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Régulation de l'activation de lymphocytes B / cellules plasmatisques pendant le rejet chronique : Le rôle de SYK dans la modulation de Mcl-1

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Regulating the activation of B-lymphocytes during chronic antibody mediated rejection: The role of SYK in modulating Mcl-1

Thèse de doctorat de
l'Université Paris-Saclay

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Aspects moléculaires et cellulaires de la biologie

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To those I hold close to my heart

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working with you, the ever-optimistic you can never do too much experiments scientist! And thank you for your input in my research, the time you took for our discussions and your help in teaching me all the things I need to know about apoptosis. And last but not least, thank you for your humor and for appreciating mine, you'll never find someone like me again ;-)

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Titre : Régulation de l'activation de lymphocyte B pendant le rejet chronique ; La rôle de SYK dans la modulation de Mcl-1

Mots Clés : Rejet médiée par les anticorps, centres germinatifs, Signalisation du BCR, SYK, Mcl-1, Apoptose

Résumé : L'insuffisance rénale est un problème majeur de santé publique et la transplantation rénale est l'option thérapeutique principale, mais elle comporte le risque de rejet d'organe. Les cellules B jouent un rôle important dans le rejet médié par les anticorps (AMR). Au cours de l'AMR chronique, les structures lymphoïdes tertiaires, semblables aux centres germinatifs (GC), apparaissent dans l'organe rejeté, associées à la production des plasmocytes et des lymphocytes B mémoires spécifiques du donneur. Ces populations de lymphocyte B sont souvent mal contrôlées par les traitements actuels. La myeloid cell leukemia 1 (Mcl-1), un membre anti-apoptotique de la famille de B-cell lymphoma 2 (Bcl-2), est essentiel pour maintenir l'organisation de GC et de la différenciation des cellules B. Nous rapportons ici l'infiltration de cellules B exprimant Mcl-1 dans le rein de patients atteints d'AMR chronique, comme cela a été observé pour les cellules (pré) GC. Suite à l'abrogation de la signalisation du récepteur des cellules B (BCR), par l'inhibition de la spleen tyrosine kinase (SYK) nous avons observé une diminution de la viabilité des cellules GC, par l'intermédiaire d'une régulation de Mcl-1. La régulation négative de Mcl-1 est coordonnée au niveau de la transcription, potentiellement par l'intermédiaire du transducteur de signal et de l'activateur de la transcription 3 (STAT3), comme cela a été observé par (1) une translocation altérée de STAT3 dans le noyau suivant l'inhibition de SYK, et (2) les niveaux inférieurs de transcription de Mcl-1. Par ailleurs, la surexpression de Mcl-1 inhibe l'apoptose après l'inhibition du SYK. Des études avec des cellules B primaires, issues d'amygdales, ont confirmé que l'inhibition de SYK a diminué la survie cellulaire. Nous avons également constaté que l'inhibition du SYK a diminué les niveaux de protéines Mcl-1 dans les cellules B primaire, et que l'activation de ces cellules a été inhibée, tel que déterminé par l'expression de CD80 et des taux inférieurs de sécrétion d'IgG dans les cellules B primaires activées in vitro. Nos travaux suggèrent que la voie SYK-Mcl-1 peut offrir de nouvelles opportunités pour le traitement et la prévention de l'AMR.

Title: Regulating the activation of B lymphocytes during chronic antibody mediated rejection: The role of SYK in modulating Mcl-1

Keywords: Antibody mediated rejection, Germinal Center, BCR signaling, SYK, Mcl-1, Apoptosis

Abstract: Renal failure is a major public health concern and renal transplantation is the main therapeutic option, however it comes with the risk of organ rejection. B-cells play an important role in antibody-mediated rejection (AMR). During chronic AMR, tertiary lymphoid germinal center (GC)-like structures appear in the rejected organ, associated with de novo production of donor-specific plasma and memory B-cells. Which are B-cell populations that are often poorly controlled by current treatments. Myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic member of the B-cell lymphoma-2 (Bcl-2) family, is essential for maintaining the GC reaction and B-cell differentiation. We report here the infiltration of B-cells expressing Mcl-1 in the kidney of patients with chronic AMR, as observed for (pre-)GC cells. The impairment of B-cell receptor (BCR) signaling, by inhibition of spleen tyrosine kinase (SYK), reduced viability and Mcl-1 protein levels in GC like cells. This downregulation is coordinated at the transcriptional level, potentially via signal transducer and activator of transcription 3 (STAT3), as shown by (1) impaired translocation of STAT3 to the nucleus following SYK inhibition, and (2) the lower levels of Mcl-1 transcription upon STAT3 inhibition. Moreover, overexpression of Mcl-1 prevented cells from entering apoptosis after SYK inhibition. In vitro studies with primary tonsillar B-cells confirmed that SYK inhibition decreased cell survival. We also found that SYK inhibition decreased Mcl-1 protein levels in primary B-cells, and that B-cell activation was inhibited, as determined by CD80 expression and lower levels of IgG secretion in tonsillar B-cells activated in vitro. Overall, our data suggest that the SYK-Mcl-1 pathway may provide new opportunities for the treatment and prevention of AMR.

Synthèse en Français

L'insuffisance rénale est un problème de santé majeur dans les pays occidentalisés. La transplantation rénale est la méthode thérapeutique la plus satisfaisante en cas d'insuffisance rénale terminale permettant une meilleure qualité de vie, une meilleure survie et à moindre coût par rapport à la dialyse. Cependant, la transplantation rénale est limitée par la survenue de rejet d'organe et en particulier au long court de rejet chronique à médiation par les anticorps (AMR). Les cellules B jouent un rôle important dans l'AMR via la synthèse d'anticorps allo-réactifs. Les allo-anticorps produits contre la greffe sont associés à une fréquence élevée de détérioration des organes et de perte de greffe malgré les traitements actuels développés dans le but de contrecarrer l'AMR. Cependant, ces thérapies échouent souvent à contrôler la production d'anticorps spécifiques du donneur (DSA) et la formation des plasmocytes et lymphocytes B mémoires spécifiques du donneur. Ce qui est observé par le fait que l'AMR est une cause principale d'insuffisance organique chronique, et il est actuellement responsable de près d'un quart des pertes de greffe totales.

La reconnaissance de l'allo-antigène par le récepteur des cellules B (BCR) joue un rôle important dans le déclenchement de la réponse immunitaire humorale et de la différenciation des lymphocytes B en cellules plasmiques productrices d'anticorps (Ab) et en cellules B mémoires. Après l'activation des lymphocytes B dépendant des lymphocytes T et la formation du centre germinatif (CG), les cellules B subissent une expansion clonale, une commutation isotypique, une hypermutation somatique, et une maturation et une sélection d'affinité. Ils quittent le GC en tant que cellules B mémoire hautement spécifiques et/ou les plasmocytes produisant un Ab. La persistance du GC et la sélection des cellules B effectrices de haute affinité sont régulées par des signaux pro- et anti-apoptotiques induits lors de la liaison l'antigène (Ag) et des interactions à médiation cellulaire. La myeloid cell leukemia 1 (Mcl-1), un membre anti-apoptotique de la famille des B-cell lymphoma (Bcl-2), est essentiel au maintien de la réaction GC et de la différenciation des lymphocytes B.

Une inflammation persistante due à une stimulation et une réponse immunitaire continue peut conduire à la formation de structures lymphoïdes tertiaires (TLS), qui ressemblent à des GC. Le développement de TLS dans lesquels une réaction fonctionnelle de GC ectopique a été mise en évidence en transplantations humaines ayant développé une AMR chronique (cAMR)

Le but de cette recherche est de caractériser davantage les cellules B infiltrant les greffes rénales avec cAMR chez les malades transplantés rénaux. Des cellules B infiltrantes ont été détectées dans des greffes rénales de cAMR, par coloration de CD19 de coupes histologiques de

reins de patients atteints de cAMR. Un double marquage avec un anticorps contre Mcl-1 a mis en évidence une colocalisation de Mcl-1 avec CD19 comme observé dans les cellules GC. Nos résultats sont compatibles avec la notion que les cellules B infiltrant les organes greffés font partie du TLS et jouent un rôle dans une réponse immunitaire locale en cours et la production de cellules B effectrices

Deuxièmement, nous avons cherché à développer des stratégies pour inhiber l'activation et la survie des cellules B activées. Nous avons donc étudié les moyens de perturber les réponses et la survie des cellules B des GC, dans lesquelles la Spleen tyrosine kinase SYK joue un rôle clé en aval du BCR. Nous avons inhibé son activité pour bloquer la signalisation du BCR. L'inhibition de l'activité de SYK diminue la viabilité des cellules et le niveau de la protéine anti-apoptotique Mcl-1 dans les cellules BL41, une lignée cellulaire de lymphome de Burkitt servant de modèle de centroblastes GC. L'inhibition de SYK a montré une réponse dépendante de la dose concernant la viabilité cellulaire. Lors de l'inhibition de SYK, la diminution de Mcl-1 est caspase indépendante et seulement partiellement protéasome dépendant. Nous avons mis en évidence que l'inhibition de SYK régule l'expression de Mcl-1 au niveau transcriptionnel, comme cela a été confirmé par la PCR quantitative en temps réel. Le Signal transducteur et activator of transcription 3 (STAT3) a été identifié comme un régulateur majeur de la transcription du gène Mcl-1. L'analyse immunohistologique a montré que STAT3 est exprimé dans le noyau des cellules BL41 dans des conditions basales, où il peut médier la transcription du gène Mcl-1. L'inhibition de SYK empêche la translocation de STAT3 du cytoplasme vers le noyau, suggérant un rôle pour STAT3 dans la régulation de l'expression du gène Mcl-1 par la signalisation BCR. L'inhibition de STAT3 conduit à une viabilité réduite des cellules BL41 qui était associée à des niveaux réduits de transcription de Mcl-1. Enfin, la surexpression du gène Mcl-1 dans les cellules BL41 traitées par des inhibiteurs de SYK et STAT3 permet de restaurer la survie des cellules indiquant l'importance de Mcl-1 dans le maintien de la viabilité des cellules B G.

Nous avons confirmé l'effet de l'inhibition de SYK sur la survie des cellules B primaires liée également à la diminution de la protéine Mcl-1, sur l'activation des lymphocytes B déterminée par l'expression de CD80 et sur la sécrétion d'IgG in vitro.

En conclusion, nos résultats mettent en évidence que l'inhibition de SYK affecte la survie cellulaire médiée par la signalisation BCR par la régulation négative de la transcription du gène Mcl-1. Dans les cellules GC « like », cette régulation de Mcl-1 semble être en partie dépendante de la régulation du gène médiée par STAT3. Ainsi dans le cas de l'AMR, SYK peut être une cible potentielle pour améliorer la survie du greffon

List of Abbreviations

A

aAMR:	Acute antibody mediated rejection
Ab:	Antibody
ABO:	Blood group A, B and O
ADCC:	Ab-dependent cellular cytotoxicity
Ag:	Antigen
AID:	Activation induced cytidine deaminase
AKT:	Protein kinase B (PKB)
AMR:	Antibody mediated rejection
APAF-1:	Apoptotic activating factor 1
APC:	Antigen presenting cell

B

Bad:	Bcl-2 antagonist of cell death
BAFF:	B-cell activating factor
Bak:	Bcl-2 Antagonist Killer 1
Bax:	Bcl-2 Associated X protein
BAY:	Bay61-3606
Bcl-2:	B-cell lymphoma 2
BCL-6:	B-cell lymphoma 6
B-CLL:	B-cell chronic lymphocytic leukemia
BCR:	B-cell receptor
BrdU:	Bromodeoxyuridine
BH:	Bcl-2 homology
Bid:	BH3 interacting-domain death agonist
Bik:	Bcl-2 interacting killer
Bim:	Bcl-2 interacting mediator of cell death
BL:	Burkitt's lymphoma
Blimp-1:	PR domain zinc finger protein 1 (PRDM1)
BLNK:	B-cell linker protein
BM:	Mature B-cells
Bmf:	Bcl-2 modifying factor
Breg:	Regulatory B-cell
BTK:	Bruton's tyrosine kinase

C

C:	Complement protein
CAD:	Caspase activated DNase
cAMR:	Chronic antibody mediated rejection
CCL:	C-C motif chemokine

CCR:	ligand C-C chemokine receptor type
CD:	Cluster of differentiation
CIN85:	Cbl-interacting protein of 85 kDa
CLT:	Cytotoxic T-lymphocyte
CSR:	Immunoglobulin class switch recombination
CTLA-4:	Cytotoxic T-lymphocyte-associated protein 4
CXCL:	C-X-C motif chemokine ligand
CXCR:	C-X-C chemokine receptor

D

D:	Diversity
DAG:	Diacylglycerol
DAMP:	Danger associated pattern
DC:	Dendritic cell
DD:	Death domain
DISC:	Death inducing signaling complex
DNA:	Deoxyribonucleic acid
DR:	Death receptor
DSA:	Donor specific HLA Ab
DZ:	Dark zone

E

EBI2:	Epstein-Barr virus-induced gene 2
EBV:	Epstein-Barr virus
ERK:	Extracellular signal-regulated kinase

F

FADD:	Fas-associated protein with death domain
FasR:	Fas receptor
FcR:	Fc receptor
FDC:	Follicular dendritic cell

G

GC:	Germinal center
Grb2:	Growth factor receptor-bound protein 2
GSK3:	Glycogen Synthase Kinase-3

H

HLA:	Human Leukocyte antigen
Hrk:	Harakiri

HGG: Hypogammaglobulinemia

I

IAPs: Inhibitors of apoptosis

ICAD: Inhibitor of CAD

ICOS: Inducible T-cell co-stimulator

ICOSL: ICOS ligand

Ig: Immunoglobulins

IKK: Inhibitor of α B kinase

IL: Interleukin

IP₃: Inositol triphosphate

IRF: Interferon-regulatory factor

ITAM: Immunoreceptor tyrosine based activation motif

IV-IG: Intravenous immunoglobulin

J

J: Joining

JAK: Janus kinase

JNK: c-Jun NH2-terminal kinase

L

LZ: Light zone

M

MAC: Membrane attack complex

MAPK: Mitogen activated protein kinases

MBC: Memory B-cells

Mcl-1: Myeloid cell leukemia-1

MEF2: Myocyte-specific enhancer factor 2

MHC: Major histocompatibility complex

MOM: Mitochondrial outer membrane

MOMP: Mitochondrial outer membrane permeabilization

mTORC1: Mechanistic target of rapamycin Complex 1

mSOS: Mammalian son of sevenless

N

NFAT: Nuclear factor of activated T-cells

NF- α B: Nuclear factor α B

NK: Natural killer cell

NLR: NOD like receptors

P

PAMP: Pathogen-associated molecular pattern

PC: Plasma cell

PD-1: Programmed cell death protein-1

PD-L1: PD ligand 1

PI3K: Phosphoinositide 3 kinase

PIP₂: Phosphatidylinositol 4,5-bisphosphate

PIP₃: Phosphatidylinositol 3,4,5-triphosphate

PKC: Protein kinase C

PLC γ 2: Phospholipase C γ 2

PRR: Pattern recognition receptors

PTLD: Post-transplant lymphoproliferative disorder
Puma: p53 up-regulated mediator of apoptosis

R

RLR: RIG like I receptors

RNA: Ribonucleic acid

S

SHC: SH2 domain-containing protein

SHM: Somatic hyper mutation

SLO: Secondary lymphoid organ

STAT3: Signal transducer and activator of transcription 3

SYK: Spleen tyrosine kinase

T

TCR: T-cell receptor

T_{FH}: T follicular helper cell

TG: Transplant glomerulopathy

TGF- β : Transforming growth factor β

Th: T helper cell

TLR: Toll like receptors

TLS: Tertiary lymphoid structures

TNF: Tumor necrosis factor

TNFR1: Tumor necrosis factor receptor-1

TRADD: TNFR1 associated DD protein

Treg: Regulatory T-cell

V

V: Variable

Z

ZAP-70: Protein ζ -chain-associated protein kinase of 70kDa

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Introduction

Introduction

Introduction

(I) Transplantation immunology

(1) General history transplantation immunology

Renal failure is a major public-health concern in developed countries, especially in France (Figure 1). Renal transplantation is the major therapeutic method in case of end-stage renal failure, due to the association with a better quality of life, longer survival time and the cost efficiency as compared to dialysis.

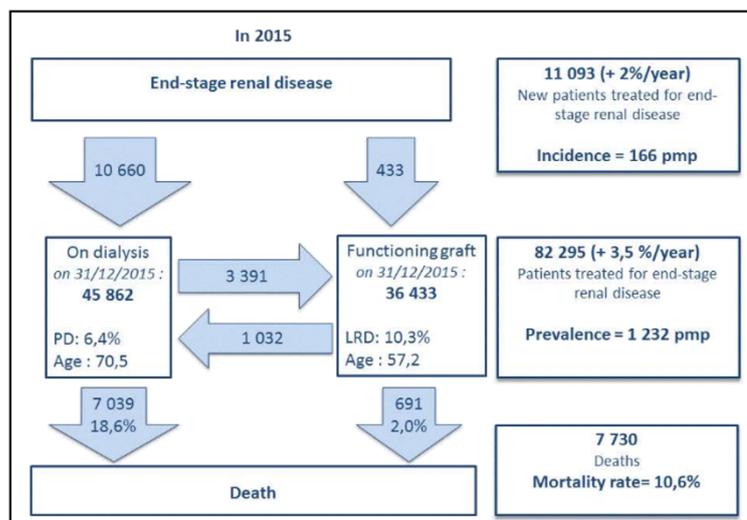


Figure 1: The overall incidence of end-stage renal disease, dialysis and organ transplantation as well as mortality in France in 2015.

Figure taken from Réseau Epidémiologie et Information en Néphrologie, rapport annual 2015. PD: peritoneal dialysis; pmp: per million population.

Organ transplantation is the act of transferring the organ of one individual to another. In the beginning of the twentieth century the idea arose to replace a non-functioning organ by a healthy functioning organ. However following organ transplantation, the immune system, which is a combination of cells and molecules and is responsible for the protection of the human body from infectious disease, can cause severe problems.

The reaction to foreign molecules coordinated by the immune system is called the immune response. According to historians the first mention of immunity came from Thucydides around 500 BC, however the ancient Chinese had a custom of using powder from skin lesions of smallpox patients to protect children against the disease. The first clear example of immunity and immunology as an experimental discipline came from Edward Jenner. He found that milkmaids who recovered from cowpox never developed the more dangerous smallpox, and by injecting a little boy with the cowpox and later with smallpox he laid the road to vaccination. (Brunham & Coombs, 1998) The importance of vaccination, and the underlining role of the immune system,

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has been shown by the world health organization when they observed in 1980 that the smallpox was the first disease to be eradicated worldwide. However, the immune system does not only recognize infectious diseases, non-infectious molecules can also induce an immune response and therefore in some cases can cause harm and disease.

In the case of organ transplantation, the immune system can cause harm leading to the rejection of the transplanted organ. A pioneer in the transplantation field is P.B. Medawar, who following his research in skin graft rejection, found that re-transplantation of skin-grafts led to a higher prevalence of graft rejection. (Medawar, 1944) Another pioneering research paper in transplant immunology using mice, showed acquired immunological tolerance to antigens (Ag; molecules that are recognized by immune cells and induce an immune response in humans) from other mice, called alloAg. (Billingham, Brent, & Medawar, 2003) The team of Joseph Murray, at the Peter Bent Brigham hospital in Boston, performed the first successful renal transplantation in 1954 by transferring the kidney from one identical twin to the other. (Murray, Harrison, & Merrill, 1956) The data acquired by several studies showed the importance of immune responses directed against the graft, and in the early 1960s by using chemical immunosuppressors the first transplantation between non-identical individuals was a fact. To date transplantation is a standard practice to treat non-functioning solid organs; such as the heart, lungs, pancreas, kidneys, intestines and liver.

(2) Hyperacute, acute and chronic rejection

There are three types of organ rejection, hyperacute, acute and chronic rejection. Hyperacute rejection occurs within the first few hours after the transplantation and is mainly caused by antibodies (Ab) recognizing foreign proteins. These are either encoded by the Human Leukocyte antigen (HLA) gene complex and expressed on the surface of cells on the graft or sugar molecules corresponding to the blood group. Proteins encoded by the HLA gene complex include major histocompatibility complex (MHC) molecules and components of the complement system. Following the recognition, Ab complexes are formed and the complement is activated leading to irreversible damage of the graft. Hyperacute rejection cannot be treated, however it can be prevented. By screening the patient for anti-blood group A, B and O (ABO) Ag and donor specific HLA Abs (DSA), followed by exclusion of donor organs that are recognized by these antibodies, as well as the removal of these HLA antibodies prior to transplantation. (Crespo et al., 2001; Higgins et al., 1996; Rydberg, 2001)

Acute rejection occurs within several days to months following the transplantation and is caused by immune cells such as dendritic cells (DC) and cells from the adaptive immune system. Activated T-cells can cause direct killing of the donor cells and Abs secreted by activated B-cells

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can cause graft injury and killing of the donor cells, inducing organ rejection. T-cells can induce an inflammatory state by the secretion of cytokines followed by the recruitment and activation of inflammatory cells, bound Abs can recruit and activate inflammatory cells as well. Acute rejection can be treated by the use of immunosuppressive drugs, and the faster the treatment the better because the physical damage to the organs is irreversible. (Alegre, Florquin, & Goldman, 2007)

Long term graft loss can occur months, even years after the transplantation and is the main cause of long term graft loss is chronic rejection, which can be a combination of both humoral and cell mediated immune responses. Chronic rejection can occur even after the acute immune responses have ceased to persist. Long term graft loss can be a multifactorial process and combination of several factors can lead to eventual graft loss. (Libby & Pober, 2001; Massy, Guijarro, Wiederkehr, Ma, & Kasiske, 1996)

(3) Innate immunity and organ rejection

The immune response can be divided into two distinct responses based on time and specificity, namely innate and adaptive immunity, or the early and late response (Figure 2).

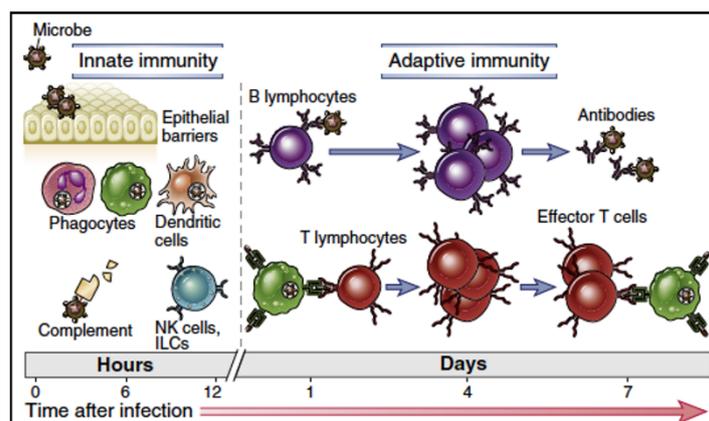


Figure 2: The innate or early and the adaptive or late immune response.

The innate immune response, which is the first defense against microbes, recognizes general molecular patterns. It consists of a physical epithelial barrier, a combination of immune cells, such as dendritic cells and phagocytes, and circulating proteins, such as the complement. The adaptive immune response is highly specific for antigens and can last for days. The main cells responsible for the adaptive immunity are cells T- and B-lymphocytes. Figure taken from Abbas et al. (Abbas, Lichtman, & Pillai, 2014, p. 3) NK: Natural Killer; ILC: Innate Lymphoid Cell

Acute inflammation is a process that happens upon innate immune activation and its function is to eliminate harmful microbes, damaged cells, and initiate tissue repair. This happens through the recruitment of special immune cells, white blood cells also known as leukocytes, and the secretion of cytokines and chemokines that help enhance the inflammatory response. During microbial/exogenous infection the first step is recognition of microbes via a limited number of

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pattern-recognition receptors (PRR); such as toll like receptors (TLR), NOD like receptors (NLR), RIG-I-like receptors (RLR) and complement receptors. These PRRs share several common trademarks such as the fact that they are highly conserved throughout evolution and encoded by the germline deoxyribonucleic acid (DNA). (Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999; J. Liu, Qian, & Cao, 2016) PRRs are expressed extra- or intracellular by various cells or are found as soluble proteins in the blood and extracellular fluids. PRRs recognize structures that are non-self and characteristic for microbes, these structures are called pathogen-associated molecular patterns (PAMP). In general, these PAMPs are essential for the survival of the microbes and therefore they are less prone to mutations making it more difficult to evade the innate immune system. (Medzhitov, 2007) Several studies have indicated the role of PRRs in the recognition of endogenous molecules, such as heat shock protein and the content of necrotic cells. TLRs and non-Ag related inflammatory responses have been highlighted in organ rejection. (Gluba et al., 2010; Goldstein, Tesar, Akira, & Lakkis, 2003; Land, 2005; Pockley, 2001) Besides to pathogen induced PRR activation, injured or dying cells are able to induce an inflammatory response by secreting danger-associated molecular patterns (DAMP). These secreted DAMPs can be recognized by several receptors present on immune and non-immune cells, such as TLRs and integrins. (Kono & Rock, 2008; Rosin & Okusa, 2011; Xiang & Fan, 2010)

Next to the induction and augmentation of the inflammatory response, and direct effect on microbes and damaged cell, another important role of the innate immune cells is phagocytosis. A process in which leukocytes ingest and process microbes, harmful molecules or dead cells. In this way phagocytes neutralize the harmful molecules and process them into peptides for Ag presentation. (Greenberg & Grinstein, 2002) Ag presentation is accomplished by loading the peptides the MHC. There are three types of MHC, namely Class I, class II and class III MHC. MHC I and II molecules play a role in presenting processed Ag on the cell surface, while MHC III molecules have a physiological role, and encode molecules such as members of the complement, cytokines and heat shock proteins. Depending on the manner of internalization of the Ag, peptides are loaded into the different MHC class I or II molecules. Endosomal/lysosomal processed proteins are loaded into class II MHC while cytosolic processed peptides are loaded into class I MHC. (Ackerman & Cresswell, 2004; Bryant, Lennon-Duménil, Fiebiger, Lagaudrière-Gesbert, & Ploegh, 2002) All nucleated cells express Class I MHC, while class II MHC are expressed by specialized Ag presenting cells (APCs) such as DCs, other leukocytes and B-lymphocytes. (Abbas et al., 2014, pp. 107–114; Hennecke & Wiley, 2001) MHC molecules play an important role in the activation of the adaptive immune response.

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Despite the fact that natural killer (NK) cells have a lot in common with the cells that comprise the adaptive immune system, they are generally classified as innate immune cells due to their lack of specific receptors. (Vivier, 2006) NKs can directly induce cell death and produce an array of cytokines inducing inflammation and activation of other immune cells. (Kitchens et al., 2006; Vivier et al., 2011) It has been shown by several transplantation studies, in which the settings were to either prevent or provoke a NK immune response, they failed to do so, indicating no direct role of NK cells in solid organ transplantation. (Heidecke et al., 1985; Markus et al., 1991; Zijlstra et al., 1992) Therefore role of NK cells has conventionally been dismissed in solid organ rejection. However, more evidence arises showing that while conventionally being dismissed there might be an important role for NK cells during rejection, for example, they are able to reject hematopoietic cells. NK cells can react to allogeneic class I MHC and NK cells that infiltrate transplanted organs have been shown to attack donor-derived cells *in vitro* in rat studies. (Petersson et al., 1997) Another mouse study showed that by depleting NK cells the rejection of cardiac transplants was significantly delayed in comparison to wild-type mice. (Maier et al., 2001) Together with several other studies it has been also been suggested that NK cells play a facilitating role in the alloreactive T-cell response, connecting the innate and adaptive immune system. (Kitchens et al., 2006) This suggest that while acting alone NK cells might not be able to induce an allo-immune response, they are involved in the process of organ rejection.

Besides the circulating cells that can recognize, kill and induce the innate immune response, there are soluble recognition and effector molecules such as immunoglobulins (Ig), also known as Abs, and members of the complement system. Even though Abs are the product of humoral immunity and thus are a part of the adaptive immune system, there are a set of circulating Abs, which are present before birth and without previous infection, vaccination or other foreign Ags, called natural Abs. These natural Abs are polyreactive and recognize a wide range of Ags and play an important role in the innate immune defense against pathogens. (Khasbiullina & Bovin, 2015) In organ transplantation the role of natural antibodies lays in the recognition of ABO- and HLA-Ags (Milland & Sandrin, 2006), antibody mediated rejection (AMR) through DSA and complement activation will be discussed in more detail.

(4) Adaptive immunity and organ rejection

Adaptive immunity, also known as specific or acquired immunity, provides a highly specific immune response as well as immunological memory. Lymphocytes and their secretion products, such as Abs and cytokines, are the main components of the adaptive immune system. (Hoffmann et al., 1999) The adaptive immune response can be divided into two responses, the cell mediated (T-cell) and humoral (B-cell) immune responses (Figure 3).

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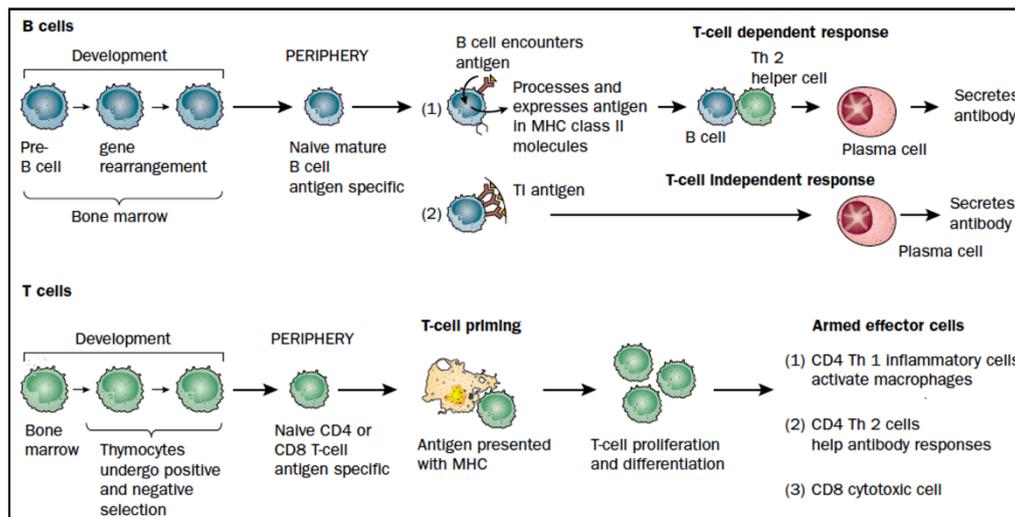


Figure 3: Summarizing overview of T and B-cell development and function.

Lymphocyte progenitors both come from the bone marrow, however the development of T-cells continues in the Thymus. After maturation, the lymphocytes migrate to the periphery where they can encounter antigens, followed by activation and the induction of the immune response. Figure taken from Parkin and Cohen. (Parkin & Cohen, 2001)
MHC: Major histocompatibility complex; TI: T-cell independent; Th: T helper; CD: Cluster of Differentiation

Several organs are important for the generation of immune cells (primary lymphoid organs) and the activation and differentiation (secondary lymphoid organs). The bone marrow and thymus, the primary lymphoid organs, is where lymphoid progenitors are generated and where B- and T-lymphocytes mature. Lymph nodes, the spleen and mucosal associated-lymphoid tissue, such as the tonsils, are secondary lymphoid organs (SLO) and are specified organs that can efficiently concentrate Ags and facilitate the adaptive immune response. (Drayton, Liao, Mounzer, & Ruddle, 2006)

SLOs are pre-programmed and formed during embryogenesis in a process called organogenesis. The organogenesis of the lymph nodes has been well documented (Mebius, 2003; Wigle et al., 2002; Y. Yang & Oliver, 2014) and seems to be a rigid and well-defined process, however, following an immune response there is a plasticity within these organs inducing a flow of immune cells. (Goeringer & Vidić, 1987; He et al., 2002; Newberry & Lorenz, 2005) The cellular organization of lymphoid cells in SLOs show a similar prominently organized micro-environment of lymphocytes. This will be demonstrated looking at the organization of lymph nodes, within the lymph nodes, distinct regions can be found based on the lymphocyte content. The lymph node can be roughly divided into 3 different regions, macrophage rich regions, B-cell follicles which contain as the name states B-cells and also follicular dendritic cells (FDC), and T-cell zones. The T-cell zones contain sophisticated structures of vessels which constantly replenish the lymphocytes and a selection of APCs from and to the periphery. (Jason G. Cyster, 1999; Y. X. Fu & Chaplin, 1999)

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Intricate studies using microscopy technologies have shown the plasticity of resting lymphocytes within the lymph nodes, indicating confined movement of T-cells, B-cells and FDCs within their assigned cellular region. (Bajénoff et al., 2006; Okada et al., 2005; Stoll, Delon, Brotz, & Germain, 2002) Showing that within lymph nodes all is optimized to enhance the possibilities for naïve lymphocytes to encounter Ags and APCs.

The adaptive immune response that occurs during solid organ transplantation is the recognition of donor alloAg, usually the MHC. Two mechanisms for allo-recognition have been proposed, the direct and indirect pathway, two independents but not mutually exclusive processes. The direct pathway follows recognition of intact donor MHC molecules on donor cells, which can be recognized by the recipient's T-cells. The indirect pathway is the representation of processed donor alloAg by the recipient's APCs followed by T-cell activation (Figure 4). (Afzali, Lombardi, & Lechler, 2008) While both processes of Ag representation are not mutually exclusive it seems that the direct pathway plays a more important role in acute rejection, while the indirect is more involved in chronic rejection. (Sayegh & Carpenter, 1996; Vella et al., 1997)

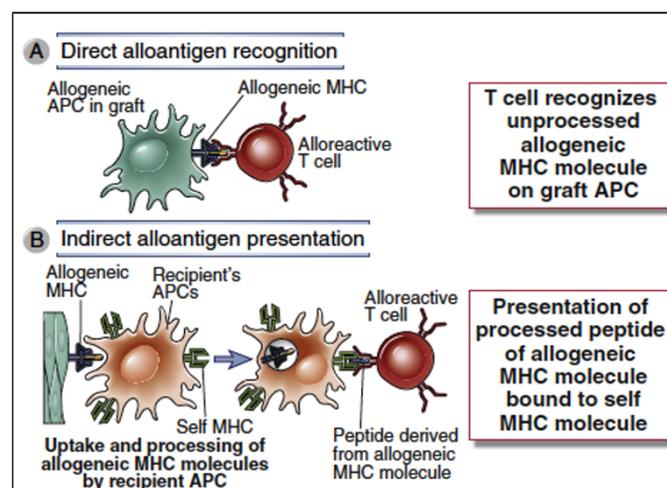


Figure 4: Mechanisms of allorecognition.

Dendritic cells originating from the donor lead to direct allorecognition while the processed alloantigens which are presented by the recipient APCs lead to indirect allorecognition Figure adapted from Abbas et al. (Abbas et al., 2014, p. 363) APC: Antigen Presenting Cell; MHC: Major Histocompatibility Complex

Evidence has arisen that there is a third mechanism of allo-antigen recognition, namely the semi-direct pathway. It is suggested that in this pathway there is a transfer of intact MHC molecules between the donor cells and recipient's APCs as well as presentation of allo-antigens through phagocytosis of necrotic cell material. (Smyth et al., 2006) Acquisition of intact MHCs can be by direct cell-to-cell contact or via release and uptake of exosomes (small vesicles secreted by the cells). (Herrera et al., 2004; Morelli et al., 2004)

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Following Ag uptake by APCs, these cells migrate to secondary lymphoid tissue for Ag presentation and activation of recipient's T-lymphocytes, a process is called sensitization. (Larsen, Morris, & Austyn, 1990) Lakkis et al. (Lakkis, Arakelov, Konieczny, & Inoue, 2000) showed that, in the case of cardiac transplantation in mice, migration to lymphoid tissue is necessary for the rejection to occur, since mice lacking secondary lymphoid organs do not show organ rejection. For the activation of naïve T-cells, DCs that encountered and processed Ags migrate to secondary lymphoid tissue where the naïve T-cells reside. T-cell subpopulations are classified based on the T-cell receptor (TCR), their specificity for Ags and their effector function. An extent of the T-cell population can be divided based on their ability to bind Ags presented by either class I MHC and class II MHC. (Carpenter & Bosselut, 2010; Taniuchi, 2016) As well as by the expression of co-receptors cluster of differentiation (CD) 8, which binds class I MHC, and CD4 that binds class II MHC, establishing distinct T-cell populations namely CD8⁺ effector T-cells and CD4⁺ T helper cells (Th). (Ellmeier, Sawada, & Littman, 1999; Taniuchi, 2016) Upon Ag recognition and activation by cytokines the naïve T-cells, both CD4⁺ and CD8⁺, undergo clonal expansion followed by differentiation leading to the formation of highly specific effector and memory T-cells. (Capece & Kim, 2016; H. Fu, Ward, & Marelli-Berg, 2016; Stein, 2015) The initiation of the immune response leads to infiltration of immune cells in the graft followed by immune mediated organ rejection (Figure 5).

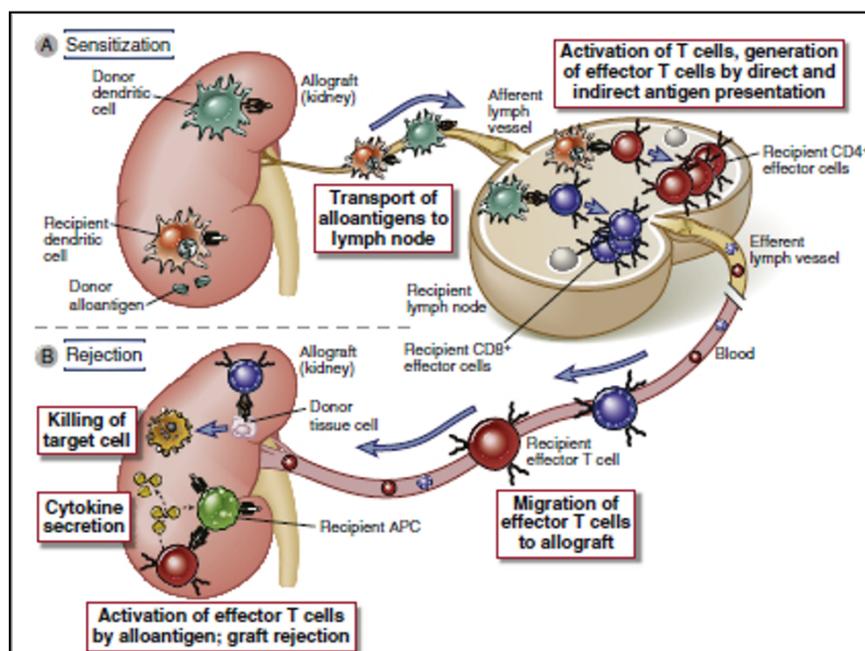


Figure 5: Cell mediated immune response during graft rejection.

The first step is the sensitization step in which DCs move to secondary lymphoid tissue where they bind and activate recipient T-cells through antigen presentation of donor MHC. Following T-cell activation, the effector T-cells migrate to the graft where they induce a local immune response leading to graft rejection. Figure adapted from Abbas et al. (Abbas et al., 2014, p. 366) DC: dendritic cell, HMC: Major histocompatibility complex

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As mentioned before there are two main types of T-cells, the CD4⁺ Th and the CD8⁺ effector T-cells, which both have different functions during the immune response. As the name hints on the function, Th-cells help by acting as a mediator of effector functions in the killing of infected cells. The Th-cells can interact with effector cells as well as secreting a specific pattern of proteins leading to different immune responses. Where Th1 mainly, but not only, induce a phagocyte dependent inflammation, Th2 induce a strong Ab response, amongst others, and can inhibit phagocytosis creating a phagocyte independent inflammation. (Abbas, Murphy, & Sher, 1996; Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 2005; Romagnani, 1991) Th17 cells are abundant in mucosal areas where they are involved in inflammatory responses through neutrophil and monocyte activation. (Littman & Rudensky, 2010; Weaver, Hatton, Mangan, & Harrington, 2007) A portion of CD4⁺ derived T-cells, CD25⁺ regulatory T-cells (Treg) play an important role in immunological tolerance and autoimmunity. (Sakaguchi, 2005) The involvement of Treg in inducing immunological tolerance for transplanted organs has been shown. In transplantation mouse models, it was shown that Tregs stimulated with alloAg against the transplanted organ improved the overall graft survival. (Joffre et al., 2008; P. A. Taylor, Lees, & Blazar, 2002) CD8⁺ T effector cells differentiate into Ag specific cytotoxic T-lymphocytes (CTL) that act on the infected cell itself leading to its elimination. (Berke, 1995; Shresta, Pham, Thomas, Graubert, & Ley, 1998) One of the main interactions of CTLs lays the binding to FasReceptor (FasR), which is a death receptor (DR) that leads to programmed cell death when engaged, inducing apoptosis of the targeted cells. (Brunner et al., 2003) Another cytotoxic effect induced by CTLs is through the secretion of perforin, creating pores in the target cells, followed by the release of granules (containing for example proteases granzyme A and B) through these pores and inducing cell death. (Trapani & Smyth, 2002) Though CTLs can directly interact and kill infected cells they also secrete cytokines that can recruit activate other effector cells, such as macrophages, and induce an inflammatory response. (Shresta et al., 1998; Harty & Bevan, 1999)

(II) Humoral immunology

(1) Defining antibody mediated rejection

Not only the cell mediated immune response, as described above, plays a role in organ rejection, and as mentioned before part of the adaptive immune response is due to differentiated B-lymphocytes and their secreted Abs. In the interest of this research the role humoral immunology will be discussed in more detail below. The discovery of B-cells was not due to the identification of a cell but more through the identification of a protein, specifically the Ab, leading to the discovery of the Ig producing plasma cell as early as 1948. (Sund et al., 2003)

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During the early 1990s the first characterizations of acute AMR (aAMR) in renal transplantation included the infiltration of neutrophils in peritubular capillaries and *de novo* DSA directed against donor MHC class I molecules. (Halloran et al., 1990; Halloran, Schlaut, Solez, & Srinivasa, 1992) Another breakthrough in recognizing anti-donor humoral activity was the identification and the deposit of complement protein (C)4d, a member of the complement pathway, as a predictive marker for aAMR. (Collins et al., 1999; Feucht et al., 1993) Later histological studies on chronically rejected organ transplants linked DSA and C4d as features of chronic AMR (cAMR) as well. (Mauyyedi et al., 2001; Regele et al., 2002) Figure 6 indicates the proposed stages of humoral graft rejection. The Banff Working Group classification system for renal allograft biopsies has been discriminating between T-cell mediated rejection and humoral rejection. The latest update of the Banff classification of AMR in 2013 necessitates the histological evidence of tissue injury, Ab interaction with vascular endothelium and the evidence of circulating DSA. (Haas, 2014; Racusen, Halloran, & Solez, 2004) The pivotal role of B-cells and Abs post transplantation becomes more and more evident. Apprehension of the underlying process of AMR is ongoing and pivotal in understanding organ rejection and improving long term organ survival.

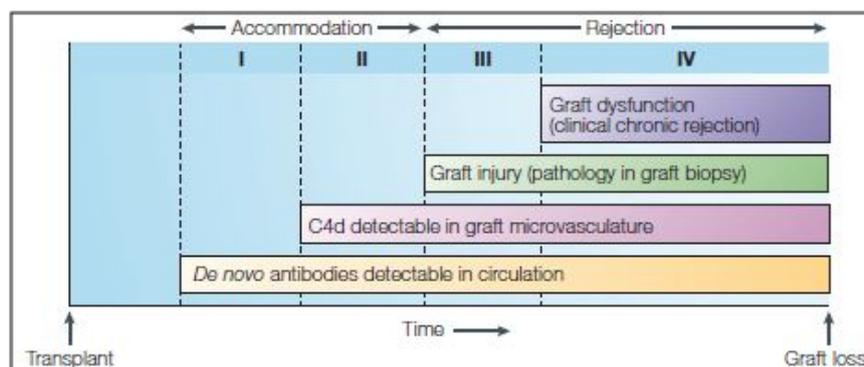


Figure 6: Proposed stages of antibody-mediated rejection based on time from transplantation to graft loss.

The timespan of the several stages of antibody-mediated rejection can be days in case of acute rejection and months or years in the case of chronic rejection. Anti-HLA antibodies can be produced at any time after organ transplantation, while the following successive stages of complement detection, graft injury and eventually graft dysfunction can appear over different timespans. During the accommodation state antibodies are present as well as complement activation, however no graft injury is observed yet, meaning there is no actual organ rejection taking place but the tell-all signs are there. Figure adapted from Colvin and Smith and Norvell et al. (Colvin & Smith, 2005; Norvell, Mandik, & Monroe, 1995) C4d: complement component 4d.

DSA in hyperacute rejected kidneys lead to cyanosis, blue discoloration of the organ due to lack of blood shunting, and necrosis of the transplanted tissue. (Williams et al., 1968) Graft injury caused by aAMR are notably necrosis, cortical infarction, acute glomerulitis, and glomerular and vascular fibrin thrombi. (Magil & Tinckam, 2003; Trpkov et al., 1996) The main injuries to kidney grafts in case of cAMR is transplant glomerulopathy (TG), which is the formation of

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multiple layers of the basement membrane of capillary walls in kidney glomerulus and arterial intimal fibrosis, which occurs due to repeated endothelial injury and healing following persistent inflammation (Colvin, 2009; Einecke et al., 2009)

Endothelial graft injury, a capillary injury, and loss of function can be due to several processes which occur when DSA bind to the grafts endothelial cells. Bound DSA recruit several members of the immune system, through binding of the Fc-portion of the Abs, Fc- α , Fc- ϵ , Fc- γ or Fc- μ depending on the Abs isotype IgA, IgE, IgG or IgM resp, to the complement and the Fc receptors (FcR; Fc α R, Fc ϵ R, Fc γ R and Fc μ R resp.), which are expressed on effector cells of both the innate and adaptive immune response. (Nimmerjahn & Ravetch, 2007)

In case of organ transplantation, the damaging role of IgG DSA has been highlighted, bound DSA can initiate the complement cascade by forming a complex with C1, through binding to the Fc- γ portion of the Ab, which is then activated and cleaved. (Lachmann & Hughes-Jones, 1984) Activated C1 can bind and activate C4 and C2 to form a membrane bound complex C4bC2a. C4 when activated is cleaved to form a soluble C4a and membrane bound C4b. C4b is subsequently cleaved in a membrane bound C4d and soluble C4c, which activates and promotes C3 cleavage into bound C3b and soluble C3a. The formation of a complement complex around C3b leads to the cleavage of C5 into bound C5b and soluble C5a, which acts as a stimulator of inflammation. C5b initiates a cascade complement reactions leading to the formation of a membrane pore, the membrane attack complex (MAC), followed by direct graft injury of the endothelial graft cells. (Müller-Eberhard, 1988) The underlining role of the complement system following organ transplantation is evident, for example it has been shown that that the production of C5b-9 by host macrophages promotes rejection (Z. Qian et al., 1999) and deficiencies in the complement can prolong graft survival (Pratt, Basheer, & Sacks, 2002) When the complement activation stops at C4, preventing the activation of C3 in the graft, there is a delay in graft injury and rejection. (Baldwin, Kasper, Zachary, Wasowska, & Rodriguez, 2004) Next to direct endothelial damage caused by complement activation and MAC formation, the cleavage products of several complement members can induce an inflammatory response through promoting the infiltration of inflammatory cells. (Morgan & Harris, 2015; Ricklin & Lambris, 2013)

For a long time the disposition of C4d in transplanted organs has been the main indicator of AMR, however the emerging role of complement independent mechanisms in AMR have led to further investigation in the role of DSA in the recruitment of immune cells as well. (Sis & Halloran, 2010; Haas, 2014) The binding of DSA to HLA on vascular endothelial cells can lead to direct effects on functional changes within these cells. Several studies have shown activation of signaling pathways that are involved in cell survival and cell proliferation, leading to neointimal

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formation and thickening of the vascular graft regions. (Y.-P. Jin et al., 2004; Jindra et al., 2008; Valenzuela & Reed, 2015)

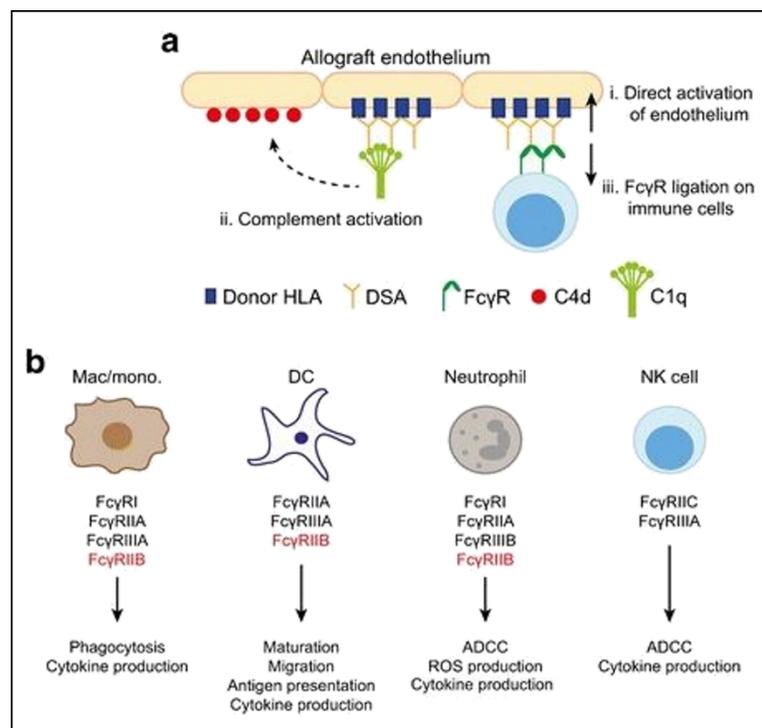


Figure 7: Multifactorial cellular and molecular effects of donor specific antibodies against the graft

When DSA bind to the endothelium of grafted organs several mechanisms can be set in motion; (i) a direct activation of the endothelium leading to direct functional changes in these cells, (ii) activation of the complement by binding of c1 to the Ig Fc region (iii) and binding of FcR expressing cells to the Ig Fc region, inducing their activation and effector functions. (a) Depending on the Ig isotype several different immune effector cells can be recruited to the graft and activated. Once activated these cells either mature and help in antigen uptake and presentation to other immune cells or induce an inflammatory response and cause endothelial damage. Figure adapted from Castro-Dopico and Clatworthy. (Castro-Dopico & Clatworthy, 2016) ADCC: antibody-dependent cellular cytotoxicity; C: complement; DC: dendritic cell; DSA: donor specific antibody; FcR: Fc receptor; Mac: macrophage; Mono: monocytes; NK: natural killer

The role of DSA mediated HLA signaling in the recruitment of monocytes, such as macrophages and neutrophils, through p-selectin upregulation on the surface of the endothelial cells has been observed as well. (Valenzuela, Mulder, & Reed, 2013; Yamakuchi et al., 2007) Valenzuela et al. (Valenzuela, Hong, et al., 2013) have shown that when P-selectin is selectively blocked there is a reduction in the infiltration of macrophages, as well as other immune cells, within the graft. On the other hand, the bound DSA itself can also recruit immune effector cells through their Fc portion which is recognized by the FcRs on these cells. For example, FcγR activated macrophages, monocytes and NKs induce phagocytosis and release cytokines such as tumor necrosis factor (TNF), interleukin-1 α (IL-1 α), IL-6 and neutrophil chemo-attractants. (Morimura

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et al., 1990) FcR engagement on DCs can lead to their maturation and promotes their migration, increase antigen uptake efficiency and presentation through the MHC molecules, and the production of inflammatory cytokines. (Menna R. Clatworthy et al., 2014; Regnault et al., 1999) Neutrophil activation through their FcR leads to Ab-dependent cellular cytotoxicity (ADCC), as well as the production of reactive oxygen species and inflammatory cytokines. (Coxon et al., 2001; Mayadas, Tsokos, & Tsuboi, 2009) FcR crosslinking of NK cells to DSA leads to ADCC and the release of inflammatory cytokines. (Legris et al., 2016; Lin, Plenter, Coulombe, & Gill, 2016)

Taken together all these effects of DSA on the transplanted organ, which are summarized in Figure 7, AMR is a complicated and multifactorial process that needs further attention. Therefore B-cell activation and differentiation leading to the formation of Ab producing plasma cells as well as memory B-cells will be discussed in further detail below.

(2) T-cell dependent B-cell activation

The humoral immune response starts with the recognition of Ag by B-cells leading to the differentiation of naïve mature B-cells in memory B-cells (MBC) or Ab producing plasma cells (PC). There are two types of humoral immune responses, the primary and secondary humoral immune response. The primary immune response occurs directly after Ag recognition and leads to activation and differentiation of naïve B-cells and the first production of Abs against the Ag. The late immune response occurs after successive Ag recognition in which MBCs induce a more rapid and specific response as well as a higher Ab titer.

As mentioned before, there are three types of allorecognition, the direct, the indirect and demi-direct pathways. Regarding the direct and indirect pathway it was Conlon et al. (Conlon et al., 2012) who gave definitive proof that the humoral immune response is mediated solely through indirect alloAg recognition. This was shown by transplantation experiments in T-cell deficient mice which were reconstituted with T-cells that either recognize only non-processed alloAg (direct) or processed alloAg (indirect). The mice reconstituted with the indirect-pathway T-cells following a heart transplantation showed alloAb production, while mice reconstituted with the direct-pathway T-cells did not. (Conlon et al., 2012) Therefore the focus will be on the B-cell activation in a T-cell dependent manner.

As previously stated there are SLOs which are specialized in concentrating Ag and inducing the adaptive immune response. Within these SLOs, upon T-cell dependent B-cell activation, transient structures, called germinal centers (GC) are formed. The germinal center reaction can be divided into several stages, one being the initiation step or antigen dependent activation, followed by Th mediated B-cell proliferation, immunoglobulin class switch recombination (CSR) and subsequent fate decision to enter the GC or not. Based on the histological appearance, the GC can

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be divided in two distinct microenvironments termed the dark zone (DZ) and light zone (LZ). Next to B-cells, the GC also contains a network of several other immune cells such as T follicular helper cells (T_{FH}), FDCs and macrophages. (C. D. C. Allen et al., 2004; C. D. Allen, Okada, & Cyster, 2007; Nieuwenhuis & Opstelten, 1984; Victora et al., 2010) Within the GC the B-cells undergo clonal expansion, somatic hyper mutation (SHM) and affinity selection, possible CSR and recirculation, or differentiation and leaving the germinal center reaction as highly specific long-lived MBCs or Ab producing PCs. This process of T-cell depended B-cell activation is summarized in (Figure 8) and will be discussed in more detail in the following chapters.

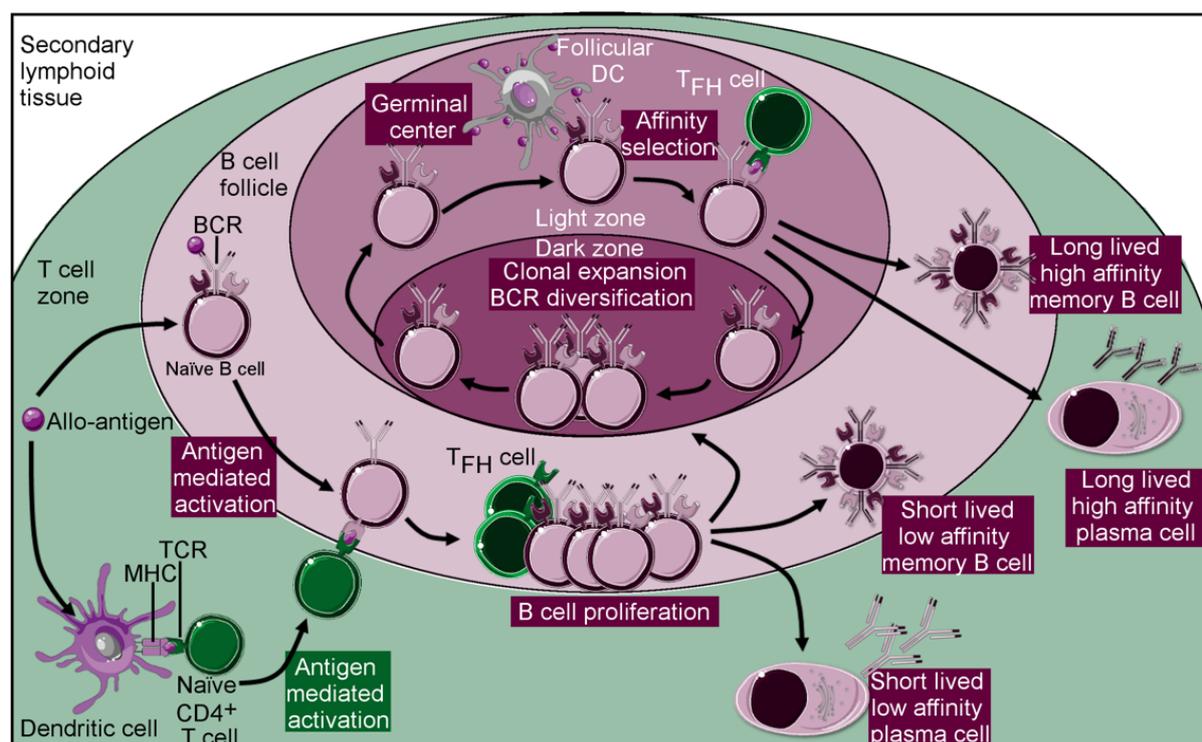


Figure 8: Overview of the cellular components and B-lymphocyte fate before, during and after the germinal center reaction.

Within secondary lymphoid structures, upon antigen activation of both naïve B and T-cells, either by direct binding of the antigen or through antigen presentation by antigen presenting cells, activated cells move to the T-cell-B-cell border. With the help of T-cells the B-cell activation will be “completed” and induce B-cell proliferation within the B-cell follicle. Following the first activation and proliferation step, these B-cells will undergo a fate decision and either leave the follicle to differentiate in short lived, low affinity plasma cells and germinal center independent memory B-cells or enter the germinal center reaction. When B-cells enter the germinal center, they expand clonally and undergo receptor mutations (dark zone reaction), followed by affinity selection with the help of follicular dendritic cells as well as T follicular helper cells (light zone reaction). The selected cells can re-enter the germinal center or leave the germinal center as highly specific long-lived memory B-cells and antibody producing plasma cells. Figure adapted from Parker. (Parker, 1993) BCR: B-cell receptor; FDC: follicular dendritic cell MHC: Major histocompatibility complex; T_{FH} : follicular T helper cell.

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(3) B-cell receptor signaling

One of the first steps in the activation of mature B-cells in the periphery is the binding of Ags to the B-cell receptor (BCR), which sets in motion a cascade of signaling pathways directing the fate of the B-cell. Therefore, it is important to understand more about the signaling pathways that are set in motion upon Ag binding. The BCR consists of a membrane bound Ig and depending on the differentiation state of the mature B-cells, either naïve or class switched such as MBCs, they can be of the IgM, IgD, IgG, IgA and IgE isotype. An important step in creating a pool of heterogeneous individual B-lymphocytes is the formation of a diversified BCR, which is composed of an identical pair of Ig heavy chains and Ig light chains. Ig heavy chains and light chains both exist of a constant region and a variable part. The diversity of the BCR is created through receptor gene rearrangement and allelic exclusion of different variable (V), diversity (D) and joining (J) Ig gene segments during B-cell ontogeny. (Brack, Hiram, Lenhard-Schuller, & Tonegawa, 1978) The BCR is associated with Ig α (CD79a) and Ig β (CD79b) subunits, which contain signaling motifs, termed immunoreceptor tyrosine-based activation motifs (ITAM). The importance of the association of the BCR with Ig α and Ig β is observed during B-cell development in which B-cells where the pre-BCR molecules fail to associate with Ig α and Ig β are arrested in development. (Papavasiliou, Misulovin, Suh, & Nussenzweig, 1995) As well as the fact that the BCRs of naïve B cells, of istype IgM and IgD, do not have any intrinsic signaling capacities and needs Src tyrosine kinases (e.g. Lyn, Fyn, Blk) for the phosphorylation of the ITAMs. Several Src kinases are expressed in B-cells however Lyn seems to play a pivotal role in the phosphorylation of the Ig α and Ig β ITAMs, which has been shown *in vitro* in B-cells and *in vivo* in mice deficient for Lyn. (Burkhardt, Brunswick, Bolen, & Mond, 1991; Chan, Lowell, & DeFranco, 1998; J. Wang, Koizumi, & Watanabe, 1996)

The phosphorylation of Ig α and Ig β ITAMs initiates a signaling cascade downstream of the BCR, starting by the recruitment spleen tyrosine kinase (SYK) which has a high affinity for double phosphorylated ITAMs. This has been shown in SYK deficient B-cells reconstituted with SYK containing mutations in the ITAMs binding site leading to obrogated BCR signaling as compared to reconstitution with wild type SYK, underlining the importance of SYK in BCR signaling. (Kurosaki et al., 1995) The central role of SYK in BCR signaling has been determined both in B-cell development as well as B-cell differentiation. (A. M. Cheng et al., 1995; Cornall, Cheng, Pawson, & Goodnow, 2000; Turner et al., 1995) Bound SYK to Ig α and Ig β is then rapidly activated through phosphorylation by Lyn as well as auto-phosphorylation, which is necessary for its kinase activity. (Furlong et al., 1997; Keshvara et al., 1998)

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Activation of SYK leads to the formation of a signalosome containing Cbl-interacting protein of 85 kDa (CIN85) and B-cell linker protein (BLNK), which is followed by the recruitment and phosphorylation of Bruton's tyrosine kinase (BTK), a non-receptor tyrosine kinase. Like SYK the kinase activity of BTK increases through auto-phosphorylation. (Engels, Wollscheid, & Wienands, 2001; S. Hashimoto et al., 1999; Park et al., 1996). Additional to the activation of SYK, Lyn leads to the recruitment of p85, a subunit of phosphoinositide 3 kinase (PI3K) to CD19 where it complexes with the catalytic p110 subunit and initiates the protein kinase B (AKT) signaling pathway through the formation of phosphatidylinositol 3,4,5-triphosphate (PIP₃). (Cambier, Pleiman, & Clark, 1994; Gold et al., 2000; Pleiman, Hertz, & Cambier, 1994) The role of CD19 in AKT signaling has been highlighted in CD19 deficient lymphoma cells as well as in CD19 deficient mice, showing that AKT signaling was greatly reduced (Otero, Omori, & Rickert, 2001) However it was observed that AKT signaling is not impaired in Lyn deficient mice, indicating another mechanism in BCR dependent AKT signaling in a CD19 independent manner. (H.-L. Li, Davis, Whiteman, Birnbaum, & Puré, 1999) It was shown that SYK able to interact with p85, and that p85 can act as an upstream activator of PI3K. (Beitz, Fruman, Kurosaki, Cantley, & Scharenberg, 1999; Moon et al., 2005) It has been shown that BTK recruitment and activation partly relies on PI3K dependent PIP₃ formation. It was demonstrated that BTK mutants failing to interact with PIP₃ lead to defective BCR responsiveness and that direct interaction with PIP₃ was enough to activate BTK. (K. Saito, Scharenberg, & Kinet, 2001; Takata & Kurosaki, 1996)

Following the formation of the BCR signalosome and the distal activation of PI3K an intricate cascade of signaling pathways is set in motion. A central role of Phospholipase C γ 2 (PLC γ 2), which is recruited and activated through the signalosome, has been elucidated. When B-cells fail to incorporate PLC γ 2 in the BCR signaling pathway, impaired calcium influx and distal signaling is attenuated. (Ishiai et al., 1999) Activated PLC γ 2 is involved in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) in inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ increases the calcium levels in the cell and elevated calcium levels are needed for the activation of transcription factors such as nuclear factor κ B (NF- κ B) and nuclear factor of activated T-cells (NFAT). (Dolmetsch, Lewis, Goodnow, & Healy, 1997; Trushin, Pennington, Algeciras-Schimnich, & Paya, 1999) Cleavage product DAG is involved in the activation of protein kinase C (PKC), which is for one involved in the NF- κ B pathway.

It has been shown that mice deficient for PKC β failed to regulate the phosphorylation and subsequent activation of inhibitor of κ B kinase (IKK), which is an inhibitory protein complex of NF- κ B, regulating its activation and nuclear translocation. (Saijo et al., 2002; T. T. Su et al., 2002) IP₃ and DAG/PKC dependent activation of mitogen activated protein kinases (MAPK), notably

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extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK, are involved in signal transduction following BCR engagement as well. (A. Hashimoto et al., 1998; Ishiai et al., 1999; Jiang, Craxton, Kurosaki, & Clark, 1998)

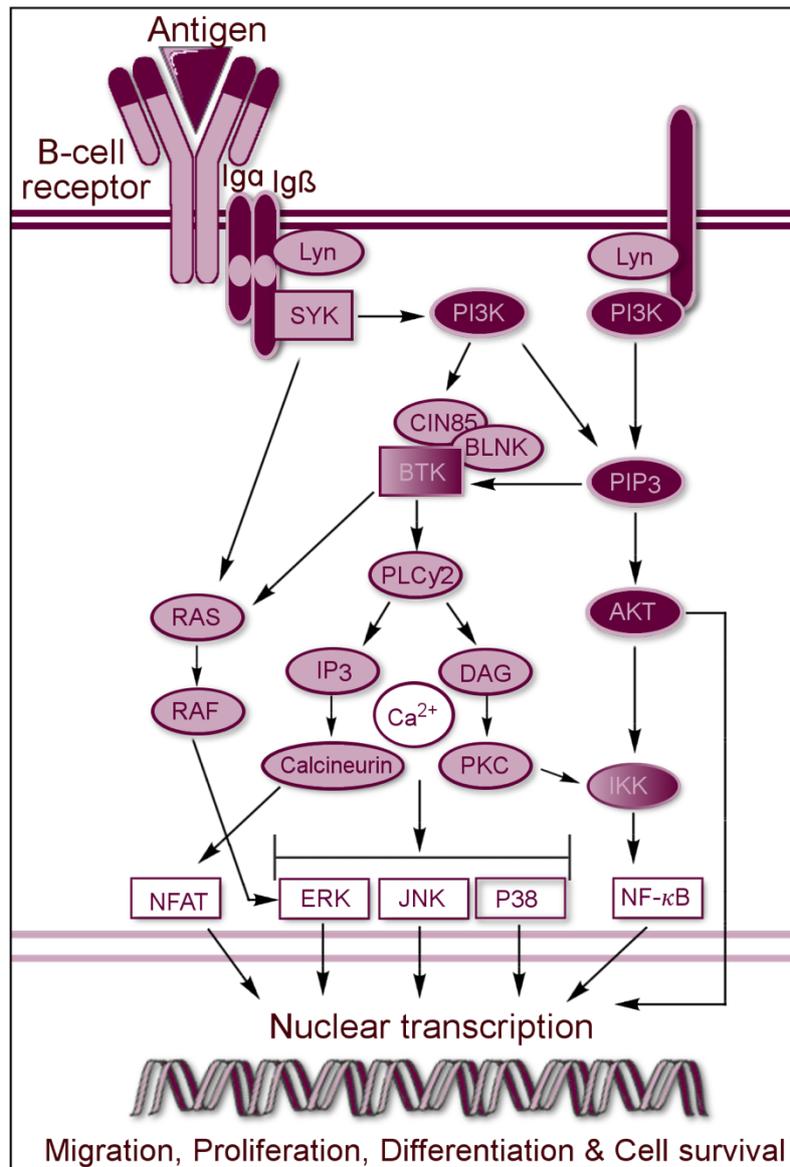


Figure 9: Simplified scheme of B-cell receptor signaling upon antigen engagement.

Antigen binding to the BCR leads to the recruitment of Src kinase Lyn, which leads to the phosphorylation of Igα and Igβ subunits associated with the BCR. Following the phosphorylation of the subunits, SYK is recruited to the BCR and activated, leading to the formation of a signalosome, distal phosphorylation of CD19 by Lyn leads to the recruitment of PI3K. Several signaling pathways, including Ras/Raf, NF-κB, MAPK and AKT, as well as an increase in calcium influx. Activation of these pathways leads to nuclear translocation of transcription factors that in turn promote cell migration, proliferation, differentiation and cell survival. Ag: antigen; BCR; B-cell receptor; CD: cluster of differentiation

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Next to the IP₃ and DAG mediated activation of ERK, another transduction signal has been proposed leading to BCR signalosome independent activation of ERK. The phosphorylation of the ITAMs in Ig α and Ig β and recruitment of SYK can recruit a complex contacting SH2 domain-containing protein (SHC), Growth factor receptor-bound protein 2 (Grb2) and mammalian son of sevenless (mSOS) which activates Ras. (D'Ambrosio, Hippen, & Cambier, 1996; Nagai, Takata, Yamamura, & Kurosaki, 1995) Activation of Ras leads to the recruitment of induces the Raf/MAPK/ERK signaling pathway inducing nuclear transcription. (Alessi et al., 1994; Yan, Roy, Apolloni, Lane, & Hancock, 1998)

As mentioned before, BCR activation leads to distal PI3K activation and the formation of PIP3 that plays a role in the activation of BTK, however PIP3 is also involved in the activation of AKT by promoting its phosphorylation. (Franke et al., 1995) Like PCK, AKT activation influences the degradation of IKK and thus regulates the activation of NF- κ B. (Kane, Shapiro, Stokoe, & Weiss, 1999) Activated AKT can also translocate to the nucleus of the cell where it can interact and affect several transcription factors involved in cell growth and proliferation. (K. Du & Montminy, 1998; Kops & Burgering, 2000; Meier, Alessi, Cron, Andjelković, & Hemmings, 1997) The engagement of the BCR and the signaling pathways that are activated upon Ag recognition (Figure 9) leads to cell migration, proliferation, differentiation and cell survival events which will be discussed below

(4) Germinal center initiation

Following Ag engagement by the TCR and the BCR, T- and B-cells undergo several chemostatic modifications and changes in expression of chemokine receptors. For example, C-C chemokine receptor type (CCR) 7 is a chemokine receptor that binds T-cell zone chemokines such as C-C motif chemokine ligand (CCL) 19 and CCL21, promoting migration to the T-cell zone. Whereas C-X-C chemokine receptor type (CXCR) 5, which binds B-cell zone chemokine C-X-C motif chemokine ligand (CXCL) 13, promotes migration to the B-cell zone. (Förster et al., 1999; Schaerli et al., 2000; Reif et al., 2002) Regulation of the expression of both CCR7 and CXCR5 by follicular T- and B-cells promotes migration from their respective niches to the T-B-cell border to facilitate their interaction (Figure 10 A)

At the T-B-cell border B-cells form cognate interaction with T-cell by presenting the processed Ag via MHC class II to the TCR. In return, the B-cells receive T-cell-derived activation signals such as cytokines and co-stimulatory interactions necessary for the formation of the GC (Figure 10 B). (D. M. Mills & Cambier, 2003) The CD40-CD40L interaction needed for proper T-cell dependent activation of B-cells, as it has been shown that mice deficient for CD40, expressed on B-cells, fail to form GCs as well as undergo isotype switching. (Kawabe et al., 1994) Reciprocally

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the same results were obtained in mice deficient for CD40L, expressed by T-cells. (Xu et al., 1994) It has been observed in thymus less mice as well, who fail to form GCs, which are rescued by adoptively transferred thymocytes before immunization. (Jacobson, Caporale, & Thorbecke, 1974) The B7-CD28/CTLA-4 costimulatory pathway is another way the B-cell and T-cell interact. Members of the CD28 family expressed by the T-cells include CD28, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), inducible T-cell co-stimulator (ICOS) and programmed cell death protein-1 (PD-1). Members of the B7 family expressed by the B-cells include, CD80 (B7-1), CD86 (B7-2), ICOS ligand (ICOSL; B7-H2) and PD-ligand 1 (PD-L1; B7-H1). The B7-CD28/CTLA-4 costimulatory pathway is essential for the activation of T-cells and thus indispensable for T-cell help. (Gimmi et al., 1991; June, Ledbetter, Linsley, & Thompson, 1990; Poirier, Blanche, & Vanhove, 2011) Without this co-stimulation pathway, activated B-cells fail to proliferate, undergo CSR, differentiate or form GCs. (Borriello et al., 1997; Coyle et al., 2000; Tafuri et al., 2001)

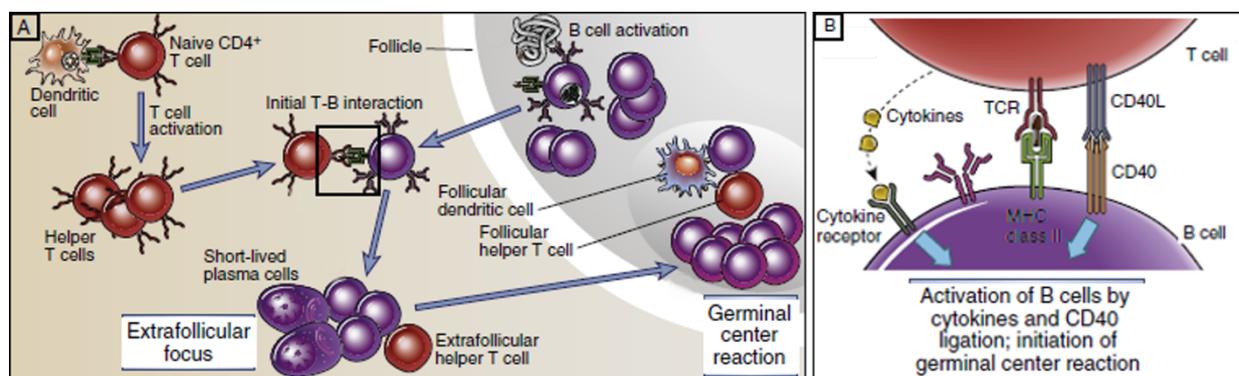


Figure 10: Germinal center initiation.

(A) Upon antigen binding by both the T-cells and B-cells, they migrate towards each other where they can form cognate interactions leading to B-cell activation and proliferation. Followed by either differentiation or entry into the germinal center. (B) Antigen presentation of the B-cell to the T-cell via the MHC and TCR induces B-cell stimulatory signals, such as cytokines and co-stimulation through CD40, leading to the initiation of the germinal center reaction. Figure adapted from Abbas et al. (Abbas et al., 2014, p. 246,248). CD: Cluster of differentiation, MHC: Major histocompatibility complex, TCR: T-cell receptor.

Following the first interaction of the T- and B-lymphocytes, a rapid induction of B-cell proliferation is induced. (Coffey, Alabyev, & Manser, 2009; K. M. Toellner, Gulbranson-Judge, Taylor, Sze, & MacLennan, 1996) It has also been shown that these activated B-cells have undergone CSR during the early onset of the humoral immune response. (K. M. Toellner et al., 1996; K.-M. Toellner et al., 1998; Pape et al., 2003) During CSR, the Ig gene is rearranged in a way that the heavy chain constant regions μ and δ are interchanged with constant regions γ , α , or ϵ to generate different Ig subclasses (IgG, IgA and IgE resp.). The major player during CSR is activation induced cytidine deaminase (AID), which is a ribonucleic acid (RNA)-editing deaminase that is

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almost uniquely expressed in activated B-cells, and abrogation of AID obstructs CSR. (Muramatsu et al., 1999, 2000; Revy et al., 2000) AID induces the change of cytosine residues in single stranded DNA to uracil which leads to mismatches and thus mutations in the DNA strand for both the C/G and A/T residues. During CSR, when these mutations occur in the constant region of the Ig gene locus, it leads to double strand breaks followed by joining and generating class switched Igs. (Chaudhuri et al., 2007; Dickerson, Market, Besmer, & Papavasiliou, 2003; Noia & Neuberger, 2007)

Antigen binding and cellular interactions lead to the induction of several molecular factors that are important in the initiation and maintenance of the GC. An important transcriptional factor that is upregulated upon antigen binding is Myc, which is known to be involved in proliferation in many cell types by regulating multiple processes such as cell cycle progression, DNA replication and telomere maintenance (Dang, 2012; Nie et al., 2012) When Myc is abrogated shortly post immunization GCs are not formed. This indicates the importance of Myc in GC formation, in centroblasts however Myc expression is drastically downregulated. (Calado et al., 2012; Dominguez-Sola et al., 2012) Other transcriptional factors that are upregulated during the GC initiation are NF- κ B, myocyte-specific enhancer factor 2 (MEF2), interferon-regulatory factor (IRF) 4 and IRF8. It was recently shown that defects in the NF- κ B signaling pathway in T-cell dependent activation of B-cells abrogated the formation of GCs. (Jacque et al., 2014) MEF2B, IRF4 and IRF8 are responsible for the regulation of transcriptional repressor B-cell lymphoma 6 (BCL-6), which is a major regulator of GC initiation and maintenance. (C. H. Lee et al., 2006; Ochiai et al., 2013; Ying et al., 2013) BCL-6 is upregulated in GC B-cells, and mice deficient for BCL-6 showed defect in T-cell dependent B-cell activation due to the fact that they failed to form GCs. (Fukuda et al., 1997; Ye et al., 1997) In the initiation of the GC, BCL-6 has been shown to be involved in cell migration into the follicle, as well as promoting T-cell B-cell interactions and T-cell differentiation into T_{FH}. (Gatto, Paus, Basten, Mackay, & Brink, 2009; Kitano et al., 2011; Pereira, Kelly, Xu, & Cyster, 2009)

(5) Germinal center reaction

The GC reaction is a dynamic Darwinian survival of the fittest situation allowing only generated B-cell clones with the highest affinity for the Ag to survive and differentiate. One of the first models of the GC mechanism, for its organization and function, was proposed in 1994 by MacLennan established by years of research ranging from tissue analysis to *in vitro* studies. The organization of the GC was divided into a DZ and LZ based on the histological appearance of the cells that build up these distinct zones (Figure 11). The GC cells inhabiting the DZ were termed

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centroblasts that were characterized by their large size, their mitotic activity and low expression of sIg. These cells were considered to undergo rapid proliferation and SHM within the variable region of the Ig genes to produce a fast variety of B-cell clones. These centroblasts were then proposed to exit the cell cycle, re-express the modified sIg, reduce in size and travel to the LZ, as cells termed centrocytes. Due to the mutations in the Ig gene these centrocytes express BCRs with a wide range in affinity for the Ag and it was proposed that in the LZ these centrocytes compete to bind Ag complexes presented by FDCs. Cells losing the competition die while the winners present Ags to T_{FH} cells which assist in the proliferation and differentiation of GC cells into MBCs and PCs. (MacLennan, 1994)

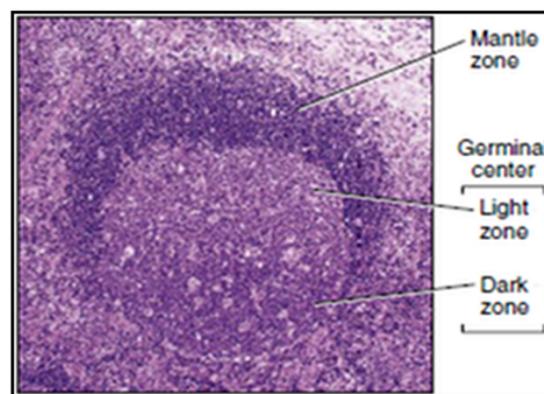


Figure 11: Histological section of a germinal center.

Hematoxylin and eosin staining of a germinal center reveals a densely packed dark zone and a light zone containing fewer cells. Figure adapted from Abbas et al. (Abbas et al., 2014, p. 250).

This model laid the basis for most of the understanding of the GC however, due to the large numbers of unknowns within the GC reaction it is an ongoing working model to elucidate the events happening within this complex dynamic cellular structure. These events will be discussed in detail below.

The notion that cell division was restricted to the DZ came from histological observations of mitotic figures which were observed in higher frequency in the DZ. (Rademakers, 1992), as well as the observation that H^3 -thymidine incorporation showed that labelled cells appeared first in the DZ. (Hanna, 1964) However several studies have overturned the idea that cell division is restricted to the DZ. Staining GC cells with proliferation marker Ki67 indicated the presence of proliferating cells in both the DZ and the LZ, as well as results obtained with bromodeoxyuridine (BrdU) uptake assays in which BrdU was taken up by cells in both the zones. (C. D. C. Allen, Okada, Tang, & Cyster, 2007; Hauser et al., 2007) Indicating that both centroblasts and centrocytes can be in a proliferating state. Nonetheless cell cycle analysis showed that while in both zone there are cells in S phase, meaning entering the cell cycle, cells in the G2/M phase are extremely rare in the LZ and

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more abundant in the DZ, indicating that the cell cycle is rarely completed in the LZ. These results were supported by the fact that G2/M cell cycle genes are upregulated in DZ cells. These results could suggest that cells can enter the cell cycle in the LZ, however this might be a trigger for the cells to leave the LZ, either for re-entry in the DZ or a post-GC fate. Supporting the notion that cell proliferation and thus clonal expansion occurs in the DZ. (C. D. C. Allen et al., 2007; Y. J. Liu, Zhang, Lane, Chan, & MacLennan, 1991; Victora et al., 2010) An important step in generating differentiated B-cells with high affinity for the Ag is receptor editing through SHM. It was observed that B-cells present in the follicle and the GC contain the same germline variable region for both the heavy and light chain of the Ig, however in the GC B-cells mutations were found in these gene segments while cells outside of the foci showed none, indicating that the GC is the site of SHM. (Jacob & Kelsoe, 1992; Jacob, Kelsoe, Rajewsky, & Weiss, 1991) As mentioned before an important enzyme for BCR editing is AID, which is an RNA editing deaminase that is involved in CSR, however in the germinal center reaction this enzyme is responsible for the SHM as well. SHM is a consequence of point mutations in the variable region of the Ig gene due to the change from cytosine residue into uracil. (Dickerson et al., 2003; Noia & Neuberger, 2007) It was observed that AID is mainly expressed in the DZ of the GC and found in only a few LZ B-cells (Figure 12), indicating that SHM occurs mainly in the DZ of the GC. (Carlotti et al., 2015; Moldenhauer et al., 2006)

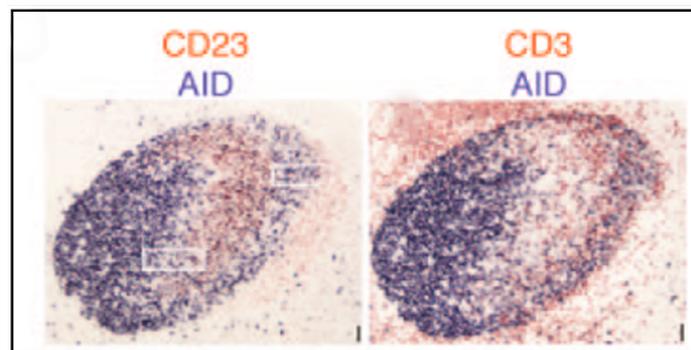


Figure 12: Localization of AID in the Germinal Center

Immunohistochemically staining of germinal centers reveal the localization of AID mainly in the dark zone, while only some cells expressing AID are found in the light zone identified based on the presence of CD23 positive follicular dendritic cells and CD3 positive T-cells. Figure adapted from Cattoretti et al., 2006. AID: activation induced cytidine deaminase, CD: Cluster of differentiation

Following clonal expansion and SHM, centroblasts from the dark zone transition into centrocytes and enter the LZ. In the classical model of GC migration, it was suggested and observed that GC cells moved from the DZ to the LZ, based on the migration of labeled cells, prior to leaving the GC. (Hanna, 1964; Y. J. Liu et al., 1991) However, the likelihood that such a

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striking high affinity is accumulated by one-step random mutations is very low. This led to theoretical modeling, and follow up experimental results which showed that affinity maturation was a stepwise process, suggesting that some of the centrocytes re-entered the DZ for further proliferation and mutation and re-selection in the LZ. (Brown et al., 1992; Kepler & Perelson, 1993; Oprea & Perelson, 1997) This was confirmed by real time imaging, which resulted in the conclusion that the GC cells moved between the DZ and LZ in both directions. (C. D. C. Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007) This involved the expression of chemokine receptors CXCR4 and CXCR5 (C. D. C. Allen et al., 2004; C. D. Allen et al., 2007; Nieuwenhuis & Opstelten, 1984; Victora et al., 2010) It was shown by Allen et al. (C. D. C. Allen et al., 2004) that centroblasts expressed high levels of CXCR4, and that their chemokine ligand CXCL12 was expressed in higher abundance in the DZ. In immunized mice, deficient for CXCR4, they observed no segregation of the DZ and LZ within the GC. On the other hand, they observed that CXCL13 was abundantly expressed in the LZ and that the expression of CXCR5 was needed to direct the cells towards the LZ. However, defects in either CXCR5 or CXCL13 were not necessary for the segregation of the GC into the DZ and LZ. (C. D. C. Allen et al., 2004) Interestingly the zonal segregation of the GC does not seem to be necessary for the centroblasts to transition into centrocytes, however CXCR4 is necessary for efficient participation in the GC. It was shown that CXCR4 deficient cells do not obtain a much mutations and are outcompeted by their wild type counterparts over time, indicating the importance of the zonal segregation and separation of SHM and affinity selection and maturation, as well as GC re-cycling. (Bannard et al., 2013)

At first the idea arose that GC B-cells are selected solely based on their affinity of the BCR for the Ag. It was believed that the limited availability of Ag led to competition for Ag binding which results in the survival of only high affinity B-cells. This coincided with the results found in several early studies that showed that a lower dose of Ag led to greater affinity. (Eisen & Siskind, 1964; Goidl, Paul, Siskind, & Benacerraf, 1968) FDCs are radioresistant cells forming a network in primary follicles and GCs, and they are seen as a histological marker for the LZ. (C. D. C. Allen & Cyster, 2008; Kosco-Vilbois & Scheidegger, 1995) FDCs were believed to be the cells driving in affinity selection of GC B-cells through direct competition, since they can retain intact Ag for a long time. The higher affinity B-cells would sequester all the antigen and the lower affinity B-cell would die due to lack of survival signals. (MacLennan, 1994; Mandel, Phipps, Abbot, & Tew, 1981) However this model fails to explain how differential BCR signaling could lead to either vigorous proliferation or induced cell death. And if the presence of Ag would be enough for selection, autoreactive GC B-cells, which can be formed by random mutations due to SHM, would flourish due to the fact that self-Ag are present in high numbers in the GC. However, under physical

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conditions autoreactive cells undergo apoptosis within the GC reaction. (A Davidson, R Shefner, A Livneh, & Diamond, 1987; B Diamond et al., 1992) Therefore the notion of affinity selection by direct Ag binding via FDCs has been challenged and it has been shown that when the Ag presentation of the FDCs is retained, the formation of the GC is not abrogated and GC cells still show signs of affinity maturation. (Chen, Koralov, Gendelman, Carroll, & Kelsoe, 2000; Hannum, Haberman, Anderson, & Shlomchik, 2000; Mitsuru Matsumoto et al., 1996; X. Wu et al., 2000) However in the absence of FDCs GC fail to form. (Mitsuru Matsumoto et al., 1996) Next to the role of antigen presentation by FDCs, they are involved in GC localization of B and T-cells. FDCs produce CXCL13, which as mentioned before directs the migration of GC B-cells to the LZ. (C. D. C. Allen et al., 2004; J. G. Cyster et al., 2000) FDCs are also responsible for the secretion of cytokines such as IL-6 and B-cell activating factor (BAFF), which are involved in the GC reaction and B-cell differentiation. (Kopf, Herren, Wiles, Pepys, & Kosco-Vilbois, 1998; Kosco-Vilbois & Scheidegger, 1995; Y. Wu et al., 2009) These observations indicate that FDCs and direct antigen binding are indispensable in the GC reaction, however not in affinity maturation as previously believed.

Therefore, another hypothesis has arisen in which competition for T-cells help is necessary for the selection of GC mutants with the highest affinity. It was shown that T-cells promote the selection of B-cells in the GC through processed Ag on MHC complexes, the higher the affinity the higher the density of Ag presentation on the B-cell surface and thus a greater share of T-cell help (Figure 13). (Depoil et al., 2005; Vitorica et al., 2010) The importance of T-cells within the GC reaction was shown by Han et al (Han, Hathcock, et al., 1995) as well, they showed that injecting antibodies blocking the CD40-CD40L interaction abrogates an ongoing GC reaction. A lineage of T-cells, T_{FH} cells have been identified within the GC LZ. These cells were identified as $CD4^+$ T-cells which express chemokine receptor CXCR5, which is involved in the localization of the T-cells in the LZ through a CXCL13 gradient. (Ansel, McHeyzer-Williams, Ngo, McHeyzer-Williams, & Cyster, 1999; Haynes et al., 2007) T_{FH} cells have been identified based on their expression of both ICOS and PD-1, which are as mentioned before involved in the B7-CD28/CTLA-4 costimulatory pathway. (Haynes et al., 2007; Hutloff et al., 1999) Interactions between GC B-cells and T_{FH} have been shown to be promoted via ICOS-ICOSL binding, and it was observed that this interaction increases the expression of CD40L on T-cells. Upregulation of CD40L on T-cells increases the CD40 stimulation B-cells receive upon T-B-cell interactions. Increased CD40 stimulation in B-cells is followed by the upregulation of ICOSL in the B-cells resulting in a feedforward loop increasing the T-cell help received by higher affinity GC B-cells as compared to their lower affinity counter parts. (Dan Liu et al., 2015)

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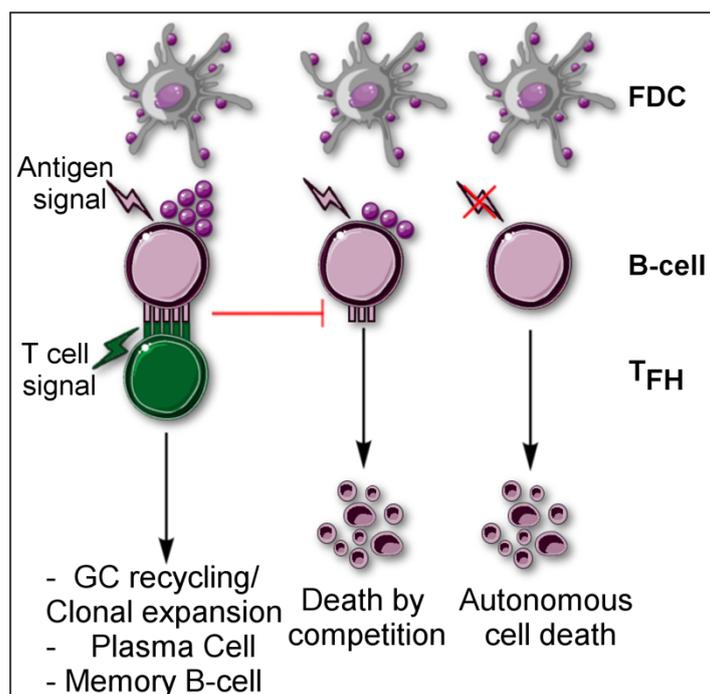


Figure 13: Antigen presentation and T-cell interaction germinal center B-cells.

A proposed model of antigen presentation and T-cell help in affinity selection of germinal center B-cells. Follicular dendritic cells present in the light zone of the germinal center reaction present antigens to the germinal center B-cells. Depending on the affinity of the B-cell receptor for the antigen the higher the density of antigens presented to the follicular T-cells and thus the greater the T-cell help. Cells that receive the most T-cell help are able to expand and differentiate in effector cells while lower affinity cells die by apoptosis. Figure adapted from Victora and Nussenzweig. (Victora & Nussenzweig, 2012) FDC: Follicular dendritic cell, GC: Germinal center, T_{FH}: Follicular T helper cell

Following the selection of GC B-cells with high affinity for the Ag, the cells will differentiate and will be exported from the GC as either MBCs or PCs. Plasma cells will secrete Abs with high affinity for the antigen and thus neutralize the threat while MBCs will be able to rapidly differentiate into PCs upon re-exposure to the Ag generating a pool of highly specific and efficient effector B-cells. Whereas the decision for GC cells to differentiate in PCs has been elucidated, the decision to differentiate into MBCs is still poorly understood. Blimp-1 (a.k.a. PRDM1: PR domain zinc finger protein 1) is seen as the master regulator of PC differentiation. It is expressed in a small subset of GC cells before they are exported, indicating their predisposition to become PCs. (Angelin-Duclos, Cattoretti, Lin, & Calame, 2000; Fooksman et al., 2010) Bone marrow PCs were shown to have an almost uniformly high affinity as compared to GC B-cells and MBCs, indicating a differential selection method for PC and MBC differentiation. (Kenneth G. C. Smith, Light, Nossal, & Tarlinton, 1997) Extensive research has been performed by the group of Brink, in which they look at Ag affinity and B-cell differentiation. They found that B-cell expressing high affinity BCRs are actively selected into PCs. By generating mice that transgenically express a

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BCR specific for hen egg lysosome and subsequent challenging these B-cells with Ags of various affinities. They observed that lower affinity Ags lead to accumulation in total SHM in GC B-cells as compared to higher affinity Ag induced GCs, they observed lower levels of secreted Ag specific IgG as well. (Brink, Phan, Paus, & Chan, 2007; Paus et al., 2006; Phan et al., 2006) Indicating that differentiation of GC cells into PCs is directly related to high absolute BCR affinity. As mentioned before Blimp-1 is an indicator for PC differentiation, while the BCR affinity seems to be the thriving force in PC selection, T-cell help has been shown to influence Blimp-1 expression via CD40 mediated activation of NF- κ B and subsequent induction of IRF4. (Klein et al., 2006; Sciammas et al., 2006) The fact that PCs and MBCs differentiate in two different ways has been highlighted by the fact that PCs seem to have an overall high affinity compared to MBCs. (K. G. C. Smith, Weiss, Rajewsky, Nossal, & Tarlinton, 1994; Kenneth G. C. Smith et al., 1997) On the other hand, disturbing the GC selection process is usually reflected by MBCs while having different effects on PCs. (Good-Jacobson & Shlomchik, 2010) The differentiation of GC cells into MBCs still remains unclear. A current hypothesis is, that MBCs randomly differentiate from GCs simply by being positively selected and surviving apoptosis. This notion is supported by the fact that when GC cells are prevented from entering in apoptosis the number of GC and memory B-cells increase disproportionately while the affinity for the Ag decreases, indicating that MBCs cell fate is directed more through selection and survival than Ag affinity. (K. G. C. Smith et al., 1994; Takahashi et al., 1999; Takahashi, Ohta, & Takemori, 2001)

(6) Germinal center independent B-cell differentiation

As mentioned before, a part of the activated B-cells will leave the follicle without entering the GC and they can either differentiate in GC independent MBCs or in extra follicular PCs. Mice deficient for Bcl-6 maintained the possibility to have differentiated MBCs in response to T-cell dependent Ags, indicating the formation of GC independent MBCs. (Toyama et al., 2002). Another study showed that when the onset of the GC is selectively blocked by inhibiting the interaction with ICOS, the recruitment of high affinity MBCs is impaired while low affinity MBCs are generated in a GC independent manner. (Inamine et al., 2005) The influence of T-cell B-cell interactions in the fate decision to become GC independent MBCs has been shown to be dependent of CD40 (Erickson et al., 2002) GC independent MBCs can either express sIgM or isotype switched sIg, however they do not show signs of SHM nor, in some cases, expression of memory marker CD27. (Toyama et al., 2002; Inamine et al., 2005; J. J. Taylor, Pape, & Jenkins, 2012)

Extra follicular PCs, are differentiated low-affinity plasma cells that have a short lifespan. (Sze, Toellner, Vinuesa, Taylor, & MacLennan, 2000) While they have upregulated expression

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levels of AID and they underwent isotype switching, there is no evidence of SHM or affinity maturation. (Jacob & Kelsoe, 1992; K.-M. Toellner et al., 2002; Marshall et al., 2011) Recent studies have shown the importance of Ag recognition and binding strength in the decision for the B-cell to differentiate in follicular plasma blasts. In which they showed that in the initial Ag recognition response the cells with higher affinity for the Ag are more likely to become short-lived, low-affinity plasma blast, as where moderate and lower affinity B-cells enter the GC to differentiate in high-affinity selected long lived differentiated B-cells. (Dal Porto, Haberman, Kelsoe, & Shlomchik, 2002; O'Connor et al., 2006; Paus et al., 2006)

The fate decision to enter the GC can be regulated at molecular levels as well, the expression of Epstein-Barr virus-induced gene 2 (EBI2), is involved in the follicular localization of B-cells. (Glynne, Ghandour, Rayner, Mack, & Goodnow, 2000; Rosenkilde et al., 2006) B-cells with upregulated expression of EBI2 favor the outer follicular region, while B-cells with downregulated EBI2 favor the inner follicular region. It has been shown that transcriptional repressor Bcl-6 is responsible for the transcriptional regression of EBI2, (Gatto et al., 2009; Pereira et al., 2009)

(III) Balancing B-cell survival and death

(1) Regulating germinal center B-cell survival

As stated previously, B-cells in the GC undergo a selection process in which their fate is decided, they either re-enter the GC, differentiate or are eliminated by apoptosis, creating a state of homeostasis for B-cells within the GC. Apoptosis is a highly regulated programmed cell death and is an important process for the maintenance of a cellular homeostasis without the induction of an inflammatory response. Apoptosis leads to the extermination and elimination of infected, damaged, self-reactive, non-reactive or redundant cells through phagocytosis. (Aderem & Underhill, 1999; Henson & Hume, 2006; Monks, Smith-Steinhart, Kruk, Fadok, & Henson, 2008)

The default cell fate of GC B-cells is to die of apoptosis. Liu et al (Y.J. Liu et al., 1989) showed that GC B-cells extracted from tonsils and maintained in culture die from apoptosis within hours if they are not activated through the BCR. This hypothesis is supported by the fact that GC B-cells lose the expression of anti-apoptotic protein B-cell lymphoma-2 (Bcl-2), which is a member of the Bcl-2 protein family involved in the regulation of apoptosis and which will be discussed in further detail below. The fact that FasR is highly expressed by GC B-cells supports this hypothesis as well. (Yoshino et al., 1994) Master regulator of the GC reaction, Bcl-6, has been identified as a repressor of Bcl-2 gene transcription. (Ci et al., 2009; M. Saito et al., 2009) On the other hand it has been shown in that overexpressing Bcl-2 alters the differentiation of the B-cells in the GC. Overexpression of Bcl-2 leads to the survival of self-reactive B-cells, which would normally be

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cleared through apoptosis. Overexpression of Bcl-2 also increases the number of isotype switched GC and memory B-cells, however it decreases the efficiency of affinity selection. (Notidis, Heltemes, & Manser, 2002; K. G. C. Smith et al., 1994)

The survival of GC cell depends on the reception of survival signals, which can induce the expression of anti-apoptotic proteins preventing apoptotic cell death. Vikstrom et al (Vikstrom et al., 2010) showed that myeloid cell leukemia-1 (Mcl-1; BCL2L3), another Bcl-2 family member, is the main anti-apoptotic regulator of the GC reaction. They confirmed by immunoblotting that Mcl-1 and Bcl-xl were upregulated in GC B-cells, therefore they created conditional Cre-Lox mice models for both Mcl-1 and Bcl-xl. In this model, they observed that while deletion of Bcl-xl did not alter the GC response upon antigen activation (Figure 14A), deletion of Mcl-1 abrogated the formation of both GC B-cells and MBCs (Figure 14 B).

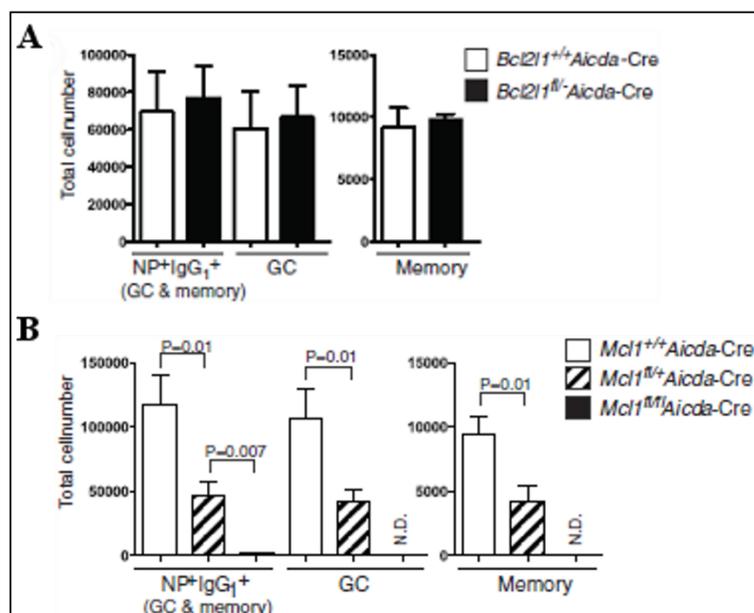


Figure 14: Effect of Bcl-xl and Mcl-1 deletion GC and Memory B-cell formation.

While conditional depletion of Bcl-xl does not alter the formation of GC as well as memory cells upon activation (A), depletion of Mcl-1 abrogates both GC and memory formation. Figure adapted from Vikstrom et al. (Vikstrom et al., 2010) Bcl-2: B-cell lymphoma-2; Ig: immunoglobulin, GC: germinal center, NP: 4-hydroxy-3-nitrophenyl

In our group (Clybouw et al., 2011), we showed that another regulator of the GC reaction is Bcl-2 homology (BH)3-only protein p53 up-regulated mediator of apoptosis (Puma). Deletion of Puma *in vitro* prolonged the survival of mitogen activated B-cells, additionally *in vivo* studies showed an accumulation of antigen specific B-cells in Puma deficient mice as compared to wild type mice. In this same study, a co-localization between Mcl-1 and Puma was observed within GCs (Figure 15).

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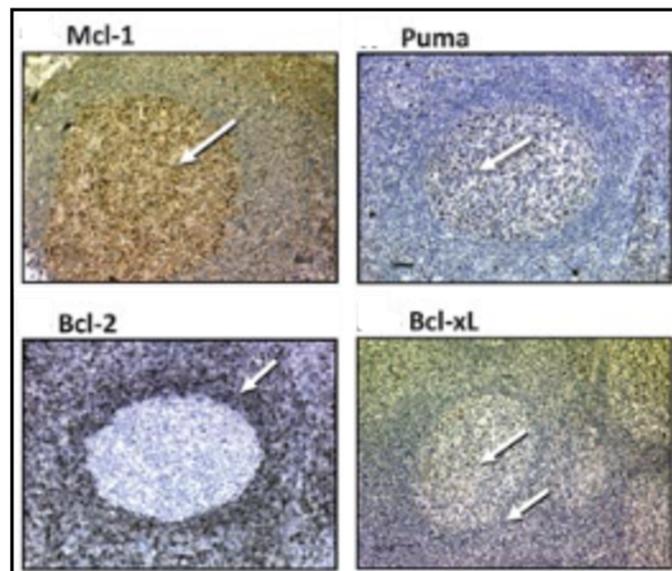


Figure 15: The localization of pro-apoptotic Bcl-2 family members in relation to Puma and germinal center structures found in human lymph nodes.

Anti-apoptotic protein Mcl-1 is mainly expressed in germinal center cells, where BH3-only activator Puma is located too. Whereas both Bcl-2 and Bcl-xL are mainly expressed by cells around the germinal centers, though Bcl-xL can be found within the germinal center as well. The identified proteins are indicated by the white arrows. Figure adapted from Clybouw et al. (Clybouw et al., 2011)

These studies indicate the importance of the Bcl-2 family in regulating the GC reaction, both in an anti- and pro-apoptotic manner, regulating B-cell survival and death and thus ensuring BCR specificity.

(2) The Bcl-2 family

The protein laying the foundation for the Bcl-2 family was, as the name suggests, Bcl-2 and was first identified in human B-cell follicular lymphoma. A chromosomal translocation $t(14;18)$ in this lymphoma caused deregulation in the Bcl-2 gene and inducing its overexpression. (Tsujimoto, Cossman, Jaffe, & Croce, 1985) Following the discovery of Bcl-2, it was shown that when overexpressed in hematopoietic cells apoptosis was prevented leading to an accumulation of lymphocytes in mice models. (McDonnell et al., 1989; Vaux, Cory, & Adams, 1988). Antonsson et al. (Antonsson et al., 1997) found that Bcl-2 is capable to prevent the induction of mitochondrial membrane permeability, known as mitochondrial outer membrane permeabilization (MOMP), and thus prevent the release of mitochondrial proteins, such as cytochrome c, involved in apoptosis. The release of pro-apoptotic proteins following MOMP is a main biochemical event observed during the intrinsic apoptotic pathway, an apoptotic pathway that will be discussed in more detail below. Bcl-2 has been shown to oppose apoptosis downstream of MOMP as well by demonstrating

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that Bcl-2 is able to partially inhibit caspase activation, even in the presence of cytochrome c. (Cosulich, Savory, & Clarke, 1999) Shortly after, other family members were identified based on similar function, structural similarities and conserved α -helical BH domains. The Bcl-2 family members share up to four BH domains, termed BH1-4, of which BH3 is found in almost all family members and is necessary for their interaction. (Youle & Strasser, 2008; Zha et al., 1997) The interactions between the family members determine the integrity of the mitochondrial outer membrane (MOM). In mammals, there are around 20 family members identified which can be divided into two groups based on their function during apoptosis, namely pro-apoptotic and anti-apoptotic (Figure 16).

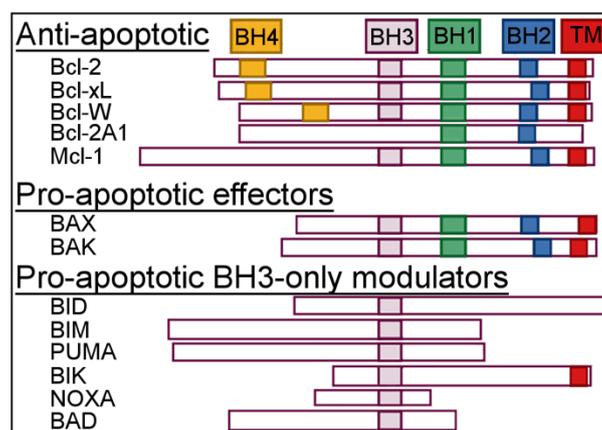


Figure 16: The Bcl-2 family of proteins.

The presence of up to 4 homologous BH domains define the Bcl-2 family members. Functionally these proteins can be subdivided in anti- and pro-apoptotic proteins of which the pro-apoptotic proteins can be further sub-divided into multidomain executioner proteins and BH3-only protein. Figure adapted from Rodriguez et al. (Rodriguez, Rojas-Rivera, & Hetz, 2011) BH3: Bcl-2 homology, TM: Transmembrane.

The anti-apoptotic family members identified up to date are Bcl-2, Bcl-xL (BCL2L1 long isoform), Bcl-w (BCL2L2), Mcl-1 and A1 (BCL2L5; Bfl-1). All anti-apoptotic Bcl-2 family members share a structural fold forming a hydrophobic pocket that stretches over the BH1-BH3 or BH1-BH4 domains. This conformation is essential for the interaction between the anti-apoptotic and pro-apoptotic Bcl-2 family members. Overexpression of the anti-apoptotic proteins blocks apoptosis by sequestering the pro-apoptotic effector proteins (Certo et al., 2006; E. H. Y. A. Cheng et al., 2001)

The pro-apoptotic Bcl-2 family members comprise two groups related to their structure and correlated functions, namely the effector proteins and BH-3 only proteins. The effector proteins BCL-2 Associated X protein (Bax) and BCL-2 Antagonist Killer 1 (Bak) are responsible for MOMP and subsequent release pro-apoptotic molecules from the mitochondria. Bax and Bak both share BH1-BH3, and like the anti-apoptotic members, they display structural conformations

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necessary for protein-protein interaction. (Moldoveanu et al., 2006; Suzuki, Youle, & Tjandra, 2000) Once activated Bax and Bak undergo conformational changes and are able to oligomerize followed by pore formation in the mitochondrial membrane. (Antonsson, Montessuit, Sanchez, & Martinou, 2001; Griffiths et al., 1999; Nechushtan, Smith, Lamensdorf, Yoon, & Youle, 2001) The BH3 domain of Bax is necessary for protein-protein interactions, either with itself or other family members, as well as for its apoptotic activity. (Simonen, Keller, & Heim, 1997) The BH3 domain of Bak is involved in the interaction with anti-apoptotic family members as well. (Sattler et al., 1997) Both Bax and Bak are necessary for an optimal induction of apoptosis upon stress signals. (Kepp, Rajalingam, Kimmig, & Rudel, 2007)

The second group of pro-apoptotic proteins, the BH3-only proteins is the largest group of Bcl-2 proteins and comprise of e.g. BCL-2 antagonist of cell death (Bad), BH3 interacting-domain death agonist (Bid), BCL-2 interacting killer (Bik), BCL-2 interacting mediator of cell death (Bim), BCL-2 modifying factor (Bmf) Harakiri (Hrk), Noxa, and Puma. In the absence of Bax and Bak the BH3-only proteins alone are unable to induce apoptosis. (Zong, Lindsten, Ross, MacGregor, & Thompson, 2001) BH3-only proteins are sensors of intracellular stress and their role in apoptosis depends on their interaction with specific Bcl-2 family members. The expression of the BH3-only proteins varies depending on the cell type and they can be restrained in different ways. They can either be regulated at transcriptional level, proteins like Bim, Puma, Noxa and Hrk are upregulated following cellular stress, or they can be present in the cell in a non-active state followed by their activation upon post-translational changes. For example, Bad is sequestered by 14-3-3 proteins, while Bid is produced as a large non-active protein which can be activated through cleavage by caspase-8 and granzyme B. (Puthalakath & Strasser, 2002) Phosphorylation of BH3-only proteins, such as Bim, Bad, Bmf and Bik may play a role in their activation state as well, however the mechanism is still poorly understood. For Bim it has been found that phosphorylation at specific serine residues following survival signals leads to its proteasome depended degradation, while phosphorylation following growth factor withdrawal can enhance apoptosis. (Harada, Quearry, Ruiz-Vela, & Korsmeyer, 2004; Putcha et al., 2003)

In a healthy cell, anti-apoptotic family members are able to repress the activity of the pro-apoptotic effector proteins, and following an apoptotic signal the balance is tipped and apoptosis is induced. This happens predominantly by an increased expression of the pro-apoptotic (mainly BH3-only) and/or inactivation and degradation of the anti-apoptotic members. This tip of the balance leads to the activation of Bax and Bak followed by MOMP.

Interactions between the family members have been extensively researched (Table 1), giving rise to several proposed models of activation of the intrinsic apoptotic pathway.

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Table 1: Interactions between Bcl-2 family members

Interactions between the Bcl-2 family members that are necessary either to prevent the cell from dying by apoptosis or induce apoptosis upon the signals received by the cell. Table adapted from Shamas-Din, Kale, Leber, & Andrews, 2013

Anti-apoptotic	Effector proteins	Anti-apoptotic binds to:	
		BH3 activators	BH3 sensitizers
A1	Bak	Bid, Bim, Puma	Bik, Hrk, Noxa
Bcl-2	Bax	Bid, Bim, Puma	Bad, Bmf
Bcl-w	Bak, Bax	Bid, Bim, Puma	Bad, Bik, Bmf, Hrk
Bcl-xl	Bak, Bax	Bid, Bim, Puma	Bad, Bik, Bmf, Hrk
Mcl-1	Bak	Bid, Bim, Puma	Hrk, Noxa

One model is called the displacement or derepressor model (Figure 17, left), in which the effector proteins Bak and Bax are constitutively expressed by the cell. However, they are rendered inactive through interactions with anti-apoptotic family members. The activation of Bak and Bax in this model depends on competitive binding of the BH3-only members to the anti-apoptotic family members, leading to the release oligomerization of Bak and Bax. (E. H. Y. A. Cheng et al., 2001; Willis et al., 2005)

A second model proposed is the direct activator model (Figure 17, middle), in which the BH3 only proteins are divided into two groups. One group are called the activators, namely Bid, Bim and Puma, and the rest of the BH3-only proteins are called sensitizers. In this model, the anti-apoptotic proteins sequester the BH3-only activators and prevent them from binding and activating Bax and Bak. Following an apoptotic signal, the sensitizers can bind to the anti-apoptotic family members leading to the release of the activator BH3-only proteins. The activators can then bind and activate the executioners Bax and Bak. (Certo et al., 2006; H. Kim et al., 2006; Letai et al., 2002) Proof for this model came from the fact that the activation of Bax and Bak was impaired in triple knockout cells for BH3-only activators Bid/Bim/Puma. (Ren et al., 2010)

The third model, the unified model (Figure 17, right), depends on the anti-apoptotic proteins that on one hand sequester the direct BH-3 activators (Mode1) and on the other hand sequester Bax and Bak directly (Mode 2). The activation of this model depends on the binding of BH-3 only sensitizers to the anti-apoptotic proteins, followed by the activation of both modes, due to the dual effect of the anti-apoptotic proteins on both the effector and BH3-only activators. Even though both inhibitions of apoptosis take place simultaneously, mode 1 is easier to bypass by BH3-only sensitizers and induce MOMP then mode 2. (Llambi et al., 2011)

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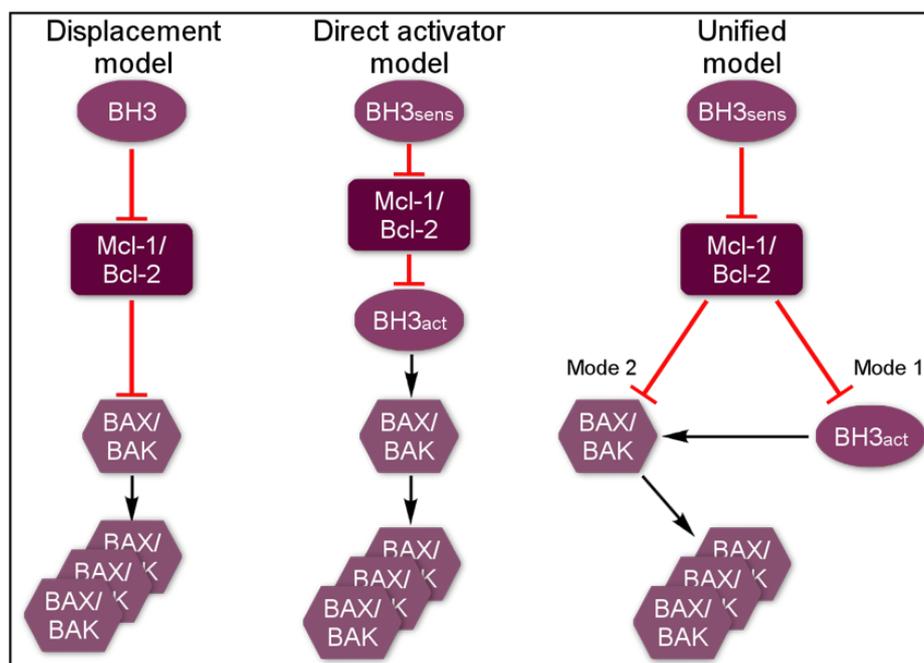


Figure 17: Proposed models of activation of mitochondrial intrinsic apoptotic pathway by the Bcl-2 family.

A graphical summary of proposed models of activation of apoptosis through interactions between Bcl-2 family members. The anti-apoptotic proteins (green) sequester and inhibit either directly the pro-apoptotic effectors (red) or the BH3-only activators (brown). Upon apoptotic signals BH3-only can interact with the anti-apoptotic proteins, leading the release of Bax and Bak directly or they release BH3-only activators that in turn activate Bax and Bak leading to their activation, oligomerization and pore formation in the mitochondrial membranes. Figure adapted from Bender and Martinou. (Bender & Martinou, 2013) Act: activator, sens: sensitizer

(3) Induction and execution of apoptosis

The apoptotic pathway consists of initiation pathways and an execution pathway. The initiation pathways lead to the activation of the so-called executioners of apoptosis, namely caspases. The human caspase protein family consists of cysteine proteases that can be divided into 3 groups based on structural similarity and their function. Group I consist of pro-inflammatory caspases -1, -4 and -5. The pro-apoptotic caspases can be divided in two groups: (I) group II, the apoptotic effector caspases, namely caspase-3, -6 and -7, and (II) group III, the initiation caspases-2, -8, -9 and -10. (Julien & Wells, 2017) Under physiological conditions the pro-apoptotic caspases are expressed as inactive procaspases, which are processed into their active form following apoptotic signals. The activation of initiator caspases can activate effector caspases amplifying the apoptotic signaling pathway. The activation of for example caspases-8, -9 and -10 comes from the dimerization of their monomeric procaspases following the recruitment into a protein scaffold and/or proteolytic cleavage. Other caspases, such as caspases-3, -6 and -7, which are constitutive dimers even in their procaspase form, are activated following cleavage by either initiation caspases or auto-proteolysis. (Riedl & Shi, 2004; Salvesen & Ashkenazi, 2011; Stennicke & Salvesen, 2000)

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Once the executioners of apoptosis are activated there is no turning back and the cell will die a programmed cell death. (G. M. Cohen, 1997) Up to date more than 400 substrates have been mentioned in literature to be cleaved by caspases. (Lüthi & Martin, 2007) The events leading to the activation and subsequent apoptotic events related to activated caspases will be discussed below.

The two main apoptotic initiation pathways, leading to the activation of caspase-3, -6 and -7, are the extrinsic pathway, that is activated upon extracellular signals, and the intrinsic pathway, that is activated by signals coming from within the cell. However, a third initiation pathway has been described to be able to induce apoptosis as well, namely the perforin/granzyme pathway.

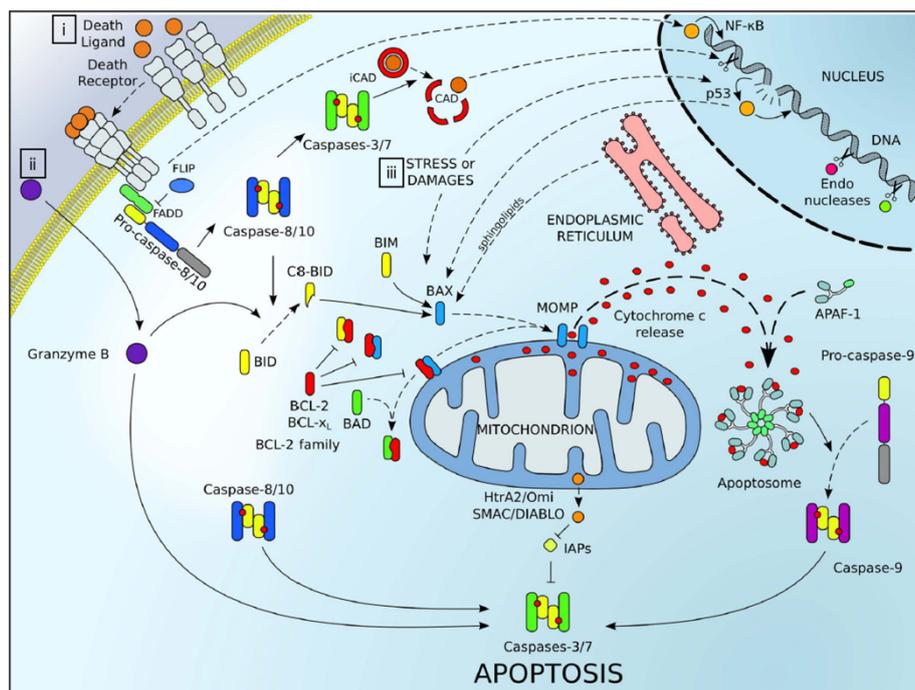


Figure 18: Initiation pathways of apoptosis

Graphic representation of the apoptotic pathways, the extrinsic pathway (i) activated upon extracellular signals, the granzyme/perforin pathway (ii) following cytotoxic T-lymphocyte signals and the intrinsic pathway (iii) activated upon signals from within the cells. The pathways lead to the activation of effector caspases 3, 6 and 7. The extrinsic pathway directly activates caspase-8 which in turn activated the effectors and granzyme B interacts directly with the effectors, the intrinsic pathway is dependent on the diminished integrity of the mitochondrial membrane regulated by the Bcl-2 family and the release of cytochrome C and other apoptotic proteins, followed by the formation of the apoptosome involving caspase-9, which activates the effectors. Inter pathway communication leads to increased apoptotic signaling. Figure taken from (Renault & Chipuk, 2013) APAF-1: apoptotic protease activating factor 1 ; CAD: Caspase activated DNase FADD: Fas-associated protein with death domain; IAPs: Inhibitors of apoptosis; MOMP: Mitochondrial outer membrane permeabilization.

The extrinsic pathway (Figure 18i) is activated upon binding of specific ligands to transmembrane DR present on the cell surface. DR are members of the TNF super family of proteins, of which the best-characterized are FasR (aka APO-1), Tumor necrosis factor receptor 1

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(TNFR1), DR3 (aka APO-3), DR4 (aka TRAILR1, APO-2) and DR5 (aka TRAILR2). These receptors have in common a cytoplasmic domain called the death domain (DD), which plays an essential role in signal transduction following the engagement of a ligand to the receptor. (Ashkenazi & Dixit, 1998; Peter & Krammer, 1998; Suliman, Lam, Datta, & Srivastava, 2001) Activation of the DR leads the recruitment of adaptor protein Fas-associated protein with death domain (FADD) directly or with the aid of a second adaptor protein TNFR1 associated DD protein (TRADD). FADD associates with the non-active procaspase-8 and -10 forming the death inducing signaling complex (DISC) leading to the activation and release of initiators caspase-8 and -10. (Kischkel et al., 1995; Sprick et al., 2002)

The perforin/granzyme pathway (Figure 18ii) plays a role in CTL cytotoxicity through the release of granzyme B into the target cell. Granzyme B is a proteasome that can cleave proteins, and it has been shown that granzyme B can induce apoptosis in several ways. For one direct targets of granzyme B are the caspases, notably caspase-10 and caspase-3. On the other hand, Granzyme B induces the activation of caspase activated DNase (CAD), an apoptotic nuclease, through interaction with inhibitor of caspase activated DNase (ICAD). (Talanian et al., 1997; Thomas, Du, Xu, Wang, & Ley, 2000)

The intrinsic pathway, or mitochondrial pathway (Figure 18iii), is activated upon signals coming from within the cell, which implicates non-receptor mediated signals. The intracellular signals that initiates the intrinsic pathway can come from either positive or negative modus operandi. The negative signals can come from the withdrawal of stimulating factors leading to the loss of apoptotic suppression and activation of the intrinsic pathway. (Elmore, 2007) The positive factors can come from stress applied to the cells, e.g. radiation, viral infection, the presence of toxins, and free radicals. These signals trigger the dis-balance between the Bcl-2 family members and initiate, leading to the release of pro-apoptotic proteins into the cytoplasm. The release of pro-apoptotic Cytochrome c from the mitochondria is the point of no return in the intrinsic pathway, followed by the activation of apoptotic protease activating factor 1 (APAF-1). Pro-caspase-9 is recruited to the Cytochrome c-APAF-1 complex leading to the formation of a complex called the apoptosome and subsequent activation of caspase-9. (Hill, Adrain, Duriez, Creagh, & Martin, 2004; J. Yang et al., 1997) The release of Smac/DIABLO and omi/Htra from the mitochondria promote apoptosis through the inhibition of inhibitors of apoptosis (IAPs) (C. Du, Fang, Li, Li, & Wang, 2000; van Loo et al., 2002) A third group of pro-apoptotic proteins released from the mitochondria are involved in chromatin condensation and nucleotide fragmentation, either in a caspase independent or dependent manner. (Enari et al., 1998; Joza et al., 2001; L. Y. Li, Luo, & Wang, 2001) The apoptotic initiation pathways are intertwined, and the activation of one can induce the

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activation of the other to amplify apoptotic signaling. For one, activated caspase-8 can cleave and activate Bid, a member of the Bcl-2 family, and establish intrinsic apoptotic signaling and thus promote cytochrome c release and activation of caspase-3. (H. Li, Zhu, Xu, & Yuan, 1998) It has been shown in some cell types that the activation of the mitochondrial pathway following FasR mediated apoptosis, through the activation of caspase-8 followed by the cleavage of Bid, is necessary to release of cytochrome C and the activation of caspase-9 to induce apoptosis (Scaffidi et al., 1998; Yin et al., 1999) For Granzyme B it has also been shown to establish intrinsic apoptotic signaling by interacting with Bid. (Sutton et al., 2000)

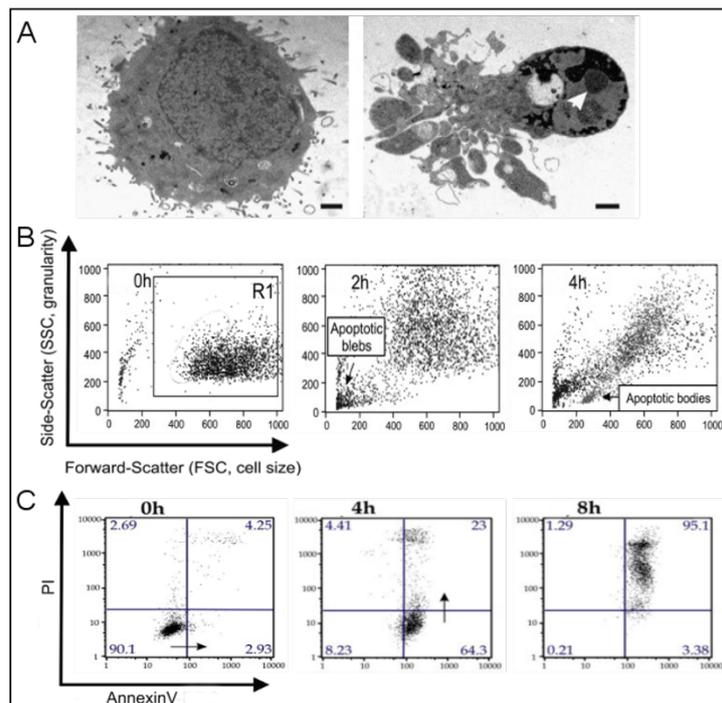


Figure 19: Identification of apoptotic cells based on morphological changes observed by electron microscopy and flow cytometry.

A) comparison of a healthy cell (left pane) to an apoptotic cell (right pane), the apoptotic cell shows condensed chromatin (arrow head) and nuclear fragmentation, as well as blebbing of the membrane and the formation of apoptotic bodies. (B) Flow cytometry can identify apoptotic cells based on the size and granularity of the cell. Apoptotic cells decrease in size while they become more granular hence the spreading of the dots. (C) Flow cytometry can identify apoptotic cells based on the permeabilization of the cells membrane, PI uptake, and flip-flop of phosphatidylserine, by binding of AnnexinV, as well. Early apoptotic cells are identified as AnnexinV positive and PI negative while late apoptotic cells are identified as AnnexinV and PI positive. Figure adapted from Krysko, Vanden Berghe, D'Herde, & Vandenabeele, 2008b. PI: propidium iodide

The final results of the induction pathways, is the activation on activator caspases-3, -6 and 7 which induces the execution phase of apoptosis. Caspases, once activated, are able to induce morphological and biochemical changes that have been well documented. Morphologically the cell

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will become smaller due to shrinking of the cytoplasm which is followed by bulging, of the cell membrane, also known as blebbing. Given that if the apoptotic cell is not immediately removed through phagocytoses it will be broken down in smaller vesicles, containing cellular components such organelles, called apoptotic bodies that are prone to phagocytoses. (Figure 19) (Kerr, Wyllie, & Currie, 1972; Krysko, Vanden Berghe, D'Herde, & Vandenabeele, 2008)

The blebbing is partly explained by a weakened cytoskeleton, due the fact that many cytoskeletal components can be cleaved by caspases, and the flow of the cytosol against unsupported parts of the plasma membrane. (R. C. Taylor, Cullen, & Martin, 2008) Some intact contracted actin seems to be necessary for the membrane blebbing and contraction of actin filaments depends on the caspase dependent activation of ROCK1. (Coleman et al., 2001; Sebbagh et al., 2001)

Biochemically, during apoptosis the nucleus is affected as observed by condensation of the chromatin, DNA fragmentation, shrinking of the nucleus and finally nuclear defragmentation (caryorrhexie). (Toné et al., 2007) The protein CAD has been found as the main player in DNA fragmentation during apoptosis. It was observed in CAD deficient cells undergoing apoptosis do not show DNA fragmentation as well as chromatin condensation. Non-active CAD is present in the cell in association with ICAD, cleavage of ICAD by caspases leads to its release and active CAD can then translocate to the nucleus to perform its role. (Enari et al., 1998; Napirei et al., 2000; Samejima, Tone, & Earnshaw, 2001) Interestingly mice deficient for CAD leading to the impairment of DNA fragmentation provoked an immune response normally absent during apoptosis, underlining the importance of CAD in efficiently clearing apoptotic cells by phagocytosis. However early chromatin condensation is not blocked in CAD deficient mice, indicating a secondary signal involved in chromatin condensation. (Samejima et al., 2001) It has been suggested that histone phosphorylation plays a role in apoptosis dependent chromatin condensation and indeed a caspase dependent activation for mammalian sterile-20, a kinase responsible for histone phosphorylation, was observed. Mutations in the caspase binding site of this kinase strongly reduced chromatin condensation observed during apoptosis. Next to the effect on nuclear contents, apoptosis leads to caryorrhexie, which is in part, like the cell membrane, due to ROCK1 dependent actin contraction. Besides being responsible for actin contraction, caspases can cleave nuclear lamins wakening the nuclear envelope, causing nuclear fragmentation. (Croft et al., 2005; Rao, Perez, & White, 1996) Caspases are able to target many proteins involved in the regulation of transcription, they able to cleave transcription and translation, shutting down the entire housekeeping of the cell. (Cheung et al., 2003; Lüthi & Martin, 2007; Ura, Masuyama, Graves, & Gotoh, 2001)

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Activated caspases are also involved in changes in the outer cell membrane, particularly the flip-flop of phosphatidylserine leading to its expression on the cell surface, causing the apoptotic cell to be recognizable by phagocytes through specific receptors. (Fadok et al., 1992, 2000) Nuclear fragmentation is involved in the recruitment of phagocytes as well, since it has been shown that nucleotides, especially adenosine triphosphate and uridine triphosphate, are potent chemoattractants. (Elliott et al., 2009) It is important for the apoptotic cell to be cleared by phagocytosis, since defects in the clearance of apoptotic cells leads to a stage called late apoptosis (secondary necrosis). During late apoptosis, the cell membrane will become more permeable and release their content to induce an inflammatory response. (Krysko, D'Herde, & Vandenabeele, 2006) The central role of caspases is summarized in Figure 20

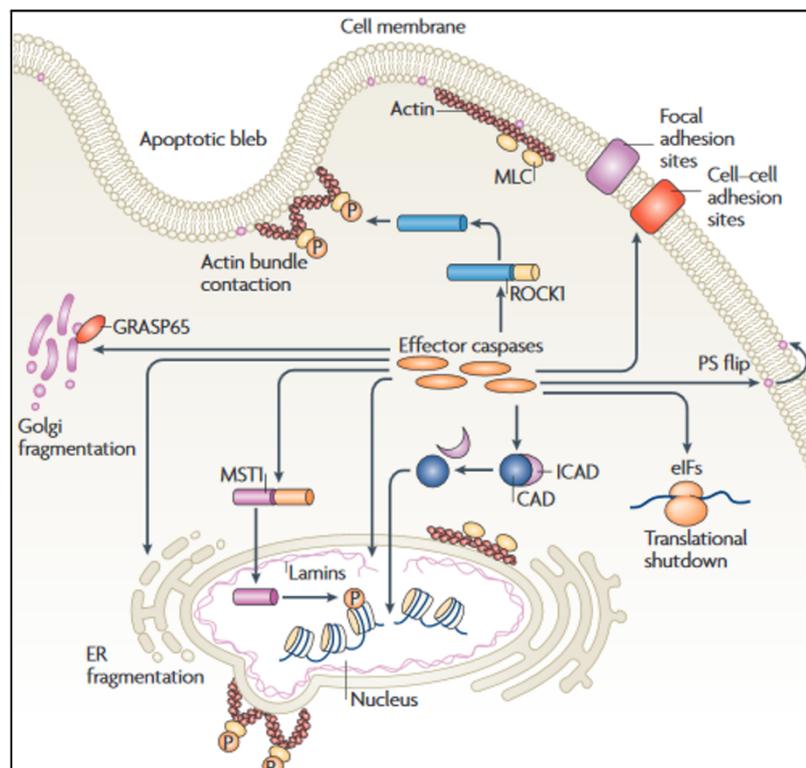


Figure 20: Effector pathway of apoptosis, the central role of caspases.

Effector caspases play a central role in the execution of apoptosis, by cleaving many proteins they can regulate their activity. By activating ROCK1, caspases lead to blebbing of the membrane observed for apoptotic cells. They can activate kinases and DNases involved in chromatin condensation and DNA fragmentation. Caspases can alter the cells housekeeping by shutting down translational activity as well as organelle fragmentation. Finally, an important role of caspases is in regulating signals to induce phagocytosis and minimize an inflammatory response. Figure taken from (R. C. Taylor et al., 2008) eIFs: eukaryotic translation initiation factors; ER: Endoplasmatic reticulum; MST1: Mammalian sterile-20 PS: phosphatidylserine

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(IV) B-cell persistence in organ transplantation

In recent years the development of new therapeutic strategies, based on the use of immunosuppressive drugs, such as calcineurin inhibitors has led to a significant improvement in the prevention and treatment of acute rejection. (D. J. Cohen, 1984; Meier-Kriesche, Schold, Srinivas, & Kaplan, 2004) However, the side effects such as increased cardiovascular risk, diabetes and induced nephrotoxicity related to the treatment have been assigned to calcineurin inhibitors. (Cosio et al., 2005; Palestine et al., 1986; Sander, Lyson, Thomas, & Victor, 1996) Leading to necessity of developing new immunotherapies to prevent acute and chronic organ rejection.

Treatments to reduce (pre-)existing HLA-Ab have been developed, for example plasmapheresis (Bonomini, Vangelista, Frascà, Di Felice, & Liviano D'Arcangelo, 1985; Slatinska, Honsova, Burgelova, Slavcev, & Viklicky, 2009) and high dose of intravenous immunoglobulin (IV-IG) (Jordan, Vo, Tyan, & Toyota, 2006; Waiser et al., 2016) to de-sensitize highly sensitized patients, however with poor efficiency since these methods do not prevent *de novo* DSA synthesis. As mentioned before DSA are involved in the activation of the complement which play a role in AMR and related graft damage. Promising effects have observed when using complement inhibitors in treating AMR, and the results suggest that that treatment with complement inhibitors might serve in severe early AMR (Orandi et al., 2014; Yelken et al., 2015). However, complement inhibitors do not prevent early AMR in all patients and they do not modulate the B-cell response that causes the AMR. (Bentall et al., 2014; Cornell, Schinstock, Gandhi, Kremers, & Stegall, 2015) Other treatments which are directed against the B-cells themselves in order to block DSA synthesis, such as monoclonal anti-CD20 antibodies and proteasome inhibitors, have been developed. CD20 is a membrane protein expressed on the cell surface of many B-cell populations. The treatment of B-cell malignancies with rituximab have been investigated and it was shown that by blocking CD20 the survival of malignant cells was reduced. This occurred via inhibition of intracellular signaling involving p38, NF- κ B, ERK and AKT anti-apoptotic signaling, depending on the type of malignancy, inducing B-cell apoptosis. (Bonavida, 2007; Jazirehi, Gan, Vos, Emmanouilides, & Bonavida, 2003; Shan, Ledbetter, & Press, 2000) In organ transplantation promising results have been observed in increasing graft survival and reducing aAMR. (Faguer et al., 2007; Kaposzta et al., 2009; Mulley et al., 2009) However, CD20 is not expressed on pro-B-cells and notably PCs, leading to rituximab's inadequacy to completely deplete the PC lineage which is responsible for the production of circulating IgG. (M. R. Clatworthy, 2011; Valeriya Zarkhin, Chalasani, & Sarwal, 2010) The effect of rituximab on the levels of DSA has been disputed as well, some studies have shown a reducing effect of rituximab on DSA levels in some particular patients, whereas others did not observe this effect. (Faguer et al., 2007; Fehr et al., 2009; Tanriover et al., 2008; V. Zarkhin

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et al., 2008) Another treatment that has emerged in preventing AMR are proteasome inhibitors, which is commonly used in plasma cell disorders. Bortezomib was shown to selectively induce apoptosis in activated PCs. (Diwan et al., 2011) Recent studies have shown promising results with Bortezomib alone or in combination with plasmapheresis, IV-IG, steroids and/or rituximab, increasing graft survival. (Aubert et al., 2014; Kizilbash et al., 2017; Waiser et al., 2016; Woodle, Walsh, Alloway, Girnita, & Brailey, 2011) However Waiser et al. (Waiser et al., 2016) observed increased side effects in patients treated with Bortezomib in combination with plasmapheresis, IV-IG and rituximab.

Nevertheless, the outcome of renal transplantation in cases of aAMR and cAMR is still poor. This is due to the inefficiency of current drugs to completely control the humoral immune response since AMR is a main cause of chronic organ failure, and it is currently responsible for nearly one fourth of total graft losses. (Durrbach, Francois, Beaudreuil, Jacquet, & Charpentier, 2010; Lodhi, Lamb, & Meier-Kriesche, 2011; Loupy, Hill, & Jordan, 2012) It is apparent that these therapies often fail to target and destroy MBCs and/or PCs responsible for both aAMR and cAMR. (Jackson et al., 2015; Leandro, 2013; Perry et al., 2008; Ramos et al., 2007).

It is known that the main source of MBCs and PCs is the GC reaction, however tertiary lymphoid structures (TLS) have been identified which show apparent similarity to GCs found in secondary lymphoid organs. Indicating that these TLS are responsible for local lymphoid-neogenesis. TLS generally arise from chronic inflammation, viral infections and cancers (GeurtsvanKessel et al., 2009; Goc et al., 2014; Kratz, Campos-Neto, Hanson, & Ruddle, 1996; Takemura, Klimiuk, Braun, Goronzy, & Weyand, 2001) The development of TLS supporting functional ectopic germinal center reaction has been evidenced within human transplants as identified by infiltrating B-cells, T-cells and follicular dendritic cells following a histological study (Figure 21). (Koenig & Thaunat, 2016; Thaunat et al., 2005) GC B-cell functionalities, such as clonality, high proliferation, and upregulation of GC related genes, have been observed in these infiltrations. (J. Cheng et al., 2011; Kerjaschki et al., 2004; Thaunat et al., 2005) The presence of TLS within rejected organs indicate that local *de novo* synthesis of effector B-cells can occur within the rejected organ.

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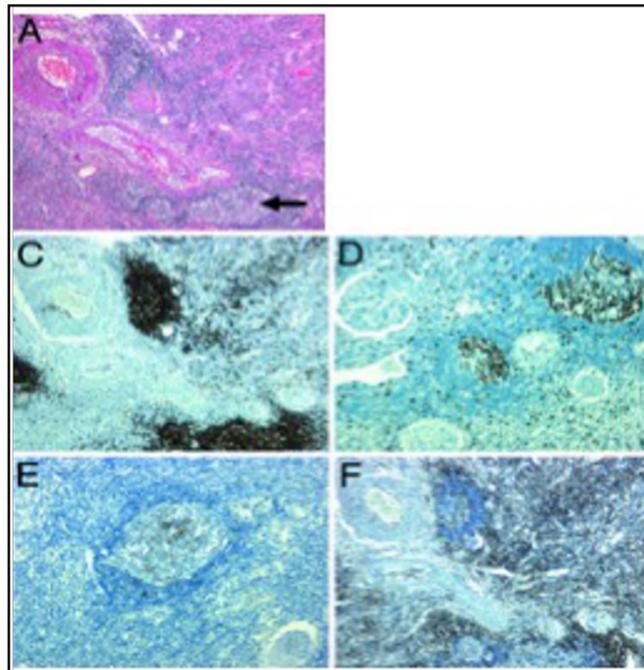


Figure 21: Histological analysis of chronically rejected kidney graft indicates the presence of tertiary lymphoid structures.

Hematoxylin and eosin staining show the presence of germinal center like structures, indicated by the arrow (A). Using specific markers such as anti-Ki-67, indicating proliferation (C), anti-CD20, a B-cell marker (D), anti-CD23, a dendritic cell marker (E) and anti-CD3, a T-cell marker (F) Thauinat et al. showed the presence of cells found in the classical germinal center, highlighting the presence of tertiary lymphoid structures. Figure adapted Thauinat et al (Thauinat et al., 2005)

(I) Objectives

Due to the high prevalence and difficulty to control B-cell lineages during (c)AMR we for this project we aim to: (1) further characterize the B-cells infiltrating cAMR kidney transplants as B-cells resembling GCs and (2) develop strategies to impair the activation and survival of GC B-cells while further elucidating the pathways activated upon BCR mediated Ag recognition in GC B-cells. Understanding the GC B-cell activation and survival will help us to prevent the differentiation into MBCs and PCs and thus the production anti-HLA antibodies to prevent and/or treat acute and chronic humoral rejection.

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(I) Article

SYK inhibition induces apoptosis in germinal center-like B-cells by modulating anti-apoptotic protein Mcl-1, affecting B-cell activation and antibody production.

Nathalie Roders, Florence Herr, Gorbachev Ambroise, Olivier Thaunat, Aimé Vazquez and Antoine Durrbach

Renal failure is a major health concern in westernized countries, and renal transplantation is the main therapeutic method in case of end-stage renal failure. This is due to the association with a better quality of life, longer survival time and the cost efficiency compared to dialysis. However, a risk associated with renal transplantation is organ rejection due to alloimmune responses directed against the grafted organs, a process called antibody mediated rejection (AMR). Alloantibodies produced against the graft are associated with a high frequency of organ deterioration and graft loss despite current treatments that are developed with the aim to counteract AMR. However, these therapies frequently fail to control donor-specific antibody (DSA) production and plasma and memory B-cell formation. Which is observed by the fact that AMR is a main cause of chronic organ failure, and it is currently responsible for nearly one fourth of total graft losses.

Alloantigen recognition by the B-cell receptor plays an important role in the initiation of the humoral immune response and B-cell differentiation into Ab producing plasma cells and memory B-cells. Following T-cell dependent B-cell activation and the formation of the germinal center (GC), the B-cells undergo clonal expansion, isotype class switching, somatic hypermutation and affinity maturation and selection. They leave the GC as highly specific long-lived memory B-cells and Ab-producing plasma cells. The persistence of the GC and the selection of high-affinity effector B-cells are regulated through pro- and anti-apoptotic signals induced upon Ag binding and cell-mediated interactions. Myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic member of the B-cell lymphoma-2 (Bcl-2) family, is essential for maintaining the GC reaction and B-cell differentiation. Persistent inflammation due to an ongoing immune response can lead to the formation of tertiary lymphoid structured that resemble GCs. The development of tertiary lymphoid structures (TLS) supporting functional ectopic germinal center reaction has been evidenced within human transplants undergoing chronic AMR (cAMR)

The aim of this research is for one to further characterize the B-cells infiltrating cAMR kidney transplants by using histological sections of kidneys from patients with cAMR. Secondly, we aimed to develop strategies to impair the activation and survival of B-cells while further elucidating the pathways activated B-cell activation. For this we used Burkitt's lymphoma cell line

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BL41 as a model for GC centroblasts and aimed to abrogate BCR signaling by inhibiting spleen tyrosine kinase (SYK) which acts very early in the signaling chain. We assessed the effect of SYK inhibition on BL41 cell viability by flow cytometry and determined the effect on anti-apoptotic protein Mcl-1, member of the B-cell lymphoma-2 (Bcl-2) protein family, by western blot and real-time quantitative PCR. We aimed to elucidate the signaling pathway involved in the regulation of Mcl-1 and determine the indispensability of Mcl-1 in maintaining cell viability in BL41 cells by retroviral transduction of these cells. We determined the effect of SYK inhibition on primary B-cells, extracted from human tonsils, by looking at cell viability, the expression of Mcl-1 and the activation state determined by CD80 expression. We also aimed to determine the effect of SYK inhibition in antibody secretion using a total IgG ELISA

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SYK inhibition induces apoptosis in germinal center-like B-cells by modulating anti-apoptotic protein Mcl-1, affecting B-cell activation and antibody production

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SYK Inhibition Induces Apoptosis in Germinal Center-like B-cells by Modulating Anti-Apoptotic Protein Mcl-1, Affecting B-cell Activation and Antibody Production

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ABSTRACT

B-cells play a major role in antibody-mediated rejection (AMR) of solid organ transplants, a major public health concern. During chronic AMR, tertiary lymphoid germinal center (GC)-like structures appear in the rejected organ, leading to the generation of donor-specific plasma and memory B-cells, which are often poorly controlled by current treatments. Myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic member of the B-cell lymphoma-2 family, is essential for maintaining the GC reaction and B-cell differentiation. We report here the infiltration of B-cells expressing Mcl-1 in the kidney of patients with chronic AMR, as observed for (pre-)GC cells. The impairment of B-cell receptor (BCR) signaling, by inhibition of spleen tyrosine kinase (SYK), reduced viability and Mcl-1 protein levels in a Burkitt's lymphoma cell line. This downregulation is coordinated at the transcriptional level, potentially via signal transducer and activator of transcription 3 (STAT3), as shown by (1) impaired translocation of STAT3 to the nucleus following SYK inhibition, and (2) the lower levels of Mcl-1 transcription upon STAT3 inhibition. Moreover, overexpression of Mcl-1 prevented cells from entering apoptosis after SYK inhibition. In vitro studies with primary tonsillar B-cells confirmed that SYK inhibition decreased cell survival. We also found that SYK

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inhibition decreased Mcl-1 protein levels in total as well as GC B-cells and that B-cell activation was inhibited, as determined by CD80 expression. We observed lower levels of IgG secretion in tonsillar B-cells activated in vitro following SYK inhibition. Overall, our data suggest that the SYK-Mcl-1 pathway may provide new opportunities for the treatment and prevention of AMR.

INTRODUCTION

B-cells play a major role in acute and chronic antibody-mediated rejection (AMR) and allograft survival. AMR following solid organ transplantation is associated with a high frequency of organ deterioration and graft loss despite treatment. Current treatments include plasmapheresis (Bonomini et al., 1985; Slatinska et al., 2009), high dose intravenous immunoglobulin (IV-IG) (Jordan et al., 2006; Waiser et al., 2016), monoclonal anti-CD20 antibodies (Faguer et al., 2007; Mulley et al., 2009), proteasome inhibitors (Waiser et al., 2016; Kizilbash et al., 2017) and complement inhibitors (Orandi et al., 2014; Yelken et al., 2015), which frequently fail to control AMR, donor-specific antibody (DSA) production and memory B-cell formation. Germinal centers (GC) drive B-cell differentiation into DSA-producing plasma and memory B-cells. (Jacob et al., 1991b) The development of tertiary lymphoid structures (TLS) supporting functional ectopic germinal center reaction has been evidenced within human transplants undergoing chronic AMR (cAMR). (Thaunat et al., 2005; Koenig and Thaunat, 2016)

B-cell activation through B-cell receptor (BCR) signaling drives B-cell survival, differentiation, anergy, or apoptosis, depending on co-signals received by the cell. Antigen (Ag)-dependent BCR activation leads to the recruitment and activation of spleen tyrosine kinase (SYK). (Cornall et al., 2000; Tsang et al., 2008) Active SYK induces the formation of a signalosome, containing kinases and adaptor proteins, which sets in motion signaling cascades such as those involving AKT, mitogen-activated protein kinases (MAPK), nuclear factor of activated T-cells (NFAT), and nuclear factor- κ B (NF κ B), resulting in translational modifications. (Dal Porto et al., 2004) Following T-cell dependent B-cell activation and the formation of the GC, the B-cells undergo clonal expansion, isotype class switching, somatic hypermutation (Berek et al., 1991; Jacob et al., 1991a; Muramatsu et al., 2000), and affinity maturation and selection (Liu et al., 1989; Tarlinton and Smith, 2000; Meyer-Hermann et al., 2006). They leave the GC as highly specific long-lived memory B-cells and antibody (Ab)-producing plasma cells. The persistence of the GC and the selection of high-affinity effector B-cells are regulated through pro- and anti-apoptotic signals induced upon Ag binding and cell-mediated interactions. (Billian et al., 1997; Tarlinton and Smith, 2000; Fischer et al., 2007)

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The B-cell lymphoma-2 (Bcl-2) protein family maintains a delicate balance between cell survival and apoptotic death. Members belonging to this family can be subdivided into three groups: (i) anti-apoptotic proteins, including myeloid cell leukemia-1 (Mcl-1), Bcl-2 and Bcl-x_{s/l}, which play an essential role in cell survival; (ii) pro-apoptotic proteins BAX and BAK, which are required to trigger downstream apoptotic processes, such as the release of cytochrome C from the mitochondria, and the subsequent activation of caspases; (iii) the so-called “BH-3-only” proteins, including Puma, Noxa, Bad, Bid and Bim, which interact with the other members of the family to control their activity. (Youle and Strasser, 2008; Shamas-Din et al., 2013; Siddiqui et al., 2015) Vikstrom et al. (Vikstrom et al., 2010) showed that no GCs or memory B-cells form in the absence of Mcl-1, highlighting the importance of Mcl-1 for GC maintenance and B-cell differentiation. In a previous study performed by our team, involving the role of PUMA in regulating mitogen-activated B-cells and memory B-cells, we observed that PUMA and noticeably Mcl-1 are expressed in GCs *in vivo*. (Clybouw et al., 2011)

In this study, we observed B-cells expressing Mcl-1, as reported for (pre-)CG B-cells, infiltrating kidneys displaying cAMR. We investigated the relationship between BCR signaling and the survival and differentiation of B-cells, by inhibiting SYK. Using Burkitt's lymphoma derived cells as a model for GC centroblasts, we showed that SYK inhibition led to the downregulation of Mcl-1 gene expression via signal transducer and activator of transcription 3 (STAT3), and a decrease in cell viability. The synthesis of immunoglobulins was impaired by SYK inhibition in primary B-cells *in vitro*; these cells also displayed lower viability and Mcl-1 protein levels, and weaker B-cell activation following SYK inhibition.

MATERIALS AND METHODS

Reagents and Antibodies

The following reagents were used: BAY61-3606 (Merck Millipore), Stattic (Torcis), Q-VD-Oph (Sigma), MG-132 (Calbiochem) and cycloheximide (Sigma).

We used the following primary Abs: hCD19-APD-Cy-7 (SJ25C1; BD Biosciences Cat# 557791 RRID: AB_396873), hCD38-BV421 (HIT2; BD Biosciences Cat# 562445 RRID:AB_11153870) hsIgD-Pe-Cy7 (IA6-2; BD Biosciences Cat# 561314 RRID:AB_10642457), hCD80-BV605 (L307.4; BD Biosciences Cat# 563315), hPhospho-SYK-AF488 (C87C1; Cell Signaling Technology Cat# 4349) and hMcl-1-FITC (Biorbyt Cat# orb15956 RRID:AB_10747574) for flow cytometry. Anti-Mcl-1 (S-19; Santa Cruz Biotechnology Cat# sc-819 RRID:AB_2144105), anti-Bcl-2 (C-2; Santa Cruz Biotechnology Cat# sc-819 RRID:AB_2144105), anti-Bcl-x_{s/l} (S-18; Santa Cruz Biotechnology Cat# sc-819

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RRID:AB_2144105), anti-GAPDH (Sigma-Aldrich Cat# G9545 RRID:AB_796208) and anti-PARP (Cell Signaling Technology Cat# 9542 also 9542S, 9542L, 9542P RRID:AB_2160739) antibodies were used for immunoblotting and anti-hCD19 (HIB19; BD Biosciences Cat# 555409 RRID:AB_395809), anti-hMcl-1 (Sigma-Aldrich Cat# HPA008455 RRID:AB_1079334) and anti-STAT3 (K-15; Santa Cruz Biotechnology Cat# sc-483 RRID:AB_632441) Abs were used for immunohistology.

Retroviral particles were generated with the following plasmids: expression plasmid pBabe-Flag-hMcl-1, a gift from Roger Davis (Morel et al., 2009) (Addgene plasmid # 25371), empty vector control plasmid pBabe-puro-IRES-EGFP, a gift from L. Miguel Martins (Addgene plasmid # 14430), envelope-expressing plasmid pCMV-VSV-G, a gift from Bob Weinberg (Addgene plasmid # 8454), and packaging plasmid pCL-Eco (Novus Biologicals).

Cell Culture

BL41 Burkitt's lymphoma cells were cultured in complete RPMI medium: RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Dominique Dutscher), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma).

HEK 293T and CD40 ligand/CD32 ligand-expressing murine fibroblasts were cultured in complete DMEM: Dulbecco's modified Eagle medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1 mg/ml Normocin™ (InvivoGen).

Primary cells were isolated from tonsillar tissue removed from patients during tonsillectomy. The tonsils were dissected and pushed through a stainless-steel strainer with a glass grinder. Cells were collected and washed with complete RPMI, then homogenized by passage through a nylon cell strainer with 100 µm pores (BD Bioscience).

Tonsillar cells were cocultured in complete RPMI, with CD40 ligand/CD32 ligand-expressing fibroblasts, in the presence of anti-µ Abs (Jackson ImmunoResearch), LPS (Sigma) and BAY61-3606. Fibroblast growth was blocked by incubation with mitomycin C (10 µg/ml, Roche) for thirty minutes at 37°C with 5% CO₂ before the addition of tonsillar cells.

Retrovirus Production and Cell Transduction

Retroviruses were generated by the transient co-transfection of HEK 293T with a three-plasmid combination, by the calcium phosphate coprecipitation method, as previously described (Gavrilescu and Van Etten, 2007). Following culture for three days, the retroviral particles were collected and concentrated with 5x PEG IT™ viral precipitation solution (System Biosciences).

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For retroviral transduction, 2×10^6 BL41 cells were collected and suspended in a suspension of retroviral particles in the presence of polybrene (Santa Cruz Biotechnology). The resulting suspension was then centrifuged at $300 \times g$ for ninety minutes. Cells were incubated at 37°C with 5% CO_2 for two hours, and fresh complete RPMI was then added. Transduced cells were selected by a series of puromycin ($1 \mu\text{g}/\text{ml}$; InvivoGen) treatments.

Western Blotting

Whole-cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA (ethylenediaminetetraacetic acid), 1% Triton X-100 and 1% Igepal/NP-40), supplemented with Halt™ Protease inhibitor cocktail (Thermo Scientific). For phosphorylation analysis, the phosphatase inhibitors β -glycerophosphate (12.5 nM), sodium orthovanadate (10 μM), sodium fluoride (0.1 mM) and N-ethylmaleimide (30 μM) were added before cell lysis. Protein determinations were performed with the micro-BCA protein assay kit (Thermo Scientific). Protein samples (equal mass) were heated for five minutes at 99°C after the addition of Tris-glycine SDS sample buffer (Life Technologies) containing 10% β -mercaptoethanol (Sigma). They were subjected to polyacrylamide gel electrophoresis and proteins were transferred onto nitrocellulose membranes (Santa Cruz). The membranes were incubated with primary Abs, and Ab binding was visualized by chemiluminescence with HRP-conjugated secondary Abs (Jackson Immunoresearch), the Immobilon western chemiluminescent HRP substrate (Millipore) and a DDC camera (LAS-4000 mini, Fujifilm).

Flow Cytometry

BL41 cell viability was assessed by flow cytometry, with a BD Accuri C6 flow cytometer (BD Biosciences). Viability was assessed by expressing the proportion of viable cells (excluding granular and shrunken cells) as a percentage of the total cell population, based on forward and side scattering profiles.

Apoptotic cells were identified with the Pacific Blue Annexin V Apoptosis detection kit and 7-AAD (Biolegend), according to the manufacturer's protocol. Cells were analyzed in a BD LSRFortessa flow cytometer (BD Biosciences).

For extracellular staining, cells were incubated with fvs620 (1:1000 dilution; BD Biosciences) in 1 x PBS for fifteen minutes at room temperature. Non-specific binding was blocked with human BD FC block ($2.5 \mu\text{g}/1 \times 10^6$ cells; BD Biosciences) in 1 x PBS supplemented with 2.5% FCS and 0.1% sodium azide, and cells were then incubated with fluorescent Abs. Intracellular staining for Abs against non-phosphorylated proteins was performed as follows: cells were fixed

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in 4% paraformaldehyde (PFA, Alfa Aesar), then quenched with NH_4Cl (100 mM), permeabilized with 0.15% saponin (VWR), and incubated with conjugated Abs in the presence of saponin.

For intracellular staining for phosphorylation analysis, saponin was replaced with 0.1% Triton X-100 (Sigma) and additional 50% methanol on ice for permeabilization. Cells were then incubated with fluorochrome-conjugated Abs and analyzed on a BD LSRFortessa flow cytometer (BD Biosciences). All flow cytometry data were analyzed with FlowJo™ (FlowJo Treestar, RRID:SCR_008520).

Enzyme-linked Immunosorbent Assay

Total Immunoglobulin G (IgG) secretion by tonsillar B-cells was assessed with the Human IgG Total ELISA Ready-SET-Go!® kit (Affymetrix, eBioscience) according to the manufacturer's protocol, with undiluted supernatant. Absorbance was read at 450 nm and 570 nm, with the FLUOstar Omega microplate reader (BMG Labtech). Secreted IgG was quantified with a linear regression line fitted to the standard curve.

Immunofluorescence

Paraffin was removed from paraffin-embedded sections by three sequential washes in xylene (Sigma). The sections were then rehydrated by passage through a series of ethanol solutions in water (100%, 90% and 70% ethanol in distilled water). Ags were retrieved by boiling samples twice, for five minutes each, in citrate buffer (pH 6) supplemented with 0.05% Tween-20 (Sigma) in a microwave oven and allowing the sample to cool to room temperature. Non-specific binding to FC receptors was blocked by incubating slides for one hour at room temperature in blocking buffer (1 x PBS, 1% FBS, 1% BSA, 1% human AB serum) supplemented with 0.1% Triton X-100, and then incubating them with primary Abs followed by AF488- or AF594-conjugated secondary Abs (Life Technologies).

BL41 cells were attached to poly-L-lysine slides (Thermo Scientific), fixed in 4% PFA and permeabilized with 0.15% Triton X-100. Samples were blocked with 10% FBS in 1 x PBS, incubated with primary Abs and then with AF488-conjugated secondary Abs (Life Technologies).

Nuclei were stained with DAPI (4,6 diamidino-2-phenylindole, 1:10,000; Life Technologies) and the sections were mounted on slides in Fluoromount-G™ slide mounting medium (Beckman Coulter) and covered with a coverslip.

Images were acquired with a Leica SP5 confocal microscope (Leica Microsystems) equipped with an x63 oil immersion fluorescence objective.

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Gene Expression Analysis

RNA was extracted from BL41 cells with the RNeasy plus mini kit (Qiagen), according to the manufacturer's protocol, including lysate homogenization with QIAshredder spin columns (Qiagen). cDNA was then generated from 1 µg of RNA, with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's protocol.

Real-time quantitative PCR was carried out with cDNA diluted 1:10, primers (0.75 µM final concentration) and the QuantiNova SYBR Green PCR Kit (Qiagen). The following primers were used: Mcl-1 (forward (fw) 5'-ATGCTTCGGAAACTGGACAT-3'; reverse (rv) 5'-TCCTGATGCCACCTTCTAGG-3') as the target gene and GAPDH (fw 5'-AATCCCATCACCATCTTCCA-3'; rv 5'-TGGACTCCACGACGTACTCA-3'), 18s (fw 5'-AGAAACGGCTACCACATCCA-3'; rv: 5'-CACCAGACTTGCCCTCCA-3') and RPS13 (5'-CGAAAGCATCTTGAGAGGAACA-3'; rv: 5'-TCGAGCCAAACGGTGAATC-3') as housekeeping (HK) genes (Sigma). PCR was performed with the Mx3005P qPCR System (Agilent Technologies)

PCR efficiency was determined with 10-fold dilutions, according to the following equation: efficiency (E) = $10^{(-1/slope)}$. Cq values were determined with the following equation: $Cq = Ct \times \log_2(E)$. The Mcl-1 expression ratio was determined as follows: $Ratio = ((2)^{\Delta Cq_{Mcl-1}}) / ((2)^{\Delta Cq_{HK}})$, where $\Delta Cq = Cq_{untreated} - Cq_{treated}$.

Statistical Analysis

We analyzed the data for BL41 cells with multiple t -tests, corrected for multiple comparisons according to the Holm-Sidak method, in Prism (Graphpad Prism, RRID:SCR_002798). Data for tonsillar B-cells were analyzed with paired t -test/within-subject analysis, in InVivoStat

RESULTS

Infiltrating B-cells Expressing Mcl-1 are Observed in Kidney Grafts Displaying Chronic Antibody-mediated Rejection

The importance of Mcl-1 in GC maintenance and B-cell differentiation has been shown. (Vikstrom et al., 2010) By analyzing non-stimulated tonsillar B-cells by flow cytometry, according to the mature B-cell (BM)1-BM5 classification, we determined Mcl-1 expression profiles of different B-cell populations. (Pascual et al., 1994) Looking at the expression of surface IgD (sIgD) and CD38. sIgD⁺CD38^{low} cells are naïve B-cells that are undifferentiated or in the early stages of differentiation (BM1-BM2), sIgD⁺CD38^{high} cells are pre-GC cells (BM2'), sIgD⁻CD38^{high} cells are

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GC cells (BM3+BM4), and sIgD⁻CD38^{-/low} cells are terminally differentiated B-cells (early BM5-BM5). We determined the population distribution of tonsillar B-cells and our findings confirm that Mcl-1 levels are high in in the early GC and GC populations (Figure 1A).

Infiltrating B-cells were detected in cAMR kidney grafts, by the staining for CD19 of histological sections of kidneys from patients with cAMR. Counterstaining for Mcl-1 revealed that this protein was colocalized with CD19. Histological sections of non-rejected human kidneys were stained in the same way, and no infiltrating B-cells were observed on sections of these kidneys; however, Mcl-1 is not exclusive to B-cells and was observed in tubular structures (Figure 1B).

These results indicate that cAMR is associated with an infiltration of the kidney graft by Mcl-1-expressing B-cells

Inhibiting SYK Activity Decreases Viability and Mcl-1 Protein Levels in Germinal Center-like Cells

We then investigated ways of disrupting GC B-cell responses. As SYK plays a key role downstream from the BCR, we treated BL41 cells, a Burkitt's lymphoma cell line serving as a model of GC centroblasts (Küppers et al., 1999), with the SYK inhibitor BAY61-3606 (Yamamoto et al., 2003), to inhibit BCR signaling. We assessed the effect of SYK inhibition on cell viability. Shrinkage and blebbing are two characteristic features of apoptotic cells (Krysko et al., 2008), we therefore identified dead cells on the basis of their small size and high granularity. Cell viability was weakened in a dose dependent manner following BAY61-3606 treatment, being reduced by 70% in the presence of 5 μ M BAY61-3606 (Figure 2A). We confirmed that BAY61-3606 reduced the level of SYK phosphorylation, at TYR525/526 in the activation loop of the kinase (Tsang et al., 2008), in non-stimulated BL41 cells (Figure 2B).

We then analyzed the expression of the anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-xs/l. SYK inhibition resulted in lower levels of Mcl-1 protein, whereas the levels of the Bcl-2 and Bcl-xs/l proteins remained stable. Mcl-1 may be either cleaved by activated caspases (Michels et al., 2004) or degraded by the proteasome (Nencioni et al., 2005). We assessed the possible caspase-dependent degradation of Mcl-1, by treating cells with caspase inhibitor Q-VD-Oph and BAY61-3606. It resulted in only a partial rescue of Mcl-1 levels while successfully inhibiting caspase activation and the induction of cell death, as shown by FACS analysis and the cleavage pattern of PARP, a known substrate of caspase-3 (Figure 2C).

The inhibition of proteasome activity with MG132 led to an increase in Mcl-1 protein levels in control cells, however Mcl-1 levels remained similar in cells treated with BAY61-3606 and

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MG132 as compared to those in control cells (Figure 2D). This suggests that SYK inhibition does not modify the rate of Mcl-1 protein degradation.

SYK Inhibition Modulates Mcl-1 Gene Expression and Alters the Cellular Distribution of STAT3

Since SYK inhibition did not accelerate Mcl-1 protein degradation, we hypothesized that the reduced-level of Mcl-1 protein observed in BL41 cells exposed to BAY61-3606 could be the result of reduced de novo protein synthesis. To test this theory, we assessed Mcl-1 mRNA levels by RT-qPCR. BAY61-3606 treatment substantially decreased Mcl-1 gene expression in BL41 cells up to four hours after treatment. The short turnover of Mcl-1 protein was confirmed by blocking protein synthesis with cycloheximide, which led to lower Mcl-1 protein levels after two hours, supporting the hypothesis that SYK inhibition affects de novo protein synthesis. (Figure 3A).

Signal transducer and activator of transcription 3 (STAT3) has been identified as a major regulator of Mcl-1 gene transcription. (Bhattacharya et al., 2005; Thomas et al., 2010) Immunohistological analysis showed STAT3 to be expressed in the nucleus of BL41 cells under basal conditions, where it can mediate Mcl-1 gene transcription. BAY61-3606 inhibits the translocation of STAT3 from the cytoplasm to the nucleus (Figure 3B), suggesting a role for STAT3 in the regulation of Mcl-1 gene expression by BCR signaling. Incubation with STAT3 inhibitor Stattic showed cell viability had decreased by 70% associated with decreased Mcl-1 protein expression. Inhibition of caspases by Q-VD-Oph rescued the cells from cell death induced by Stattic, whereas the protein levels of Mcl-1 were only partially rescued, suggesting that STAT3 inhibition does not result in caspase dependent cleavage of Mcl-1 as observed with BAY61-3606. The lack of PARP cleavage confirmed the effectiveness of Q-VD-Oph as shown in Figure 2C. We assessed Mcl-1 gene expression by RT-qPCR. We found that four hours of treatment with Stattic decreased the level of Mcl-1 gene expression (Figure 3B). Taken together, these results indicate that STAT3 is involved in regulating Mcl-1 gene expression in BL41 cells.

Overexpression of the Mcl-1 gene Counteracts the Inhibition of Both SYK and STAT3

We investigated whether overexpression of Mcl-1 could rescue BL41 cells from apoptotic cell death. Cells were transduced with retroviral particles containing (or not, control) a Mcl-1 construct. As expected the overexpression of Mcl-1, induced by retroviral particles, was not influenced neither by BAY61-3606 nor Stattic in transduced BL41 cells. (Figure 4A). The same effect was observed for gene expression levels of Mcl-1 (Figure 4B). The effect of BAY61-3606 and Stattic on apoptosis was then assessed on both types of BL41 cells using a flow cytometry

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assay based on the extracellular expression of phosphatidylserine and the uptake of 7-AAD. The proportion of apoptotic cells increased for cells transduced with the empty vector, whereas no such increase upon SYK or STAT3 inhibition was observed in cells overexpressing Mcl-1 (Figure 4C). We conclude that the downregulation of Mcl-1 in BL41 cells following SYK inhibition is necessary to induce apoptotic cell death.

SYK Inhibition in Tonsillar B-cells Decreases Viability, Activation and Mcl-1 Expression Resulting in Lower Levels of Immunoglobulin Secretion

We then sought to confirm the general effect of SYK inhibition on primary B-cells activated *in vitro*. Analysis of SYK phosphorylation revealed that BAY61-3606 reduced the phosphorylation state of SYK in activated B-cells. (Figure 5A).

After three days in culture, Tonsillar B-cells (CD19+) were analyzed by flow cytometry. Activated B-cells with impaired BCR signaling had a viability 60% lower than that of control cells. (Figure 5B).

Lower B-cell viability was associated with a decrease in Mcl-1 protein as well as reduced B-cell activation as determined by CD80 expression. This effect was observed in total B-cells as well as in the pre-GC and GC (BM2' and BM3+BM4) populations determined by the BM1-BM5 classification. (Figure 5C)

We then investigated the effects of SYK inhibition on immunoglobulin (Ig) production, by assessing IgG secretion after three days in culture. Activated cells secreted about 150 ng of IgG per ml, whereas less IgG was secreted by activated cells treated with BAY61-3606 (Figure 5D).

These results suggest that SYK inhibition reduces cell viability associated with lower Mcl-1 protein levels as well as impaired B-cell activation and IgG secretion.

DISCUSSION

The GC reaction is important for the generation of effector B-cells with a high Ag affinity. During the GC reaction, apoptosis is required to eliminate B-cells with low Ag affinity and self-reactive B-cells. (Liu et al., 1989; Han et al., 1995; Shokat and Goodnow, 1995) Conversely, the positive selection of high-Ag affinity GC B-cells and the promotion of their survival are essential for the generation of effector B-cells. (Liu et al., 1989; Tarlinton and Smith, 2000) The role of anti-apoptotic Bcl-2 family members, including Bcl-2, Mcl-1 and Bcl-xl, during the GC reaction has been investigated. Yoshino et al. (Yoshino et al., 1994) observed that resting and mantle-zone B-cells expressed Bcl-2, whereas GC B-cells did not. This finding was confirmed by the identification of Bcl-6 as a repressor of Bcl-2 transcription. (Ci et al., 2009; Saito et al., 2009) We confirmed in a

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previous study by in situ staining of human lymphocytes, that GC cells do not express Bcl-2, whereas Mcl-1 is co-expressed with PUMA and Bcl-xl in these cells. (Clybouw et al., 2011) There is growing evidence to suggest that Mcl-1 is a key player in the survival of activated B-cells. Vikstrom et al. (Vikstrom et al., 2010) showed, with a conditional Mcl-1 knockout model in mice, that the absence of Mcl-1 resulted in defective GC and memory cell formation and impaired Ig secretion. In the same study, no effect on the GC reaction of memory B-cell formation was observed in Bcl-xl knockout mice. We confirm here that Mcl-1 is strongly expressed in the BM3+BM4 population of tonsillar B-cells, corresponding to GC B-cells (Pascual et al., 1994).

A recent study demonstrated that GCs may develop outside secondary lymphoid organs and form TLS which generate effector and memory B-cells; such TLS have been reported in cases of persistent inflammation. (Kratz et al., 1996; Takemura et al., 2001) Thauinat et al. (Thauinat et al., 2005) detected TLS in the kidney allografts in case of cAMR, with the identification of infiltrating B-cells, T-cells and follicular dendritic cells. GC B-cell characteristics, such as clonality, high proliferation, and the upregulation of GC related genes, have been observed in these infiltrations. (Kerjaschki et al., 2004; Thauinat et al., 2005; Thauinat et al., 2010; Cheng et al., 2011) We found that the B-cells infiltrating cAMR kidney expressed Mcl-1, consistent with the notion that the B-cells infiltrating grafted organs are part of TLS and play a role in an ongoing local immune response and effector B-cell production. Despite various immunosuppressive treatments have been developed to prevent and treat acute cellular rejection, these treatments are clearly ineffective at controlling cAMR, which remains a leading cause of chronic organ failure. (Durrbach et al., 2010; Lodhi et al., 2011; Loupy et al., 2012) Therefore there is an urgent need to develop new treatments that effectively alter GC-like cell viability and differentiation to reduce the prevalence of cAMR.

BCR signaling during B-cell development leads to the elimination of self-reactive B-cells by programmed cell death (Yurasov and Nussenzweig, 2007; Yarkoni et al., 2010), whereas, later in the GC reaction, Ag affinity-linked BCR engagement leads to the apoptosis of cells with a low Ag affinity and the differentiation of cells with a high Ag affinity into B-effector cells (Liu et al., 1989; Tarlinton and Smith, 2000; Meyer-Hermann et al., 2006) SYK acts very early in the signaling chain, and is therefore an attractive candidate regulator of BCR signaling. In this study, we aimed to impair BCR signaling, by inhibiting the kinase activity of SYK, to decrease GC B-cell viability. In the BL41 GC centroblast cell line, inhibition of the constitutively active SYK kinase resulted in a downregulation of Mcl-1 and decreased cell viability. We show that preventing Mcl-1 protein degradation in experiments with caspase or proteasome inhibitors only partially rescues Mcl-1

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protein levels, and we confirmed by qPCR that Mcl-1 downregulation was controlled at the transcriptional level.

Akgul et al. (Akgul et al., 2000) identified several binding sites for transcription factors, including STAT3, within the promotor region of the Mcl-1 gene. The role of STAT3 in the GC response remains unclear, but this transcription factor has been shown to influence the T-cell-dependent IgG response, and STAT3 deficiencies impair the generation of human memory B-cells. (Fornek et al., 2006; Avery et al., 2010) Ding et al. (Ding et al., 2016) recently showed, with a STAT3 knockout mouse model, that STAT3 is dispensable for GC initiation but essential for maintenance of the GC reaction. The number of apoptotic GC B-cells was higher in immunized STAT3 knockout mice than in normal mice, and this higher level of apoptosis was associated with lower levels of Mcl-1 gene expression. (Ding et al., 2016) STAT3 has already been reported to be involved in Mcl-1 regulation, but most of these observations were based on experiments in which the JAK/STAT pathway was activated by cytokine- and growth factor-induced signaling. (Puthier et al., 1999; Epling-Burnette et al., 2001; Mott et al., 2007) We show here, in BL41 cells, that SYK inhibition leads to impaired translocation of STAT3 to the nucleus, preventing its binding to the promoter of Mcl-1, whereas this transcription factor remains constitutively nuclear in the absence of treatment. STAT3 inhibition mimicked the effects of SYK inhibition in terms of Mcl-1 expression and cell death. We show here that BCR signaling is associated with the STAT3-modulated regulation of Mcl-1 protein levels. The BCR-mediated modulation of STAT3 levels is supported by data from studies reporting a JAK-independent link between BCR signaling and STAT3. (Wang et al., 2006; Uckun et al., 2010) We also show that overexpression of Mcl-1 prevents cells from entering apoptosis in the presence of both SYK and STAT3 inhibitors. These results suggest that the downregulation of Mcl-1 is necessary to induce apoptosis., confirming the key role of Mcl-1 in maintaining the GC reaction, as reported in previous studies. (Vikstrom et al., 2010; Clybouw et al., 2011; Vikstrom et al., 2016)

The damaging role of DSAs produced following organ transplantation has been studied in detail. Preexisting and *de novo* IgG DSAs are associated with acute and chronic solid graft injury (Lee et al., 2002; Loupy et al., 2012; Kaneku et al., 2013), whereas DSAs of the IgM and IgA types have no such effects on their own (Arnold et al., 2013; Everly et al., 2014). We show here that SYK inhibition in activated B-cells reduces cell viability *in vitro*. Our results are in agreement with the findings of Flynn et al (Flynn et al., 2015), they observed that PBMC extracted B-cells from patients with active graft versus host disease were more susceptible to apoptotic cell death as compared to B-cells extracted from patients with inactive or no graft versus host disease. We observed that, in BL41 cells this lower viability is associated with the downregulation of Mcl-1, which we confirmed

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by experiments with primary tonsillar B-cells. We also show that the SYK inhibition is associated with a state of lower activation, as shown by the level of CD80 expression. SYK inhibition thus affects the GC reaction and B-cell differentiation as well as antibody production as shown in primary cells activated *in vitro*, where BAY61-3606 completely inhibits total IgG secretion. These results also suggest that SYK inhibition influences the GC reaction and B-cell differentiation.

In conclusion, our data show that SYK inhibition affects BCR signaling-mediated cell survival through the downregulation of Mcl-1 gene transcription. We demonstrate that SYK inhibition impairs B-cell responses by altering B-cell reactivity to Ags and decreasing Ab production. These results suggest that, in case of cAMR, SYK could be targeted in new therapeutic tools for improving graft survival by manipulating the humoral immune response.

AUTHOR CONTRIBUTIONS

AD, AV and NR designed the researched study; NR, FH, GA and AP performed experiments; FH and GA had input into the study design; OT contributed to obtaining the histological slides of cAMR kidney samples; NR prepared the manuscript and the other authors commented on the manuscript.

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Results

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FIGURE LEGENDS

Figure 1: Mcl-1-expressing B-cells are found in kidney grafts in chronic antibody-mediated rejection.

(A) Non-stimulated tonsillar cells were stained with CD19, sIgD, CD38 and Mcl-1 and analyzed by flow cytometry. B-cell populations were identified according to the BM1-BM5 classification based on the expression of CD19, sIgD and CD38 and an additional staining with Mcl-1 was performed. The boxplot represents the distribution between donors and the quadrant indicates the values between the 5th and 95th percentiles, n=7. (B) Immunofluorescence staining of paraffin-embedded chronically rejected kidney using anti-CD19 and anti-Mcl-1. Images were obtained with a Leica confocal microscope fitted with a 63x objective. sIgD: surface immunoglobulin D, CD: cluster of differentiation, BM: mature B-cell.

Figure 2: SYK inhibition in BL41 cells (Burkitt's lymphoma cells) reduces viability and decreases levels of the anti-apoptotic protein Mcl-1

(A) Cell viability was determined by flow cytometry analyses of cell morphology (forward and side scatter) following a dose gradient treatment with BAY61-3606 for 16 hours. Data represent mean (\pm SEM, n=3). (B) The phosphorylation status of SYK was determined by flow cytometry following SYK inhibition with BAY61-3606 (5 μ M; 20 min). (C) BL41 cells were treated with BAY61-3606 (5 μ M; 4 hours) and Q-VD-Oph (10 μ M; 4 hours). Protein levels of anti-apoptotic proteins, Mcl-1, Bcl-2 and Bcl-x_{s/l}, were determined by western blot, and quantified by protein densitometry and adjusted for GAPDH. Data represent mean (\pm SEM, n=3). Q-VD-Oph efficiency was determined by western blot analysis of PARP cleavage (4 hours) and inhibition of cell death (16 hours), data represent mean (\pm SEM, n=5) (D) BL41 cells were treated with BAY61-3606 (5 μ M; 4 hours) and MG-132 (10 μ M; 4hours) and Mcl-1 protein levels were determined by western blot and quantified by protein densitometry and adjusted for GAPDH. Data represent mean (\pm SEM, n=5). Bay: BAY61-3606, N-I: non-inhibited, I-C: isotype control, QVD: Q-VD-Oph, ns: not significant, MG: MG132.

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Figure 3: STAT3 as a potential regulator of Mcl-1 gene transcription in BL41 cells (Burkitt's lymphoma cells) upon SYK inhibition

(A) BL41 cells were treated with BAY61-3606 (5 μ M) for 1, 2 and 4 hours and Mcl-1 gene expression levels were determined by RT-qPCR. Data represent mean (\pm SEM, n=5), the P-value indicates the significance for comparisons with cells in the absence of BAY61-3606. BL41 cells were treated with cycloheximide (10 μ M) for 1, 2 and 4 hours and Mcl-1 protein levels were determined by western blot and quantified by protein densitometry and adjusted for GAPDH. Data represent mean (\pm SEM, n=3), the P-value indicates the significance for comparisons with cells in the absence of BAY61-3606 (B) BL41 cells were treated with BAY61-3606 (5 μ M; 60 minutes) the cellular location of STAT-3 was determined by immunofluorescence. Images were obtained with a Leica confocal microscope equipped with a 63x objective. (C) The viability of BL41 cells was determined by flow cytometry treatment with the STAT3 inhibitor Stattic (5 μ M; 16 hours) in the presence or not of Q-VD-Oph (10 μ M) and apoptotic cells were identified based on their size and granularity, according to forward and side scatter data. Data represent mean (\pm SEM, n=6). Mcl-1 protein levels were assessed by western blot following treatment with Stattic (5 μ M; 4 hours) and Q-VD-Oph (10 μ M); Mcl-1 levels were quantified by protein densitometry and adjusted for GAPDH. Data represent mean (\pm SEM, n=3). Q-VD-Oph efficiency was determined by western blot analysis of PARP cleavage (4 hours) and inhibition of cell death (16 hours), data represent mean (\pm SEM, n=6). Mcl-1 gene expression levels were determined by RT-qPCR. Data represent mean (\pm SEM, n=4), the P-value indicates the significance for comparisons with cells without BAY61-3606. CHX: cycloheximide, N-I: non-inhibited, Bay: BAY61-3606, QVD: Q-VD-Oph, ns: not significant, MG: MG132.

Figure 4: Overexpression of Mcl-1 protects BL41 cells from BAY61-3606 induced apoptosis

BL41 cells were transduced with retroviral particles containing a Mcl-1 construct (pMcl-1), or an empty vector (pIRES) as a control. (A) Cells transduced with Mcl-1 or the empty vector were treated with BAY61-3606 or Stattic (5 μ M; 4 hours). Mcl-1 protein levels were determined by western blot and quantified by protein densitometry and adjusted for GAPDH. Data represent mean (\pm SEM, n=3) (B) RT-qPCR showed that Mcl-1 gene expression was determined by RT-qPCR following treatment with BAY61-3606 (5 μ M; 4hours or Stattic (5 μ M; 4 hours). Data represent mean (\pm SEM, n=4), the P-value indicates the significance for comparisons with cells without inhibitor. (C) Apoptotic cells were identified on the basis of their extracellular expression of phosphatidylserine, detected by Annexin V staining, and plasma membrane permeability with their uptake of 7-AAD after treatment with BAY61-3606 (5 μ M; 8 hours) or Stattic (5 μ M; 8 hours).

Results

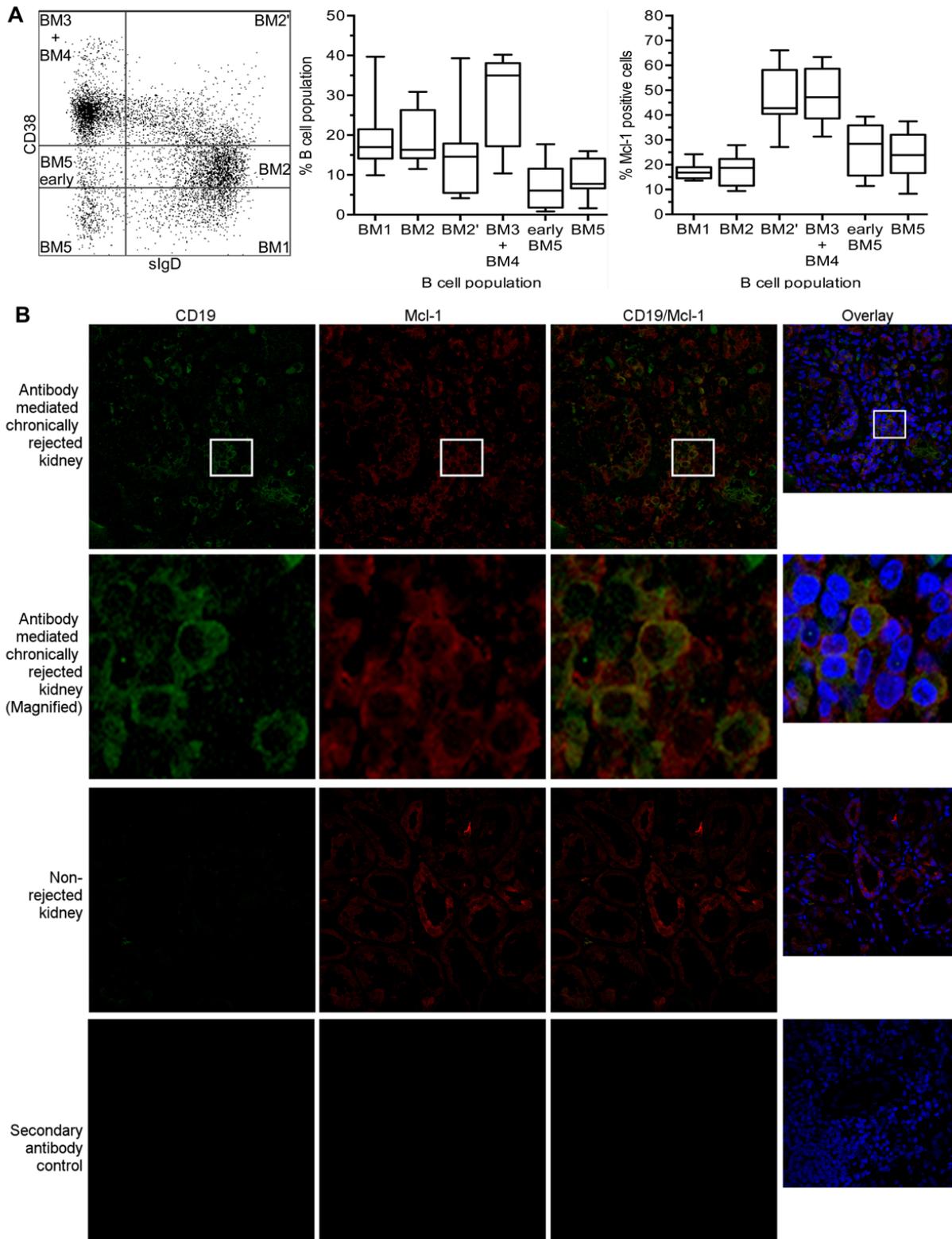
Data represent mean (\pm SEM, n=4). Bay: BAY61-3606, N-I: Non-inhibited; ns: not significant; Stat: Statistic.

Figure 5: Syk Inhibition in tonsillar B-cells decreases viability, activation and effector functions

Tonsillar cells activated *in vitro* by incubation with CD40L fibroblasts (1:10 ratio, fibroblasts: tonsillar cells), anti- μ antibody (10 μ g/ml) and LPS (1 μ g/ml) (A) Following activation (20 minutes) in the presence or not of BAY61-3606 (5 μ M), B-cells were identified based on the expression of CD19 and the phosphorylation status of SYK was determined by flow cytometry (B) Following three days of culture, cells were stained with fixable viability stain and CD19 identified by flow cytometry. Dead B-cells were discriminated by flow cytometry using fixable viability stain. The boxplot represents the distribution between donors and the quadrant indicates the values between the 5th and 95th percentiles, n=7. (C) Following three days of culture, cells were stained with fixable viability stain, CD19, sIgD, CD38, CD80 and Mcl-1, and analyzed by flow cytometry. Viable cells were identified as fixable viability stain negative. Mcl-1 levels were determined in CD19-positive cells as well as pre-GC (BM2⁺) and GC B-cells (BM3+BM4) based on the expression of sIgD and CD38. The activation state was determined by the expression of CD80 in CD19-positive cells as well as pre-GC (BM2⁺) and GC B-cells (BM3+BM4). The boxplot represents the distribution between donors and the quadrant indicates the values between the 5th and 95th percentiles; the P values shown on the graphs indicate the significance for comparisons between all stimulated cells and those treated with BAY61-3606 n=7. (D) The total IgG secreted by the cells following three days of culture was analyzed by ELISA. The boxplot represents the distribution between donors and the quadrant indicates the values between the 5th and 95th percentiles, n=9. Stim: stimulated, Bay: BAY61-3606, N-A: Non-activated, CD: cluster of differentiation

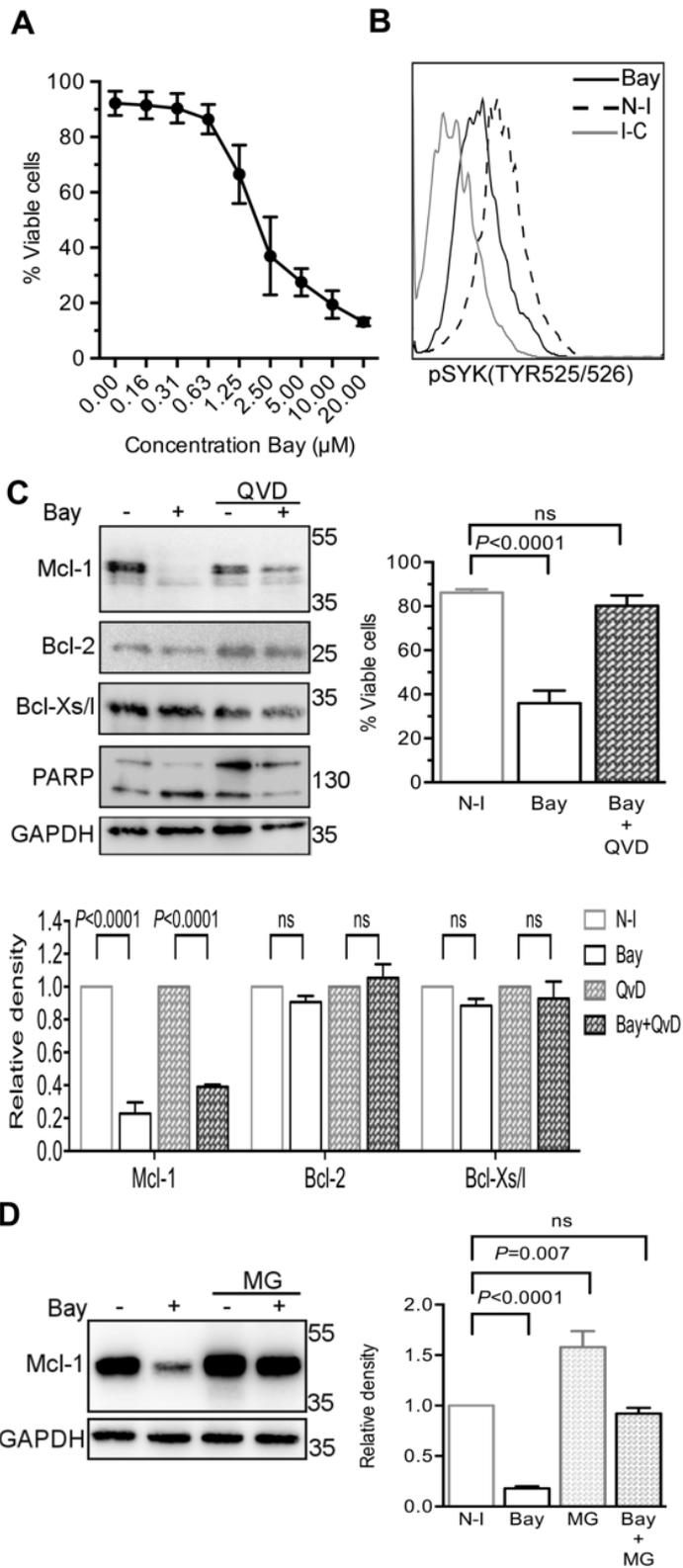
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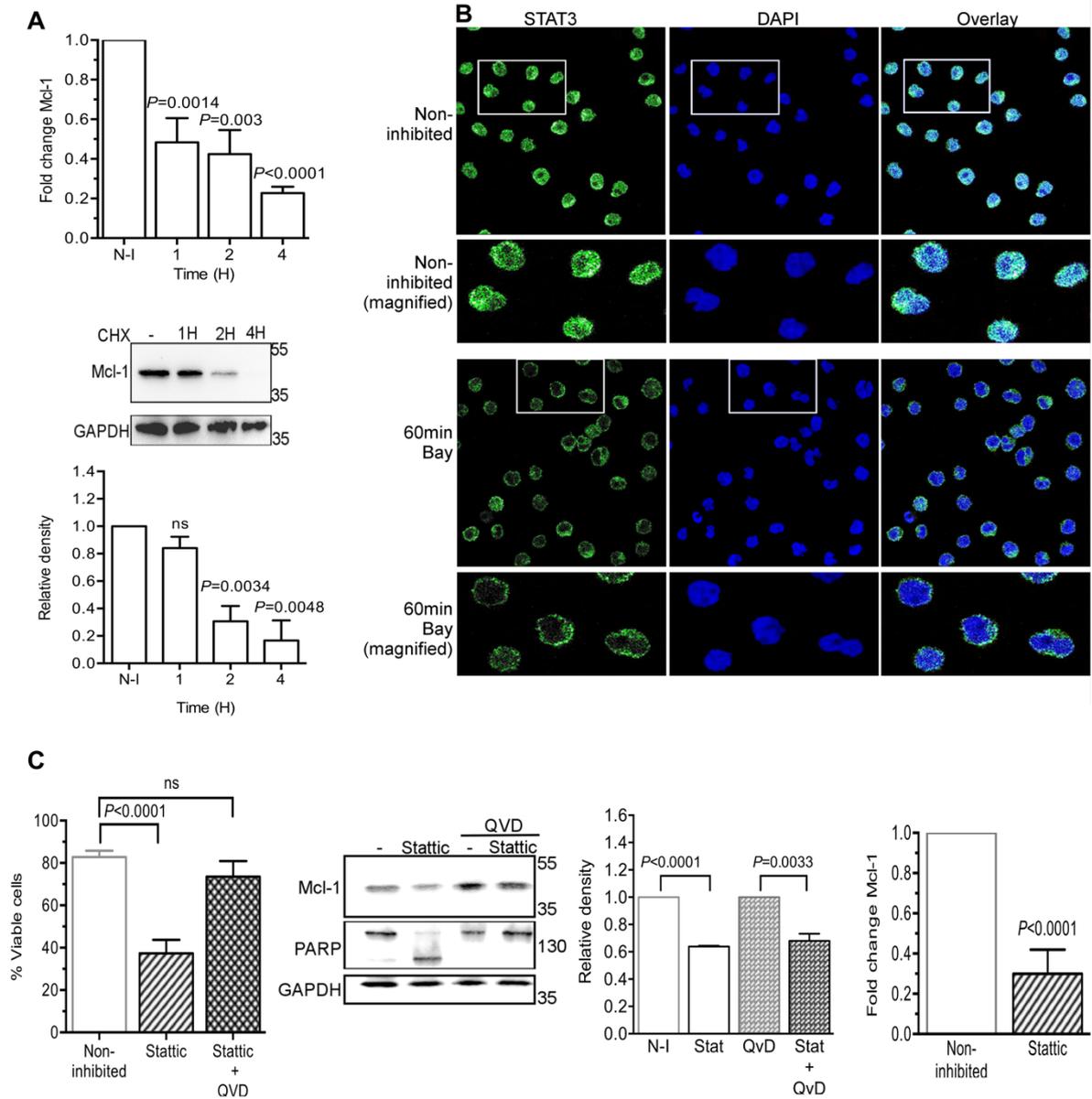
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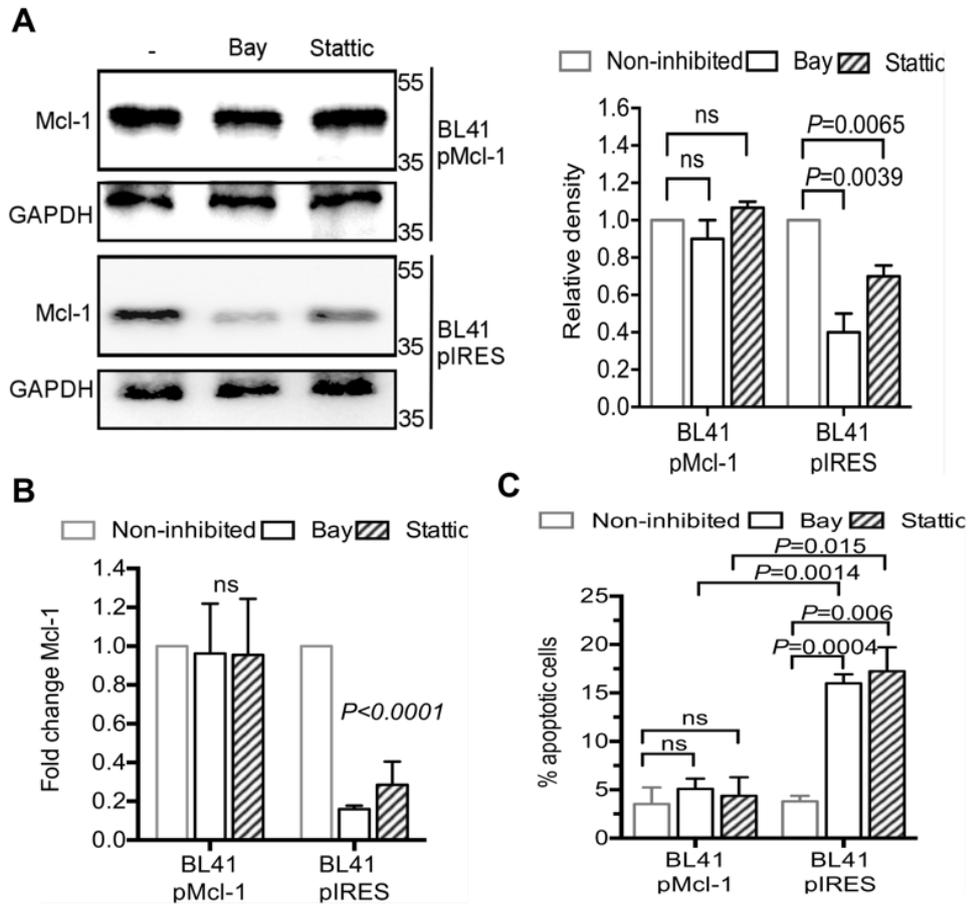
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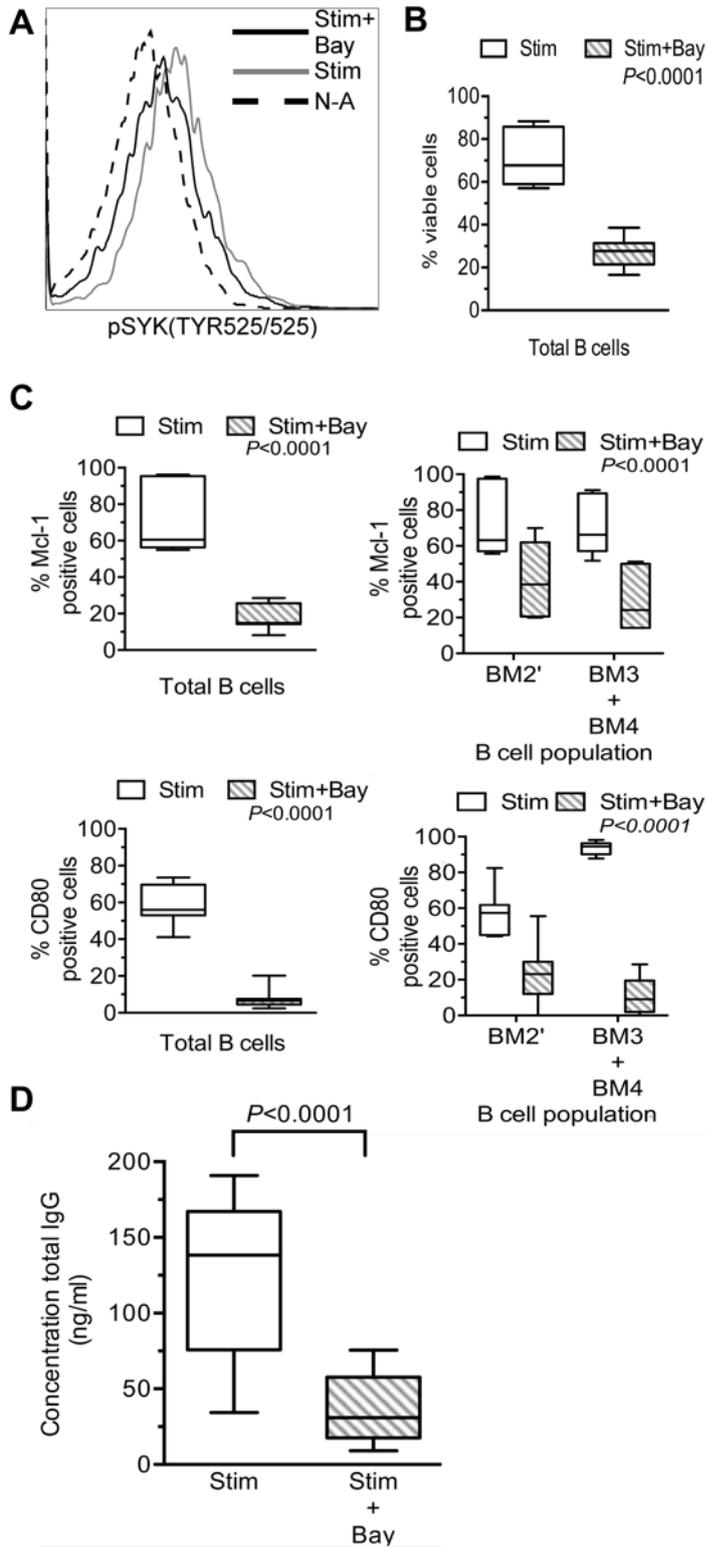
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Figure 4



Results

Figure 5



Results

(II) Supplemental results

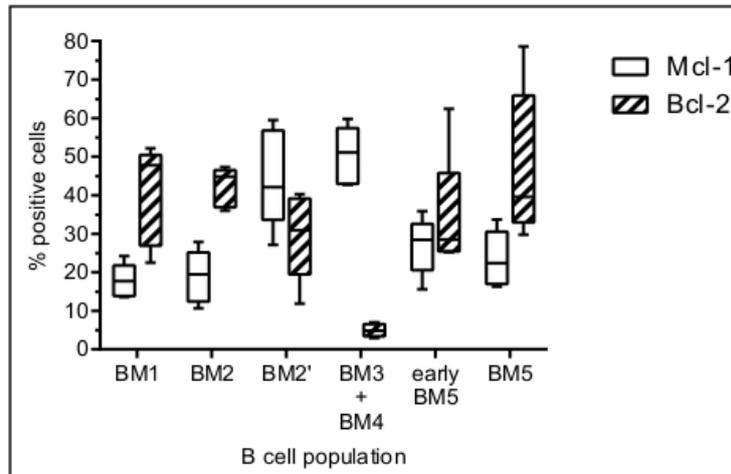


Figure SR 1: Mcl-1 expression is higher in (pre-)germinal center cells while Bcl-2 expression is higher in naïve mature and differentiated B-cells.

Mature B-cell populations (CD19^{pos}) were identified by flow cytometry according to the BM1-BM5 classification based on the expression of sIgD and CD38. sIgD^{pos}CD38^{-/low} represent naïve non-differentiated and early differentiating B-cells (BM1-BM2), sIgD^{pos}CD38^{high} represent pre-GC cells (BM2'), sIgD^{neg}CD38^{high} represent GC cells (BM3+BM4), while terminally differentiated B-cells are sIgD^{neg}CD38^{-/low} (earlyBM5-BM5). Mcl-1 is strongly expressed by activated follicular B-cells, while Bcl-2 expression is higher in naïve and early activated and differentiated B-cells. The boxplot represents the distribution between donors and the quadrant indicates the values between the 5-95 percentile, n=5. Bcl-2: B-cell lymphoma-2; BM: Mature B-cell, CD: cluster of differentiation; GC: germinal center; Mcl-1: multiple myeloma-1; sIgD: surface immunoglobulin

Results

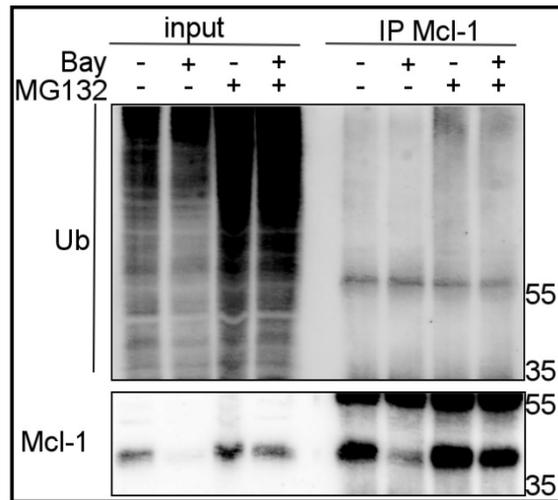


Figure SR 2: SYK inhibition in BL41 cells does not increase Mcl-1 ubiquitination to promote proteasome dependent degradation.

Ubiquitination of Mcl-1 in BL41 cells was determined following a 2-hour incubation with SYK inhibitor BAY61-3606, in the presence or not of proteasome inhibitor MG132. Cells were lysed to obtain the protein content followed by an immunoprecipitation of Mcl-1 (using Protein G-sepharose beads from SIGMA in the presence of 2g of Mcl-1 antibody). Samples were boiled in the presence of 10% β -mercaptoethanol, separated on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with primary Abs (Ub (P4D1): sc-8017; Mcl-1 (S-19): sc-819), and bound Abs was visualized by chemiluminescence using HRP-conjugated secondary Abs. While Mcl-1 is degraded in cells treated with BAY61-3606, there is no apparent increase in Mcl-1 ubiquitination. To confirm that there is no increase in ubiquitination the cells were treated with proteasome inhibitor MG132 to prevent the degradation of Mcl-1. Under these conditions Mcl-1 does not show increased ubiquitination as in cells treated with Bay61-3606 alone. Bay: BAY61-3606; IP: immunoprecipitation; Ub: Ubiquitin

Results

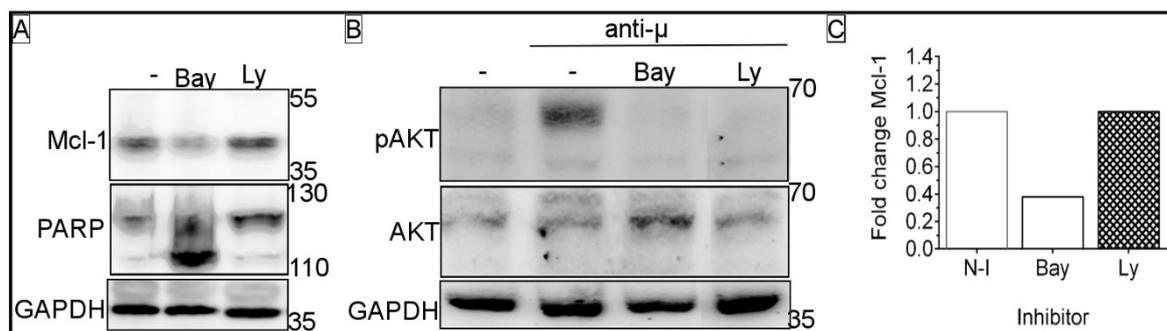


Figure SR 3: Inhibition of AKT does not affect Mcl-1 expression or induce PARP cleavage in BL41 cells as compared to SYK inhibition.

(A) Mcl-1 expression was determined in BL41 cells treated with either SYK inhibitor BAY61-3606 or Pi3K inhibitor LY294002 for 4-hours. While treatment with SYK reduces the expression of Mcl-1, inhibition of Pi3K does not alter the expression of Mcl-1. Caspase activity assessed by PARP cleavage shows that SYK inhibition leads to the activation of caspase-3 and thus the cleavage of PARP, while Pi3K inhibition does induce PARP cleavage, indicating no activation of caspase-3. (B) To determine the effect of the inhibition on AKT, phosphorylation of AKT was assessed by western blot. While BL41 cells under basal conditions show a slight phosphorylation of AKT, activation of the BCR with anti- μ increase AKT phosphorylation. Both BAY61-3606 and LY294003 reduce the phosphorylation of AKT, indicating both inhibitors are able to regulate the activity of AKT. (C) Real time qPCR results of Mcl-1 gene expression following inhibition, while SYK inhibition reduces the gene levels of Mcl-1, the inhibition of Pi3K does not seem to affect Mcl-1 gene expression. Note: n=1, experiments need to be repeated to confirm results. Bay: BAY61-3606; Ly:LY294002

Discussion & Perspectives

Discussion & Perspectives

(1) Identifying infiltrating B-cells; Mcl-1 expression in GCs and TLS infiltrating cAMR kidney

In cAMR, tertiary structures mimicking GCs in which B-cells play an important role, have been observed. Therefore, we wanted to further investigate properties of B-cells infiltrating and participating in a local immune response in cAMR kidney grafts focusing on the presence of anti-apoptotic protein Mcl-1.

The GC reaction is important for the generation of effector B-cells with a high Ag affinity. During the GC reaction apoptosis is necessary for eliminating low Ag affinity and self-reactive B-cells (Han, Zheng, Porto, & Kelsoe, 1995; Y.-J. Liu et al., 1989; Shokat & Goodnow, 1995) while on the other hand positive selection of high Ag affinity GC B-cells, and promoting their survival, is essential for effector B-cell generation (Y.-J. Liu et al., 1989; Tarlinton & Smith, 2000). The role of anti-apoptotic Bcl-2 family members, including Bcl-2, Mcl-1 and Bcl-xl during the GC reaction have been under investigation. It was observed by Yoshino et al. (Yoshino et al., 1994) that while resting B-cells as well as mantle zone B-cells express Bcl-2, GC B-cells do not. This was confirmed when Bcl-6, the master regulator of the GC reaction was identified as a repressor of Bcl-2 transcription. (Ci et al., 2009; M. Saito et al., 2009) In this work, we confirmed the expression profile of Bcl-2 in tonsillar B-cells and observed that Bcl-2 is absent from the GC population while it is expressed both in naïve mature and differentiated MBCs (supplemental results S1). In a previous study performed by our lab we observed an association of pro-apoptotic protein Puma and GC like B-cells, and that Puma plays a role in the regulation of the memory B-cell population. In that study, we confirmed with in situ staining of human lymph nodes that Puma is co-expressed with Mcl-1 and Bcl-xl within the GCs, while Bcl-2 is absent. (Clybouw et al., 2011) More evidence is arising that Mcl-1 is a key player in the survival of activated B-cells. As mentioned before Vikstrom et al. (Vikstrom et al., 2010) showed with a conditional knock-out mice model for Mcl-1, that the absence of Mcl-1 resulted in defects in GC formation, defects in the generation of MBCs and reduced Ig secretion. In the same study Bcl-xl knockout mice did not abrogate the GC reaction nor the formation of memory B-cells. Thus, even though anti-apoptotic family member Bcl-xl is present within the GC it is not essential in maintaining the GC reaction, suggesting the prominent role of Mcl-1 in GC survival. Vikstrom observed in a follow up study that Mcl-1 is required for survival throughout B-cell development, while Bcl-xl promotes survival of immature B-cells. (Vikstrom et al., 2016) In this study, we confirmed using B-cells extracted from human tonsils that Mcl-1 expression is elevated in the mature B-cells (BM)2' corresponding to pre-GC B-cells and BM3+BM4 population, corresponding to GC B-cells.

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Recent studies demonstrated that GCs can develop outside of SLOs allowing the generation of effector and memory B-cells, these TLS have been reported in persistent inflammation. (Kratz et al., 1996; Takemura et al., 2001) In solid allograft rejection, Thauinat et al. (Thauinat et al., 2005), found infiltrating B-cells, T-cells and FDCs within the rejected organ, indicating the presence of possible TLS and thus suggesting a possible local lymphoid neogenesis. Several groups observed that infiltrating lymphoid cells in the rejected organs were highly proliferative by looking at the expression of proliferation marker Ki-67. (Kerjaschki et al., 2004; Thauinat et al., 2005) In a follow up study Thauinat et al. (Thauinat et al., 2010) further analyzed the cellular and molecular phenotype of these infiltrating B-cells. They observed CD20^{pos} cells lacking the expression of sIgD and Bcl-2 but expressing Bcl-6 and AID, like GC B-cells. They also identified the expression of GC genes, by RT-qPCR, such as CXCL13, CXCR5, CCL19, CCL21 and CCR7, proteins involved in the localization of lymphoid cells in GCs. Cheng et al. (J. Cheng et al., 2011) observed that B-cells infiltrating kidney grafts express the genes necessary for SHM as found in GC cells, as well as a clonality of B-cells originating from these infiltrating lymphoid clusters. These observations show that a local humoral response similar to the GC reaction is occurring in chronically rejected organs. Our results show that B-cells infiltrating cAMR kidney express Mcl-1, which is a mentioned before essential for GC formation and B-cell differentiation, thus strengthening the notion that B-cell infiltrating grafted organs are part of TLS and play a role in a local humoral immune response, lymphoid neogenesis and differentiation into effector B-cells.

(2) Targeting BCR signaling; SYK inhibition induced GC like cell death associated with Mcl-1 gene regulation

BCR signaling during B-cell development leads to the elimination of self-reactive B-cells by programmed cell death. (Yarkoni, Getahun, & Cambier, 2010; Yurasov & Nussenzweig, 2007) While later in the GC reaction, Ag affinity-linked BCR engagement mediates the apoptosis of cells with a low Ag affinity and the differentiation of cells with a high Ag affinity into B-effector cells. (Y.-J. Liu et al., 1989; Meyer-Hermann, Maini, & Iber, 2006; Tarlinton & Smith, 2000) We hypothesized that the decrease in BCR signaling leads to reduced B-cell viability. Therefore, we aimed to abrogate BCR signaling to influence GC B-cell viability by inhibiting the kinase activity of SYK. Since SYK acts very early in the signaling chain making it an attractive candidate regulator of BCR signaling.

Significant clinical activity of SYK inhibition has been observed for non-Hodgkin and B-cell chronic lymphocytic leukemia (B-CLL). (Friedberg et al., 2010; Delong Liu & Mamorska-Dyga, 2017; Sharman et al., 2015). Fostamatinib disodium, the pro-drug version of SYK inhibitor R406,

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was used in a Phase 1/2 trial (n=68). The outcome of this trial showed that treatment induced tumor cell death and significant clinical responses. Common toxicities included neutropenia, thrombocytopenia, diarrhea, fatigue, cytopenias, hypertension, and nausea, however they seemed to be reversible. (Friedberg et al., 2010) Another phase 2 clinical trial using SYK inhibitor entospletinib (n=186), which showed a greater selectivity as compared to R406 (Currie et al., 2014), showed a median progression free survival of 13.8 months. Patients who achieved a partial response remained free from progression at 6.5 months. The toxicities observed during this trial were similar to those observed in the fostamatinib trial (Sharman et al., 2015)

For our experimental settings, we use SYK inhibitor BAY-613606 (BAY) whose pharmacological profile was tested *in vitro* and *in vivo* by N. Yamamoto et al. (Yamamoto et al., 2003) They showed that BAY selectively blocks SYK activity, which was associated with reduced BCR and FcR signaling mediated cell functions *in vitro*. *In vivo* associated effects were observed in cutaneous allergies and bronchitis in rat models, in which allergic effects were reduced in animals treated with BAY. (Yamamoto et al., 2003) We observe a constitutively active SYK in Burkitt's lymphoma (BL) cell line BL41, as a model for GC centroblasts, BAY effectively reduces SYK phosphorylation in these cells. We demonstrate that inhibiting the kinase activity of SYK induces cell death in BL41 cells under basal conditions. Our results are in agreement with the results found by Schmitz et al (Schmitz et al., 2012), who observed reduced viability of cells when shRNA against CD79a and SYK were used to abrogate BCR signaling in several Burkitt's lymphoma cell lines, including BL41. We observe a reduction of Mcl-1 associated with SYK inhibition in BL41 cells under basal conditions while the expression anti-apoptotic proteins Bcl-2 and Bcl-xl are not affected. Constitutive activation of SYK was observed B-CLL as well, SYK inhibition in these cells leads to reduced viability and reduced expression of Mcl-1, similarly as to what we observe in BL41 cells. (Baudot et al., 2009; Gobessi et al., 2009) Gobessi et al., (Gobessi et al., 2009) observed that while Mcl-1 was reduced following SYK inhibition, Bcl-2 expression was not modified as we observed in BL41 cells, suggesting that in these models SYK seems to be involved in the regulation of anti-apoptotic Mcl-1 alone.

It is known that Mcl-1 is a substrate of apoptotic effector caspases-and that activation of caspase-3 can induce Mcl-1 degradation. (Michels et al., 2004) We observe that inhibiting caspase activity only partially restored Mcl-1 protein expression, suggesting that Mcl-1 degradation occurs before the activation of caspases and is thus caspase-independent following SYK inhibition. Baudot et al., (Baudot et al., 2009) observed the same effect in B-CLL, SYK inhibition in the presence of Q-VD-Oph did not restore Mcl-1 expression. These findings supporting the notion

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that SYK inhibition reduces Mcl-1 protein expression levels, independent of caspase activation, leading to apoptotic cell death.

In the same study, it was observed that inhibiting the activity of the proteasome, which is responsible for Mcl-1 protein degradation (Nencioni et al., 2005), restored Mcl-1 expression following SYK inhibition. (Baudot et al., 2009) The stability of Mcl-1 can be regulated via phosphorylation followed by ubiquitination and proteasome dependent degradation. This was shown by the generation of a Mcl-1 mutant for the phosphorylation site S159. Phosphorylation of Mcl-1 at S195 has been shown to be induced by Glycogen Synthase Kinase-3 (GSK3) and inhibition of GSK3 prevents cells from entering in apoptosis. It was shown that AKT, which is one of the kinases activated downstream of the BCR, can inhibit the activity of GSK3 and thus regulate Mcl-1 stability. (Q. Ding et al., 2007; Maurer, Charvet, Wagman, Dejardin, & Green, 2006) Baudot et al. (Baudot et al., 2009) suggested that SYK inhibition in B-CLL leads to proteasome dependent degradation of Mcl-1 which related to increased GSK3 activation due to decreased AKT activity. We show that when preventing proteasome dependent Mcl-1 protein degradation by proteasome inhibitor MG132 protein levels of Mcl-1 are restored following SYK inhibition, suggesting that SYK inhibition induces proteasome dependent degradation of Mcl-1. However, we observe that while there is no degradation of Mcl-1 in SYK inhibited cells with impaired proteasome activity, there is no accumulation of newly synthesized Mcl-1 either. If SYK inhibition would directly impair Mcl-1 protein stability by probing it for the proteasome one would expect an accumulation of Mcl-1 in SYK inhibited cells, as observed in cells with functional SYK, under proteasome impaired conditions. To confirm this hypothesis, we also look at the ubiquitination levels of Mcl-1 in BL41 cells treated with SYK, with functional or impaired proteasome activity. We observe no increase in the ubiquitination levels of Mcl-1 in the presence of BAY, supporting the notion that SYK inhibition does not actively induce Mcl-1 protein degradation in our model (supplemental results S2). These results suggest that SYK inhibition impairs *de novo* synthesis of Mcl-1 in BL41 cells, which we confirm by RT-qPCR, showing reduced Mcl-1 gene expression levels following SYK inhibition. To confirm that SYK inhibition regulates Mcl-1 gene expression and that SYK induced down regulation of Mcl-1 gene expression is necessary to induce apoptosis, we overexpressed Mcl-1 in BL41 cells. While apoptosis was induced upon SYK inhibition in wild type cells this effect was counteracted in cells overexpressing Mcl-1 by retroviral transduction, and Mcl-1 expression, both at protein and genomic levels, was not reduced.

Taken together, our results suggest that SYK inhibition in BL41 cells leads to reduced down-regulation of anti-apoptotic protein Mcl-1 at transcriptional levels which is associated with apoptotic cell death.

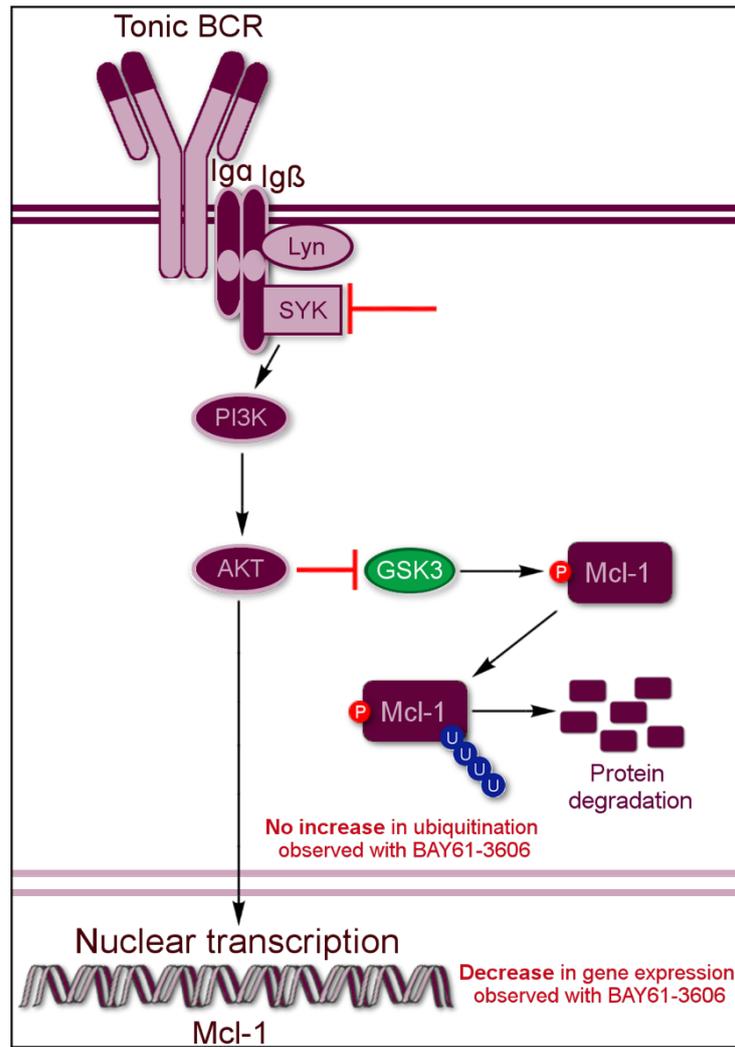


Figure 22: Potential role of SYK in regulating Mcl-1.

BCR signaling can regulate Mcl-1 gene transcription and Mcl-1 protein stability. Downstream of the BCR, active SYK induces the activation of AKT through PI3K. Degradation of Mcl-1 by the proteasome is regulated through ubiquitination of Mcl-1. Phosphorylation of Mcl-1, which precedes its ubiquitination, can be regulated by GSK3 whose activity is suppressed by active AKT. Inhibiting AKT activation by abrogating SYK activity can lead to activation of GSK3, followed by phosphorylation of Mcl-1 probing it for proteasome mediated degradation. However, in our model, SYK inhibition using BAY61-3606 does not increase Mcl-1 ubiquitination suggesting SYK inhibition does not alter Mcl-1 protein stability. We observed that SYK inhibition in BL41 cells leads to decreased levels of Mcl-1 mRNA.

(3) Regulating Mcl-1 in GCs; The role of SYK in STAT3 mediated Mcl-1 gene transcription

Since our results suggest that SYK inhibition regulates Mcl-1 at transcriptional levels we aimed to further understand the signaling pathways involved in Mcl-1 transcription. As mentioned before, the binding of Ag leads to the activation and differentiation of mature B-cells, this type of signaling is called active signaling. Another type of BCR signaling has emerged, which is important

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for maintaining mature B-cells in the absence of antigen, this signaling is called tonic signaling. The first evidence of tonic BCR signaling arose from the research of Lam et al. (Lam, Kühn, & Rajewsky, 1997), using a conditional Cre-Lox mouse model in which the BCR on mature B-cells can be removed. They showed that conditional removal of the BCR reduces the number of mature B-cells and induces apoptotic cell death of these cells. (Lam et al., 1997) The same group confirmed in a follow up study that the BCR subunits CD79a/b, necessary for the recruitment and phosphorylation of SYK to the BCR, are essential for this tonic BCR signaling and survival of mature B-cells in the periphery. (Kraus, Alimzhanov, Rajewsky, & Rajewsky, 2004) Active BCR signaling regulates the activation of several signaling pathways, such as NF- κ B, ERK, NFAT and AKT. The group of Rajewski found in their conditional mouse model that constitutively active Pi3K rescues mature BCR negative B-cells. In the same conditional model for BCR ablation, they showed that constitutive activation of the NF- κ B nor the ERK pathways were able to rescue mature B-cells from cell death induced by BCR depletion in mature B-cells, suggesting that Pi3K signaling downstream of the BCR is essential for tonic BCR mediated cell survival. (Srinivasan et al., 2009) Schmitz and all. (Schmitz et al., 2012) suggested that the BCR signaling in Burkitt's lymphoma stems from chronic tonic signal transduction involving active SYK and AKT mediated signaling.

The activation of AKT can, as mentioned before, inhibit the activation of GSK3 and increase Mcl-1 protein stability. The Pi3K/AKT pathway can also regulate the expression of Mcl-1 at transcriptional and post transcriptional levels as well. Longo et al (Longo et al., 2008) showed, by transfection of constitutive active AKT in B-CLL cells and subsequent quantification of Mcl-1 gene expression, an increase in the fold change of Mcl-1. While Mills et al (J. R. Mills et al., 2008) showed in E μ -myc mouse model, which are mice transgenic for a c-myc gene driven by the IgH enhancer, with constitutively active mechanistic target of rapamycin Complex 1 (mTORC1), that the AKT pathway can regulate Mcl-1 translation via mTORC1. In our experimental model, we did not observe an effect of AKT inhibition on Mcl-1 protein expression as compared with SYK inhibition. While the results we found with real time qPCR seems to confirm that Pi3K inhibition does not affect Mcl-1 expression. These results need to be duplicated to be confirmed (supplemental results S3). Taken together, these results suggest that there is another mechanism involved in Mcl-1 gene regulation following SYK inhibition in our model which is partly Pi3K/AKT independent.

Akgul et al (Akgul, Turner, White, & Edwards, 2000) identified several transcription-factor binding sites within the promotor region of Mcl-1 including signal transducer and activator of transcription 3 (STAT3). In the current study, we observe that STAT3, under basal circumstances, is expressed in the nucleus of BL41 cells where it can therefore actively transcribe the Mcl-1 gene.

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We observe that, under basal condition, SYK inhibition leads to impaired translocation of STAT3 to the nucleus. While the involvement of STAT3 in the regulation of Mcl-1 has been precisely observed, these observations were made mainly through the activation of the JAK/STAT pathway regarding cytokine and growth factor induced signaling. (Epling-Burnette et al., 2001; Mott, Kobayashi, Bronk, & Gores, 2007; Puthier, Bataille, & Amiot, 1999) An interaction between Pi3K/AKT signaling and STAT3 activation has been reported by Han et al (Han 2010) in iMyc^{Flu} B-cell lymphomas, and the inhibition of Pi3K with the inhibitor Ly290042 reduced the phosphorylation state of STAT3. Transformation of murine fibroblastic cells with a catalytically active p110 subunit of pI3K unveiled an upregulation of genes that are under STAT-mediated transcription. (Jonathan Ross Hart, Liao, Ueno, III, & Vogt, 2011) In a follow up study Hart et al (Jonathan R. Hart, Liao, Yates, & Vogt, 2011) observed that Pi3K inhibition in these transformed cells showed reduced STAT3 phosphorylation. They observed that reducing STAT3 phosphorylation prevented oncogenic transformation of these cells, indicating the important role of STAT3 downstream of Pi3K induced oncogenesis. Interestingly inhibition of JAK under these conditions did not reduce STAT3 phosphorylation. However as mentioned before inhibition of Pi3K in our model does not regulate Mcl-1 expression suggesting Pi3K independent regulation of STAT3.

BCR mediated modulation of STAT3 has been observed in several studies, and stimulation of the BCR leads to an increase in STAT3 phosphorylation (L. Su, Rickert, & David, 1999; L. Wang, Kurosaki, & Corey, 2006) as well as STAT3 DNA binding activity (Fan & Rothstein, 2001). Buchner et al (Buchner et al., 2009) showed that in B-CLL have constitutively active STAT3 due to constitutive BCR signaling. B-CLL cells treated with SYK inhibitors showed reduced levels of STAT3 phosphorylation. As mentioned before Mcl-1 gene regulation was observed in JAK/STAT dependent signaling, however there is no apparent evidence that the BCR provides a docking place for JAK. It was shown by Uckun et al (Uckun, Qazi, Ma, Tuel-Ahlgren, & Ozer, 2010) that STAT3 can be a substrate for SYK, and that JAK inhibition, under oxidative stress circumstances, does not abrogate STAT3 phosphorylation. Wang et al (L. Wang et al., 2006) showed that BCR mediated STAT3 signaling is JAK independent as well, since BCR stimulation in the presence of JAK inhibitors did not reduce STAT3 phosphorylation. We show that inhibiting STAT3 in BL41 cells mimicked the effects observed with SYK inhibition regarding Mcl-1 expression as well as cell death, suggesting that BCR signaling is associated with STAT3 modulated regulation of Mcl-1. We showed that by overexpressing Mcl-1 the effects induced by both SYK and STAT3 inhibition were abrogated. suggesting that BCR mediated STAT3 signaling is involved in Mcl-1 gene transcription STAT3 (Figure 23).

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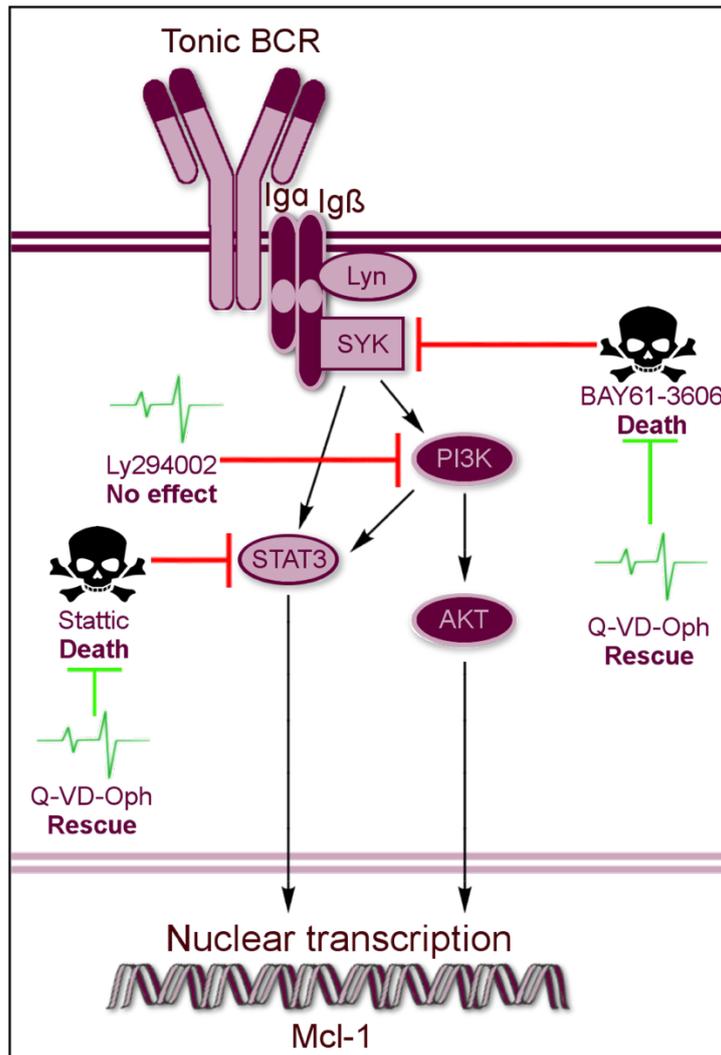


Figure 23: Proposed model for regulating Mcl-1 following SYK inhibition in BL41 cells.

Toni chronic BCR signaling in Burkitt's lymphomas leads to the activation of the Pi3K/AKT pathway, which has been linked to Mcl-1 gene transcription. In our model of BL41 and abrogation of the BCR signal following SYK inhibition we observed reduced cell viability and Mcl-1 gene transcription. However, abrogation of the Pi3K signaling pathway does not alter Mcl-1 expression, suggesting a partly pi3K/AKT independent regulation of Mcl-1. We observed that STAT3 is constitutively expressed in the nucleus of BL41 cells and that SYK inhibition induces a translocation from the nucleus, suggesting a role for STAT3 in the regulation of Mcl-1. The Pi3k/AKT pathway has been implied to regulate STAT3 activation and SYK has been shown to be able to regulate STAT3 activity as well. We propose that in our experimental model that SYK mediated activation of STAT3 is involved in the regulation of Mcl-1, possibly in a Pi3K dependent and independent manner.

While the role of STAT3 in the GC response remains controversial, it was shown that STAT3 is involved in B-cell differentiation. Fornek et al. (Fornek et al., 2006) showed in conditional STAT3 knock out mice model that STAT3 is essential for a T-cell dependent terminal IgG B-cell responses. Avery et al. (Avery et al., 2010) showed that B-cells from patients with autosomal dominant hyper IgE syndrome, which have a mutated STAT3, are unable to produce a high titer

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of high affinity Ig as compared to healthy controls. While fewer MBCs were generated in *in vitro* experiments with STAT3 deficient cells, STAT3 mutation did not affect isotype switching nor SHM in this experimental model. However, IL-21 induced STAT3 activation is essential for naïve B-cells to differentiate in PCs and STAT3 deficiency lead to reduced Ig secretion as compared to healthy controls. A recent study performed by Ding et al (C. Ding et al., 2016) underlined the importance of STAT3 in the GC. They found using a STAT3 knockout mice model, that while STAT3 might not be essential for GC initiation it is essential for maintaining the GC reaction. The number of apoptotic GC B-cells were higher in immunized mice knock out for STAT3, as compared to normal mice, which was associated with lower gene expression levels of Mcl-1.

Taken together, our results suggest that in BL41 cells abrogating SYK kinase activity reduces STAT3 dependent Mcl-1 transcription and supports the results that STAT3 might play an important role in the GC reaction by promoting Mcl-1 mediated cell survival.

It should be stated that the results found in BL41 cells are based on basal tonic BCR activation. SYK inhibition following BCR activation with anti- μ in BL41 cells, lead to abrogated ERK and NF- κ B phosphorylation as well (data not shown), showing that other pathways are influenced by SYK inhibition. Indicating the broad effect of SYK inhibition on BCR signaling, under both tonic as well as active BCR signaling conditions. In a tonsillar B-cell model, with T-cell dependent B-cell activation conditions *in vitro*, we observed that SYK inhibition, under active BCR signaling, induced with anti- μ and co-stimulation with CD40-Ligand and LPS, lead to B-cell death and reduced Mcl-1 protein expression.

(4) The effect of SYK inhibition in altering B-cell responses; SYK as a potential target in organ transplantation

The damaging role of DSA during organ transplantation have been extensively studied and preexisting as well as *de novo* DSA correlates with acute and chronic solid graft injury. (Drachenberg & Papadimitriou, 2013; Kaneku et al., 2013; P. Lee et al., 2002; Loupy et al., 2012) DSAs of the IgM and IgA isotypes have been observed in patient showing signs of AMR, however as compared to IgG DSA they are not as frequent and do not seem to have extreme deteriorating effects on their own as compared to IgG. (Arnold et al., 2013; Everly et al., 2014; Warner et al., 2015) As mentioned before, IgG DSA can activate the complement and cause graft deterioration. Several studies using complement inhibitors have shown promising results regarding severe early acute rejection (Orandi et al., 2014; Yelken et al., 2015), however this treatment does not solve the problem of *de novo* DSA production and tackles the problem at the end of the road, not the beginning. The studies in which rituximab is used to treat AMR, the effect on the production of

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DSA have been debated. (Faguer et al., 2007; Fehr et al., 2009; Tanriover et al., 2008; V. Zarkhin et al., 2008) This could be partly explained by the fact that CD20, the target of rituximab, is not expressed on all B-cell populations, and in particular not on plasma cells which are responsible for the production of DSA. (M. R. Clatworthy, 2011; Valeriya Zarkhin et al., 2010) It is apparent that these therapies often fail to target and destroy MBCs and/or PCs responsible for both aAMR and cAMR. (Jackson et al., 2015; Leandro, 2013; Perry et al., 2008; Ramos et al., 2007). PCs secrete Abs and that one of the main DSA isotypes involved in AMR is IgG (Castro-Dopico & Clatworthy, 2016). MBCs influence the outcome of organ transplantation as well. For example, preformed anti-HLA MBCs predict a poor outcome in kidney organ transplantation, increasing the risk for sensitized patients. (Lúcia et al., 2015) These findings underline the importance of the GC reaction leading to the formation of differentiated B-cells and *de novo* DSA production. Therefore, it is important to find new therapeutic methods that effectively block B-cell viability, differentiation and DSA production.

In this study, we show that SYK inhibition in *in vitro* activated B-cells reduces cell viability. Our results support the findings of Flynn et al (Flynn et al., 2015) observed that PBMC extracted B cells from patients with active graft versus host disease were more susceptible to apoptotic cell death as B-cells extracted from patients with inactive or no graft versus host disease. We show that the reduced viability of primary tonsillar B-cells is associated with the down-regulation of anti-apoptotic protein Mcl-1. Abrogating BCR signaling leads to reduced expression of Mcl-1 in total B-cells as well as in the BM3+BM4 population as compared to stimulated B-cells. The inhibition of SYK is associated with a reduced activation state, as assessed by CD80 expression, in total B-cells as well. We observe a similar expression profile for CD80 in BM3+BM4 cells. We show that abrogating BCR signaling following SYK inhibition of *in vitro* activated primary B-cells inhibits total IgG secretion, thus suggesting that SYK is a potential therapeutic target for reducing the production of IgG DSA.

Taken together our data showed that SYK inhibition in *in vitro* activated B-cells affects BCR signaling-mediated cell survival through the downregulation of Mcl-1 gene transcription. We also demonstrate that SYK inhibition impairs B-cell responses by altering B-cell reactivity to Ags and decreasing Ab production.

It has to be noted, that blocking BCR signaling for longer times can possibly lead to the development of B-cell immunodeficiency's. For example, Bruton's disease, which is caused by a mutation in the BTK gene leading to reduced or no PCs and thus reduced (hypogammaglobulinemia) or no (agammaglobulinemia) Igs. BTK plays a role in the maturation from pro-B-cells to pre-B-cells, and individuals having Bruton's disease have failures in B-cell

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development. (Ochs & Edvard Smith, 1996; Tao, Boyd, Gonye, Malone, & Schwaber, 2000) These individuals have higher risks in contracting infections however, studies have shown that treatment with IV-IG in these patients reduced infections and related mortality. (Quartier et al., 1999) It has been observed that immunotherapies targeting B-cells can be linked to the onset of hypogammaglobulinemia (HGG), characterized by a reduction in all types of gamma globulins. A retrospective review made by Makatsori et al. (Makatsori et al., 2014) revealed several patients that were treated with rituximab for autoimmune diseases and hematopoietic malignancies developed HGG. Casulo et al. (Casulo, Maragulia, & Zelenetz, 2013) investigated the incidence of HGG in patients with B-cell non-Hodgkin lymphoma treated with Rituximab. They observed that maintenance rituximab treatment increased the incidence of HGG as compared to chemoimmunotherapy and immunotherapy. However, the occurrence of HGG (38% *de novo* HGG and 72% progressive HGG) was higher than the incidence of symptomatic hypogammaglobulinemia (approximately 14% for both *de novo* and progressive HGG). They also observed an increased incidence in patients treated with higher doses of Rituximab. (Casulo et al., 2013) Since SYK inhibition induces apoptosis in B-cells and reduces the levels of secreted IgG therefore prolonged use of SYK inhibitors might interfere with normal B-cell function and lead to HGG. Hence, it is important to determine the length of the treatment. Since SYK inhibition induces the cell death of the B-cells short treatment could be sufficient, and once the B-cells responsible for AMR are eliminated the treatment could be stopped or receive less intense immunosuppressive therapies. However, the treatment time needs to be determined and the effect on the development of HGG needs to be further explored.

In conclusion, taken together all our data on SYK inhibition, we show that SYK inhibition affects BCR signaling mediated cell survival through the downregulation of Mcl-1 gene transcription. In GC like cells this regulation of Mcl-1 seems to be in part dependent on STAT3 mediated gene regulation of Mcl-1. By showing that SYK inhibition can modify B-cell viability, altering B-cell reactivity to antigens and antibody production we demonstrate that in case of AMR SYK can be a potential target to improve graft survival due to the humoral immune response.

(5) Perspectives

There are still aspects of SYK inhibition on the effect on different B-cell lineages, other immune cells and graft cells that need to be investigated further.

(I) One population of B-cells have not been discussed until here, which are de regulatory B-cells (Breg). Bregs, like Tregs, play a role in the regulation of the inflammatory response to prevent excessive inflammation. Following an immune response, which leads to the clearance of

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the pathogen, the inflammatory response is abrogated by the production of anti-inflammatory mediators and cytokines. Cells producing these anti-inflammatory mediators are called regulatory, hence Treg and Breg. (Nathan & Ding, 2010) The regulatory effect of Bregs is complex, for example they are able to bias T-cell differentiation to favor the production of T-cells with a regulatory phenotype or can directly produce anti-inflammatory cytokines, events observed both in mice and human. (Carter et al., 2011; Flores-Borja et al., 2013) Part of the effect of Bregs on the regulation of T-cell differentiation is related to the IL-10 producing capacity of Bregs (Carter et al., 2011; Carter, Rosser, & Mauri, 2012; Flores-Borja et al., 2013), however direct cognate interactions between Breg and T-cells have been suggested to play a role too. (Mann, Maresz, Shriver, Tan, & Dittel, 2007; Yoshizaki et al., 2012) Next to the expression of IL-10 Bregs have been shown to express other regulatory-cytokines such as transforming growth factor β (TGF- β), which is able to induce apoptosis in CD4⁺ T-cells and anergy in CD8⁺ T-cells. (Parekh et al., 2003; Tian et al., 2001) Bregs are able to reduce the pro-inflammatory response of DCs. (Masanori Matsumoto et al., 2014; Sun, Deriaud, Leclerc, & Lo-Man, 2005)

While most of the research on Bregs focusses on autoimmune disease, there have been some studies that focuss on the role of Breg in the context of transplantation. An evident role for Bregs was observed both in mice and human studies showing that B cell depletion, using anti-CD20 antibodies, lead to increased cellular rejection. (Menna R. Clatworthy et al., 2009; Marino et al., 2016) Interestingly, patients showing tolerance post transplantation had higher numbers of B-cells as compared to patients showing chronic rejection or patients that received immunosuppression. (Chesneau et al., 2014; Newell et al., 2010; Pallier et al., 2010) The same observation was made in an established rat kidney transplant model of tolerance. Animals showing tolerance had increased B-cell numbers. While there were B-cell infiltrating the graft, these B-cells were of the unswitched IgM isotype, which was associated with decreased complement activation. In the same study, it was observed that transferring B-cell from tolerant animals induced tolerance in new hosts. (Le Texier et al., 2011) These studies show that specific B-cell populations in the context of transplantation can be beneficial.

Several different Breg population have been described, and it seems that any B-cell e.g transitional B-cells, marginal zone B-cells, plasma blast, has the potential to differentiate in to Breg. (Rosser & Mauri, 2015; Wortel & Heidt, 2017) It is suggested that Bregs are activated in the same inflammatory responses that give rise to autoimmune diseases. (Evans et al., 2007; Mizoguchi, Mizoguchi, Takedatsu, Blumberg, & Bhan, 2002) It was demonstrated that Bregs emerge in the presence of pro-inflammatory cytokines such as IL-1 β , IL-6 and IL-21. (Rosser et al., 2014; Yoshizaki et al., 2012) Next to the non-cognate inflammatory stimuli cognate interaction seem to

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influence Breg formation as well. For example, CD40-CD40L interactions between B-cells and T-cells or regulatory DCs have shown to induce Breg differentiation, increase the number of Bregs and anti-inflammatory cytokine production. (Blair et al., 2009, 2010; L. Qian et al., 2012) There is evidence for a pool of Ag-specific Bregs as well, which is suggested by the fact that Ag primed Bregs were able to reduce symptoms of experimental autoimmune encephalitis whereas naïve B-cell were not. (Matsushita, Horikawa, Iwata, & Tedder, 2010) And inhibiting BCR signaling by knocking down CD19 or Stromal interaction molecule 1 and 2 either reduced the prevalence of B10 cells or reduced the production of IL-10. (Masanori Matsumoto et al., 2011; Yanaba, Bouaziz, Matsushita, Tsubata, & Tedder, 2009) The role of SYK has been touched upon by Jin et al. (G. Jin et al., 2013) who observed that SYK inhibition lead to a partial but not complete reduction in the expression of IL-10 by splenic B-cells. Interestingly Barr et al. (Barr et al., 2012) observed, using PBMCs from patients from a clinical trial with SYK inhibitor fostamatinib, no deteriorating effect on the number of IL-10+ B-cells however this study was performed shortly after the introduction of the SYK inhibitor, long term effects have not been determined.

As mentioned before there are several different populations of Bregs spanning different developmental stages observed in B-cells and there seems to be a partial effect of SYK inhibition on Breg development. Therefore, it would be interesting to determine the effect of SYK inhibition on the specific Breg populations, determine the effect of SYK inhibition on the production of anti-inflammatory cytokines and if SYK inhibition can influence Breg mediated tolerance.

(II) For this research project, the effect of SYK inhibition is mainly focused around the BCR mediated signaling, however SYK is involved in other ITAM based immune receptor signaling involving the TCR, FcRs, integrins and PRR as well.

Regarding T-cells and TCR signaling, mature-T immunoreceptor possess ITAMS, however the dogma seems to be that in these cells the ITAM based signaling is dependent SYK related protein ζ -chain-associated protein kinase of 70kDa (ZAP70) and not SYK. (Au-Yeung et al., 2009, 2014) though it has to be mentioned that for example in lupus patients it has been found that they lack the ζ -chains and that SYK seems to take over the role of ZAP-70 in mature T-cells in these patients. (Moulton & Tsokos, 2011) Therefore, the role of SYK in TCR signaling should not be readily dismissed in all cases.

Regarding FcRs, as mentioned before, they are involved in Ab mediated signaling, following the binding of Ag-Ab complexes. Bound DSA recruit several members of the immune system, through binding of the Fc-portion of the Abs, which are expressed on effector cells of both the innate and adaptive immune response. (Nimmerjahn & Ravetch, 2007) It has been shown that IgG mediated signaling plays a role in hypersensitivity reactions through binding of IgG-

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antigen complexes FcγRs on cells of the innate immune system. Three of these, FcγRI, FcγRIIA and FcγRIIIA, are coupled to the activation of Syk through ITAMs. (Sánchez-Mejorada & Rosales, 1998) Bound IgG can trigger phagocytosis and the generation of reactive oxygen species. If this process is triggered by non-self-molecules which are of no danger for the individual it can cause unnecessary tissue damage. And it was shown in SYK deficient macrophages fail to phagocytose IgG coated particles, while SYK deficient neutrophils fail to undergo oxidative burst. (Kiefer et al., 1998; Ziffle & Lowell, 2009) Recognition of IgG to self-Ag contribute to inflammatory diseases such as rheumatoid arthritis. Phase I/II trials using fostamatinib in patients with rheumatoid arthritis promising results were obtained regarding decreased joint inflammation. (Genovese et al., 2011; Weinblatt et al., 2008) There have also been studies that showed a role of SYK in ischemia reperfusion and SYK inhibition in a mouse model reduced tissue damage after ischemia reperfusion, which was associated with reduced IgM and C3 deposition and reduced infiltration of neutrophils. (Ishizuka et al., 2013; Pamuk et al., 2010)

Therefore, one can speculate that SYK inhibition cannot only affect AMR by regulating the B-cell response but also affect the DSA mediated graft deterioration due to DSA mediated recruitment and activation of inflammatory cell

(III) Co-stimulation blockade to prevent T-cell activation has shown promising results organ transplantation. It has been that shown that by selectively blocking the interaction between CD28 family members and their B7 family ligands, greatly enhances both short term and long-term graft survival. (Lenschow et al., 1992; Turka et al., 1992; Ogawa et al., 2001) The new molecule Belatacept, a CTLA-4 blocking fusion protein has shown promising results following a long-term trial. It improved renal function, and reduced risk of mortality and graft loss in patients treated with Belatacept as compared to calcineurin inhibitors. In the same long-term study, it was observed that Belatacept treatment lead to reduced DSA levels as compared to patients treated with Cyclosporine. (Durrbach et al., 2016; Rostaing et al., 2013; Flavio Vincenti et al., 2016)

The mechanism of how Belatacept affects DSA production have yet be elucidated and are under investigation. Leibler et al (Leibler et al., 2014) have investigated the effect of Belatacept on peripheral blood B-cell populations, and they observed that patients treated with Belatacept higher levels of B-cells having a naïve transitional phenotype and hence lower levels of differentiated MBCs as compared to calcineurin treated patients. Interestingly, in a follow up study, it was observed that these transitional B-cells were enriched in immunoregulatory phenotype. (Bigot et al., 2016) Kim et al. (E. J. Kim et al., 2014) have observed in a rhesus macaque an obstruction in GC formation and B-cell differentiation following Belatacept treatment. However, it needs to be noted that these results were obtained under T-cell depleted experimental settings, when treated

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with Belatacept alone, without T-cell depletion, this effect on GC formation was not observed. (Haggerty & Proctor, 2012; E. J. Kim et al., 2014) And concerns have been raised during the Belatacept trials. For one a higher rate of acute rejection was observed, which were increased 2-fold as compared to cyclosporine treated patients, and the severeness of the acute rejection seemed to be increased as well. (F Vincenti et al., 2005; F. Vincenti et al., 2010) A second concern that has been raised was the increase in post-transplant lymphoproliferative disorder (PTLD), caused by B-cell proliferation which can give rise to Epstein-Barr virus (EBV)-associated lymphomas, in patients treated with Belatacept as compared to cyclosporine treated patients. The development of PTLD seemed to occur mainly in EBV seronegative patients. (F. Vincenti et al., 2012)

Since the humoral and cellular immune response are not mutually exclusive, and as observed with Belatacept, targeting the cellular arm can result in effects on the humoral arm. Therefore, one might speculate that blocking the humoral arm of the immune response could affect the cellular immune cells as well. Activation of naïve B-cells leads to the upregulation of costimulatory molecules involved in T-cell interactions, rendering Ag activated B-cells better at interacting with Th-cells. (Depoil et al., 2005; Victora et al., 2010) We show that the inhibition of SYK activation is associated with a reduced CD80 expression, which is a member of the B7 family, however we did not look at other co-stimulatory molecules expressed by the B-cells. B-cells act as APCs as well. Ag binding to the BCR induces internalization of the Ag-BCR complex followed by Ag processing and Ag presentation to T-cells via MHC class II. (Lankar et al., 1998; Ma et al., 2001) Therefore, it would be interesting to investigate the effect of SYK inhibition on T-cell. For example, by investigating the expression profile of other B7-CD28/CTLA-4 family members by B-cells, determine the effect on Ag presentation and T-cell proliferation and differentiation following SYK inhibition.

Therefore, it would be interesting to investigate the effect of SYK inhibition on the Ag presenting capacities of these cells and their ability to activate T-cells in the context of organ transplantation and whether SYK inhibition can influence this process.

(IV) It is necessary to perform transplant mouse experiments to determine the effect of SYK inhibition on B-cells *in vivo*. Regarding the effect of SYK inhibition on GC formation and B-cell differentiation in context of organ transplantation as well as the effect on Ig secretion and the formation of TLS. On the other hand, it is also important to determine the effect of SYK inhibition during an ongoing rejection and determine the effect on ongoing GC/TLS reaction and B-cell differentiation as well as the effect on Ig mediated inflammatory response and tissue damage.

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Annexes

(I) Annex 1

**Subcellular localization of PUMA
regulates its pro-apoptotic
activity in Burkitt's lymphoma B cells**

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Subcellular localization of PUMA regulates its pro-apoptotic activity in Burkitt's lymphoma B cells

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ABSTRACT

The BH3-only protein PUMA (p53-upregulated modulator of apoptosis) is a major regulator of apoptosis. It belongs to the Bcl-2 family of proteins responsible for maintaining mitochondrial outer membrane integrity by controlling the intrinsic (mitochondrial) apoptotic pathway. We describe here a new pathway regulating PUMA activation through the control of its subcellular distribution. Surprisingly, neither PUMA upregulation in normal activated human B lymphocytes nor high levels of PUMA in Burkitt's lymphoma (BL) were associated with cell death. We show that PUMA is localized to the cytosol in these cells. By contrast, various apoptosis-triggering signals were found to promote the translocation of PUMA to the mitochondria in these cells, leading to their death by apoptosis. This apoptosis was associated with the binding of mitochondrial PUMA to anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Mcl-1. This translocation was caspase-independent but was prevented by inhibiting or knocking down the expression of the MAPK kinase p38. Our data suggest that the accumulation of PUMA in the cytosol may be important for the participation of this protein in apoptosis without the need for prior transcription. This regulatory pathway may be an important feature of differentiation and tumorigenic processes.

INTRODUCTION

Apoptosis is regulated by two major groups of proteins: caspases and the proteins of the Bcl-2 family. Bcl-2 family proteins regulate the integrity of the mitochondrial outer membrane (MOM) and can be classified into three different groups: pro-apoptotic, anti-apoptotic and BH3-only regulators. The pro-apoptotic molecules Bax and Bak are directly responsible for the formation of pores in the MOM, allowing the exit of apoptogenic factors, such as cytochrome-*c*. The anti-apoptotic molecules, such as Bcl-2, Bcl-x1, Bcl-w, A1 or Mcl-1, can inhibit Bax/Bak-dependent MOM permeabilization (MOMP). They can be classified into two subgroups, Mcl-1/A1 and Bcl-2/Bclx1/Bclw, according to their sensitivity to BH3-only proteins and to various inhibitors, such as ABT-737. The third group, BH3-only proteins, contains proteins regulating the other two groups

and controlling the pro/antiapoptotic balance. This group can also be classified into two subgroups: (i) BH3-only proteins binding only one subgroup of anti-apoptotic molecules (e.g. Bad and Bik interact with and inhibit the Bcl-2 subgroup whereas Noxa and Bmf interact with the Mcl-1 subgroup) and (ii) BH3-only proteins able to interact with all anti-apoptotic molecules, such as tBid, Bim and PUMA. These two subgroups also differ in their capacity to activate pro-apoptotic proteins directly. Only the proteins of the second subgroup (tBid, Bim and PUMA) have been reported to interact with and activate Bax and Bak directly [1-3].

PUMA (p53-upregulated modulator of apoptosis), also known as Bbc3, was described simultaneously by several different groups [4] [5]. Its gene encodes four different isoforms (α , β , γ and δ). Only, the α (23 kDa) and β (18 kDa) isoforms have apoptotic properties. Both these isoforms contain the BH3 domain, essential for

homotypic interaction with other Bcl-2 family members, and the C-terminal domain, which includes a putative transmembrane domain [4]. PUMA gene transcription is regulated by p53-dependent and p53-independent pathways including various transcription factors, such as Foxo3A, C/EBP and E2F1 [4, 6-8]. PUMA can promote apoptosis by either direct interactions with and inhibition of all anti-apoptotic molecules or the direct activation of Bax and Bak, leading to MOMP [4, 9]. Triple-KO mice (Bid/Bim/PUMA) have the same phenotype as Bax/Bak KO mice, whereas Bim/Bid double-KO mice do not, highlighting the importance of PUMA in the mitochondrial apoptotic pathway [10]. PUMA has been implicated in various apoptotic responses, including DNA damage, growth factor/cytokine withdrawal and the response to glucocorticoids [11, 12] [13]. PUMA has also recently been shown to play a major role in the control of memory T- and B-lymphocyte survival [14, 15]. In this context, activated B cells produce large amounts of PUMA, which is associated with cell activation rather than cell death [15]. This raises questions about the mechanisms preventing PUMA-induced apoptosis in activated lymphocytes. We show here that this paradoxical situation is due to localization of PUMA to the cytosol in these cells. We also show that, in response to apoptotic stimuli, PUMA is translocated from the cytosol to the mitochondria, where it can bind anti-apoptotic molecules, including Bcl-2 and Mcl-1, leading to cell death.

RESULTS

PUMA is present in the cytosol of activated B cells

We previously observed that PUMA expression was strongly upregulated in response to mitogenic activation *in vitro*, in normal human B lymphocytes (in which PUMA is barely detectable in the absence of such activation) (Figure 1A). This finding was confirmed *in vivo*: PUMA (mostly the 18 kDa β isoform) was found to be present in large amounts in germinal center B cells and their tumoral counterparts, Burkitt's lymphoma cells, such as BL41 cells (ref 15 and Figure 1B). Surprisingly, this significant increase in PUMA expression was found to be associated with cell proliferation, as assessed by thymidine uptake, rather than cell death (Figure 1A). This paradoxical observation suggested that the pro-apoptotic activity of PUMA was somehow inhibited, allowing the cells to grow and preventing them from dying. However, we previously observed that the amounts of Bcl-2, Bcl-XL and Mcl-1 were stable after mitogenic activation, indicating that the increase in PUMA expression was not counterbalanced by the production of large amounts of the anti-apoptotic counterparts of PUMA (see figure 1A in ref. 15). We checked that the protein observed was indeed

PUMA, by assessing the specificity of the anti-PUMA antibody (Ab) targeting the C-terminal end of PUMA used in two ways: (i) Following immunoprecipitation of the FLAG-tagged recombinant α or β isoforms of PUMA with an anti-FLAG antibody, our anti-PUMA Ab was able to recognize both these isoforms (which were absent from the control Ig immunoprecipitate; Supplementary Figure 1A & 1B). (ii) Using two different siRNAs, we observed a knockdown of the expression of both the α (23 kDa) and β (18 kDa) isoforms of endogenous PUMA (Supplementary Figure 1C). The activity of some Bcl-2 family members is known to be regulated by their localization to particular compartments within the cell (e.g. Bax, Bid and Bad [16-18]). We therefore investigated the distribution of PUMA within these cells. Using a commercial kit (Calbiochem), we showed that PUMA was concentrated in the cytoplasmic fraction (fraction 1) of SAC-activated B cells. Almost no PUMA was found in the heavy membrane fraction (fraction 2), which included mitochondrion-associated proteins, such as VDAC, the BH3-only protein Bim and the anti-apoptotic protein Mcl-1. No PUMA was present in the fractions corresponding to the nucleus (fraction 3) or the less soluble fraction associated with the cytoskeleton (fraction 4) (Figure 1A). A similar distribution of PUMA and Bim was observed when BL41 cells were fractionated with the same kit (Figure 2A). For confirmation of these observations, we performed another type of fractionation, leading to the separation of cytosolic fractions (S25 or S) and mitochondrion-enriched fractions (P5 or P) (Figure 1C). Again, PUMA was present in the cytosolic fractions (S), whereas Bim and VDAC were present in the heavy membrane fractions (P) prepared from B41 cells (Figure 1B). PUMA was found in the cytosol of other BL cell lines, including CA46, Daudi and Ramos. EBV infection did not alter either the expression or the cytosolic localization of PUMA: no difference was observed between EBV-infected BL cells, such as BL41 95.8 or Ramos AW, and uninfected BL41 or Ramos cells (Figure 1D). As previously reported [19], Bim, unlike PUMA, displayed a strong downregulation of expression in the presence of EBV (Figure 1D). We then studied the cellular distribution of PUMA by immunofluorescence methods. Confocal microscopy showed that, unlike Mcl-1, PUMA did not colocalize with the mitochondria (TOM20). This finding confirmed that PUMA was cytosolic rather than located at the mitochondrial surface in BL41 cells (Figure 1E). Moreover, with the exception of the BH3-only protein Bid and the pro-apoptotic protein Bax, the cytoplasmic localization of which has been clearly established, the other Bcl-2 family members present in BL41 cells (the BH3-only proteins Bim, Noxa, Bik, the pro-apoptotic Bak and the anti-apoptotic proteins Mcl-1, Bcl-2 and BclxL) were all found in the mitochondrial fractions (Figure 1F).

Thus, PUMA had an unexpected, never before reported distribution in non-apoptotic activated human

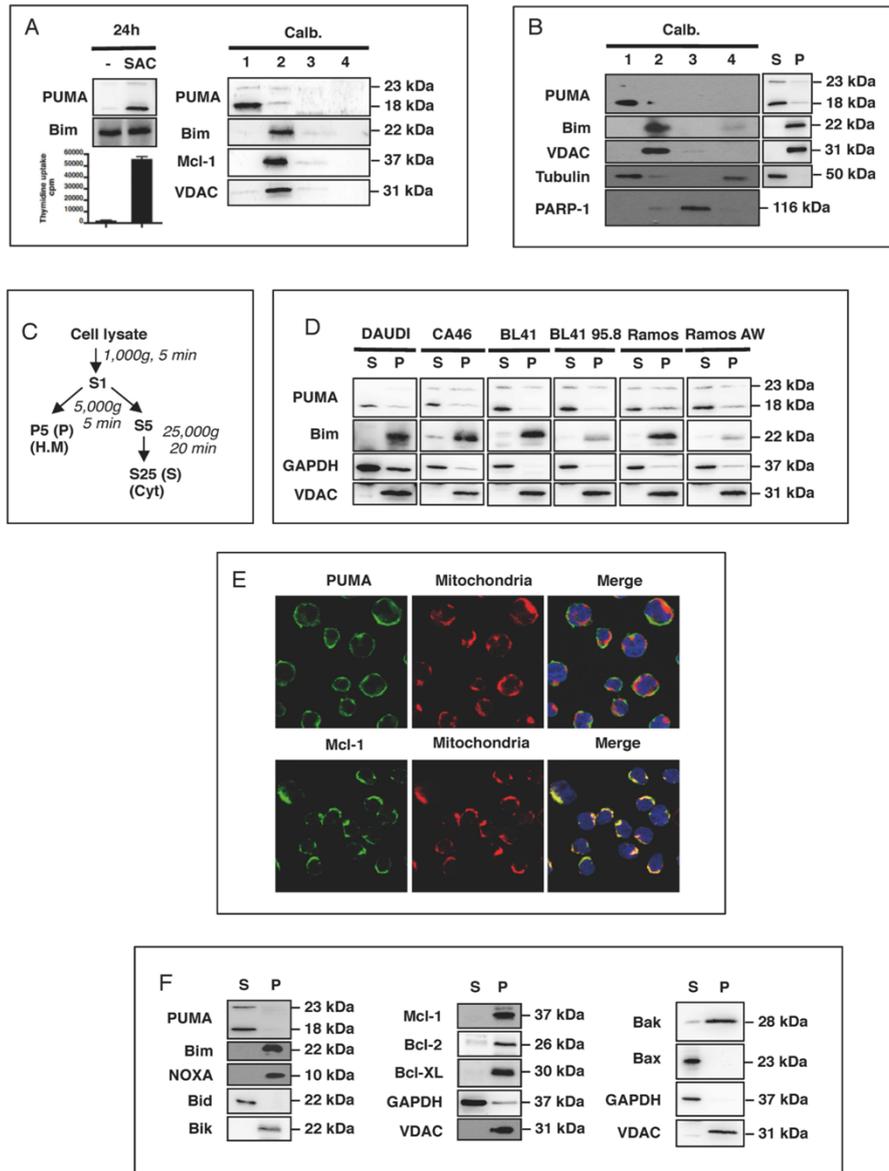


Figure 1: PUMA is present in the cytosol of activated B cells. **A.** Human tonsillar B cells were treated with mitogenic doses of *Staphylococcus aureus* strain Cowan 1 (pansorbin: SAC) at a dilution of 1/10,000 for 24 h. Proliferation was assessed by measuring DNA synthesis, as assessed from ³H-thymidine incorporation during the last 16 h of culture, in counts per minute (cpm). Cells were fractionated with the Calbiochem® extraction kit and western blotting was used to determine the subcellular localization of PUMA, Bim and Mcl-1 in the fractions (1: cytosol; 2 heavy membrane; 3: nucleus and 4: less soluble material associated with the cytoskeleton). **B.** Resting BL41 cells were fractionated as in **A.** and the subcellular localization of PUMA, Bim, VDAC, tubulin and PARP was assessed by western blotting. **C.** Purification of the cytosol (S) and heavy membrane (P) fractions by differential centrifugation of cell lysates. **D.** Cell lysates from DAUDI, CA46, BL41 and Ramos Burkitt's lymphoma cell lines and their EBV-positive counterparts (BL41 95.8 for BL41 and RamosAW for Ramos) were fractionated and the S and P fractions tested for PUMA, Bim, GAPDH and VDAC by western blotting. **E.** BL41 cells were stained with anti-PUMA, anti-Mcl-1 and anti-TOM20 primary antibodies with their corresponding fluorochrome-conjugated secondary antibodies, green for PUMA and Mcl-1 or red for TOM20, and the subcellular distribution of PUMA and Mcl-1 was analyzed by confocal microscopy (**E**, right panel). **F.** Resting BL41 cells were fractionated as shown in **C.** and the subcellular distributions of the various Bcl-2 family members were analyzed by western blotting.

B cells and in Burkitt's lymphoma cells, in which it was localized to the cytosol.

Apoptosis is associated with the translocation of PUMA to the mitochondria

We then investigated whether apoptotic signals affected the distribution of PUMA. The induction of BL41 cell apoptosis through BCR-mediated activation with cross-linked anti- μ Abs [20], drugs such as anisomycin [21] or by the UV treatment of HeLa cells [22] was associated with the translocation of PUMA from the cytosol to the mitochondria (Figure 2A, 2B, 2C and Supplementary Figure 2). This finding was confirmed by confocal microscopy, showing that anisomycin triggered the translocation of PUMA to the mitochondria, together with Bax expression in the mitochondria, and the release of cytochrome *c* into the cytosol (Supplementary Figure 2A & 2B). This suggests that the translocation of PUMA to the mitochondria may play a role in mitochondrial activation, leading to apoptosis. Consistent with this hypothesis, the translocation of PUMA to the mitochondria was also observed as early as 2 h after the exposure of HeLa cells to UV light, before appearance of apoptotic features, such as caspase-3 activation (manifested by PARP-1 cleavage) and cell shrinkage (Figure 2C). Furthermore, the siRNA-mediated downregulation of PUMA was correlated with the inhibition of anisomycin-mediated apoptosis in BL41 cells, strongly suggesting that this mitochondrial translocation of PUMA played an important role in the apoptotic process (Figure 2D). We checked that this translocation was directly associated with the mitochondrial apoptotic pathway, by activating HeLa cells with recombinant TRAIL. TRAIL-mediated apoptosis was not associated with the mitochondrial translocation of PUMA or the release of mitochondrial cytochrome *c* (Figure 2E panels a and b). These data provide strong support for our conclusion that PUMA translocation is associated with the mitochondrial apoptotic pathway. Nevertheless, it remains possible that the mitochondrion-associated PUMA was a newly synthesized protein rather than that localized to the cytosol in non-apoptotic cells. We addressed this question in two ways: (i) we quantified PUMA gene transcription, by carrying out qPCR on RNA isolated from BL41 cells activated with anisomycin or anti- μ antibody, or not activated. We observed no difference in PUMA mRNA levels between these cells (Supplementary Figure 3 panel a), (ii) we investigated the translocation of PUMA to the mitochondria in the presence of the protein synthesis inhibitor cycloheximide. We observed no decrease in the amount of PUMA in the mitochondria in the absence of protein synthesis (Supplementary Figure 3 panel b). Overall, these data fully support the conclusion that the PUMA present in the mitochondria came from the pre-

existing cytosolic pool and was not synthesized *de novo*.

PUMA overproduction is associated with mitochondrial localization and apoptosis

We overproduced PUMA in HeLa cells, to determine whether the translocation of PUMA to the mitochondria led to the induction of apoptosis. Both biochemical separation (Figure 3A, panel a) and confocal microscopy (panel b), showed that the overproduced PUMA was present at the mitochondria. This mitochondrial localization was associated with cytochrome *c* release (panel c) and cell shrinkage (panel a), suggesting that the presence of PUMA at the mitochondria was sufficient to induce cell death. We investigated the mechanisms underlying this translocation, by studying the localization and the pro-apoptotic activity of various truncated PUMA proteins. Constructs including the N-terminal or C-terminal ends of the β isoform of PUMA, including the BH3 domain, were overproduced in HeLa cells, and their cellular distribution was determined (Figure 3B panel a). The C-terminal fragment, like the full-length protein (PUMA β), was present at mitochondria (panels b and c), and this mitochondrial localization was associated with apoptosis (panel d). By contrast, the N-terminal fragment was found mostly in the cytosol of the transfected cells (Figure 3B panels b and c) and was not associated with cell death (panel d). We therefore verified that the N-terminal fragment was well produced in the presence (Figure 3B panel c) or the absence of the caspase-inhibitor Q-VD-OPh (Figure 3B panel d and Supplementary Figure 4 panel a). The data obtained showed that PUMA-mediated apoptosis was dependent on its mitochondrial localization and also implicated the C-terminal domain of PUMA in this localization. We also found that deletion of the BH3 domain of the C-terminal fragment abolished its apoptotic properties (Supplementary Figure 4 panel b). This suggests that, once translocated to the mitochondria, PUMA binds other Bcl-2 family proteins *via* its BH3 domain.

Mitochondrial PUMA binds to and may inhibit Mcl-1 and Bcl-2 in BL41 cells

The apoptosis-mediating activity of PUMA, like that of all BH3-only proteins, is due to its binding, *via* its BH3 domain, with anti-apoptotic members of the Bcl-2 family (such as Mcl-1 or Bcl-2) and/or with the pro-apoptotic proteins Bax and Bak [9, 23-25]. We therefore investigated the association of PUMA with Mcl-1 and Bcl-2 in both activated and non-activated BL41 cells. No association between PUMA and these anti-apoptotic proteins (Figure 4A panel a) was observed in healthy cells, consistent with the cytosolic location of PUMA and the mitochondrial location of the anti-apoptotic proteins (Figure 1F). However, following activation with

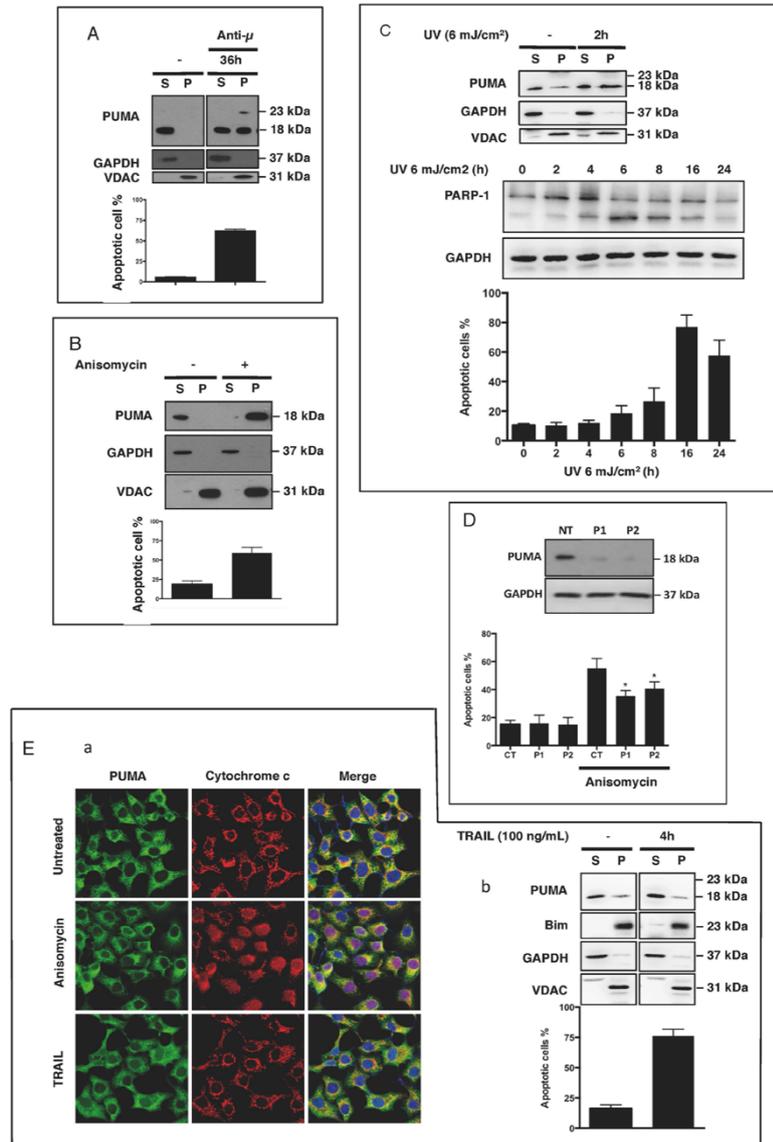


Figure 2: PUMA is found at the mitochondria when apoptosis is triggered. **A.** BL41 cells were stimulated for 36 h with mouse anti-human μ antibodies (5 $\mu\text{g/ml}$) cross-linked with anti-mouse IgM antibodies (28 $\mu\text{g/ml}$). Western blotting was used to test the cytosol (S) and heavy membrane fractions (P) for PUMA. Apoptosis was assessed by flow cytometry and cells were considered apoptotic if they were shrunken, with high side scatter and low forward scatter. The data shown are means \pm SD for triplicate experiments. **B.** BL41 cells were stimulated with anisomycin (2 $\mu\text{g/ml}$) for 4 h. The S and P fractions were tested for PUMA, GAPDH and VDAC by western blotting, and apoptosis was assessed by flow cytometry. **C.** HeLa cells were exposed to UV (6 mJ/cm²) and cultured for 2 h. Cell lysates were fractionated and the fractions were tested for PUMA, GAPDH and VDAC by western blotting. Cell shrinking and PARP-1 cleavage were assessed by flow cytometry and western blotting, respectively, at the indicated times following UV exposure. The data shown are means \pm SD for triplicate experiments. **D.** BL41 cells transfected with a non-targeting siRNA (CT) or a PUMA-targeting siRNA (P1 and P2) for 76 h were treated for 4 h with anisomycin (2 $\mu\text{g/ml}$) or left untreated (controls). Apoptosis was analyzed by flow cytometry (means \pm SD for triplicate experiments) and PUMA knockdown efficiency was analyzed by western blotting with GAPDH as a loading control. **E.** HeLa cells were or were not activated with recombinant TRAIL (100 ng/ml) for 4 h. (panel a) Cells were stained with anti-PUMA and anti-cytochrome *c* primary antibodies with their corresponding fluorochrome-conjugated secondary antibodies, green for PUMA and red for cytochrome *c*, and the subcellular localization of PUMA and cytochrome *c* was analyzed by confocal microscopy. (panel b) S and P fractions were tested for PUMA, Bim, GAPDH and VDAC by western blotting, and apoptosis was assessed by flow cytometry.

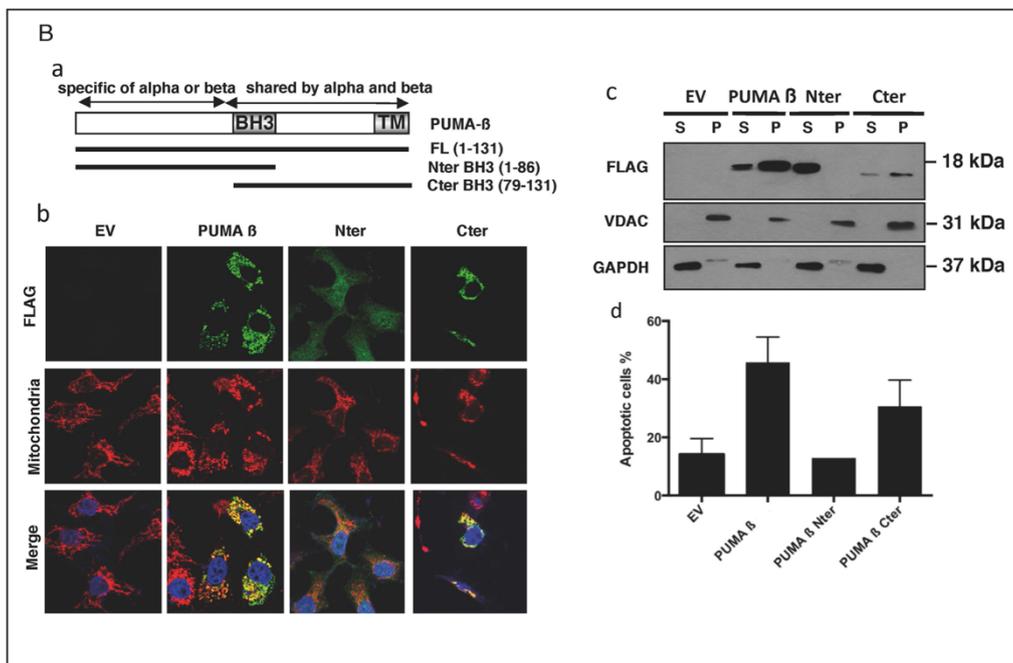
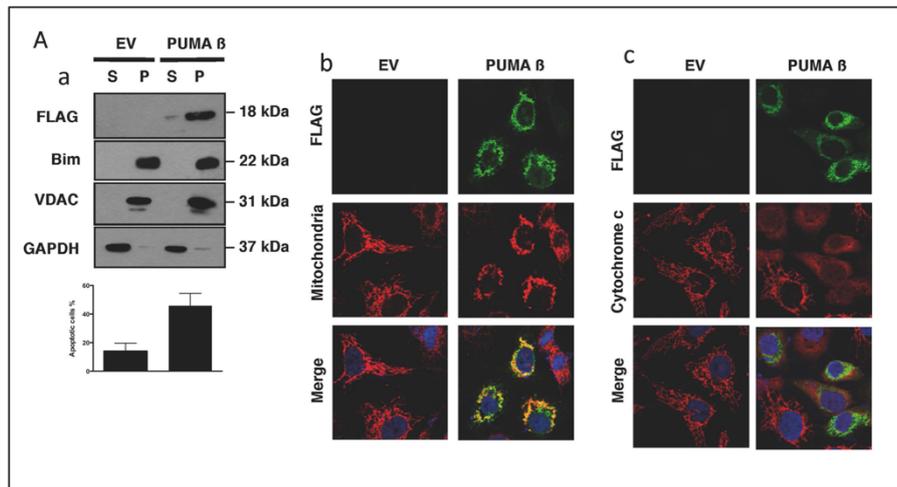


Figure 3: When overproduced, PUMA is found at the mitochondria and induces apoptosis. **A.** HeLa cells, left untreated or treated for 30 min with 10 μ M QVD-OPh, were transfected with a plasmid encoding a full-length Flag-tagged PUMA β (Puma β) or an empty vector (EV) and incubated for 24 h. The subcellular distributions of PUMA β , Bim, VDAC and GAPDH in QVD-OPh-treated cells were analyzed by western blotting (panel a). The data shown are means \pm SD for triplicate experiments. EV- or PUMA β -transfected HeLa cells were stained with anti-FLAG and anti-HSP60 (panel b) or with anti-FLAG and anti-cytochrome *c* (panel c) primary antibodies, together with the corresponding fluorochrome-conjugated secondary antibodies, green for PUMA and red for HSP60 or cytochrome *c*. **B.** HeLa cells, treated for 30 min with QVD-OPh, were transfected with an empty vector (EV) or the full-length PUMA β (Puma β), N-terminal (Nter) or C-terminal (Cter) constructs of PUMA (panel a) and incubated for 24 h. Cells were stained with anti-FLAG and anti-HSP60 (mitochondria) primary antibodies, together with the corresponding fluorochrome-conjugated secondary antibodies, green for the various PUMA constructs and red for HSP60. Cytosolic (S) and mitochondrion-enriched (P) fractions were studied by western blotting (panel c), to determine the subcellular distribution of the proteins. HeLa cells were transfected with the various constructs, in the absence of QVD-OPh, incubated for 24 h and apoptosis was assessed by flow cytometry (panel d). Mean values \pm SD for triplicate experiments are reported.

anisomycin, PUMA was translocated to the mitochondria, where it interacted with Mcl-1 and Bcl-2 (Figure 4A panel b). Likewise, the mitochondrial PUMA β recombinant protein, which strongly induced cell death (Figure 3A and 3B), was able to bind Mcl-1, as demonstrated by cross-immunoprecipitation of transfected cell lysates with anti-FLAG (PUMA) and anti-Mcl-1 Abs (Figure 4B). Unlike the full-length PUMA β and the C-terminal fragment, the N-terminal fragment, which was located in the cytosol, did not bind Mcl-1 (Supplementary Figure 4 panel c). Similar associations between PUMA and Bax or Bak proteins were not detected in our experimental conditions, either in activated BL41 cells or in transfected HeLa cells (data not shown). Nevertheless, both proteins seem to be required for the apoptotic properties of PUMA.

Indeed, transfection of deficient MEF cells for Bax or Bak promotes an intermediate level of apoptosis in these cells whereas double deficient cells for both Bax and Bak were resistant to apoptosis mediated by PUMA β recombinant (Supplementary Figure 5). Altogether, these data show that (i) translocation of PUMA to mitochondria leads to its association with the anti-apoptotic proteins Mcl-1 and/or Bcl-2 and (ii) that apoptosis resulting from this translocation depends on both Bax and Bak.

The translocation of PUMA to the mitochondria is caspase-independent but p38-dependent

We assessed the dependence of PUMA translocation on caspases, given the key role that caspases are known

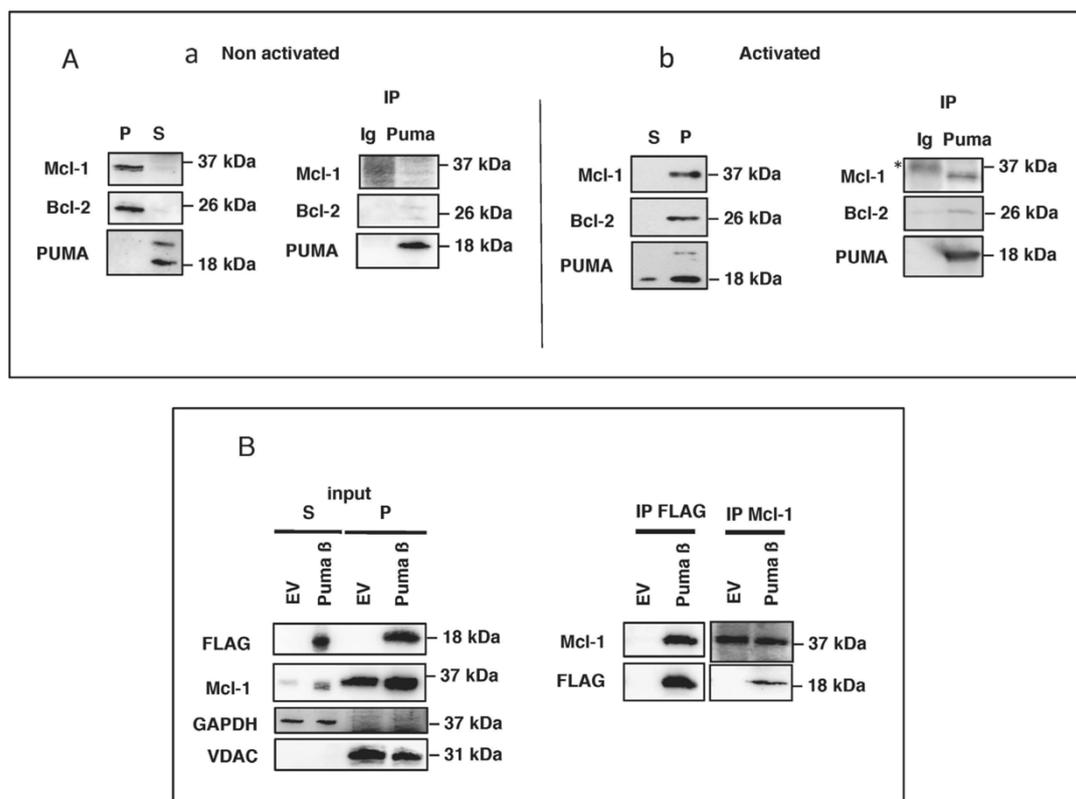


Figure 4: Mitochondrial PUMA binds to Mcl-1 and Bcl-2 in BL41 cells. **A.** BL41 cells were left untreated or were treated for 4 h with anisomycin (2 μ g/ml). Cell lysates were prepared and fractionated. S and P fractions from non-activated (panel a) and activated (panel b) cells were tested for Mcl-1, Bcl-2 and PUMA by western blotting. PUMA was immunoprecipitated (IP) from the S fraction of unstimulated cells (panel a) and the P fraction of stimulated cells (panel b); the immunoprecipitate was tested for Bcl-2 and Mcl-1 by western blotting (* non-specific band). **B.** HeLa cells pretreated with 10 μ M QVD-OPh were transfected with an empty vector (EV) or a full-length FLAG-tagged PUMA construct (PUMA β), incubated for 24 h and lysed; cell lysates were separated into S and P fractions. The P fraction was subjected to IP with anti-FLAG or Mcl-1 antibodies. The resulting immune complexes were analyzed by western blotting with antibodies against PUMA (FLAG) or Mcl-1. VDAC and GAPDH were used as fraction purity controls for mitochondria and cytosol, respectively.

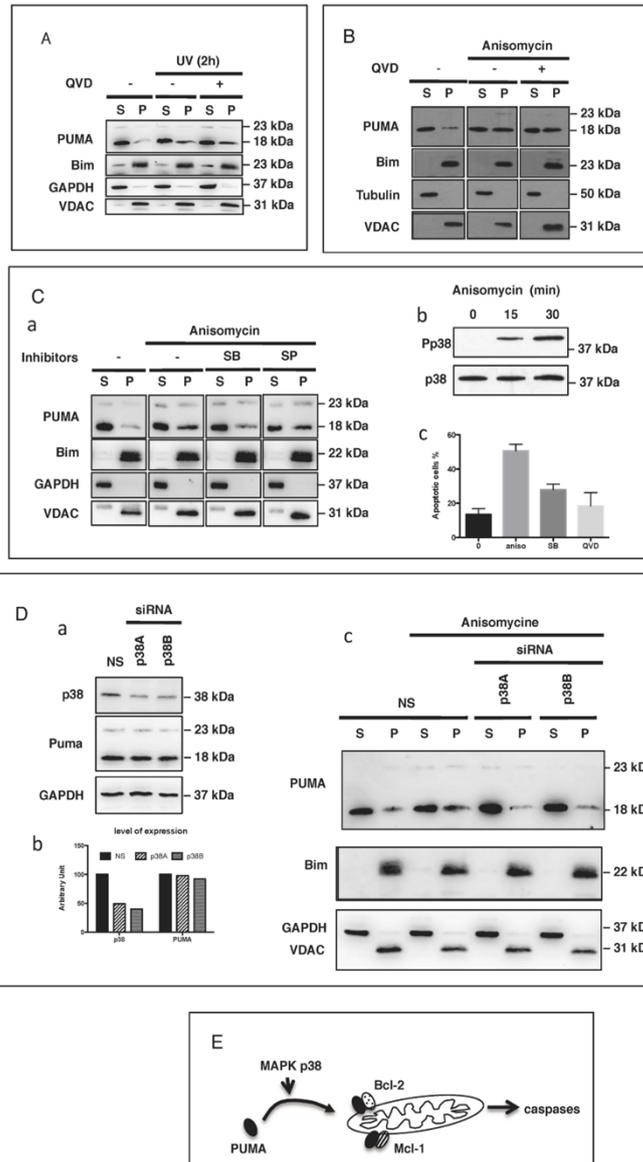


Figure 5: PUMA mitochondrial translocation (PMT) is caspase-independent but p38-dependent. **A.** HeLa cells were treated or mock-treated with QVD-Oph (10 μ M) for 30 min then exposed to UV (6 mJ/cm²) and cultured for an additional 2 h. The subcellular distribution of PUMA, Bim, GAPDH and VDAC was determined by subjecting western blotting the S and P fractions. **B.** BL41 cells treated or mock-treated with QVD-Oph (10 μ M) were stimulated with anisomycin (2 μ g/ml) for 4 h and the subcellular distributions of PUMA, Bim, tubulin and VDAC were determined by fractionation and western blotting. **C.** BL41 cells were treated or mock-treated with 10 μ M SB203580 (SB) or 10 μ M SP600125 (SP) for 30 min then stimulated for 4 h with anisomycin (2 μ g/ml). The subcellular distributions of PUMA, cytochrome *c*, GAPDH and VDAC were analyzed by fractionation and western blotting (panel a). BL41 cells were treated with anisomycin (2 μ g/ml) for 0, 15 or 30 min. Phosphorylated p38 levels were assessed by western blotting with an anti-phospho-p38 antibody (Pp38) and total p38 levels were assessed with an anti-p38 Ab (p38) (panel b). BL41 cells were treated or mock-treated with 10 μ M SB203580 (SB) or 10 μ M QVD-Oph for 30 min, then stimulated for 4 h with anisomycin (2 μ g/ml), and apoptosis was assessed by flow cytometry (panel c). **D.** BL41 cells transfected with a non-targeting siRNA (NS) or a p38-targeting siRNA (p38A and p38B) for 76 h were treated for 0 (NS) or 4 h with anisomycin (2 μ g/ml). The subcellular distributions of PUMA, Bim, GAPDH and VDAC were determined by western blotting the S and P fractions, and p38 knockdown efficiency was analyzed by western blotting with GAPDH as a loading control. **E.** Proposed model for the pathways regulating the translocation of PUMA to the mitochondria and role in apoptosis induction.

to play in apoptotic cell death. Prior treatment of BL41 cells with the pan-caspase inhibitor Q-VD-OPh did not prevent the mitochondrial translocation of PUMA following UV exposure (Figure 5A) or treatment with apoptotic doses of anisomycin (Figure 5B). These findings suggest that caspase activation occurs downstream from the translocation of PUMA to the mitochondria. Various members of the MAPK family of kinases play a major role in early signaling. We investigated their possible role in controlling the translocation of PUMA to the mitochondria. We found that, following anisomycin treatment, the translocation of PUMA to the mitochondria was significantly decreased by the MAPK p38 inhibitor SB203580, whereas a more specific inhibitor of JNK (SP600125) had only a very weak effect (Figure 5C panel a). As a control, we checked that anisomycin promoted the phosphorylation and activation of p38 in our experimental conditions (Figure 5C panel b) and that the inhibition of p38 decreased anisomycin-mediated apoptosis (Figure 5C panel c). Furthermore, the siRNA-mediated downregulation of p38 was associated with inhibition of the anisomycin-induced translocation of PUMA to the mitochondria (Figure 5D). We also observed a very similar pattern in HeLa cells treated with UV: (i) p38 was phosphorylated (Supplementary Fig. 6 panel a) and (ii) the decrease of p38 expression with specific siRNA (Supplementary Figure 6 panel b) inhibited the mitochondrial translocation of PUMA (Supplementary Figure 6 panel c). In this context, no change was observed in the levels of anti-apoptotic proteins, such as Bcl-XL which is present in large amounts in HeLa cells (Supplementary Figure 6 panel d). These findings are consistent with the notion that apoptotic stimuli (such as anisomycin or UV treatment) can induce the translocation of cytosolic PUMA to the mitochondria, in a p38-dependent manner. After reaching the mitochondria, PUMA can bind to and inhibit Mcl-1 and/or Bcl-2, thereby promoting mitochondrial activation and the subsequent activation of caspases and apoptosis (Figure 5E).

DISCUSSION

We describe here an original mechanism controlling PUMA activation: its translocation from the cytosol to the mitochondria. The pro-apoptotic activity of PUMA is believed to be largely controlled by the transcriptional and posttranslational regulation of its expression, through the modulation of its degradation. The general consensus is that once produced, PUMA is targeted to the mitochondria, where it can act as a pro-apoptotic protein [26], as observed in our experimental conditions after the synthesis of recombinant PUMA, eventually inducing cell death. However, in some conditions, the upregulation of PUMA may be associated with cell activation and proliferation rather than apoptosis. This situation has been observed, for example, during the activation of human B lymphocytes *in*

vitro following stimulation of the BCR and CD40 receptor, or activation with mitogens, such as SAC [15]. *In vivo*, germinal center B cells and Burkitt's lymphoma cells (their tumoral counterpart) also produce large amounts of PUMA without the occurrence of cell death [15]. We show here that the role of PUMA in these non-apoptotic cells is dependent on its cytosolic localization. Our results also demonstrate that the translocation of PUMA from the cytosol to the mitochondria, in the absence of transcriptional regulation, is an alternative pathway for the expression of the pro-apoptotic activity of PUMA, at least in some cell types, including B lymphocytes. Our findings indicate that, in our system, PUMA must be "activated" by translocation to the mitochondria, to induce apoptosis. Several unanswered questions remain about the maintenance of PUMA in the cytosol. Other BH-3-only proteins are located in the cytoplasm or associated with skeleton structures, such as tubulin and actin [27, 28], respectively, whereas other proteins are sequestered in the cytoplasm through direct interaction with the chaperone protein 14-3-3 (Bad) or through interactions with unknown partners or in the absence of partners, as for Bid [18, 29, 30]. The subcellular distribution of endogenous PUMA has not been extensively studied. PUMA is generally described as mitochondrial, but one group reported interactions between PUMA and the cytosolic domain of the activated EGFR and EGFRvIII proteins, resulting in its cytosolic sequestration in glioblastoma cells [31]. We detected no interactions, in our cells, between PUMA and other members of the ErbB family (EGFR is not expressed by B cells), such as ErbB1, ErbB2, ErbB3 or ErbB4, which may be present at various levels in some B cells, such as multiple myeloma cells [32]. We have not yet identified any putative cytosolic partners of PUMA in B cells, although mass spectrometry analysis has suggested various potential candidates for further investigation. Alternatively p38 may mediate an increase in PUMA transcription, by activating p53, with or without c-Abl [33-35]. It therefore remains possible that the PUMA in the mitochondria was synthesized *de novo* rather than being translocated from the cytosol of non-apoptotic cells. However, this seems unlikely in our model, because (i) we observed no increase in PUMA mRNA levels in our experimental conditions and (ii) the mitochondrial translocation of PUMA was not prevented by the inhibition of protein synthesis.

Our data indicate that the C-terminal domain, rather than the BH3 domain, is crucial for the targeting of PUMA to mitochondria. Indeed, the N-terminal fragment containing the BH3 domain was located in the cytosol, whereas the C-terminal fragment was associated with mitochondria and able to induce the death of transfected cells. The BH3 domain is therefore not sufficient to target PUMA to mitochondria, whereas the C-terminal domain common to the α and β isoforms of PUMA and containing a putative transmembrane domain, is necessary for the

mitochondrial localization of the protein [36]. These findings suggest that the mitochondrial localization of PUMA involves the insertion of PUMA into the mitochondrial outer membrane rather than simple contact with mitochondrial outer membrane proteins [37]. Three issues need to be addressed: (i) the molecular structure of cytosolic PUMA, (ii) the molecular basis of its translocation to the mitochondria and (iii) the nature of the mitochondrial partners of PUMA.

Phosphorylation regulates the activity of many proteins. The MAPK family of kinases plays an important role in regulating the expression and localization of various BH3-only proteins. Indeed, Erk can phosphorylate Bim, driving its degradation by the proteasome [38] [20]. Phosphorylation by JNK may control the release of Bim and Bmf from tubulin and actin, respectively, and JNK-mediated Bid phosphorylation prevents Bid cleavage by caspase-8 and, thus, its activation [27, 28, 39-41]. Phosphorylation have been reported to affect Bim and PUMA degradation [20, 42, 43]. Sandow et al. recently reported that IKK-mediated phosphorylation of the PUMA S10 residue can induce the degradation of this protein by the proteasome [44]. Interestingly, the β isoform, major isoform present in human B cells ([15] [21] and this paper), lacks the S10 residue, suggesting other residues may be involved in a similar way. Indeed, Fricker et al. described also other phosphorylation sites besides S10 (the predominant one) whose effect on PUMA expression or function remains unknown [43]. It has also been suggested that p38 may trigger an increase in PUMA transcription *via* p53 (with or without c-Abl activation [33] [35] [34]). This suggests that the PUMA associated with mitochondria might be newly synthesized rather than translocated from the cytoplasm. This is unlikely in our model because (i) there was no increase in PUMA mRNA levels in our experimental conditions and (ii) the translocation of PUMA to the mitochondria was observed in the presence of the protein synthesis cycloheximide. Thus, the translocation of PUMA to the mitochondria is dependent on p38 activation. We are therefore currently trying to determine whether (i) PUMA is phosphorylated upon apoptotic stimuli and (ii) a direct phosphorylation of PUMA by p38 is required for its translocation, and, if not, which targets of p38 might be involved

Once translocated to the mitochondria, PUMA can associate with other members of the Bcl-2 family [26]. Our data clearly show that, following apoptotic stimulation, PUMA can bind to and potentially inhibit both Mcl-1 and Bcl-2 in BL41 cells. This finding is consistent with previous demonstrations that the pro-apoptotic activity of PUMA depends on its capacity to interact with, and thereby inhibit the biologic activity of anti-apoptotic proteins of the Bcl-2 family, such as Mcl-1 and Bcl-2. However, there is evidence that PUMA may also associate with and directly activate the pro-apoptotic molecules Bax and Bak [23, 26, 45], although this remains to be

demonstrated definitively. In our experimental conditions, we detected no direct association between PUMA and Bax or Bak. This implies that (i) no such association occurred in our experimental conditions or, more probably, (ii) no such association was detectable in the physiological conditions used (almost all the experiments showing such an interaction were performed with recombinant proteins) [23, 45, 46]. This is consistent with the "hit and run" model involving a transient interaction of PUMA with Bax and Bak [47-49]. Nevertheless, our data show that PUMA-mediated apoptosis is directly dependent on the production and function of both Bax and Bak, because, in the absence of these molecules, the mitochondrial translocation of PUMA does not lead to apoptosis.

PUMA, along with Bid and Bim, is a major regulator of apoptosis involved in the responses of various types of cells to a large panel of apoptotic stimuli. It is therefore important to determine whether the original mechanism observed in Burkitt's cells is also found in other cell types. We also observed the same cytosolic localization of PUMA in various multiple myeloma cell lines, the tumoral counterpart of normal plasma cells of the B-cell lineage (data not shown). We are currently studying the distribution of PUMA in a wide variety of tumor cells, to determine whether the presence of PUMA in the cytoplasm is restricted to lymphoid cells or is a more general feature as suggested by our observations in HeLa cells. Our data suggest that the accumulation of PUMA in the cytosol may be an important feature in the differentiation and tumorigenesis processes, allowing this protein to participate in the apoptotic process without the need for additional transcription.

MATERIALS AND METHODS

Antibodies and reagents

We used primary antibodies against the following proteins for immunoblotting (IB) and immunofluorescence staining (IF): PUMA (C-Term, 1652-1, clone EP512Y) and Bim (1036-1, clone Y36) from Abcam; Bax (N-20, sc-493), Bcl-2 (C-2, sc-7382), Bcl-XL (S18, sc-634), Bik (FC-160, sc-10770), HSP60 (N-20, sc-1052), Mcl-1 (S-19, sc-819), Tom20 (F-10, sc-17764), and tubulin (TU-02, sc-8035) from Santa Cruz Biotechnology; VDAC (AB10527) from Merck Millipore; cytochrome *c* (IB: 556433; IF: 556432) and PARP-1 (556362, clone C2-10) from BD Pharmingen; FLAG (F1804, clone M2) and GAPDH (G9545) from Sigma-Aldrich; NOXA (IMG-349A) from Imgenex; Bid (AF860) from R&D Systems; Bak (38145) caspase-3 (9662) and P-p38 (9211) from Cell Signaling Technology.

Anti-human IgM (DA.44) monoclonal antibodies from the American Type Culture Collection (ATCC) were

purified on protein A-Sepharose columns from Pharmacia Biotech. Anti-mouse IgM antibody was obtained from Jackson Immunotech.

The following reagents were used: anisomycin (A9789) and cycloheximide from Sigma-Aldrich; Q-VD-OPh (OPH001) and recombinant TRAIL from R&D Systems; N-tosyl-Phe chloromethyl ketone (TPCK, 616387) and SP600125 (420119) from Calbiochem and SB203580 (PHZ1253) from Life Technologies.

RNA interference

PUMA/BBC3 silencing was achieved with two small interfering RNAs (siRNA), BBC3HSS146895 and BBC3HS178326 referred to as "P1" and "P2" respectively. Medium GC duplex (12935-112) was used as a negative control. All these siRNA oligomers were purchased from Life Technologies. P38 silencing was achieved with 2 siRNAs, SASI_Hs01_00018465 and SASI_Hs01_00018464, referred to as "p38A" and "p38B" respectively, from Sigma-Aldrich. We assessed siRNA knockdown efficiency by western blotting.

Plasmid construction

The full-length cDNA for the human β isoform of PUMA (kindly provided by Dr P. Juin, Nantes) was inserted into p3XFLAG-CMV-10 (Sigma-Aldrich, E7658). The sequences encoding N-terminal-BH3 (1-258) and C-terminal-BH3 (232-395) were amplified by PCR and ligated into the same plasmid. All constructs were checked by DNA sequencing.

Cell culture and DNA and siRNA transfections

The BL41, BL41 95.8, Ramos, Ramos AW, DAUDI and CA46 cell lines were cultured in RPMI 1640 medium with GlutaMAX™, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate and 1 x nonessential amino acids (Life Technologies). HeLa and MEF cells were cultured in DMEM (Sigma, D0819) supplemented with 10% heat-inactivated FBS, 0.1 mg/ml Normocin™ (InvivoGen) and antibiotics. SV-40-transformed Bax^{-/-}, Bak^{-/-}, DKO and the corresponding wild-type (WT) MEF cells were generously provided by Stanley J Korsmeyer (Harvard Medical School, Boston, MA, USA).

BL41 cells were transfected with siRNA in the BTX-Harvard Apparatus ECM 830 Square Wave Electroporation System (Fisher Scientific). For each siRNA, 4x10⁶ BL41 cells were washed twice in RPMI 1640 without GlutaMAX™ and resuspended in 400 μ l of medium in a cuvette, with 80 nmol of the appropriate

siRNA. Cells were then electroporated at 240 V for 10 ms, and transferred to six-well plates in a final volume of 4 ml. The experiments were carried out 72 h later.

HeLa cells were transiently transfected with empty and PUMA-encoding p-CMV-neo vectors, with Fugene HD (Promega, E2311), according to the manufacturer's protocol or with 5 mol of the appropriate siRNA, with oligofectamine (Invitrogen, 12252-011). Cells were treated with Q-VD-OPh for 30 min, then transfected with DNA.

qPCR

RNA was extracted from the cultured cells with the RNeasy Plus mini kit (Qiagen), according to the manufacturer's protocol. Cell lysates for RNA extraction were obtained by homogenization with QIAshredder spin columns (Qiagen). We synthesized cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). In brief, 5 μ g RNA was combined with oligo (dT)₁₈ primer (10 μ M), H₂O, 5x reaction buffer, 10 mM dNTP mix, RiboLock RNase inhibitor (20 U/ μ L) and RevertAid H Minus M-MuLV reverse transcriptase (200 U/ μ L). The mixture was incubated at 42°C for 60 minutes and then at 70°C for five minutes to stop the reaction. We carried out qPCR in a volume of 20 μ l containing 5 μ l undiluted cDNA, primers (0.75 μ M final concentration) and Power SYBR® Green Master Mix (Applied Biosystems). The following primers were used: PUMA (fw 5'-GACGACCTCAACGCACAGTA-3'; rv 5'-CTAATTGGGCTCCATCTCG-3')* and 18s (fw 5'-AGAAACGGCTACCACATCCA-3'; rv 5'-CACCAGACTTGCCCTCCA-3')** (SIGMA). PCR was performed on an Mx3005P qPCR System (Agilent Technologies), as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s. PCR efficiency was determined with 10-fold dilutions, as follows: Efficiency $E = 10^{(-1/\text{slope})}$. The ratio of PUMA expression in treated cells to PUMA expression in untreated cells was determined as follows: Ratio = $((E_{\text{PUMA}})^{\Delta\text{Ct PUMA (control-treated)}})/((E_{18s})^{\Delta\text{Ct 18s (control-treated)}})$.

Immunofluorescence (IF) staining and confocal microscopy

Cells were fixed by incubation with 4% paraformaldehyde (Alfa Aesar, 43368) for 15 min, then permeabilized by incubation with 0.15% Triton-X100 (Sigma-Aldrich, X100) for 15 min. Samples were blocked by incubation with 2% BSA (Sigma, A9576) for 30 min. The cells were incubated with relevant primary antibodies at a dilution of 1:400 for 1 h at room temperature or overnight at 4°C, then with the corresponding secondary antibodies conjugated with fluorescent dyes (Alexa Fluor®, Life Technologies), at 1:800, for 1 h at room temperature, in the dark. DAPI (Life Technologies,

D1306) was then incubated with the cells, at a dilution of 1:10,000 for five minutes, for nuclear staining and cells were mounted on cover slips in 7 μ L of Fluoromount-G™ slide mounting medium (Beckman Coulter, 731604). Cells were washed three times with PBS between steps. Images were acquired with a Leica SP5 confocal microscope (Leica Microsystems) equipped with a x63 oil immersion fluorescence objective.

Western blot analysis

Whole-cell lysates were prepared by incubation of the cells with TNT buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton and 1% Igepal/NP-40), supplemented with Halt™ protease inhibitor cocktail (Thermo Scientific, 1861279) for 30 min. The lysates were clarified by centrifugation at 10,000 x g for 10 min. Cells were fractionated with the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, 539790), according to the manufacturer's protocol. At least 4x10⁶ cells were lysed mechanically in 27G 1/2 Tuberculin syringes (Sigma, Z192082-100EA), in 200 μ L of H60 buffer (20 mM HEPES, 1.5 mM MgCl₂, 60 mM KCl) supplemented with the same protease inhibitor cocktail (18 back and forth movements), and fractionated by differential centrifugation (procedure detailed in Figure 1C). The P5 pellets were washed once in H60 buffer and lysed in TNT buffer with 1% SDS for 30 min. Protein quantity was measured with the microBCA protein assay kit (Thermo Scientific, 23235). Protein samples (usually 5 μ g) were then boiled for 5 min at 99°C after the addition of Tris-Glycine SDS sample buffer (Life Technologies, LC2676) with 10% β -mercaptoethanol (Sigma, M3148), separated on gradient 5-20% polyacrylamide gels and transferred to nitrocellulose membranes (Santa Cruz, sc-201698). The membranes were incubated with specific antibodies, and bound antibody was visualized by chemiluminescence with the Immobilon western chemiluminescence HRP substrate (Millipore, WBKLS0500) and a DDC camera (LAS-4000 mini, Fujifilm).

Immunoprecipitation

500 μ l of whole cell lysates, cytosol fractions (S25) or mitochondria-enriched fractions (P5) were pre-cleared by adding 40 μ l of protein G-sepharose beads (Sigma-Aldrich, P3296) and placed on a spinning wheel at 4°C. After 30 min, the beads were pulled down by centrifugation at 15,000g for 10 sec. The supernatant was collected and 2 μ g of relevant antibody and 40 μ l of protein G-sepharose beads were added, and the samples were spun at 4°C for 2h. The beads were pulled down by centrifugation, and the supernatant discarded. Bound proteins were eluted from the beads with 30 μ l of Tris-Glycine SDS sample buffer with 10% β -mercaptoethanol.

Samples were then analyzed by western blotting.

Detection of apoptotic cells

The dot-plot scatter profiles of the cells were analyzed by flow cytometry with a BD Accuri C6 Flow Cytometer® (BD Biosciences). Shrunken cells with high side scatter (SSC) and low forward scatter (FSC) were considered apoptotic. The number of apoptotic cells is expressed as a percentage of the total population.

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CONFLICTS OF INTEREST

The authors have no competing financial interests relating to this work to declare.

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(II) Annex 2

Co-stimulation Blockade Plus T-cell Depletion in Transplant Patients: Towards a Steroid- and Calcineurin Inhibitor-Free Future?

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Co-stimulation Blockade Plus T-Cell Depletion in Transplant Patients: Towards a Steroid- and Calcineurin Inhibitor-Free Future?

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Abstract Long-term survival of solid allografts depends on both immunosuppressive efficacy and reducing the side effects associated with these therapies. Immunotherapies developed over the past 15 years to prevent organ rejection have greatly improved cardiovascular and renal function compared with classical therapies, such as calcineurin inhibitors and corticosteroids. Immunotherapies that target T cells through the co-stimulation blockade (CTLA-4-Ig) improve renal function and the survival of grafts and patients, but are associated with higher rates of T-cell-mediated acute rejection. Improvements to safe and efficacious therapeutic options could combine a co-stimulation blockade with a depleting immunotherapy. Herein, we describe the clinical outcomes and the likely causes of defects in the co-stimulation blockade, and comment on new therapeutic strategies to overcome these. Great progress has been made to optimize immunotherapy using the co-stimulation blockade, but the therapeutic combinations should be assessed further.

Key Points

Cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4)-Ig improves graft survival and renal function, and decreases cardiovascular risk compared with a calcineurin-inhibitor-based regimen.

However, CTLA-4-Ig increases the risk of acute rejection episodes. A combination of co-stimulation blockade with depletion therapies, by inhibition of cells implicated in acute-rejection episodes, could decrease steroid and calcineurin inhibitor use.

1 Introduction

Rejection of a solid allograft can be caused by numerous and overlapping mechanisms involving the innate and adaptive immune system of the recipient. Detection of a ‘danger signal’ by innate immune cells leads to activation of the whole immune system [1]. Ischemia reperfusion and tissue damage induce expression of damage-associated molecular patterns, such as the high-mobility group box-1 protein, reactive oxygen species, and heat-shock proteins, which are recognized by receptors of pathogen-associated patterns [2]. Infiltration of innate cells into transplanted organs can then lead to an inflammatory response through cytokine production, involving tumor-necrosis factor- α and interleukin (IL)-1 β [3], causing further damage by producing reactive nitrogen and oxygen species [4]. Release of chemo-attractant molecules through inflammation, at the

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same time as activation of antigen-presenting cells (APCs), is the first step towards activation of the adaptive immune system. T cells are central in this process due to their capacity for allo-recognition, which induces clonal expansion, effector differentiation, and an ability to migrate within tissues. Moreover, CD4 T cells allow full activation of cytotoxic CD8 and alloantibody production by B cells. Thus, CD4 T-cell activation is central to cellular-graft rejection [5].

Immunosuppressive drugs, such as calcineurin inhibitors (CNIs), significantly reduce acute rejection [6, 7]. However, these immunosuppressive drugs exhibit side effects, such as increasing cardiovascular risk factors (including hypertension) [8], dyslipidemia [9], and diabetes mellitus [10, 11], as well as inducing nephrotoxicity [12, 13]. During the last decade, efforts have been made to reduce these cardiovascular risk factors, which appear to be strongly associated with the mortality of patients with chronic renal failure and transplant patients. The risk of death is increased during the 6 months following transplantation, but decreases inducing an overall survival benefit as compared with patients remaining on dialysis. However, cardiovascular risk remains elevated compared with the healthy population.

Immunosuppressive regimens can directly and indirectly participate in the increased risk of developing a cardiovascular disorder. CNIs increase blood pressure compared with a regimen without CNIs [14]. Similarly, some CNIs participate in elevating cholesterol levels and favor de novo diabetes mellitus [15, 16]. Based on studies that have focused on non-renal solid-organ transplantation, when chronic renal failure develops after transplantation with a CNI-based regimen, it appears clearly that CNIs impair creatinine clearance, which then correlates with increased cardiovascular risk [17].

Among the classical combined therapies used to prevent acute renal failure after renal transplantation, steroids can cause sodium retention, hypertension [18], participate in de novo diabetes mellitus [19], and are associated with CNIs in increasing cardiovascular risk factors [20]. In non-immunized patients, randomized trials have demonstrated that steroid avoidance is associated with an acceptable risk of acute rejection, especially when steroids are stopped early after transplantation. Although studies have not yet shown benefits to the cardiovascular system or to bone density, some have reported intermediate markers of improvement [21, 22]. Although a few patients already receive a steroid-free regimen, there is increasing effort to minimize or avoid steroids altogether with CNI-based regimens. A meta-analysis that assessed steroid avoidance and withdrawal emphasized the benefits of reducing cardiovascular risk, despite the increased risk of acute rejection [23]. A recent trial on rapid steroid withdrawal vs. maintenance did

not find a difference in organ survival at 5 years between these groups [24].

New molecules have been developed to replace CNIs, such as mammalian target of rapamycin inhibitors, but are associated with hypercholesterolemia and de novo diabetes mellitus [25, 26]. Since the introduction of sequential therapies, consisting of induction with anti-IL-2 receptor monoclonal antibodies and CNIs, mycophenolate-mofetil, and steroids, the incidence of acute rejection has decreased. Thus, consideration could be given to minimize or replace some of these therapies. Although minimizing the use of CNIs will probably reduce the risk of cardiovascular events, no benefits in terms of graft- or patient-survival rates have been demonstrated. Indeed, questions have emerged regarding the risk of developing anti-donor-specific antibodies, which are then associated with a higher incidence of antibody-mediated rejection. Therefore, the development of new therapies that can replace current treatments is needed [27]. Herein, we discuss the advances made in co-stimulation blockade immunotherapy, the clinical outcomes, and the perspectives for the immunomodulation of transplant patients.

2 Co-stimulation Blockade Strategies

Based on our knowledge that activation of T cells is central in the allogeneic response, several molecules have been developed over the past 20 years to inhibit these cells. T-cell activation results in three signals from APCs, mainly dendritic cells (DCs), which steer T cells towards effector function and orientation. The first signal occurs through an interaction between the major histocompatibility complex (MHC) and the T-cell receptor: this signal is required for specificity and is dependent on the T-cell-receptor repertoire. The second or co-signal can produce a positive or negative signal to T cells and participates in their activation or inhibition [28–30]. In the presence of these two activation signals, T cells can then secrete cytokines with paracrine and autocrine functions. A third signal that activates T cells can then activate the mammalian target of rapamycin pathway, which controls the cellular division of T cells.

Co-signaling involves many different interactions between ligands and receptors from APCs to T cells. The most investigated co-stimulation signal for therapeutic purposes is B7-CD28, which brings together two ligands on APCs (CD80 and CD86: the B7 family) and two receptors on T cells (cytotoxic T-lymphocyte-associated molecule-4 [CTLA-4] and CD28). CD28 is expressed by naive T cells and is involved in T-cell activation, proliferation, and survival concurrent with signal 1. Activated T cells then express CTLA-4, which inhibits T-cell activation and acts

as a retro-control. Thus, co-signaling involves overlapping activation and inhibitory signaling (in T cells and APCs), and changes according to the subpopulation involved and the activation state [31]. Thus, interactions between CD80 and CTLA-4 or PD-L1 (a ligand of programmed cell death 1) by T cells provides an inhibitory signal, whereas CD80/86 or the ligand of the inducible T-cell co-stimulator (ICOS) can lead to T-cell proliferation through CD28 [32, 33].

CD28 signaling is also a key factor in the activation and homeostasis of conventional T cells and natural regulatory CD4+ T cells (Tregs) [34, 35]. In contrast, CD28 enhances early signals induced by the T-cell receptor through increased phosphorylation and recruitment of some of the signaling components [36, 37]; however, CD28 also induces its own signal [38, 39]. Overall, CD28 is implicated in IL-2 secretion, proliferation, survival, and rescue from anergy of newly activated T cells [40–42]. Activated conventional and regulatory T cells can then up-regulate CTLA-4 transcription and expression [43, 44]. The inhibitory signal of CTLA-4 is still being discussed, but it seems to inhibit CD3 or the phosphorylation of a later mediator [45, 46] (Fig. 1).

Different co-signals have been targeted by therapies to fine tune the T-cell response in terms of amplitude and orientation [47, 48]. The CD28/CTLA-4/B7 signal is the major co-signal investigated for its potential therapeutic benefits because CD28 is expressed by naive T lymphocytes before activation, whereas ICOS expression, amongst

others, is induced by activation. In CD28/CTLA-4/B7 co-signaling, the target molecule and the method of blocking are important. First, B7 consists of two proteins: CD80 and CD86. CD80 has a two-fold higher affinity for CD28 than CD86, and is slowly dissociated from CTLA-4 [49]. In addition, blocking the signal from APCs using anti-B7 should inhibit the fixation of both molecules in a balanced manner. The avidity of CTLA-4 for CD80 and CD86 is higher than that of CD28. This discrepancy in avidity and kinetics is necessary for CTLA-4 to inhibit CD28-mediated T-cell activation, but is a conflict point in the development of anti-CD28- or CTLA-4-blocking antibodies [49, 50].

Various strategies have been assessed since the 1990s to inhibit T-cell activation and prevent co-stimulation in transplantation as well as autoimmune disorders [48]. Blocking anti-CD28 antibodies was first considered as an interesting option because it preserves interactions between CTLA-4 and its ligands, thus allowing a homeostatic signal. However, all conventional anti-CD28 antibodies developed have an antagonist effect due to dimerization of CD28, which leads to a positive signal [51]. In contrast, a monomeric fragment of the anti-CD28 antibody (Fab) does not retain this activation property [52, 53], but has a short half-life that is not compatible for in vivo use [54].

Another way to block CD28 signaling is to use anti-B7 antibodies. Expression of B7 molecules by APCs and a balance between CD80 and CD86 expression is dependent on their activation states. As an example, immature DCs express a higher level of CD86 than CD80, which are then

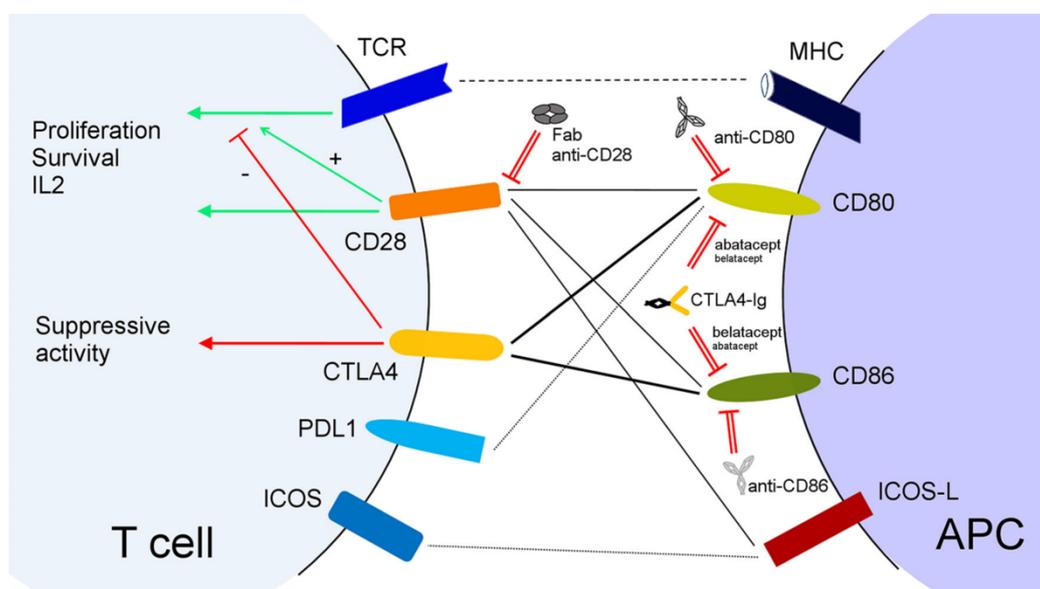


Fig. 1 Schematic of the B7/CD28/CTLA4 axis in T-cell activation. APC antigen presenting cell, CTLA4 cytotoxic T-lymphocyte-associated molecule-4, Fab antigen-binding fragment, ICOS inducible

T-cell co-stimulator, IL2 interleukin-2, MHC major histocompatibility complex, PDL1 programmed death-ligand 1, TCR T-cell receptor

both up-regulated by an activator [55]. Moreover ligation of CD80 or CD86 does not activate the same signaling pathway in DC or the same T-cell phenotype [56]. CD80 could direct Th1 orientation whereas CD86 could induce Th2 polarization because of the higher proportion of IL4 secreted [57, 58]. Furthermore, CD80 and CD86 induce signaling in APCs and have different affinities to CD28 and CTLA-4. Finally, blocking both CD28 and CTLA-4 signals implies inhibition of the second signal of activation, but also the signal of homeostasis given by CTLA-4 and the regulatory effect of CTLA-4. However, blocking CD80/CD86 should also be considered; successful results have been obtained in non-human primates (see below).

CTLA-4 has a natural advantage compared with CD28 in its ability to bind CD80/86, which makes it more suitable for *in vitro* bioengineering. The most complete investigation concerns the CTLA-4-Ig fusion protein. The first CTLA-4-Ig (abatacept) has demonstrated great efficacy in rheumatoid arthritis [59] but has shown disappointing results in transplantation in non-human primates [60], possibly because of its better affinity to CD80 than CD86, inducing a change in the Th1/Th2 balance [49, 61]. Larsen et al. have described the development of belatacept from CTLA-4-Ig to improve its avidity to CD86 [62].

3 Regulatory T-Cell Activation and Activity Through the CD28/CTLA-4/B7 Axis

We have described that, unlike CD28, CTLA-4 expression is restricted to activated conventional and regulatory T cells [63]. The importance of CTLA-4 expression to regulate T-cell activity has been suggested in a study on CTLA-4^{-/-} mice with a lymphoproliferative disorder with organs infiltrated by T cells [64]. A further study on conditional CTLA-4 knockout (KO) mice demonstrated normal development and survival of Tregs, but impaired function of these cells [65, 66]. Nevertheless, other studies have demonstrated a role for the CD28/CTLA-4/B7 axis in Treg homeostasis (Fig. 2). *In vivo*, a decrease in peripheral Tregs was observed in CD80 and CD86 conditional KO mice (KO restricted to DCs) [67]. Likewise in a mouse model of heterotopic heart transplantation treated with CTLA-4-Ig, a decrease in peripheral natural Tregs was observed and was linked to rejection in the MHC-II, but not class-I mismatches [68]. Similarly, another study described increased skin rejection being associated with a decrease of IL-2-induced Tregs in CTLA-4-Ig-treated mice. In the same model, the number of Tregs could be restored by treating mice with agonist anti-CD28 [69]. This decrease in Tregs could be explained by the reduced proliferation of Tregs, as shown with CD80/86 KO mice DCs *in vitro* [70] and with blocking antibodies against CD80/86 *in vivo* [71].

However, this proliferation is not totally abolished, suggesting an interaction between a second signal, such as the glucocorticoid-induced tumor-necrosis factor receptor-related protein.

Apart from the inhibition of CD28 signaling through ligand competition [72], CTLA-4 contributes to the suppressive functions of Tregs through signaling, as demonstrated in mice with a mutation on the CTLA-4 phosphorylation site [44]. In mixed lymphocyte reactions (MLR), blocking CD80 and/or CD86 showed that CD86 stimulation of Tregs inhibited their function whereas CD80 increased suppressive activity [73]. However, we could not distinguish CD28 from CTLA-4 in this effect. Another mechanism of suppression through CTLA-4 by Tregs is the alteration of B7 expression on APC via trans-endocytosis [74], which involves the transfer of B7 from APCs to Tregs after interaction with CTLA-4, and internalization of the ligand-receptor complex's induced degradation of B7. Furthermore, surprisingly, evidence shows that blocking trans-endocytosis and signaling within CTLA-4-Ig does not inhibit the suppressive function of Tregs, thus highlighting another mechanism. A positive effect of CTLA-4-Ig on Treg function has been described in mice; this study described enhancement of a suppressive function that was dependent on nitric oxide secretion by macrophages and transforming growth-factor- β secretion [75].

Another inhibitory effector to consider is DCs. Indoleamine 2,3-dioxygenase (IDO) secretion by DCs is a mediator of suppression; interactions between B7-CD28/CTLA-4 combined with interferon- γ can induce the synthesis of IDO by DCs in murine MLR [76, 77]. In anti-CD28 treatments, this could lead to decreased DC-induced regulation. However, activating anti-CD80, anti-CD86, or CTLA-4-Ig treatment could increase secretion of IDO and the regulatory effect of DCs. Furthermore, indirect modulation of Treg homeostasis and activity could impact on DC activity as their maturation can be reversed by Tregs through CTLA-4 [78].

In a study that assessed CTLA-4-Ig on human Tregs, belatacept inhibited *in vitro* Treg proliferation and generation [79]. This was confirmed *in vivo*, in patients with rheumatoid arthritis and treated with abatacept [80]. In contrast, a phase II trial on kidney-transplant patients treated with CTLA-4-Ig and the anti-IL-2 receptor showed no difference in the proportions of CD4⁺ CD25⁺ cells in peripheral blood compared with healthy donors [81]. This *in vivo* analysis is supported by data from MLR [82] where CTLA-4-Ig could convert naive T cells in Tregs [83]. The described consequences of CTLA-4-Ig on Treg homeostasis are dependent on context. Nevertheless, assessment of the regulatory function of CD4⁺ T lymphocytes in patients treated with CTLA-4-Ig could overcome these divergent results.

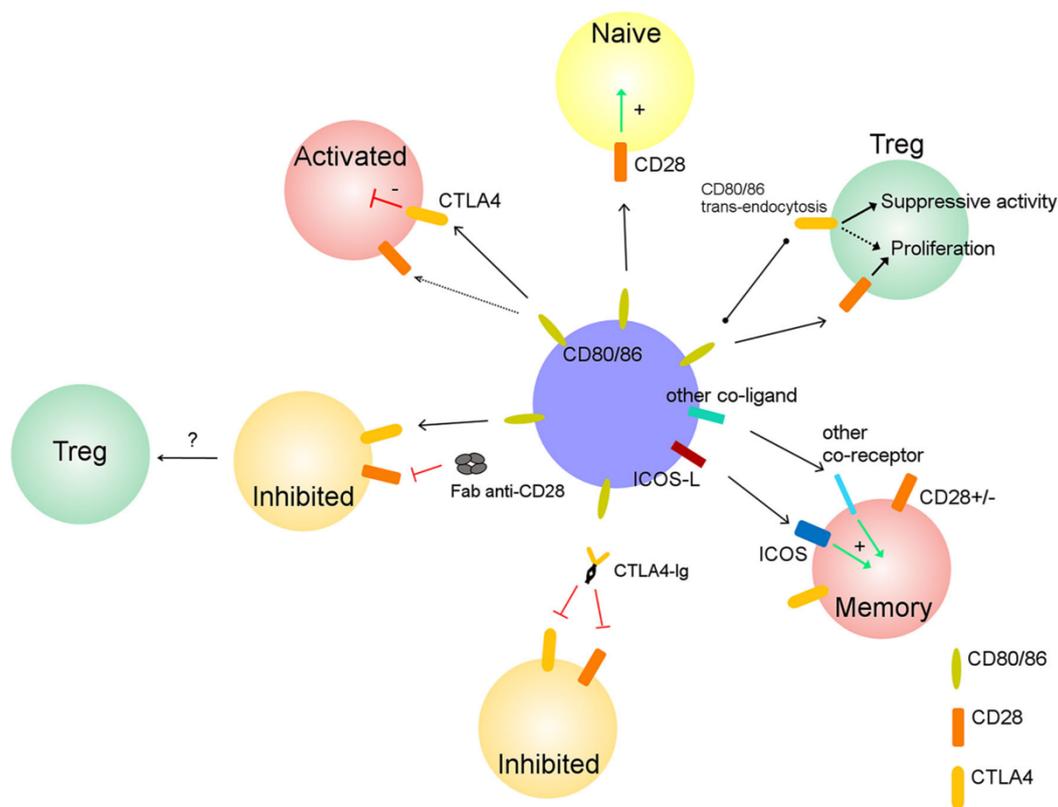


Fig. 2 Schematic of CD28/CTLA4 co-stimulation in T-cell populations. *CTLA4* cytotoxic T-lymphocyte-associated molecule-4, *Fab* antigen-binding fragment, *ICOS* inducible T-cell co-stimulator, *Treg* regulatory CD4+ T cells

4 In Vivo CD28/CTLA-4/B7 Co-signaling Blockade

A blocking strategy that disrupts CD80/86 and CD28 interactions and prevents early activation of T cells has been evaluated in vivo. In mouse models, treatment with anti-CD80 or anti-CD86 antibodies after heterotopic heart transplantation, as well as anti-CTLA-4 treatment, decreased the survival of animals [84]. Nevertheless, repeated treatment of monkeys with humanized anti-CD80- or anti-CD86-specific antibodies slightly inhibited the rejection of transplanted kidneys, and combining these antibodies delayed rejection further; however, anti-donor antibody production was not abrogated [85]. In a rhesus-monkey model, the use of combined anti-CD86 and anti-CD80 was not antagonized by cyclosporine or steroids, but increased survival was achieved when sirolimus was added [86, 87]. In contrast, the association of rapamycin with anti-B7 antibodies did not increase graft survival compared with anti-B7 antibodies alone in a kidney-graft model of baboons [88]. These antibodies did not prevent early severe acute rejection and did not induce graft tolerance in cynomolgus monkeys [86]. More recently, anti-CD80/86

antibodies have been used to generate Tregs in a cell therapy given after living-donor liver transplantation. This study showed promising results regarding the induction of operational tolerance [89].

Following improvements to the affinity of CTLA-4-Ig, with a two-point mutation in the CTLA-4 domain, modified CTLA-4-Ig (belatacept) has been tested in vivo to replace CNIs as a cornerstone immunosuppressive molecule, associated with mycophenolate-mofetil, steroids, and basiliximab. A first trial demonstrated the non-inferiority of belatacept concerning rejection compared with cyclosporine [90]. A larger study then compared two belatacept regimens with cyclosporine in two cohorts of kidney-transplant patients over a 7-year period. Analyses at 1, 3, and 5 years confirmed similar patient- and graft-survival rates in the belatacept groups compared with those receiving cyclosporine [91–95]. After 7 years, patients treated with belatacept showed better renal function, as expected [96]. Similarly, belatacept resulted in a reduced risk of mortality and reduced graft loss in those that had received a standard kidney [97]. Despite this being the first long-term analysis (7 years) performed, one could speculate that there was bias regarding the selection of patients at

the end of the study (i.e., 3 years) followed by their subsequent enrollment in the long-phase extension study. This occurred despite most patients treated with belatacept being enrolled in the extension study. Further analyses of cohorts of patients could confirm these interesting results. A side effect induced by belatacept was a higher risk for a lymphoproliferative disorder in patients that were not infected by the Epstein–Barr virus before transplantation or had been treated with an intensive regimen of belatacept. However, the lymphoproliferative disorders were not directly associated with the lymphocyte-depleting therapies [91, 92].

5 Acute Rejection and the Co-stimulation Blockade

Even though belatacept has shown superiority regarding graft function and cardiovascular protection, it increases the risk for and severity of acute-rejection episodes beyond 1-year post-transplantation compared with cyclosporine. However, these early events do not affect longer term survival [91, 95]. Interestingly, a higher incidence of acute rejection was observed in patients receiving a kidney from a standard-criteria donor, but not from an extended-criteria donor. In general, the incidence of acute rejection is higher in this population, possibly because of the increased lesions caused by ischemia/reperfusion. In addition, anti-B7 treatment after renal grafting in cynomolgus monkeys was associated with a high incidence of severe rejection [87]. The occurrence of acute rejection has been also described by Ville et al. in a study that compared FR104 (anti-CD28 Fab) and belatacept given to baboons that had undergone kidney transplantation: two of the five animals treated by FR104 and sirolimus, and four of the five animals treated by belatacept and sirolimus encountered an acute rejection, whereas only those treated with belatacept alone had a steroid-resistant rejection [98].

The occurrence of an acute rejection impacts on the outcome of transplantation. It is associated with lower graft function in patients that have developed an acute rejection independently of their immunosuppressive regimen. In two phase III studies, BENEFIT and BENEFIT-EXT, belatacept was compared with cyclosporine as recommended by the authorities when the studies were designed. However, the treatment for the control arm is no longer the standard of care in many countries since the publication of the SYMPHONY study [99]. Therefore, we can speculate that the number of acute-rejection episodes would be lower in a comparative group treated with tacrolimus instead of cyclosporine, and renal function would be lower in the group treated with belatacept compared with tacrolimus [100]. However, it is noteworthy that, in the conversion

study (IM103010), patients that were switched from tacrolimus to belatacept, or from cyclosporine to belatacept, had similar improvements in renal function at 2 years [101].

Thus, comparative studies between tacrolimus and belatacept are needed to confirm the beneficial effect of belatacept on CNI-based regimens and, more specifically, tacrolimus-based regimens. Interestingly, the occurrence of an acute rejection in belatacept-treated groups was not significantly associated with the presence of anti-donor antibodies, which is a major event leading to the development of an acute or chronic antibody-mediated rejection [93, 102]. Indeed, the incidence of donor-specific antibodies in belatacept-treated patients was significantly lower than in patients treated with cyclosporin over the longer term, and was independent of having developed an acute rejection. Similarly, neither belatacept nor FR104-treated animals developed anti-donor specific antibodies. This lower incidence of donor specific antibodies could be the result of better adherence of patients to treatments given by intravenous infusion or be a consequence of the mechanisms of action of molecules blocking T- and B-cell activation. As an example, Grimbert et al. showed that B cells, in patients receiving belatacept, more frequently had a transitional phenotype when compared with B cells in patients treated with cyclosporine [103].

6 T Cells Associated with Acute Rejection in the Co-stimulation Blockade

The higher incidence of acute-rejection episodes in belatacept-treated patients suggests that some T cells are resistant to the co-signal blockade or are independent of CD28 activation and are then not targeted by CTLA-4-Ig therapy. Memory T cells are a significant component of the cellular-rejection phenomenon because of their high frequency and prompt cross-activation, despite non-exposure to tissues from the donor [104, 105]. The involvement of memory CD8+ T cells in allograft rejection within the co-stimulation blockade in treated non-human primates has already been described, highlighting the B7 co-stimulatory signal independence of these cells [106, 107].

The importance of co-signaling for naive T-cell activation and expansion is known [108]. In contrast, the dependency of memory T cells on B7 co-stimulation is still under discussion. The first in vitro studies described the independence to semi-dependence of memory T cells to B7 stimulation [109, 110]. However, these results have been challenged in vivo. In mice, CTLA-4-Ig within memory CD4 T cells showed defective proliferation and a bias for central memory T cells compared with effector memory T cells, even though early proliferation was not affected

[111]. However, other pathways have been proposed to activate memory T cells, including CD40/CD40L and ICOS/ICOS-L, which are independent of CD80/86-CD28 (Fig. 2).

To identify cells associated with acute rejection in belatacept-treated patients, Allan Kirk's group and ourselves investigated the biomarkers of patients that had encountered this event, focusing specifically on markers related to memory T lymphocytes. A higher frequency of memory-related CD4 CD57+ PD1- T-cells on day 0 of transplantation was observed in kidney-transplant patients treated with belatacept [112]. In addition, in a single observation, de Graav et al. reported a patient with vascular rejection requiring nephrectomy, mostly associated with intra-graft CD4 and CD8 effector memory T cells that synthesized interferon- γ and granzyme B [113].

CD57 expression by peripheral T lymphocytes is currently associated with clonal exhaustion, mainly for the CD8+ subset. This marker (CD57+ T cell) increases in frequency with age and in individuals with chronic immune activation, such as cytomegalovirus or human immunodeficiency virus infection [114–116]. Nevertheless, expression of CD57 is not exclusive to memory T-cell populations and its expression by non-senescent T cells has been observed [117]. Indeed proliferation of CD8+ CD57+ T cells has been reported [118] and the CD4+ CD57+ population is associated with autoimmune disease. Similarly, we observed that in vitro CD4+ CD57+ T cells proliferate in MLR and were not inhibited by belatacept, whereas their proliferation was impaired by CNIs. In addition, CD4+ CD57+ PD1- T cells expressed perforin and were able to degranulate in the presence of target cells, suggesting their cytotoxic capabilities. Moreover, they were found to infiltrate the graft during rejection.

Apart from CD4+ CD57+ T cells, another memory T-cell population of interest is CD28- T cells. Borowski et al. assessed recall in B7 KO mice in the presence of anti-CD28 blocking antibodies in influenza and herpes simplex virus infections. They reported a decreased number of specific CD8 T cells and longer persistence of the virus [119]. Despite the anomalous recall of memory cells in the absence of CD28 activation, this was not abrogated. The CD28- compartment of memory T cells may be involved in this activation because of a lack of inhibition by CTLA-4-Ig or anti-CD28 treatment. This population of cells increases with age in autoimmune diseases, immunodeficiency, and chronic viral infections [120–123], but also in patients with a transplant and chronic rejection [124, 125]. A recent study by De Graav et al. described the proliferation of CD28+ T cells in patients with end-stage renal disease in the presence of belatacept: surprisingly, some of these proliferating cells became negative [126].

7 Perspectives on Belatacept-Based Therapies

To reduce the rate of acute-rejection episodes in belatacept-based regimens, several new associations of immunosuppressive drugs have been suggested to control alloreactive T cells that are not targeted by belatacept. These therapeutic strategies include transient co-administration of a current therapy (CNIs, mycophenolate-mofetil, steroids) during initiation of belatacept treatment (i.e., <6 months), as CD4+ CD57+ PD1- is not associated with acute-rejection episodes in CNI-based regimens. However, the effect on improving renal function needs to be evaluated as the greatest benefit was observed early after renal transplantation, but was lower in studies that switched from CNI to belatacept [101]. Another option is to replace an induction therapy with basiliximab, which seems to be ineffective at preventing acute rejection in belatacept regimens in humans, but not in non-human primates, where it impaired regulation by Treg depletion.

Anti-thymoglobulin (ATG) polyclonal antibodies, used as an induction therapy, have demonstrated their efficacy to promote graft survival [127]. Low-dose (non-depleting) rabbit ATG is also associated with Treg induction in vivo in mice and in vitro in humans [128, 129]. Another option is to use a monoclonal agent such as alemtuzumab, a CD52-depleting monoclonal antibody, instead of polyclonal ATG. CD52 is expressed by mononuclear cells and alemtuzumab mainly depletes T and B lymphocytes [130]. This monoclonal antibody has demonstrated its equivalent efficacy compared with ATG with regard to graft- and patient-survival rates and delayed graft function in kidney-transplant patients (assessed in a meta-analysis [131]). Another interesting molecule is the fusion protein LFA3-Ig, alefacept. LFA3 is the ligand of CD2 and is expressed by activated T lymphocytes. LFA3-Ig inhibits proliferation of T cells [132] and induces activated T-cell depletion through natural killer activation and antibody-dependent cell-mediated cytotoxicity [133]. This mechanism is also reported with a rat anti-CD2 antibody and its humanized form. In a murine xenogenic graft-transplant model, it demonstrated decreased rejection through natural killer cells and monocyte activation of antibody-dependent cell-mediated cytotoxicity [134].

The advantage of induction using depletion therapies would be to eliminate memory T cells. It has been demonstrated in diverse trials and studies that ATG is associated with a lower rate of acute rejection than anti-IL-2 receptor-blocking antibodies in highly immunized patients [135, 136]. In vitro, the partial inhibition of CD8 T-cell proliferation by belatacept can be overcome by alefacept [107]. Interestingly in a rhesus-monkey model of kidney transplantation, alefacept, combined with sirolimus

and belatacept, depleted memory T cells but did not delay the occurrence of acute rejection and did not induce allograft tolerance: it actually favored cytomegalovirus reactivation [137]. In contrast, another study that used the same model showed increased survival of animals treated with alefacept/sirolimus/belatacept compared with animals receiving only sirolimus and belatacept, and a decrease in effector memory T cells after alefacept treatment [106].

In humans, the use of an induction therapy with ATG in conjunction with belatacept and sirolimus has been combined with steroid withdrawal [138]. This regimen was compared with belatacept plus mycophenolate-mofetil or mycophenolate-mofetil plus tacrolimus, with all patients receiving ATG as the induction therapy. After 1 year, lymphocyte reconstitution was similar in all three groups. As expected, the glomerular-filtration rate was better in belatacept-treated patients compared with the control group, and a higher rate of acute rejection was observed in those receiving mycophenolate-mofetil, but not in those receiving sirolimus. This suggests that the association of sirolimus, belatacept, and an induction therapy of ATG is an interesting option for belatacept-based regimens. For the same group of patients, no alteration in the CD4+ Foxp3+ CD25+ population was observed when patients received ATG as the induction therapy, followed by belatacept and sirolimus or mycophenolate-mofetil treatment: this was in contrast to the combination of belatacept, mycophenolate-mofetil, and the anti-IL-2 receptor antibody. Moreover, ATG/sirolimus/belatacept treatment increased the ratio of Treg cells to memory T cells [139].

Based on these strategies and to minimize long-term immunosuppression, withdrawal of sirolimus after 1 year of treatment with belatacept, sirolimus, and an induction therapy of alemtuzumab, plus a donor bone-marrow infusion, was evaluated in kidney-transplant patients. Results from this proof-of-concept study showed that alemtuzumab induction was associated with a low risk of acute rejection. The addition of an infusion of donor bone marrow did not reduce the risk of acute rejection. The phenotype of lymphocytes included in this study shows that their reconstitution following induction was associated with an increase in B regulatory cells, Tregs (CD25+ Foxp3+), and CD8 T-cell-naïve populations, but no increase in CD28- T cells [140].

A temptation would be to also further withdraw steroids in belatacept-based regimens. All long-term studies have included maintenance low-dose steroids. However, we need to consider that steroid withdrawal is associated with an increased risk of rejection in CNI-based regimens, and because a higher risk of acute rejection was observed in belatacept-based regimens, a pilot study needs to explore this possibility [23].

A recent report from Kirk et al. shows, in non-human primates, that the association of belatacept and sirolimus reduced the risk of acute rejection without the need for an induction therapy [137]. However, this strategy needs to be evaluated in humans, as the early introduction of sirolimus could be associated with longer delayed graft function and post-transplant complications. This would favor the use of this association with a short induction therapy, at least for patients with a higher risk of sirolimus-associated complications [141, 142].

Research into the co-stimulatory blockade could be increased with the development of a new anti-CD28 blocking agent based on increasing the half-life of anti-CD28 Fab [143]. Currently, the most advanced anti-CD28 Fab, FR104, is covalently modified with polyethylene glycol and inhibits rejection in baboons and prevents activation of memory T cells [144, 145].

8 Conclusion

The co-stimulation blockade in renal transplantation is associated with better renal function and better graft- and patient-survival rates. The higher rate of acute rejection in belatacept-based regimens is associated with non-targeted T cells. These events could be prevented by using combination therapies according to the clinical situation. New trials assessing sirolimus plus belatacept, with or without induction using a T-cell-depleting agent and steroids, could be promising for renal transplantation.

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Compliance with Ethical Standards

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Titre : Régulation de l'activation de lymphocyte B pendant le rejet chronique ; La rôle de SYK dans la modulation de Mcl-1

Mots Clés : Rejet médiée par les anticorps, centres germinatifs, Signalisation du BCR, SYK, Mcl-1, Apoptose

Résumé : L'insuffisance rénale est un problème majeur de santé publique et la transplantation rénale est l'option thérapeutique principale, mais elle comporte le risque de rejet d'organe. Les cellules B jouent un rôle important dans le rejet médié par les anticorps (AMR). Au cours de l'AMR chronique, les structures lymphoïdes tertiaires, semblables aux centres germinatifs (GC), apparaissent dans l'organe rejeté, associées à la production des plasmocytes et des lymphocytes B mémoires spécifiques du donneur. Ces populations de lymphocyte B sont souvent mal contrôlées par les traitements actuels. La myeloid cell leukemia 1 (Mcl-1), un membre anti-apoptotique de la famille de B-cell lymphoma 2 (Bcl-2), est essentiel pour maintenir l'organisation de GC et de la différenciation des cellules B. Nous rapportons ici l'infiltration de cellules B exprimant Mcl-1 dans le rein de patients atteints d'AMR chronique, comme cela a été observé pour les cellules (pré) GC. Suite à l'abrogation de la signalisation du récepteur des cellules B (BCR), par l'inhibition de la spleen tyrosine kinase (SYK) nous avons observé une diminution de la viabilité des cellules GC, par l'intermédiaire d'une régulation de Mcl-1. La régulation négative de Mcl-1 est coordonnée au niveau de la transcription, potentiellement par l'intermédiaire du transducteur de signal et de l'activateur de la transcription 3 (STAT3), comme cela a été observé par (1) une translocation altérée de STAT3 dans le noyau suivant l'inhibition de SYK, et (2) les niveaux inférieurs de transcription de Mcl-1. Par ailleurs, la surexpression de Mcl-1 inhibe l'apoptose après l'inhibition du SYK. Des études avec des cellules B primaires, issues d'amygdales, ont confirmé que l'inhibition de SYK a diminué la survie cellulaire. Nous avons également constaté que l'inhibition du SYK a diminué les niveaux de protéines Mcl-1 dans les cellules B primaire, et que l'activation de ces cellules a été inhibée, tel que déterminé par l'expression de CD80 et des taux inférieurs de sécrétion d'IgG dans les cellules B primaires activées in vitro. Nos travaux suggèrent que la voie SYK-Mcl-1 peut offrir de nouvelles

Title: Regulating the activation of B lymphocytes during chronic antibody mediated rejection: The role of SYK in modulating Mcl-1

Keywords: Antibody mediated rejection, Germinal Center, BCR signaling, SYK, Mcl-1, Apoptosis

Abstract: Renal failure is a major public health concern and renal transplantation is the main therapeutic option, however it comes with the risk of organ rejection. B-cells play an important role in antibody-mediated rejection (AMR). During chronic AMR, tertiary lymphoid germinal center (GC)-like structures appear in the rejected organ, associated with de novo production of donor-specific plasma and memory B-cells. Which are B-cell populations that are often poorly controlled by current treatments. Myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic member of the B-cell lymphoma-2 (Bcl-2) family, is essential for maintaining the GC reaction and B-cell differentiation. We report here the infiltration of B-cells expressing Mcl-1 in the kidney of patients with chronic AMR, as observed for (pre-)GC cells. The impairment of B-cell receptor (BCR) signaling, by inhibition of spleen tyrosine kinase (SYK), reduced viability and Mcl-1 protein levels in GC like cells. This downregulation is coordinated at the transcriptional level, potentially via signal transducer and activator of transcription 3 (STAT3), as shown by (1) impaired translocation of STAT3 to the nucleus following SYK inhibition, and (2) the lower levels of Mcl-1 transcription upon STAT3 inhibition. Moreover, overexpression of Mcl-1 prevented cells from entering apoptosis after SYK inhibition. In vitro studies with primary tonsillar B-cells confirmed that SYK inhibition decreased cell survival. We also found that SYK inhibition decreased Mcl-1 protein levels in primary B-cells, and that B-cell activation was inhibited, as determined by CD80 expression and lower levels of IgG secretion in tonsillar B-cells activated in vitro. Overall, our data suggest that the SYK-Mcl-1 pathway may provide new opportunities for the treatment and prevention of AMR.