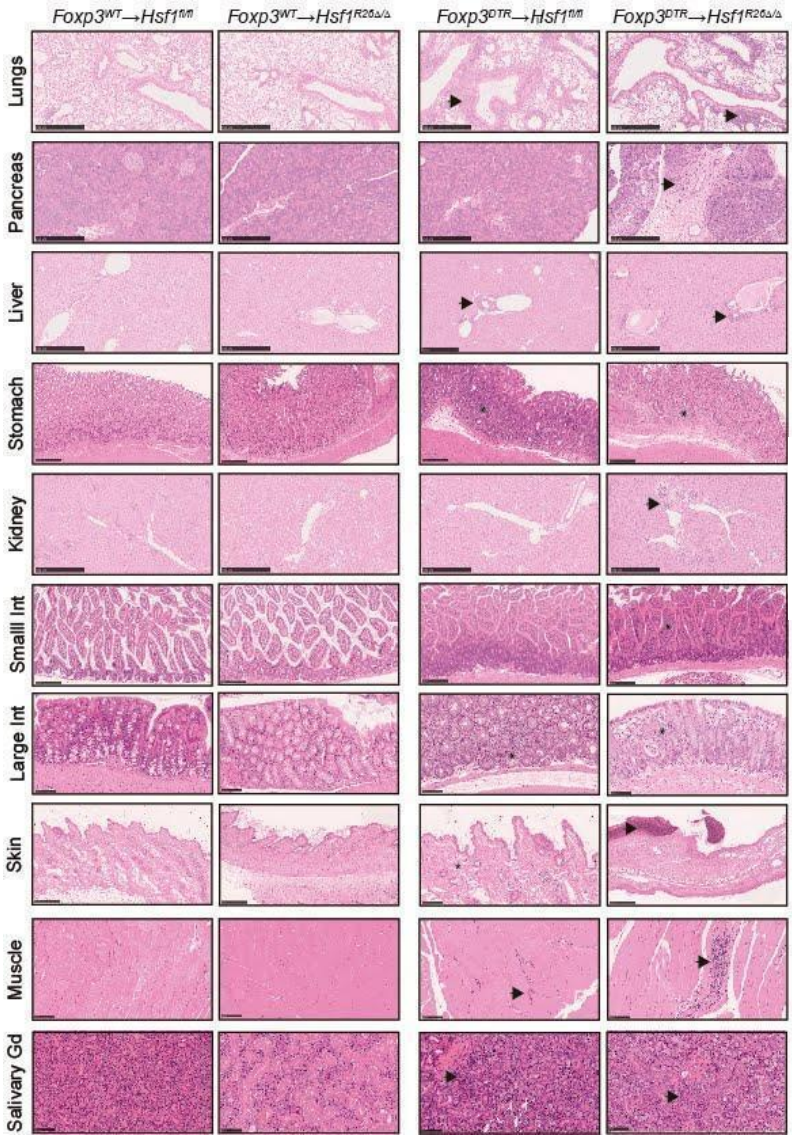

Figure 6.5. *Hsf1* deletion impacts on the severity of organ targeting

H&E pictures representative of different organs from each chimeric group of mice on day 26 after begin diphtheria toxin administration: *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ n=9; *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ n=14. Lungs from *Foxp3*^{WT} and *Foxp3*^{DTR} chimeras present mild perivascular and peribronchial inflammation (arrow). *Hsf1* deleted chimeras, which were reconstituted by *Foxp3*^{DTR} bone marrow show in the pancreas, moderate inflammation associated with extensive acinar atrophy (arrow). In the liver, *Foxp3*^{DTR} chimeras disclose mild portal to lobular inflammation (arrow). The gastric mucosa presents mild to moderate inflammation in *Foxp3*^{DTR} reconstituted chimeras. The kidney displays a minimal leukocyte infiltration (arrow) in *Hsf1* deleted and T_{REG} depleted chimeras. Small and large intestines (Int) present a mild inflammation in the mucosa and submucosa. Chimeras lacking *Hsf1* and T_{REG} unveil moderate dermal inflammation with diffuse infiltration of lymphocytes, plasma cells and neutrophils covered by serocellular crusts (arrow). On the other hand, in the presence of *Hsf1*, *Foxp3*^{DTR} chimeric mice show only mild skin inflammation with perivascular infiltration of lymphocytes, plasma cells and neutrophils. Muscle and salivary glands (Gd) are mildly infiltrated by inflammatory cells (arrows) in T_{REG} depleted chimeras.

We then asked whether non-hematopoietic *Hsf1* expression modulates systemic (auto)immune activation driven by T_{REG} cell depletion. We found no differences in the expression of $CD4^+$ and $CD8^+$ T cell activation markers (*i.e.,* $CD44^{hi}CD62^{lo}$) nor the proportion of $CD19^+$ B cells in the spleen of chimeric *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ mice, when compared to control chimeric *Foxp3*^{DTR}→*Hsf1*$^{fl//fl}$ or *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ mice (Fig.6.6A-F).

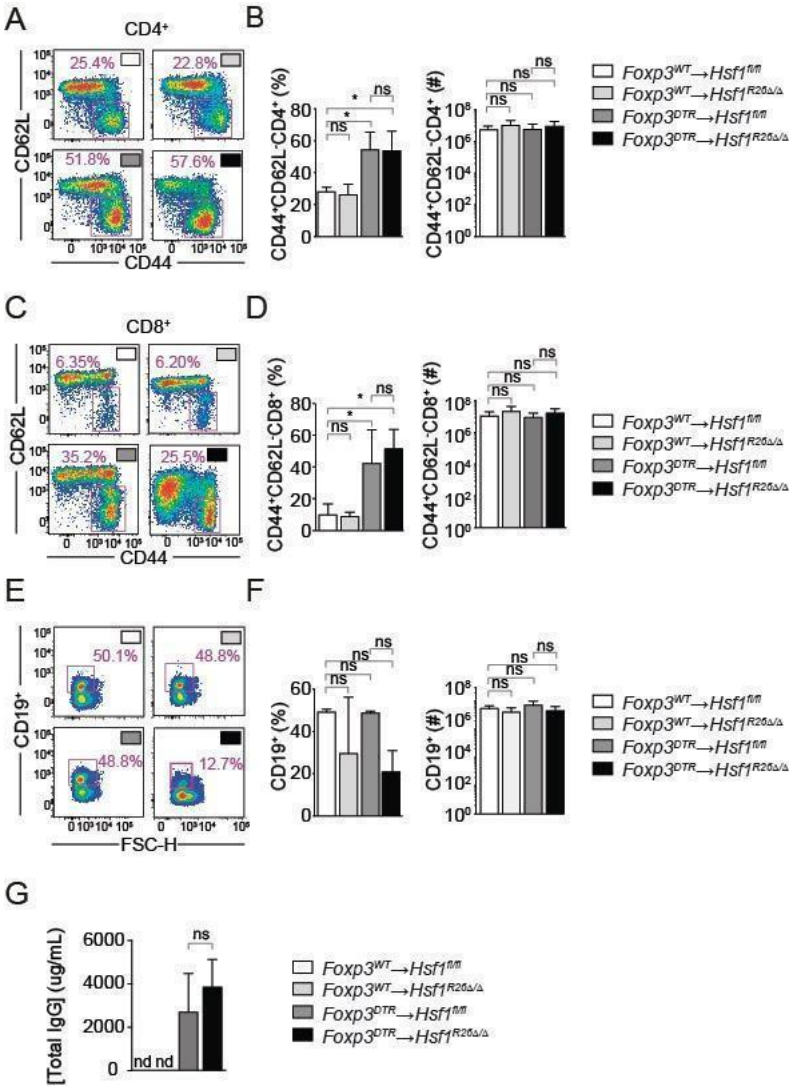

Figure 6.6. Impact of non-hematopoietic *Hsf1* deletion on immune activation

Splenic effector memory ($CD62L^{low}$ and $CD44^{high}$) $CD4^+$ (**A, B**) and $CD8^+$ (**C, D**) T cells in chimeric mice•, generated as described in Figure 6.2, analysed by flow cytometry, 26 days after DT administration (T_{REG} cell depletion). (**E-F**) Splenic B ($CD19^+$) cells, in the same mice as (A-D). (**G**) Concentration of circulating IgG of the same mice as (A-D; n=3). *p < 0.05 determined by ANOVA test. ns, not significant (p>0.05). Standard bars represent mean ± SD. •(*Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ n=6). Data from 2 independent experiments.

Levels of circulating IgG were also similar in chimeric *Foxp3*^{DTR}→ *Hsf1*$^{R26\Delta/\Delta}$ mice *vs.* control chimeric *Foxp3*^{DTR}→*Hsf1*$^{fl//fl}$ or *Foxp3*^{WT}→ *Hsf1*$^{R26\Delta/\Delta}$ mice (Fig.6.6G). This was consistent with the number of Tfh cells and expression of the Tfh activation marker ICOS being induced to a similar extent in chimeric *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ mice, *vs.* control chimeric mice (Fig.6.7C-D).

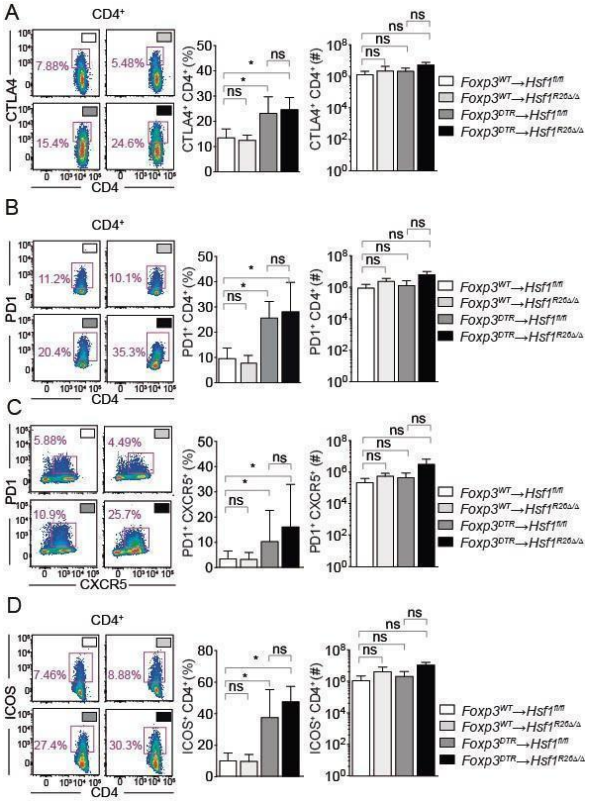

Figure 6.7. Non-hematopoietic *Hsf1* does not modulate the activation status of the immune system during autoimmunity development

Splenic CTLA4$^+$ CD4$^+$ (A), PD1$^+$ CD4$^+$ (B), ICOS$^+$ CD4$^+$ (D) and PD1$^+$ CXCR5$^+$ (C) cells analysed by flow cytometry from mice as describe in 6.4A: *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ n=6. *p < 0.05 determined by ANOVA and Holm-Sidak's multiple comparison tests. ns, not significant (p>0.05). Standard bars represent mean ± SD. Data from 2 independent experiments.

The percentage of PD1$^+$ and CTLA4$^+$ expressing cells was also increased to a similar extent in the spleen of chimeric *Foxp3*^{DTR}→ *Hsf1*$^{R26\Delta/\Delta}$ mice *vs.* control chimeric mice (Fig.6.7A-B). There were also no detectable differences on the number of splenic neutrophils (Fig.6.8A). We then conclude that non-hematopoietic *Hsf1* deletion in non-hematopoietic tissues does not interfere with adaptive and innate immune activation in the context of T_{REG} cell depletion.

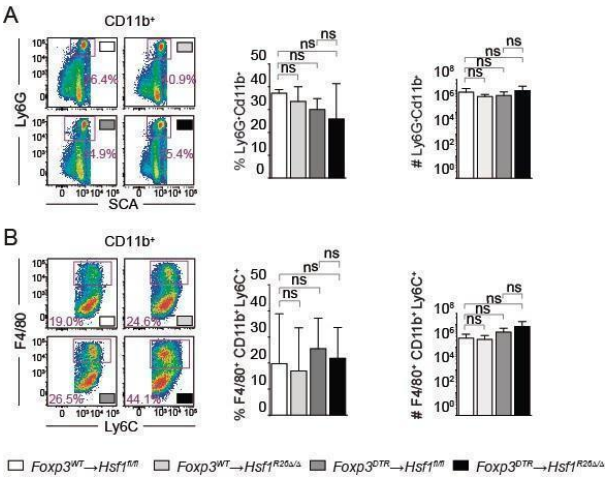

Figure 6.8. *Hsf1* deletion in non-hematopoietic tissues does not impact on innate immune activation

(A,B) Splenic neutrophils (Ly6G$^+$CD11b$^+$) (A) and macrophages (F4/80$^+$CD11$^+$Ly6C$^+$) (B) of chimeric mice as described in 6.4A: *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{WT}→*Hsf1*$^{R26Δ/Δ}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ n=4; *Foxp3*^{DTR}→*Hsf1*$^{R26Δ/Δ}$ n=6. *p < 0.05 determined by unpaired Student's t test. ns, not significant (p>0.05). Standard bars represent mean ± SD. nd, not determined. Data from 2 independent experiments.

We then compared the capacity to immune cells from chimeric *Foxp3*^{DTR}→*Hsf1*$^{R26Δ/Δ}$ mice *vs.* control chimeric mice to trigger autoimmunity upon adoptive transfer to *Rag2*$^{-/-}$ mice (Fig.6.9A). We found that *Rag2*$^{-/-}$ mice receiving splenocytes from *Foxp3*^{DTR}→*Hsf1*$^{R26Δ/Δ}$ were more likely to succumb from autoimmune disease (Fig.6.9B) when compared to control chimeric *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ mice. This suggest that autoimmune disease is transferable with a trend to more severe disease to develop when transferring cells from a chimeric mouse that lacks Hsf1 in parenchyma cells. This would imply that the expression of Hsf1 and induction of a HSR in parenchyma cells does modulate the development of autoimmunity, via a mechanism that is not clear. Overall, these observations suggest that when expressed in non-hematopoietic cells the induction of the HSR by *Hsf1* confers tissue damage control in the heart, preventing autoimmune cardiac dysfunction. This protective effect is exerted via mechanism that regulates somehow the activation of innate or adaptive immunity.

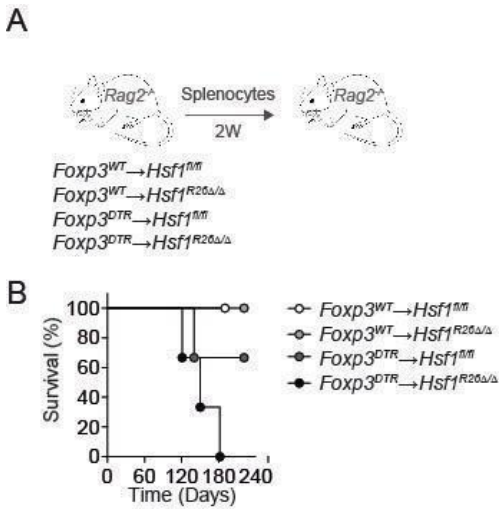

Figure 6.9. Impact of non-hematopoietic Hsf1 deletion on immune function.

(**A**) Schematic representation of adoptive transfer of splenocytes from mice described in (6.2.A) into naïve Rag2 deficient recipients. Note that donor chimeric mice received Diphtheria toxin (DT) for 2 weeks (2W) to deplete T_{REG} cells, before adoptive transfer of splenic cells. (**B**) Survival of *Rag2*$^{-/-}$ after splenocytes adoptive transfer, as previously described; *Foxp3*^{WT}→*Hsf1*$^{fl/fl}$ n=1; *Foxp3*^{WT}→*Hsf1*$^{R26\Delta/\Delta}$ n=1; *Foxp3*^{DTR}→*Hsf1*$^{fl/fl}$ n=3; *Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$ n=3.

6.3. Discussion

The transcriptional response to proteotoxic stress is controlled by several HSFs, with a preeminent role for HSF1 [3]. Initially discovered as a transcription factor that upregulates genes encoding HSPs, HSF1 also regulates the expression of several "other" genes controlling diverse stress induced processes, such as autophagy, cell growth arrest, chromatin remodelling, endoplasmic reticulum unfolded protein response and ubiquitin-proteasome system or programmed cell death by apoptosis [21]. This broad functional diversity of HSF1 led us to explore whether this transcription factor regulates tissue damage control responses that supress the pathogenesis of and progression of autoimmune diseases.

Immunopathology is catalysed by non-resolving inflammation, underlying the development of the clinical manifestations of autoimmune diseases. We reasoned that the transcriptional program regulated by HSF1 might act as a tissue damage control mechanism against inflammatory stress inflicted upon cells in tissues and organs targeted by autoimmune responses. In support of this hypothesis several HSPs regulated by HSF1 are cytoprotective [1]. Moreover, HSPs also act as alarmins to regulate inflammation, in some cases through the action of HSP-specific T_{REG} cells [22].

We found that the expression of *Hsf1* in non-hematopoietic (*i.e.,* parenchyma) cells of a mouse protects the heart from autoreactive immune damage (Fig.6.4B-F).

This protective effect was associated with a marked reduction of leukocyte extravascular infiltration in the heart (Fig.6.4D) as well as with cardiomyocyte cytoprotection, revealed by lower accumulation of troponin I in plasma (Fig.6.4E). Moreover, we have shown that *Hsf1* expression in parenchyma cells is also protective against cardiac dysfunction, as assessed by arterial pressure measurement (Fig.6.4F). The mechanism via which *Hsf1* exerts these protective effects is not yet clear.

Cardiomyocyte contraction and hence cardiac function relies on mitochondrial energy production to fuel the well-coordinated contractile apparatus of cardiomyocytes. These structures rely on the correct folding and assembly of sarcomeric transmembrane proteins that exchange ATP and Ca^{2+}, supporting cardiomyocyte contraction. As reviewed by Christians et al. several genetic conditions associated with protein miss folding are linked to cardiac diseases [23], such as long QT syndrome 2, polyglutamine related cardiac diseases and cardiomyopathy caused by desmin, actin or myosin mutations [10].

Our findings are consistent with the notion that the protective effect of *Hsf1* being exerted via the induction of the expression of one or several small 12-43kDa HSPs [10]. In support of this hypothesis, small HSPs are expressed preferentially in the heart where they counter the development of cardiac dysfunction [23], as illustrated for HSP5 (CryAB) and HSP7 (CVHSP) [10,23]. Moreover, these and other chaperone proteins such as HSP22 can regulate cell signalling by signal transducers and activators of transcription (STAT)s [11], including STAT1 and STAT3, which are cytoprotective in cardiomyocytes during ischemia and reperfusion injury [24]. These chaperones have been proposed to promote optimal STAT activity by serving a scaffolding molecule, stabilizing STAT proteins in their active, phosphorylated configuration [25]. Of note, the HSP22 chaperone is upregulated via HSF1 during cardiac stress, sustaining cardiomyocyte mitochondrial function [26] and protecting the myocardium from undergoing post-ischemic or H_2O_2-progremmed cells death via a mechanism associated with STAT signalling [17].

The STAT family of transcription factors is activated downstream of cytokine receptors, through phosphorylation by Janus kinases (JAKs) and Mithogen Activated Protein (MAP) kinase families on conserved tyrosine and serine residues on their C-terminal domains, respectively [18]. Interferon-γ (IFN-γ) is a potent activator of STAT1, while interleukin-6 (IL6) activates STAT3 [18]. IFN-γ induces Hsp70 and Hsp90

transcription via a mechanism that acts on a HSE in the promoter of these genes and relies on STAT1 interaction with HSF1 [8]. This is antagonized by STAT3 activation in response to IL-6, without interacting directly with HSF1 [19].

It is possible therefore that HSF1 and STAT act in a concerted manner to support cardiomyocyte function and limit the extent of cardiac dysfunction imposed by autoimmunity. This idea is further supported by the finding that pro-inflammatory cytokines that play a central role in the pathogenesis of autoimmune disease such as IFN-γ, inducing the formation of a STAT1/HSF1 complex that binds to DNA HSE to promote the transcription and expression of several cytoprotective genes [8]. Moreover, interaction of STAT3 with HSF1 also increases STAT3 activity and protects cardiomyocytes from undergoing programmed cell death by apoptosis [24]. Whether interaction of HSF1 with one or several STAT family members underlies the protective effect of HSF1 against autoimmune-driven cardiac dysfunction remains however to be established.

Regarding organs such as pancreas, stomach and liver, as previously reported (Fig.6.4B), were expectedly targeted, although not independently of *Hsf1* presence. These results imply that *Hsf1* has no role in preventing disease development in these organs under an aseptic context. The same principle shouldn't be applied for other organs such as the lungs, colon or salivary glands. The preliminary deletion results showed that these organs aren't fully deleted (Fig.6.1F). Indeed, the lack of deletion in these organs may be due to a highly variation of *Hsf1* expression portrayed by the large standard deviation intervals. The same reasoning can't be applied to the lungs. In this organ, we foresee that *Hsf1 locus* might not be accessible to Cre recombinase, preventing DNA excision.

Different tissue targeting between $Hsf1^{R26\Delta/\Delta}$ vs $Hsf1^{fl/fl}$ could be justified by differences in the systemic immune activation. However, this has not been the case (Fig.6.6). We do report activation of $CD4^+$, $CD8^+$, B cell response (total IgG), similar innate immune cells amounts (neutrophils and activated macrophages) and down-regulation of the immune system towards a new self-tolerance equilibrium (increased of $PD1^+$ and $CTLA4^+$ $CD4^+$ cells). Following Roudier et al [27] identification of Hsp70, a downregulated protein of Hsf1, in alleles carrying susceptibility for autoimmune diseases to be the ligand of a highly homologous amino acid motif in the third hypervariable region of the MHC DRß1 chain, we could have expected differences in the immune system activation due to compromised antigen presentation. Hsp70

participates in the intracellular trafficking and peptide association of these DRß1 chains in humans [27], consequently, one may consider that a compromise of Hsp70 expression could influence the immunologic processes. Since *Hsf1* and its downstream transcripts, including Hsp70, are involved in protein folding, hence, also in MHC–peptide assembly, their expression might influence processing and presentation of non-hematopoietic antigen presenting cells, possibly leading to a quantitatively and qualitatively altered antigenic repertoire. However, more studies have to be done to ascertain it.

In addition, finally we asked whether lacking *Hsf1* in non-hematopoietic cells would result in a worse outcome upon disease transfer to an empty $Rag2^{-/-}$ mouse (Fig.6.9). We found that mice grafted from sick *Hsf1* non-hematopoietic deleted donors (*Foxp3*^{DTR}→*Hsf1*$^{R26\Delta/\Delta}$) were more likely to die (2 out of 3 adoptive transfers). The small numbers of mice do not allow us to take a conclusion, however, this result brought forward the notion that the grafted immune system was originally activated and diverse enough to lead to a fatal event.

In conclusion, we found that *Hsf1* deletion in non-hematopoietic cells promoted a more severe form of autoimmune cardiac damage elicited upon T_{REG} cell depletion. The expression of *Hsf1* in parenchyma cells did not interfere directly with autoimmune activation nor did it interfere with the ability of autoreactive B and/or T cells to prompt autoimmune disease, when adoptively transferred to *Rag2*$^{-/-}$ mouse. These findings reveal that regulation of the HSR in parenchyma cells confers tissue damage control and contributes to establish disease tolerance to autoimmunity.

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Chapter 7

Contribution of the DNA Damage Response To Tissue Damage Control in Autoimmune Diseases

Abstract

DNA damage can arise from exposure of cells to a variety of exogenous forms of stress, with DNA double-strand breaks (DSB) being the most cytotoxic form of DNA damage. Once sensed, DNA damage elicits a complex signal transduction network known as the DNA damage response (DDR) [1]. While the ataxia-telangiectasia mutated (ATM) kinase is a central player in the orchestration of DDR, its putative role, if any, in the pathogenesis and progression of systemic autoimmune diseases remains unknown. In this Chapter, we tested the hypothesis that regulation of the DDR by the expression of ATM in parenchyma cells contributes to tissue damage control and limits the pathogenesis of an autoimmune disease. To test this hypothesis, we generated a chimeric mouse model allowing for inducible *Atm* deletion in parenchyma cells. We found that *Atm* deletion in non-hematopoietic cells was associated with a more severe autoimmune pancreatic inflammation caused by T_{REG} cell depletion. This was characterized by atrophy and destruction of exocrine pancreatic tissue without affecting Langerhans cells. This is likely explained by the high turnover rate of exocrine pancreatic cells [2] whereby autoimmune inflammation promotes DNA replication errors. Our findings support the notion that ATM contributes to protect exocrine pancreatic tissue cells during autoimmune injury, suggesting that polymorphisms in *ATM* may regulate susceptibility to autoimmune disease.

7.1. Introduction

7.1.1. DNA damage response.

On a daily basis, each cell of an organism is subjected to exogenous forms of stress, such as caused by exposure to ultra-violet (UV) light or chemical mutagens, which pose a threat to DNA integrity and can impair normal cellular function. The same is true for endogenous stimuli, such as metabolic genotoxins, recombination and replication defects. DNA double-strand breaks (DSB) arising from cell exogenous (*i.e.,* ionising radiation or chemical) or endogenous (*i.e.,* ROS) are probably the most cytotoxic forms of DNA damage, interfering with cell division and DNA replication [3].

Sensing of DNA damage by sensor proteins, such as the ones forming the MRN complex, recruit and activate transducer kinases to elicit a complex DNA damage response (DDR) damage [1]. The effector response consists in repairing DNA using different strategies, depending on the type of DNA lesion. Moreover, DNA damage signalling checkpoints provide the time for repair, slow down or arrest cell cycle progression through the inhibition of cyclin–CDK complexes [1].

The activation of the DDR is essential to sustain genomic stability and prevent the accumulation and propagation of DNA damage to the progeny. Failure of the DDR to repair DNA is coupled with signal transduction pathways inducing programmed cell death by apoptosis. Alternatively, cells carrying unresolved DNA lesions undergo a process of senescence or progress to genomic instability and carcinogenesis [1].

7.1.2. The Ataxia-telangiectasia mutated – a DNA damage response

In the last decades of the 20th century, the ataxia-telangiectasia mutated (*ATM*) gene was identified as a key regulator of the pathophysiology of ataxia-telangiectasia disease [4]. ATM is a member of a large phosphoinositidyl 3-kinase-related protein kinase (PIKK) family. This family includes the mammalian target of rapamycin (mTOR)/FKBP-rapamycin-associated protein (mTOR/FRAP), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), A-T and Rad3-related protein (ATR) and ATX/SMG [5]. In response to DNA damage, these kinases induce cell cycle arrest and signalling DNA repair mechanisms according to the type of damage and phase of the cell cycle [6].

Repair of DSB occurs via a signal transduction pathway involving the ATM kinase. ATM induces DNA repair via three mechanisms: 1) non-homologous end joining

(NHEJ) for DNA-end protection, processing and bridging by 53BP1 protein and histone H2AX; 2) homologous recombination (HR) via Mre11-Rad50-NBS1 (MRN complex), C-terminal interacting protein (CtIP) and BRCA1 activation; and 3) alternative end-joining (alt-EJ) also through MRN complex [6,7].

7.1.2.1. ATM – a central transducer of double-strand breaks signalling and more

Under steady-state conditions ATM is expressed as an inactive dimer/tetramer, being activated by the MRN complex at DNA DSB [8]. Upon activation, ATM phosphorylates different components of the MRN complex, forming a positive feedback loop sustaining ATM activity and inducing different branches of the DDR [9]. Moreover, ATM activation coordinates DNA repair with arrest of cell division.

ATM activation in response to oxidative stress and/or DSBs is followed by the phosphorylation of a variety of proteins acting as cycle checkpoints, controlling the intra-S cycle checkpoint via SMC1, the G1-S cell-cycle checkpoint through the tumour suppressor protein p53 and the G2/M checkpoint via protein kinase Chk2 [3,5,10]. During the S phase and G2 checkpoint, ATM-dependent activation conduces to single stranded DNA (ssDNA) generation through DNA resection, allowing double-stranded DNA (dsDNA) end to ssDNA [11]. Numerous ubiquitin ligases are also targeted by ATM to orchestrate transcription and repair (e.g. RNF20-RNF40) [3].

ATM can promote inflammation through activation of the NF-κB pathway [12]. This occurs via a mechanism whereby ATM phosphorylates the NF-κB essential modulator (NEMO), resulting in its ubiquitylation and degradation by the 26S proteasome, therefore promoting NF-κB activation and sustaining inflammation [12]. ATM can also induce programmed cell death by apoptosis [3] in response to DNA lesions such as DSB, however, this cytotoxic pathway is kept in check by the activation of p53[3].

7.1.2.2. ATM role in adaptive immunity

Abnormal T and B lymphocyte numbers and inadequate antibody responses are often observed in ataxia-telangiectasia (A-T) patients, revealing an essential role for T and B cells development. In leading with this notion, both class switch recombination in B cells and V(D)J recombination in T and B cells are dependent on ATM [13].

7.1.2.3. ATM at the organismal level: tissue damage control

The critical physiologic relevance of ATM is perhaps best highlighted during the development of ataxia-telangiectasia disease in individuals carrying loss-of-function mutations in ATM result. These patients are prone to develop different types of tumours, neurodegenerative disease as well an array of autoimmune and inflammatory syndromes [14].

Cells in the skin, small intestine, pancreas, thyroid, liver, breast, ovary, neurons and hematopoietic system are particularly vulnerable to DNA damage, yet cells in other tissues, like the colon, are considerably more resistant [15]. Moreover, cells in different tissues are more or less prone to different types of DNA damage imposed by tissue-specific microenvironments [16]. Given that ATM is activated in response to DNA DSB, ATM physiologic function is expected to have a predominate role in cells from tissues where this type of DNA damage is predominant.

In summary, while ATM is a central player in the DDR its contribution to the development of autoimmune diseases remains unknown. In this Chapter, we tested the hypothesis that the expression of ATM in parenchyma cells contributes critically to support tissue damage control and regulate the pathogenesis and outcome of autoimmune disease.

7.2. Results

To establish whether *Atm* partakes in the pathogenesis, progression and/or outcome of systemic autoimmune disease, we generated *Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$ mice, allowing for inducible *Atm* loss of function in mice lacking adaptive immunity (Fig.7.1A). *Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$ and control *Rag2*$^{-/-}$*Atm*$^{fl/fl}$ genotypes were identified by PCR (Fig.7.1C-E). Tamoxifen-inducible expression of the Cre^{ERT2} recombinase, under the control of the *Rosa 26* (*R26*) promoter, removed *LoxP* site-flaked exons 57-58 from the *Atm*$^{fl/fl}$ allele of *Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$ mice (Fig.7.1B), as confirmed by qRT-PCR in several organs, from *Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$ *vs.* control *Rag2*$^{-/-}$*Atm*$^{fl/fl}$ mice expressing physiologic levels of *Atm* (Fig.7.1F).

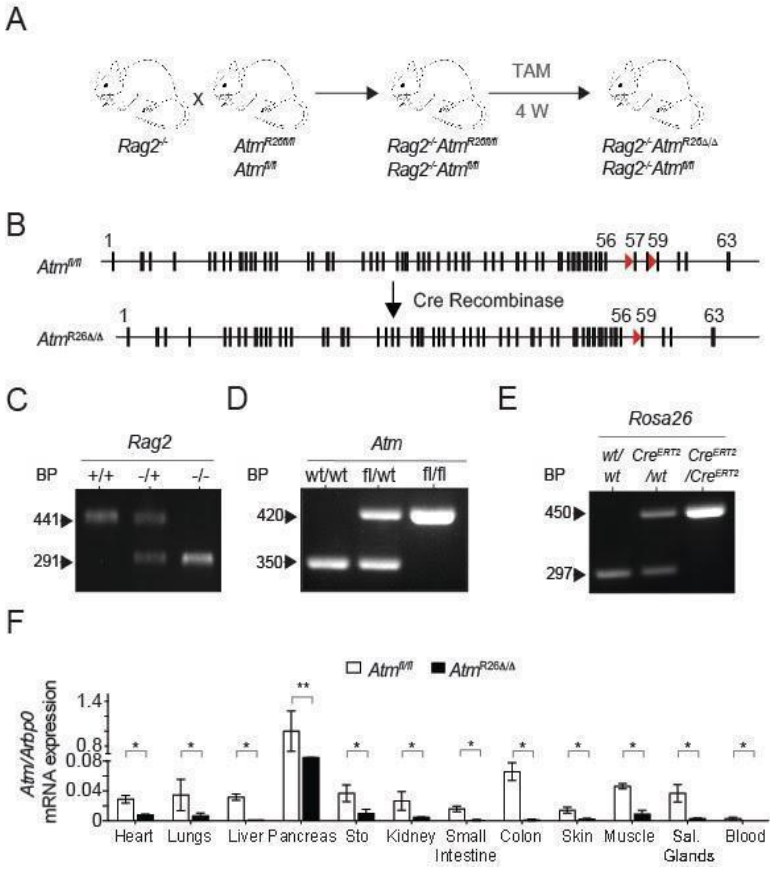

Figure 7.1. Atm deletion in Rag2 deficient mice

(A) Schematic representation of the strategy used to generate Rag2 deficient (*Rag2*$^{-/-}$) mice in which the transcription factor ataxia-telangiectasia mutated (*Atm*) is globally deleted, under the control of the *Rosa26* (R26) promoter, four weeks (W) after feeding on Tamoxifen (TAM). **(B)** Schematic representation of *Atm* locus containing two *LoxP* sites (red triangles) at the introns between exons 56 and 59 (black rectangles). Deletion of exons 57-58 is achieved by Cre recombinase activity. **(C-E)** Electrophoresis of PCR products amplified from genomic DNA of mice, generated as depicted in (A) and carrying: (C) a wild type (+/+), heterozygous (+/-) or homozygous *Rag2* allele, (D) a wild type (wt/wt), heterozygous (+/-) or homozygous *Atm* allele containing LoxP sites (fl), and (E) a wild type (wt/wt), heterozygous (Cre^{ERT2}) or homozygous (Cre^{ERT2}/Cre^{ERT2}) Cre^{ERT2} allele inserted in the *Rosa26* locus. **(F)** Quantification of *Atm* mRNA expression by qRT-PCR in different organs from mice receiving TAM, as indicated in (A). Data is represented as mean ± SD (n=3/group). p-value was determined by unpaired Student's t-test. *p< 0.05.

To delete the *Atm*$^{fl/fl}$ allele specifically in non-hematopoietic cells, we generated bone marrow chimeric mice (Fig.7.2A). Briefly, bone marrow cells from *Foxp3*^{DTR} mice were adoptively transferred into non-lethally irradiated *Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$ (*Foxp3*^{DTR}→*Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$) or control *Rag2*$^{-/-}$*Atm*$^{fl/fl}$ (*Foxp3*^{DTR}→*Rag2*$^{-/-}$*Atm*$^{fl/fl}$) mice (Fig.7.3A). Similar to described in Chapter 3, bone marrow cells from *Foxp3*^{DTR} mice were used to allow for depletion of T_{REG} cells in response to diphtheria toxin (DT) administration. As additional controls, we used chimeric *Foxp3*^{WT}→*Rag2*$^{-/-}$*Atm*$^{fl/fl}$ and *Foxp3*^{WT}→*Rag2*$^{-/-}$*Atm*$^{R26fl/fl}$ mice receiving bone marrow cells from *Foxp3*^{WT}, mice expressing physiologic numbers of T_{REG} cells. This experimental system allows testing the putative protective effect of ATM expression in parenchyma cells, against autoimmune disease triggered upon T_{REG} cell depletion.

Similarly to Chapter 3, T_{REG} cell depletion led to the development of overt systemic autoimmune disease, as assessed 26 days after the first administration of DT (Fig.7.2; Fig.7.3C; Fig.7.4).

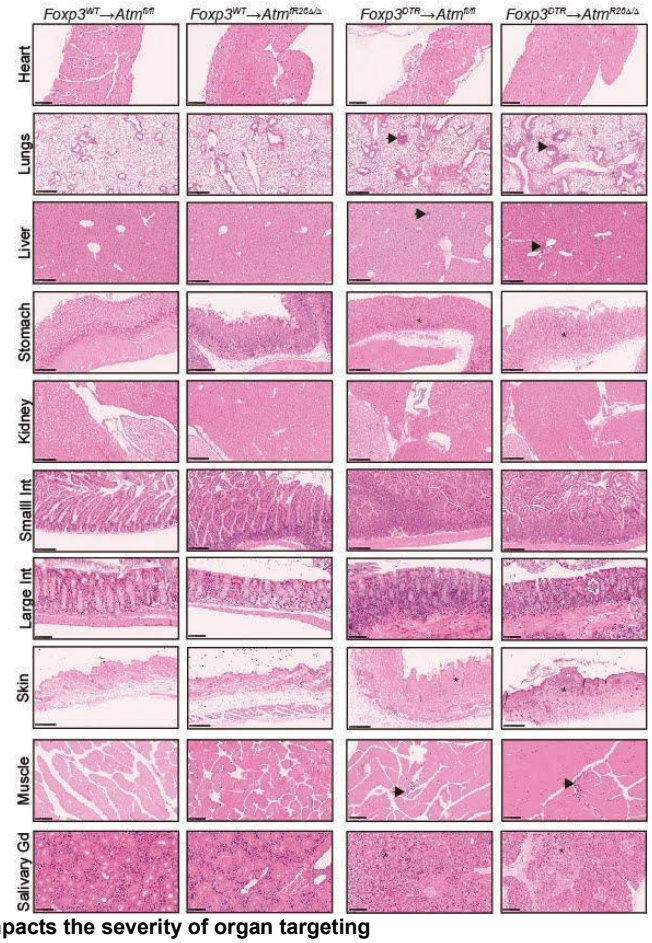

Figure 7.2. *Atm* deletion impacts the severity of organ targeting

H&E pictures representative of different organs from each chimeric group of mice on day 26 after diphtheria toxin administration began. (*Foxp3*^{WT}→*Atm*$^{fl/fl}$ n=4, *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ n=4, *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ n=10, *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ n=19) Scale bar = 500 µm Lungs from chimeric mice were very mild to mild infiltrated by mononuclear and polynuclear cells around the peribronchial (arrow) and perivascular areas. Liver of *Foxp3*^{DTR} chimeras (T_{REG} depleted) presents a mild portal to lobular inflammation (arrow). The forestomach mucosa, corpus and antrum are mildly inflamed with aggregates of mononuclear cells (*) in *Foxp3*^{DTR} chimeras compared with normal mucosa in *Foxp3*^{WT} chimeras. Similar results are shown for the heart, kidney, small intestine, muscle (arrow) and salivary glands (*) with scattered lymphocytes multifocally in T_{REG} depleted chimeras. The skin of the same chimeric mice depicts a mild inflammation with increased numbers of mononuclear and granulocytes multifocally dispersed.

Atm deletion in non-hematopoietic cells led to more severe pancreatic leukocyte infiltration and damage in chimeric *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ mice, as compared to control *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ mice expressing physiologic levels of Atm (Fig.7.3.B,C). As expected, *Atm* deletion in parenchyma cells of *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ mice was not sufficient *per se* to elicit pancreatic leukocyte infiltration and damage, similar to control *Foxp3*^{WT}→*Atm*$^{fl/fl}$ mice in which T_{REG} cells were not deleted and *Atm* was expressed at physiologic levels (Fig.7.3.B,C). Leucocyte infiltration in the lung, liver, stomach, skin, muscle and salivary glands was similar in *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ *vs.* control *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ mice (Fig.7.2).

The development of severe pancreatic inflammation in the chimeric Foxp3^{DTR}→*Atm*$^{R26\Delta/\Delta}$ mice was characterized by the destruction and atrophy of the exocrine pancreas, while preserving Langerhans islands (Fig.7.3.D). This suggests that the onset of autoimmune pancreatitis required two events to occur simultaneously, namely a dysregulation of peripheral immune tolerance (*i.e.* T_{REG} cell depletion) and failure to activate the DDR in parenchyma cells (*Atm*$^{R26\Delta/\Delta}$).

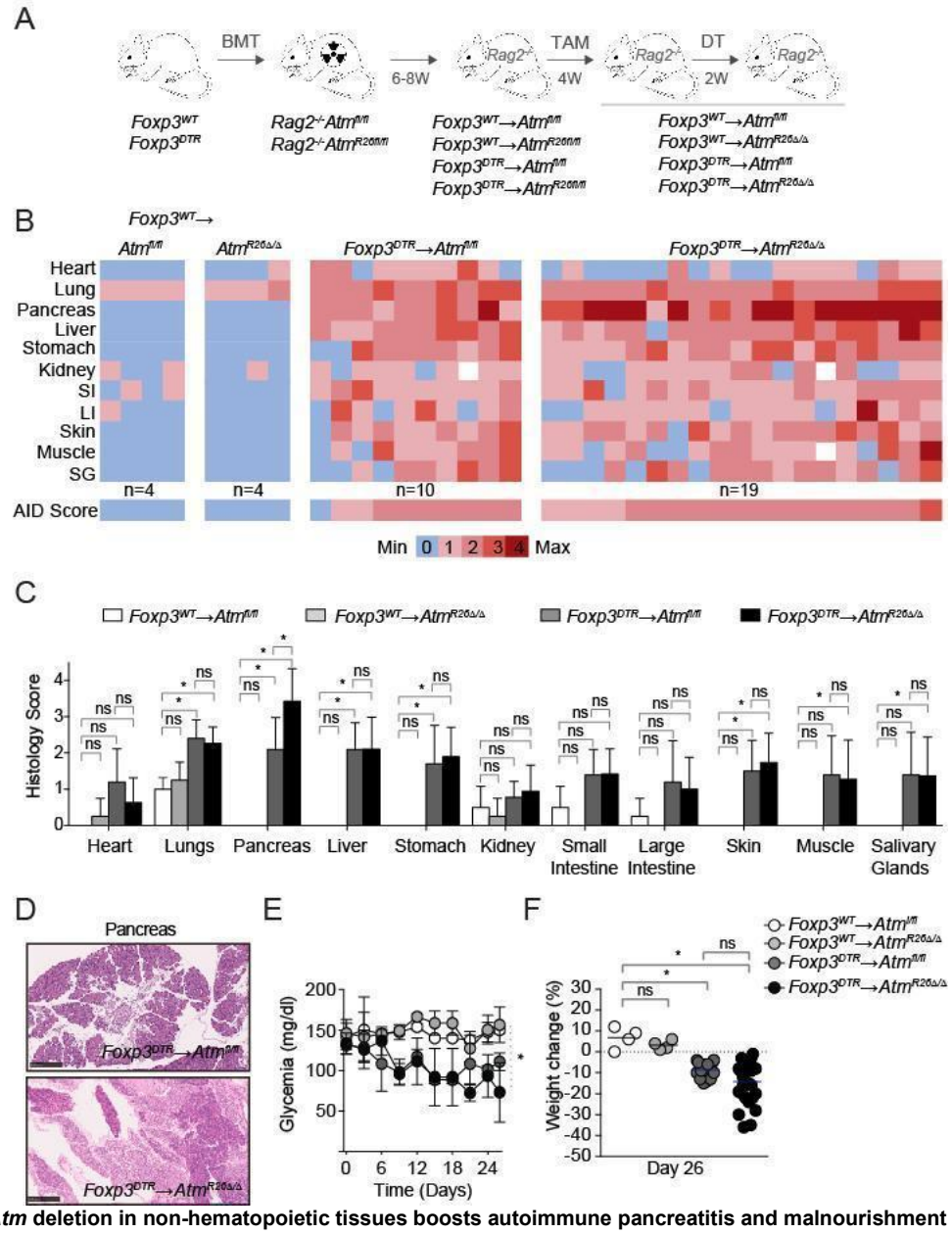

Figure 7.3. *Atm* deletion in non-hematopoietic tissues boosts autoimmune pancreatitis and malnourishment

(A) Schematic representation of the strategy used to generate chimeric mice in which the transcription factor ataxia-telangiectasia mutated (*Atm*) is globally deleted except in the hematopoietic tissues. Non-lethally irradiated *Rag2* deficient mice carrying a homozygous *Atm* floxed allele and *Rosa26* locus with Cre^{ERT2} homozygous allele (*Rag2*$^{-/-}$ *Atm*$^{R26fl/fl}$) or wt allele (*Rag2*$^{-/-}$ *Atm*$^{fl/fl}$) were grafted with bone marrow (BTM) from mice harbouring a diphtheria toxin receptor on regulatory t cells (*Foxp3*^{DTR}) or from wt mice (*Foxp3*^{WT}). Reconstitution lasted for 6-8 weeks, when chimeric mice (BMT→recipients) were fed on tamoxifen (TAM) for four weeks (W) to delete *Atm* by Cre recombinase activation (*R26*fl/fl→*R26*Δ/Δ). Thenceforth, diphtheria toxin (DT) was administrated for two weeks to induce depletion of regulatory T cells (T_{REG}), and finally, mice were analysed on day 26 after DT

started. **(B)** Histological pathology score in organs of mice described in (A). Each column represents a single mouse, and each line represents an organ. Mean Autoimmunity Disease score is depicted in the bottom horizontal line. SI: small intestine, LI: large intestine, SG: salivary glands. **(C)** Quantification of AID scores *per* organ *per* genotype of the mice represented in (B) and shown mean ± SD. *p < 0.05, as determined by bootstrapping resampling method. Data from 4 independent experiments. **(D)** Representative H&E staining of the pancreas from regulatory T cells depleted chimeric mice (Foxp3^{DTR}), developed as described in (A). Scale bar=250μm. Data from 4 independent experiments. **(E)** Glycemia concentration during follow-up time after DT administration, as indicated in (A). *Foxp3*^{WT}→*Atm*$^{fl/fl}$ (n=3), *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ (n=3), *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ (n=4), *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ (n=6) Data from 2 independent experiments. **(F)** Weight change on day 26 after T_{REG} depletion begin. *Foxp3*^{WT}→*Atm*$^{fl/fl}$ (n=4), *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ (n=4), *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ (n=14), *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ (n=29) Data from 4 independent experiments. *p < 0.05, as determined by two-way ANOVA test and Tukey's multiple comparisons test. ns, not significant.

We then asked whether protection of the exocrine pancreas against autoimmune injury was associated with better maintenance of pancreatic function. The pancreas is an endocrine gland with two main functions: i) exocrine secretion of enzymes that partake in food digestion, including proteases such as trypsin and chymotrypsin, amylase for carbohydrate digestion and lipases for breakdown of lipids and ii) endocrine secretion of insulin and glucagon by the islets of Langerhans to control organismal glucose metabolism. While glycemia was reduced to a similar extent in *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ *vs.* control *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ mice, there was a tendency for a more pronounced reduction of body weight in *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ *vs.* control *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ mice, albeit not statistically significant (Fig.7.3E,F). To what extent these relate to diet malabsorption is however, not clear.

As expected, *Atm* deletion in parenchyma cells of *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ mice was not sufficient *per se* to elicit a reduction of glycemia or body weight (Fig.7.3E,F) and was also not associated with pancreatic leukocyte infiltration (Fig.7.2) and damage (Fig.7.3B), similar to control *Foxp3*^{WT}→*Atm*$^{fl/fl}$ mice in which T_{REG} cells were not deleted and *Atm* was expressed at physiologic levels (Fig.7.3E). These observations suggest that *Atm* expression in parenchyma cells, most likely in the exocrine pancreas, confers tissue damage control and disease tolerance to autoimmune pancreatitis (Fig.7.3B-F).

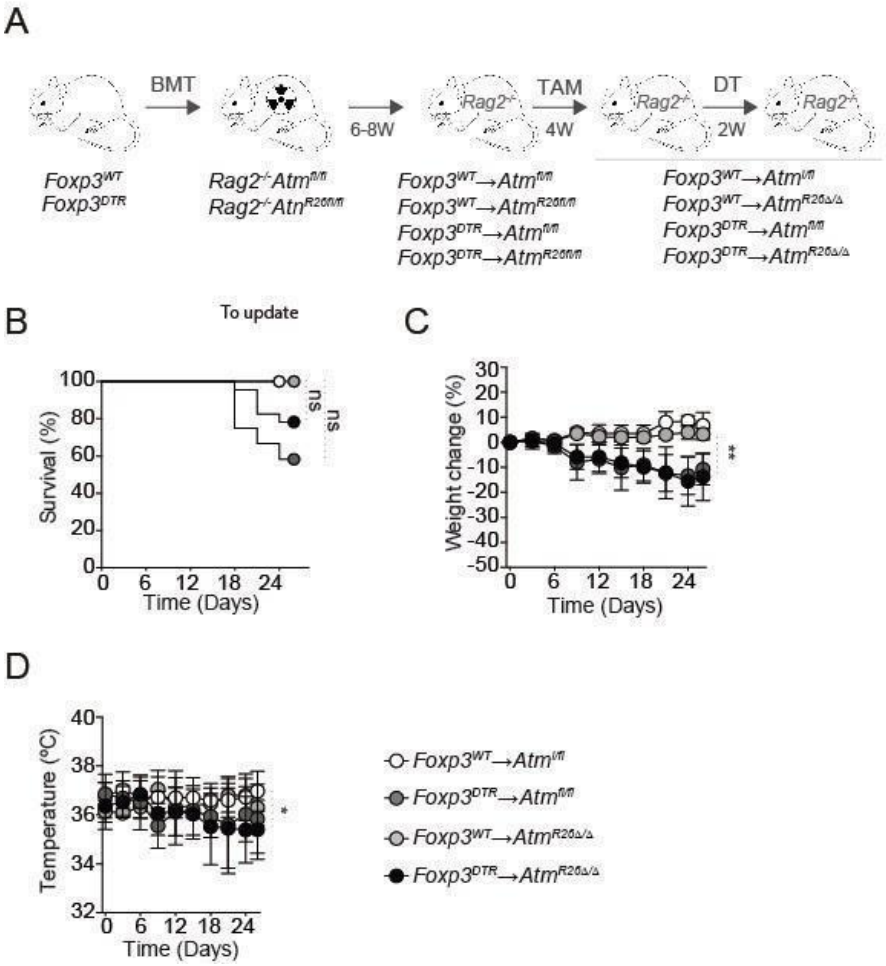

Figure 7.4. Upon T_{REG} cell depletion, *Atm* deletion does not interfere with mice vital parameters

(A) Schematic representation of the strategy used to generate chimeric mice in which the transcription factor ataxia-telangiectasia mutated (*Atm*) is globally deleted except in the hematopoietic tissues. Non-lethally irradiated *Rag2* deficient mice carrying a homozygous *Atm* floxed allele and *Rosa26* locus with Cre^{ERT2} homozygous allele (*Rag2*$^{-/-}$ *Atm*$^{R26fl/fl}$) or wt allele (*Rag2*$^{-/-}$ *Atm*$^{fl/fl}$) were grafted with bone marrow (BTM) from mice harbouring a diphtheria toxin receptor on regulatory t cells (*Foxp3*^{DTR}) or from wt mice (*Foxp3*^{WT}). Reconstitution lasted for 6-8 weeks, when chimeric mice (BMT→recipients) were fed on tamoxifen (TAM) for four weeks (W) to delete *Atm* by Cre recombinase activation (*R26*fl/fl→ *R26*Δ/Δ). Thenceforth, diphtheria toxin (DT) was administrated for two weeks to induce depletion of regulatory T cells (T_{REG}), and finally, mice were analysed on day 26 after DT started. **(B-E)** Survival (B), weight change (C) according to initial day of T_{REG} depletion and rectal temperature (D) throughout the disease development: *Foxp3*^{WT}→*Atm*$^{fl/fl}$ n=7, *Foxp3*^{WT}→*Atm*$^{R26Δ/Δ}$ n=7, *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ n=21, *Foxp3*^{DTR}→*Atm*$^{R26Δ/Δ}$ n=28. *p < 0.05, as determined by one-way ANOVA test and Tukey's multiple comparisons test. ns, not significant. Data from 4 independent experiments.

Similar results, as previously mentioned in Chapter 3, regarding weight loss, temperature drop and survival decrease were observed in these chimeric groups, regardless of *Atm* expression (Fig.7.4B-D).

We then asked whether non-hematopoietic *Atm* expression modulates systemic immune activation induced by T_{REG} depletion. We identified no differences amongst $CD4^+$ and $CD8^+$ activation markers ($CD44^{hi}CD62^{lo}$) and $CD19^+$ cells

proportions and total numbers in the spleen and found no differences among chimeric mice, independently of *Atm* expression (Fig.7.5A-F).

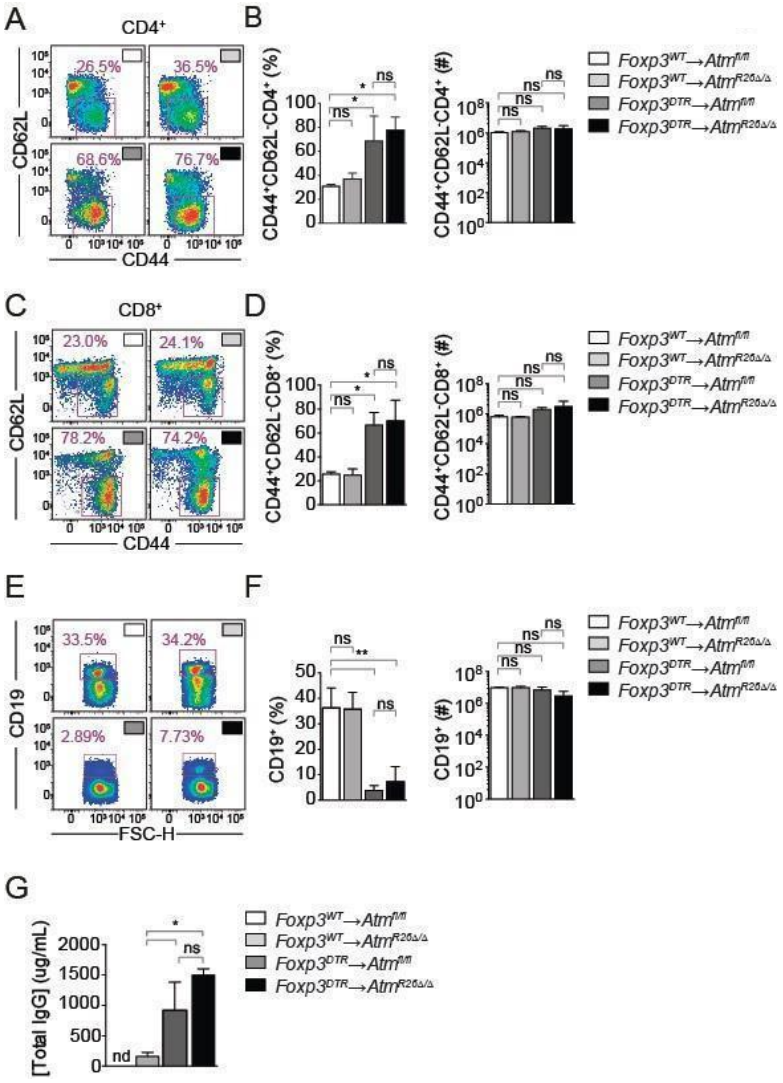

Figure 7.5. Non-hematopoietic *Atm* deletion does not impact systemic immune activation during autoimmunity development

Flow cytometry analysis of splenic effector memory (CD62L^{low} and CD44^{high}) CD4$^+$ **(A,B)** and CD8$^+$ **(C,D)** T cells from chimeric mice as described in Figure 7.3A. **(E,F)** Splenic B cells (CD19$^+$) in the same mice as (A-D). **(G)** Serum immunoglobin G concentration in the same mice as (A-F; n=3). *p < 0.05, as determined by two-way ANOVA test and Tukey's multiple comparisons test. ns, not significant. Standard bars represent mean ± SD. ♦*Foxp3*^{WT}→*Atm*$^{fl/fl}$ (n=3), *Foxp3*^{WT}→*Atm*$^{R26Δ/Δ}$ (n=3), *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ (n=4), *Foxp3*^{DTR}→*Atm*$^{R26Δ/Δ}$ (n=4).

Seric levels of total immunoglobulin G were also similar in Foxp3^{DTR} chimeric mice whether competent or deficient for *Atm* (Fig.7.5G). Activation marker ICOS and CD69$^+$ cells were also increased in regulatory T cell-depleted mice, regardless of *Atm* deletion (Fig.7.6C-D). We next asked whether these mice faced a defective immune

tolerance re-balance by ascertaining the known immune checkpoints PD1 and CTLA4. We found an increased percentage of PD1^{+} and CTLA4^{+} cells (Fig.7.6A-B) in T_{REG} depleted chimeric mice, despite *Atm* expression, meaning that immune equilibrium is being attempted by downregulating the immune system and promoting self-tolerance. Moreover, there were also no detectable differences on the number of splenic neutrophils and macrophages (Fig.7.7A-B). We then concluded that non-hematopoietic *Atm* deletion in non-hematopoietic tissues does not interfere with adaptive and innate immune activation in the context of T_{REG} cell depletion.

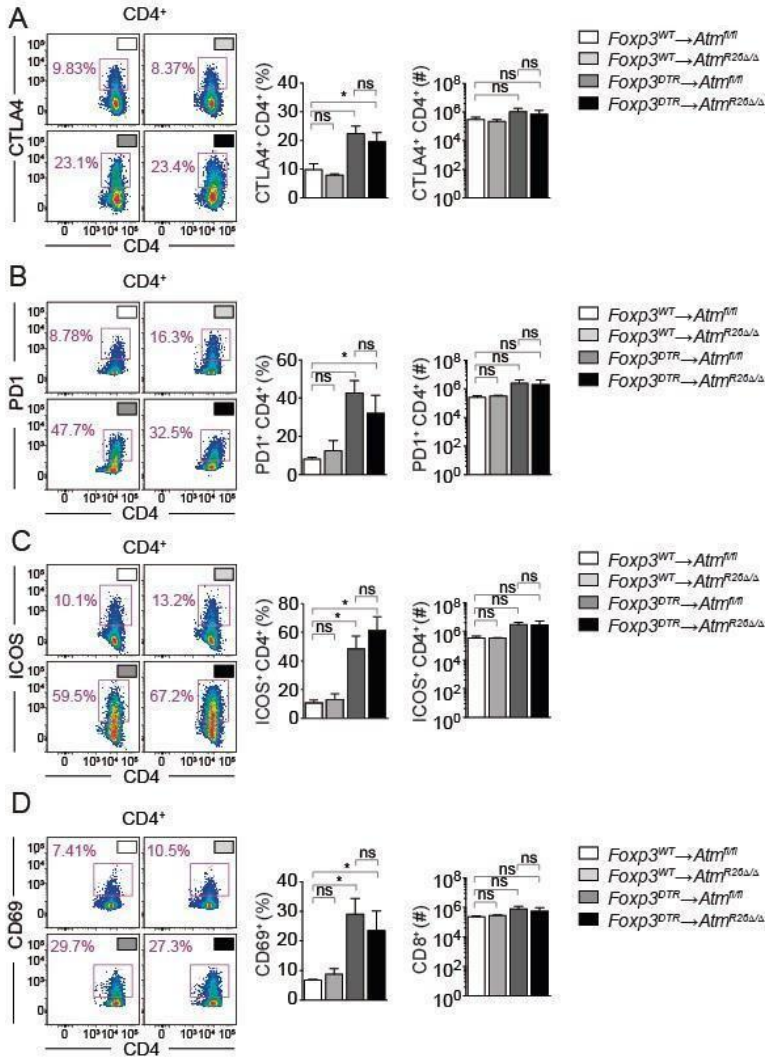

Figure 7.6. Non-hematopoietic *Atm* deletion does not modulate systemic adaptive immune activation during autoimmunity development

Splenic activated CTLA4^{+} **(A)**, PD1^{+} **(B)**, ICOS^{+} **(C)** and CD69^{+} **(D)** in CD4^{+} T cells analysis by flow of four chimeric groups on day 26, from the same mice as depicted in Figure 6.3: *Foxp3*^{WT}→*Atm*$^{fl/fl}$ n=3, *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ n=3, *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ n=4, *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ n=4. *p < 0.05, as determined by two-way ANOVA test and Tukey's multiple comparisons test. ns, not significant. Standard bars represent mean ± SD.

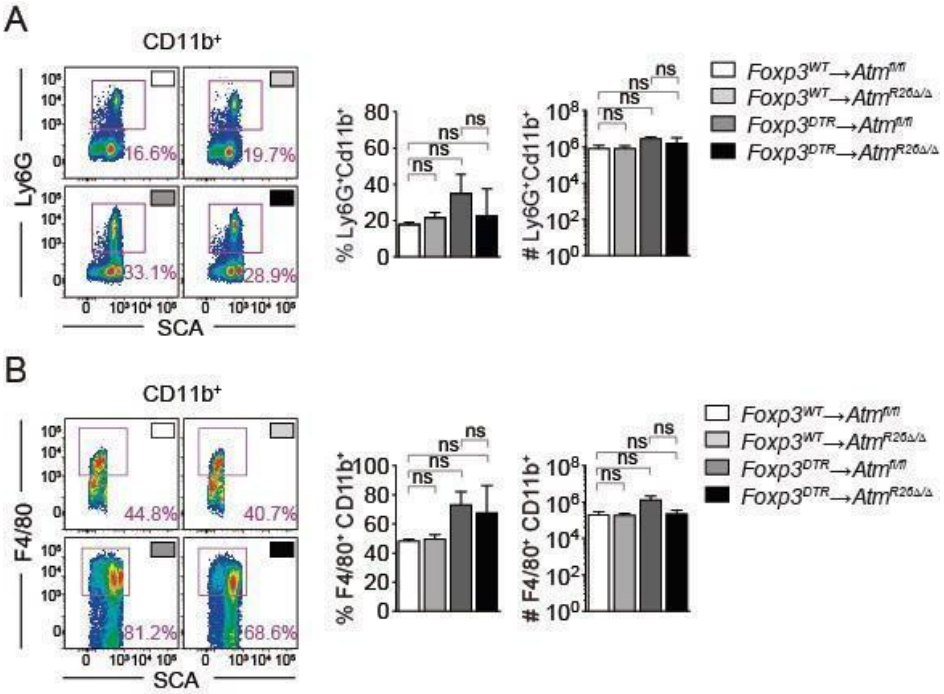

Figure 7.7. Non-hematopoietic *Atm* deletion does not modulate systemic adaptive immune activation during autoimmunity development

(A,B) Flow cytometry plots representative of splenic neutrophils (Ly6G$^+$ CD11b$^+$) (A) and macrophages (F4/80$^+$ CD11$^+$) (B) representative of four chimeric groups on day 26, , from the same mice as depicted in Figure 6.3: *Foxp3*^{WT}→*Atm*$^{fl/fl}$ n=3, *Foxp3*^{WT}→*Atm*$^{R26\Delta/\Delta}$ n=3, *Foxp3*^{DTR}→*Atm*$^{fl/fl}$ n=4, *Foxp3*^{DTR}→*Atm*$^{R26\Delta/\Delta}$ n=4. *p < 0.05, as determined by two-way ANOVA test and Tukey's multiple comparisons test. ns, not significant. Standard bars represent mean ± SD.

7.3. Discussion

We reasoned that the chronic inflammatory response associated with the development of autoimmune diseases maybe associated with oxidative stress and genotoxicity, inducing DDR in parenchyma cells. This idea is supported by the previous association between the production of auto-antibodies recognizing double-strand DNA in autoimmune disease [17].

ATM is a critical component of the DDR responsible for DSB repair and *ATM* polymorphism have been associated with the development of rheumatoid arthritis [18]. In this Chapter, we found that ATM expression in parenchyma cells protects the exocrine pancreas from autoimmune injury (Fig.7.3B-F).

The pancreas is a glandular organ functionally divided into an endocrine and an exocrine compartment. The endocrine compartment is formed by islet of Langerhans composed of α, β, δ, ε and γ cells, which produce glucagon, insulin and amylin, somatostatin, ghrelin and pancreatic polypeptide, respectively. The exocrine compartment is composed by acinar and duct tissues which secrete water,

bicarbonate and store inactive zymogen granules of digestive enzymes, including proteases, lipases and amylase to be released in the duodenum and assist digestion. Our findings suggest that ATM protects the exocrine pancreas from autoimmune dysfunction and injury without affecting the endocrine pancreas (Fig.7.3D-E).

Our findings are consistent with glandular epithelial cells of the exocrine pancreas being more subjected to stress, presenting a high cellular turnover rate [2]. Therefore, in the absence of ATM, DNA replication errors may accumulate in this cell compartment. This is in keeping with the relatively higher level of ATM expression in the pancreas compared to other organs (Fig.7.1F), as previous reported [19,20]. Moreover, the exocrine pancreas has also been shown to express relatively higher levels of *ATM* mRNA, when compared to the endocrine counterpart [19] and ATM was associated with the pathogenesis of pancreatic cancer [21–23].

The mechanism via which ATM provides tissue damage control on the exogenous pancreas remains to be established. Under steady state conditions, DNA DSB trigger DDR machinery via the recruitment of MRN complex along with the ATM kinase, which activates p53 to upregulate NKG2DL and DNAX accessory molecule-1 (DNAM1L), favouring the sequestration of immune cells such as NK, NKT, γδT and $CD8^+$ T cytotoxic cells. P53 induces the expression of ICAM1, CXCR5 chemokine receptor, Lymphocyte function-associated antigen (LFA)-1, MHC class I polypeptide–related sequence A (MICA), UL16 binding protein 2 (ULBP2) and CD155 expression that recruit the immune system [24–27].

P53 has also been described to be induced by several mechanisms [28,29], interestingly, also activated by metabolic stress [30]. In our model, cells target of autoimmune attacks are expected to be exposed to an increased level of metabolic stress associated with a higher turnover rate which combined would contribute to activation of p53, increase of DNA errors, accumulation of DSBs and decreased negative feedback loop due to ATM gene deletion in those tissues [31]. Moreover, the expression of NKG2D ligands by inflamed tissue and the NKG2D-dependent activation of immune cells have been linked to several chronic inflammatory diseases, such as rheumatoid arthritis, celiac disease, and type I diabetes [32] which might also be the case in our experimental system. First, an intricated mechanism of activation of oxidate stress known to activate this pathway may have been triggered, for which the lack of negative feedback by ATM may have contributed to the worsen outcome.

Second, DSBs are sensed by the stimulator of interferon genes (STING), as described originally in the context of viral infections [14] but also in in the context of autoimmunity [33]. STING promotes the synthesis and release of type I IFN and in doing so induces the production of other proinflammatory cytokines, such as CXCL10, sustaining inflammation and the activation of the adaptive immune system [33]. We propose that ATM might regulate this inflammatory response in the exocrine tissue upon deletion of T_{REG} cells, without interfering with the systemic activation of autoreactive $CD4^+$ T_H cells, cytotoxic $CD8^+$ T cells or the production of circulating autoreactive IgG (Fig.7.5A-G and Fig.7.7A-B).

In conclusion, the inflammatory response associated with the development of autoimmune diseases can lead to DSB in the exocrine pancreas. This is sensed by PRR such as STING [34,35] that orchestrate a Type I IFN response promoting further inflammation and tissue damage. Our results support the idea that ATM contributes to repair DSB in parenchyma cells subjected to autoimmune inflammation and, in doing so, limits the extent of inflammation causing damage to the exocrine pancreas. This finding suggests that polymorphic ATM tissue expression may result in different organ targeting in individuals susceptible to autoimmune disease.

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Chapter 8

General Discussion

General Discussion

Autoimmunity refers to immune cells and/or antibodies that react with self-antigens, eventually leading to tissue dysfunction, damage and autoimmune disease. The global burden of these chronic immune mediated inflammatory diseases affects 5% of the world population, mostly around working age [1].

A precise understanding of the molecular mechanisms underlying the pathogenesis of autoimmune diseases, and therefore their prevention and treatment, has not yet been achieved. Autoimmune diseases are thought to develop from a combination of both genetic predisposition and environmental factors, with several genetic mechanisms acting on immune cells being proposed to contribute to the pathogenesis of autoimmune diseases [2], including MHC haplotypes susceptibility [3], super-antigen promoting non-specific activation of T cells resulting in polyclonal T cell activation and massive cytokine release, molecular mimicry by shared similarities between host and exogenous antigens [4], idiotype cross-reaction to host receptors [5], cytokine dysregulation [6] or epitope spreading when the target of initial immune response is extended to other epitopes on the same protein or other proteins in the same tissue [7]. Ultimately, two main hypotheses sustain an actual systemic autoimmune inflammation: 1) disruption in the barrier control between innate and adaptive immunity, incited by continuous inflammation by a positive feedforward loop, and 2) flawed reactivity of adaptive immunity which reactivates memory autoreactive lymphocytes, including defects of tolerance checkpoints [8].

Our work brings an additional layer of complexity in the understanding of the development of autoimmune diseases, in that we show that parenchyma cells are not merely bystanders in the pathogenesis of autoimmune diseases. Similar to what was originally demonstrated in the context of organ transplantation [9–12], we found that when targeted by autoimmune cells and/or antibodies, parenchyma cells activate stress and damage responses that provide tissue damage control and ultimately mitigate the pathogenesis, progression and/or outcome of autoimmune diseases. We refer to this defence strategy as tolerance by analogy to disease tolerance to infection, originally defined in ecology, as a process that limits the impact on parenchymal cells, host health and fitness, allowing a tolerance towards internal and external elicitors without interfering with them [13]. In the context of autoimmunity, disease tolerance is explained

as a tissue tolerance to the autoreactive immune cells which aim at inducing damage or other fitness costs [14].

Parenchyma cells rely on an intrinsic ability to tolerate some degree of stress, damage or malfunction. This ability to tolerate is provided by effector genes regulated by a transcriptional stress and damage response network [15]. In this thesis, we focused on four transcriptional master regulators of this stress and damage response network, namely HSF1, HIF1a, NRF2 and ATM, which respond to proteoxic stress [16], hypoxia[17], oxidative stress [18] and DNA damage [19], respectively.

Oxidative stress results from the accumulation of ROS beyond a threshold that can be neutralised under physiological conditions [44,45]. As described in chapter 4, this elicits a defence mechanism led by the transcription factor NRF2 and aiming at sustaining redox homeostasis and cytoprotection [46]. In chapter 4, we found that the expression of *Nrf2* in parenchyma cells is protective against autoimmune hepatitis.

Perhaps the most convincing experimental evidence that NRF2 plays a central role in the pathogenesis of autoimmune diseases is the “spontaneous” development of lupus-like nephritis and shortened lifespan of female *Nrf2*-deficient mice [47]. This is associated with an overall reduction of antioxidant capacity and increased lipid peroxidation as well as DNA oxidation, owed to lower glutathione levels (*i.e.,* GSH and GSH/GSSG ratio) [48,49]. Moreover, these mice also present splenocyte apoptosis, circulating antibodies against double-strand DNA and the Smith antigen, along with myocarditis, vasculitis, glomerulonephritis and hepatitis [50]. Moreover, disease activity index and extent of organ damage in SLE patients was also associated with a decrease in GSH and GSH/GSSG ratio, and lower levels of SOD and catalase expression [51–54]. SLE patients also exhibit alterations in repair mechanisms of oxidative DNA damage [55], high serum levels of oxidised proteins, apolipoprotein, C3[53], oxidised phospholipids, and autoantibodies against oxidatively modified lipoproteins[56].

In many other autoimmune diseases such as rheumatoid arthritis [46,57–59], inflammatory bowel disease, thyroiditis, vitiligo [60–62], multiple sclerosis, systemic lupus erythematosus [63,64] and Sjogren syndrome [63], NRF2 involvement has been reported in their pathogenesis. In the case of rheumatoid arthritis, oxidative stress has been suggested to emanate from an increase in mitochondrial ROS production [65,66] and a reduction in the activity of antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx). This was associated with an

abnormal increase in inflammatory cytokines as TNFα, IL-6, IFN-γ, IL-1α and with synovial infiltration by macrophages, T_H1 and T_H2 cells [67]. Similar results were obtained in mice models of this joint damage disease [57,58,68], where *Nrf2* deletion increases vulnerability to joint alterations by dramatically increasing the release of pro-inflammatory cytokines and chemokines underlying leukocyte tissue infiltration. This was further associated with induction of cyclooxygenase-2 and inducible nitric oxide synthase as well as with ROS production [57,58,68]. In experimental autoimmune encephalitis (EAE), a model of multiple sclerosis (MS), *Nrf2* deletion exacerbates disease development [69], while the induction of *Nrf2*, genetically by *Keap1* deletion [70] or pharmacologic [71] reduced disease severity [72]. Damaged neurons use this oxidative stress response to increase their survival [72]. Moreover, treatment with pro-oxidants, such as dimethyl fumarate, has proven to reduce the severity of MS symptoms. Dimethyl fumarate, an enhancer of Nrf2 activation, was approved for MS because it decreases the expression of integrins on memory $CD4^+$ T cells, preventing the trafficking of myelin-specific memory cells into the CNS of MS patients, and prevents, in vitro, the differentiation of naïve CD4 T cells into effector T_H1 cells [73].

In chapter 4, we describe that the expression of NRF2 in parenchyma cells limits the pathogenetic effects of autoimmune mediated liver injury in a systemic autoimmunity mouse model (Fig 4.3). In keeping with the central role of NRF2 in detoxification and reducing oxidised proteins it is perhaps not surprising that deletion of *Nrf2* in parenchyma cells increases susceptibility to autoimmune hepatitis (Fig.4.3). The liver is the main organ for detoxification and is considered a vital organ, essential for filtration, digestion, metabolism and protein synthesis. Such relevance is also translated by survival loss observed in our experimental model (Fig.4.10), in which liver injury may lead to liver failure and death. These results do not exclude however that the expression of *Nrf2* in parenchyma cells does not contribute to limit autoimmune injury in other organs such as the lung or heart. This remains however to be tested, such as using other experimental models of spontaneous models of autoimmunity lung or cardiac injury.

As mentioned, NRF2 was demonstrated to be crucial for mice survival under an autoimmune context (T_{REG} depletion) in which grafted hematopoietic cells expressed physiological *Nrf2* levels while parenchymal cells lack that gene (Fig.4.10D). These findings highlight a possible role for tissue residents T_{REG} cells. We

did not verify whether resident T_{REG} were depleted during our experiments and furthermore the implication of this fact to the outcome of the disease. In the experimental model where we find both donor and host potentially depletable T_{REG} (cells expressing DTR - Foxp3^{DTR}), survival worsens, which opens a new scope of investigation, out of the aim of this thesis, understanding the relevance of resident T_{REG} in the development of autoimmunity.

The hypoxic stress occurs when O_2 supply falls below the threshold level required to meet cellular metabolic requirements. As described in chapter 5, in response to hypoxia, cells activate the transcription factor HIF1α, eliciting a profound remodulation of their gene transcription and inducing an adaptive metabolic response to this stress [32]. In chapter 5 we found that the expression of *Hif1α* in parenchyma cells promotes, rather than represses, autoimmune inflammation and tissue damage exacerbating autoimmune pancreatitis, hepatitis, gastritis and dermatitis as well as muscle injury (Fig.5.2B-D).

Our findings are in keeping with autoimmune mediated inflammatory diseases being associated with tissue hypoxia as observed for example in the synovial tissues of rheumatoid arthritis patients [33]. This suggests that HIF1α acts as a player in the pathogenesis of rheumatoid arthritis and a potential therapeutic target [34]. Activation of HIF1α takes part in IL-33-dependent regulatory circuit, involving the activation of p38 MAPK and ERK pathways in synovial cells, which sustains inflammatory arthritis [34]. HIF1α activity also contributes to bone erosion, cartilage destruction and pannus invasion causing arthritis induced by the matrix metalloproteinase Ets-1 observed in autoimmunity models [33,35]. Similarly, through VEGF, its receptors, and the Tie-angiopoietin system, HIF1α regulates angiogenesis contributing to the development and perpetuation of autoimmune disease such as rheumatoid arthritis [33,36].

Also consistent with our finding that HIF1α promotes, rather than represses, the pathogenesis of autoimmune diseases is the observation that mice in which *Hif1*α is deleted specifically in myeloid cells decrease tissue-macrophage infiltration in response to collagen-induced arthritis [37]. This suggests that HIF1α contributes to the pathogenesis of inflammatory arthritis.

Experimental mice models of psoriasis strengthen an active involvement of HIF1α in the pathogenesis of autoimmune diseases showing that HIF1α is required to support myeloid cell infiltration in the skin [37]. HIF1α has been shown essential for TNF-

α, IL-6, CXC chemokine ligand 8 and cathelicidin IL-37 production that contribute to the chronic inflammation of the skin [38]. Moreover, another HIF1α target, GLUT1 is upregulated in psoriatic skins promoting proliferation of epidermal keratinocytes [39].

Systemic sclerosis is yet another autoimmune disease where HIF1α partakes a role alluded by an HIF1α increased expression along with VEGF observed in skin biopsies of systemic sclerosis when compared with normal skin [40]. Fibrinogenesis mediators such as cytokine connective tissue growth factor (CTGF) and metalloproteinases are regulated by HIF1α [41], enhancing HIF1α contribution for this key feature of systemic sclerosis pathogenesis

Systemic lupus erythematosus is a systemic multi-organic autoimmune disorder where the kidneys are a primary target of pathology and where induction of HIF1α correlates with the severity of renal injury [42]. HIF1α promotes the mesangial cells growth, as observed in proliferative glomerulonephritis, through the induction of proliferation and inhibition of apoptosis [42]. Hence, HIF1α inhibition contributes to damper the renal disease almost completely reversing renal cortical hypoxia [43].

The abovementioned examples of HIF1α involvement in autoimmune diseases demonstrate this hypoxia inducible factor importance in different hematopoietic and non-hematopoietic cells. Its function in the regulation of homing and engraftment of immune cells in the tissue as discussed in chapter 5 can be one of the reasons for the obtained results (Fig.5.2B-D). Additional research in endothelial vascular system observing vascular permeability and inflammatory cell recruitment factors such as integrins and chemoattractants may grant added interpretation and understanding of HIF1α role in autoimmune pathogenesis.

As described in chapter 6, HSF1 is a transcription factor that regulates the expression of a number of HSP, which are essential to maintain cellular function and integrity in response to proteotoxic stress [20,21]. Different forms of stress, such as imposed to parenchyma cells in the context of autoimmunity, can elicit conformational changes to mature proteins. These are structurally complex and highly versatile, requiring a correct conformation to sustain their function [22]. Cells, in order to prevent cellular malfunction, rely on a network of integrated sensors of individual structure-function relationship, which regulate protein translation, chaperone assisted protein folding and protein degradation pathways, eliciting a heat shock response [22].

We found that the expression of *Hsf1* in parenchyma tissue is cardioprotective against autoreactive injury (Fig.6.3B-F) conferring tissue damage control and autoimmune disease tolerance (Fig.6.3D). This cardioprotective effect (Fig.6.3E) is essential to support cardiac function in the context of systemic autoimmunity, such as elicited upon dysregulation of peripheral self-tolerance upon T_{REG} cell depletion (Fig.6.3F). Our findings are in keeping with the cardioprotective effect of HSP reported in the literature [23], as illustrated by HSP70, which reduces cardiac infarct size [24] as well as for crystallin alpha B (CRYAB; HSP beta5) which reduces cardiomyocyte apoptosis during hypertrophy and myocardial infarction [25] by eliciting sarcomeric elasticity, mitochondrial integrity and also balancing redox equilibrium through an increased recycling of oxidized glutathione [26–28].

The expression of HSPs is induced in several autoimmune diseases, such as demonstrated for HSP90 in SLE patients [29] which correlates with an increase in IL-6[30] and the production of anti-HSP90 autoantibodies [31]. Whether the induction of HSP in autoimmune disease patients, including HSP90, relates to tissue dysfunction and damage or a tissue damage control mechanism against the pathogenesis of autoimmune disease is yet not clear. This could be tested using a spontaneous experimental model of autoimmune disease to test whether the induction of HSP precedes the clinical manifestation of the pathology and thus would serve as a biomarker in a clinical setting. To this aim, further research to characterise heart transcriptomic in chimeric mice that express Hsf1 in parenchyma cells may provide further information on the downstream HSP acting in a cardioprotective manner in the context of autoimmune disease.

As described in chapter 7, DNA damage response is critical to preserve cellular genetic inheritance [74] relying on sensors that that recognise different types of DNA damage such as DSB. These, for example, activate a complex signal transduction network involving the ATM [74], a central kinase in the response to genotoxic stress, together with DNA-PK and ATR. These kinases trigger the MRN complex upon identification of DSBs.

Other forms of stress can also activate ATM in an MRN-independent manner[74], including oxidative stress [74], which can impose oxidative DNA damage (*i.e.,* 8-oxo-dG), while interfering with the function of proteins that take part in the DDR machinery. This suggests that there is a bi-directional interplay between oxidative stress, DNA

damage and the DDR [75]. Moreover, patients deficient in ATM exhibit increased susceptibility to insulin resistant diabetes and impaired glucose metabolism [19]. This link between ATM and glucose metabolism is also a path for ATM to promote antioxidant responses through the induction of the pentose phosphate pathway (PPP) meanwhile regulating mitochondrial homeostasis response to ROS or mitochondrial quality control genes and proteins, such as PTEN-induced putative kinase 1 (PINK1), Parkin or BH3-interacting domain death agonist (BID) [76].

In Chapter 7, we found that ATM expression in parenchyma cells subjected to autoimmune inflammation limits the extent of inflammation causing damage to the exocrine pancreas. Our findings are consistent with the idea that DDR might play a central role in the pathogenesis and/or progression of systemic autoimmune diseases, such as SLE [77–80], systemic sclerosis [79] and RA [81]. This notion is supported by the genetic association of polymorphisms in genes involved in DDR with increased frequency of autoimmune diseases [81].

Downregulation of molecular components implicated in the DNA repair machinery and upregulation of genes driving programmed cell death by apoptosis are also frequent among autoimmune disease patients [82]. For example, rheumatoid arthritis patients have reduced *ATM* mRNA expression in T cells, which is associated with the induction of T cell programmed cell death via apoptosis [80]. This might impose a lymphopenia-induced proliferation, associated with premature immune senescence and autoimmune-biased T cell repertoire [80]. Impaired ATM-p53 function in T cells, associated with a shift of $CD4^+$ T cell activation toward T_H1 and T_H17 effector cells rather than T_{REG} cells were also observed in patients with this condition, generating an arthrogenic T cell response and imposing a hyperinflammatory phenotype in the synovial tissue [81]. Moreover, when *ATM* is deleted in rheumatoid arthritis subjects, $CD8^+$ T cells show a hyperactive mTORC1 signaling that is activation of protein translation. This hyperactive complex activation results in inflammatory cytokine production and memory T cell development, hence, an acceleration of the autoimmune genesis [81].

In chapter 7, we describe that the expression of *Atm* in parenchyma cells promotes tissue damage control and prevents from autoimmune injury (Fig.7.2B-F). Our findings suggest that *Atm* is a player in tissue susceptibility to autoimmune disease development. Furthermore, we concluded that deletion of *Atm* in non-hematopoietic cells elicit pancreatic tissue susceptibility to immune-mediated disease

to a potential accumulation of DNA replication errors that may release DAMPs igniting the inflammatory response. The accumulation of DNA replication errors and release of self-DNA will trigger immune activation, inflammation and secretion of pro-inflammatory factors through cGAS-STING pathway, the ZBP1 pathogen sensor, the AIM2 and NLRP3 inflammasomes activation [83]. Additionally, DNA damage response may induce senescence, an enduring state of cell cycle arrest, by initiating mechanisms to limit cell division. During this state, senescent cells secrete strong immune modulators called senescence-associated secretory phenotype (SASP) factors, furthermore contributing immune cell signalling [83]. However, a thorough mechanistical understanding of this path remains to be covered. Future perspectives may endeavour whether ATM contribution to the tissue susceptibility to autoimmune disease development is due to any other sensoring function, for instance, oxidative stress. To rule out the contribution of oxidative stress sensors, applying experimental conditions that disturb other sensoring path in our experimental model, could provide evidence of its role. Furthermore, a transcriptomic and metabolomic analysis comparing WT and ATM deleted pancreas in our experimental setting would contribute to a more precise awareness of the ATM role in autoimmune disease development.

The pathogenesis of autoimmune diseases is complex but probably shares common etiological (*i.e.,* genetic) factors that manifest within an array of clinical signs and symptoms according to the tissue structure and organs targeted by autoimmunity. Our findings support the notion that parenchyma cells in different tissue or organs subjected to autoimmune injury play a critical role in the pathogenesis and outcome of autoimmune diseases, as demonstrated originally in the context of organ transplantation [84–87] and thereafter in for a variety of infectious diseases.

Cells have an intrinsic ability to tolerate some degree of stress and damage, preventing malfunction through the activation of stress and damage responses, which regulate the expression of effector genes providing cellular adaptation to specific forms of stress and damage, respectively [14,15,88–90]. The effector genes involved in these protective responses are expressed in a cell-, stress- and damage-specific manner, in keeping with our findings that different transcriptional master regulators of these stress and damage responses act in different organs to regulate autoimmune inflammation and the development of autoimmune diseases.

The stress and damage-responsive transcriptional network is composed of a restricted number of stress and damage-responsive transcription factors, including Nrf2, HIF1-a and HSF-1, regulating the expression of a larger number of effector genes that mitigate the negative impact of specific forms of stress and damage[15]. The network has a hierarchical structure, with few core effector genes (≈3) being regulated by a relatively large number (≈7) of transcription factors, while most other effector genes are regulated by relatively fewer (<7) transcription factors[15]. Some of the transcription factors in the network, such as *HSF1* and *NRF2* regulate the expression of shared genes [91], such as the heme catabolizing enzyme heme oxygenase 1 (HO-1) [92], HSP70 [93] or the autophagy cargo protein sequestosome 1 (p62) [94]. Moreover, *HSF1* can activate the expression of *NRF2* via a mechanism whereby p62 that displaces NRF2 from its inhibitor KEAP1 [94]. This suggests that the heat shock response regulated by *HSF1* also interacts with the oxidative stress response regulated by *NRF2*, illustrating an important feature of the transcriptional network, namely, the functional interrelationship between the effector genes providing cellular adaptation to different forms of stress and damage.

As another example of this functional interrelationship, when activated in response to oxidative stress and/or DNA damage, ATM phosphorylates the heat-shock protein 27 (HSP27), suggesting that the DDR interacts with the heat shock response's regulated by *HSF1*. Moreover, HSP27 phosphorylation by ATM induces the expression of the glucose-6-phosphate dehydrogenase (G6PD), a rate limiting enzyme in the pentose phosphate pathway [95], which synthesizes nicotinamide adenine dinucleotide phosphate (NADPH), an essential regulator of cellular redox [95,96]. This suggests that ATM may act via the phosphorylation of HSP27 to induce NADPH, as part of a protective response to oxidative stress [97]. Of note, ATM can also be activated in response to hypoxia, independently of DNA damage and the MRN complex [98] via double-strand-break repair protein rad21 protein (MCD1), a mediator independent of NBS1 and MRE11, suggesting that ATM can interact with the stress response to hypoxia, controlled by *HIF1α*. In further support of this idea, the *HIF1α* promoter has at least one ARE DNA-binding site recognized by NRF2, suggesting that the oxidative stress response regulated by *NRF2* interacts with the stress response to hypoxia regulated by *HIF1α*. This interaction appears to be required to shift cellular glucose metabolism towards anaerobic glycolysis while providing glucose-6-phosphate towards the PPP to produce NADPH [99,100]. Mechanistically, basal level of

Nrf2 binds to the promoter and positively regulates constitutive HIF1α to mRNA expression thereafter inhibited by PHDs. R. Potteti et al. observed that upon kidney tubular hypoxia, PHDs are inhibited and with HIF1α activation Nrf2 levels are downregulated via mitochondrial complex I [101].

Analogous pathway interactions are observed between HSF1 and HIF1α in the cancer context. Gabai et al. reported that Hsf1$^{-/-}$ mice develop smaller tumours when compared to control mice due to a defect in *HIF1α*-dependent angiogenesis [102]. These authors show that HIF1α is suppressed at translation level due to an RNA-binding protein controlled by HSF1, HuR protein.

This level of interaction between different elements of the stress and damage-responsive transcriptional networks suggests that effector genes associated with specific stress and/or damage responses may cooperate functionally to regulate the pathogenesis and/or outcome of autoimmune diseases. Therefore, future research considering these mechanisms of control of autoimmunity may consider the combined targeting of several stress and damages responses. For example, the combined action of ATM and NRF2 may act synergistically to provide tissue damage control and the inflammatory lesion underlying the pathogenesis and/or progression of autoimmune diseases. This can be tested using combined genetic loss of function approaches in the same experimental autoimmunity mouse models used in this Thesis.

There are several limitations in the studies undertaken through this Thesis. First, following the concept of disease tolerance in infectious conditions where tissues recover to a homeostatic balance without impacting on pathogen load, in an autoimmunity context, where autoreactive immune cells are the menace to the tissue, it would be reassuring to identically assess tissue autoreactive immune cells load in our models to further support the concept. Second, we used a whole-body deletion of stress and/or damage transcriptional regulators model instead of a tissue specific manner. Employing such model, tissue and cell-cell interactions and communications by direct contact, paracrine, autocrine or endocrine signaling are not taken in consideration. The impairment in one parenchymal cell type may have an impact in another cell type and ultimately be translated in a different outcome. Thus, tissue-specific gene deletion would provide a more precise answer to our initial hypothesis that specific tissue stress and damage responses modulate the pathogenesis of autoimmune diseases.

In conclusion, increasing awareness of autoimmune diseases is contributing to further research interest. The complexity of these diseases, including aetiology, pathogenesis, organs targeted and clinical manifestations, constrain their study. Understanding that autoimmunity happens regardless of disease development shaped our rationale to question whether tissues could modulate the development of autoimmune diseases by regulating tissue dysfunction in autoimmunity conditions. We identified several genes previously described in stress and damages responses that contribute critically to the pathogenesis of experimental autoimmune diseases in mice. These are essential in the regulation of organ dysfunction imposed by autoimmunity, thus regulating disease development. Yet, this work might have contributed to new avenues of research there is still a long path for a better understanding, control and treatment of autoimmune diseases.

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