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The role of IDO induced tryptophan depletion and kynurenine accumulation in inhibition of translation initiation and apoptotic decline in activated human T-cells

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Science cannot solve the ultimate mystery of nature. And that is because, in the last analysis, we ourselves are part of nature and therefore part of the mystery that we are trying to solve

Max Planck, 1932

My family

and

Lukas

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is responsible for the catalysis of tryptophan to tryptophan metabolites, which triggers a depletion of this essential amino acid and kynurenine accumulation in the microenvironment of T-cells.

Tryptophan depletion through IDO was suggested as a molecular mechanism of IDO mediated immunosuppression and was also shown to activate the integrated stress response (ISR) via the GCN2 pathway in mice. This thesis aims to explore whether the effects of IDO on the GCN2 pathway are valid also in primary human T-cells, and whether the activation of the GCN2 pathway is associated with the impaired proliferative capacity in primary human T-cells upon sensing tryptophan deprivation.

Our findings first confirmed that tryptophan deprivation accounts for the inhibition of proliferation in human T-lymphocytes. Although human primary T-cells are able to respond to an activation stimulus by highly expressed CD25 cell surface molecules, proliferation is inhibited. T-cell inhibition associated with tryptophan deprivation was accompanied by an increased susceptibility to apoptosis.

Furthermore our data clearly indicated that, in contrast to the murine system, the integrated stress response was not induced upon tryptophan deprivation in primary human T-cells. Moreover, autophagy, an alternative stress related pathway, was not induced upon tryptophan withdrawal in the primary human system. Yet, the mammalian target of rapamycin (mTOR) pathway was inducible upon exposing primary human T-lymphocytes to enforced stress (tryptophan exhaustion, prolonged cell culture).

In conclusion, in primary human T-cells, the initiation of the ISR and autophagy pathway apparently do not contribute to the cellular adaption to tryptophan deprivation. However, the mTOR pathway and increased apoptotic susceptibility may account for the T-cell inhibitory effects evoked by IDO activity.

KURZFASSUNG

Indoleamine 2,3-dioxygenase (IDO) katalysiert die Umsetzung von Tryptophan zu Tryptophanmetaboliten. Dieser Prozess führt zur Erschöpfung dieser essentiellen Aminosäure in der direkten Umgebung der T-Zellen.

In Mäusen wurde gezeigt, dass Tryptophanabbau durch IDO den „Integrated Stress Response“ auslöst und somit als molekularer Mechanismus für die IDO vermittelte Immunsuppression fungiert.

Ziel dieser Arbeit war, die Effekte von IDO im besonderen Bezug auf den GCN2 Signalweg zu erforschen und infolgedessen auch den Einfluss von Tryptophanmangel resultierend aus der Aktivierung des GCN2 Signalweges auf die Proliferation von humanen T-Zellen zu ergründen.

Unsere Resultate haben bestätigt, dass die Ursache für eine inhibierte T-Zellproliferation im humanen System durch Tryptophanmangel hervorgerufen wird. Obwohl primäre humane T-Zellen durch hohe CD25 Zelloberflächen Expression im Stande sind, auf Aktivierung zu reagieren, sind sie in ihrer Proliferation gehemmt. Neben dieser Inhibierung durch Tryptophanmangel wurde zusätzlich noch ein erhöhter Anteil an apoptotischen Zellen verzeichnet.

Weiters verdeutlichten unsere Daten, dass der „Integrated Stress Response“, im Vergleich zum murinen System, in primären humanen T-Zellen nicht durch Tryptophanmangel hervorgerufen wird.

Darüber hinaus wurde Autophagie, ein alternativer, durch Stress induzierter Signalweg, nicht durch Tryptophanentzug induziert.

Dahingegen wurde der mTOR Signalweg durch erhöhten Stress, wie zum Beispiel Tryptophanmangel und längere Kultivierung im Medium, in primären humanen T-Lymphocyten aktiviert

Zusammenfassend haben die Ergebnisse dieses Projektes gezeigt, dass der „Integrated Stress Response“ und Autophagie Signalweg in primären humanen T-Zellen offenbar keine Rolle in Bezug auf die zelluläre Antwort, bedingt durch Tryptophanmangel, spielt. Nichtsdestotrotz könnten der mTOR Signalweg und erhöhte Apoptose, hervorgerufen durch IDO spezifischen Tryptophanentzug, eine wichtige Bedeutung hinsichtlich der T-Zellinhibierung darstellen.

TABLE OF CONTENTS

A - Introduction	- 9 -
1 Immune System	- 9 -
1.1 Innate Immunity	- 9 -
1.1.1 Dendritic Cells	- 10 -
1.2 Adaptive Immunity	- 12 -
1.2.1 T-cells	- 12 -
2 Hematopoietic Stem Cell Transplantation	- 16 -
2.1 Hematopoietic Stem Cells	- 16 -
2.1.1 Bone Marrow derived Stem Cells	- 17 -
2.1.2 Peripheral Blood Stem Cells	- 17 -
2.1.3 Umbilical Cord Blood Stem Cells	- 18 -
2.2 Transplantation process	- 18 -
2.3 Graft-versus-Host Disease	- 19 -
2.3.1 Genetic impact factors for the development of GvHD	- 20 -
2.3.2 Non-HLA dependent factors for GvHD	- 21 -
2.3.3 Course of acute GvHD	- 21 -
2.3.4 Symptoms of acute GvHD	- 23 -
2.4 Cells involved in GvHD	- 23 -
2.4.1 APCs	- 23 -
2.4.2 T-cell populations	- 25 -
2.4.3 NK-cell population	- 25 -
2.4.4 B-cell population	- 25 -
3 Tolerance	- 27 -
3.1 Central Tolerance	- 27 -
3.2 Peripheral Tolerance	- 28 -
4 Indoleamine 2,3-dioxygenase	- 30 -
4.1 Theories about IDO	- 32 -
4.1.1 Tryptophan Starvation Theory	- 32 -
4.1.2 Tryptophan Metabolite Theory	- 32 -
4.2 Tryptophan Degradation Pathway	- 33 -

4.3	IDO and Tolerance	- 33 -
5	Translation Process	- 36 -
5.1	Ribosome Structure	- 36 -
5.2	Translational Mechanism	- 36 -
5.2.1	Translation Initiation	- 36 -
5.2.2	Elongation	- 38 -
5.2.3	Termination	- 39 -
5.3	Translation Inhibition.....	- 39 -
6	The ISR and their main players	- 40 -
6.1	General Control Non-derepressible 2	- 42 -
6.2	Eukaryotic initiation factor 2.....	- 43 -
6.2.1	Biological function of eIF2	- 45 -
6.3	Activating Transcription Factor 4	- 45 -
6.4	C/EBP Homologous Protein	- 48 -
7	Alternative Pathways for the Adaption to Nutrient Deprivation	- 50 -
7.1	Autophagy	- 50 -
7.2	PI3K/mTOR Pathway.....	- 52 -
B	Hypothesis	54
C	Materials and Methods	- 55 -
1	Isolation and purification of human peripheral blood mononuclear cells with subsequent enrichment of T-lymphocytes	- 55 -
1.1	Isolation and purification of human PBMCs	- 55 -
1.2	Enrichment of T-lymphocytes via MACS (Magnetic Cell Separation)	- 56 -
1.3	CFSE Labeling.....	- 57 -
1.4	T-cell Culture Conditions	- 58 -
2	Isolation and purification of murine splenocytes	- 59 -
2.1	Enrichment of murine T-cells via MACS Separation	- 59 -
3	Sample Preparation	- 60 -
3.1	Protein Samples	- 60 -
3.2	RNA Samples	- 60 -
3.2.1	RNA Isolation	- 60 -

3.2.2	Reverse Transcription.....	- 60 -
3.3	Flow Cytometric Samples.....	- 61 -
4	Analyses Methods.....	- 62 -
4.1	Protein Analyses.....	- 62 -
4.1.1	SDS PAGE	- 62 -
4.1.2	Western Blotting.....	- 62 -
4.2	RNA Analyses	- 64 -
4.2.1	Real Time PCR.....	- 64 -
4.3	Flow Cytometric Analyses	- 64 -
D -	Results	- 65 -
1	The Integrated Stress Response and Autophagy Induction in the murine system .	- 65 -
1.1	Optimal culture conditions for murine splenocytes	- 65 -
1.2	Initiation of the integrated stress response in murine splenocytes upon tryptophan starvation.....	- 67 -
1.3	Assessment of autophagy in murine splenocytes upon tryptophan starvation .	- 69 -
2	The Integrated Stress Response and Autophagy Induction in the Jurkat T-lymphocyte cell line	- 70 -
2.1	Characterization of Jurkat T-lymphocytes	- 70 -
2.2	Initiation of the integrated stress response in human Jurkat T-lymphocytes	- 72 -
2.3	Autophagy in Jurkat T-lymphocytes upon TRP deprivation	- 76 -
3	The Integrated Stress Response and alternative pathways for the adaption to nutrient deprivation in primary human T-lymphocytes	- 77 -
3.1.1	Apoptotic response of primary human T-lymphocytes to TG treatment	- 77 -
3.2	Effects of TRP deprivation on the proliferative response of primary human T-lymphocytes	- 78 -
3.3	Induction of the ISR in primary human T-lymphocytes	- 83 -
3.4	Alternative pathways for the adaption to nutrient deprivation in primary human T-lymphocytes.....	- 93 -
3.4.1	Induction of autophagy in primary human T-lymphocytes.....	- 93 -
3.4.2	Initiation of the mTOR pathway in primary human T-lymphocytes (Exp. 38)-	95
	-	

3.5 Summary	- 99 -
E - Discussion and conclusion	- 101 -
F - Index of Figures and tables	- 109 -
1 Table of Figures.....	- 132 -
2 List of Tables	- 136 -
G - List of abbreviations.....	- 137 -
H - Bibliography.....	- 109 -

A - INTRODUCTION

1 Immune System

1.1 Innate Immunity

The innate immune system is the most ancient defense mechanism of the human body, comprising germ-line encoded receptors for the recognition of microorganisms. ^[5]

In the course of pathogen invasion through the skin, respiratory or gastrointestinal tract, pathogen associated molecular patterns of bacteria, viruses, protozoa and fungi, so called PAMPS (e.g. cell wall components of bacteria) are recognized by pattern recognition receptors (PRR) on immune cells. ^[6]

This recognition is achieved by specialized PRRs, namely Toll-like receptors (TLRs), which were originally found and first described in *Drosophila* as the Toll gene. Later a homologue of it was discovered in the mammalian organism. ^[7] Toll-like receptors belong to type I transmembrane receptors comprising an extracellular leucine-rich motif, being responsible for the ligand binding, and an intracellular Toll/interleukin IL-1 receptor (TIR) domain, mediating the intracellular signaling. ^[8] So far 13 different mammalian TLRs are known, with TLR-4 being the first one discovered, playing a pivotal role in binding of the bacterial cell wall component lipopolysaccharide (LPS). ^[9]

TLRs are expressed on immune cells like macrophages and dendritic cells (DCs), but can also be found on various tissues like endothelial, epithelial and muscle cells. ^[10]

During an innate immune response, the first line defense comprises immune cells like macrophages, neutrophils and natural killer (NK) cells. Macrophages are able to phagocytose invading pathogens, thereby releasing inflammatory cytokines and chemokines, which lead to the permeabilization and enhanced expression of adhesion markers on vascular vessels for the recruitment of circulating leukocytes. ^[11]

Neutrophils, which constitute around 70% of peripheral blood leukocytes, carry toxic metabolites, antimicrobial proteins and proteolytic enzymes, to override invading pathogens. ^[12]

NK cells are participating in intrinsic killing via intracellular perforin and the extracellular surface molecule FasL. ^[13]

A – INTRODUCTION

If a PAMP binds to a Toll-like receptor, adaptor proteins are recruited to the cell membrane to associate with the intracellular domains of the TLR, finally leading to the activation of transcription factors (e.g. NF κ B), which control and adapt gene expression (Fig. 1).^[5]

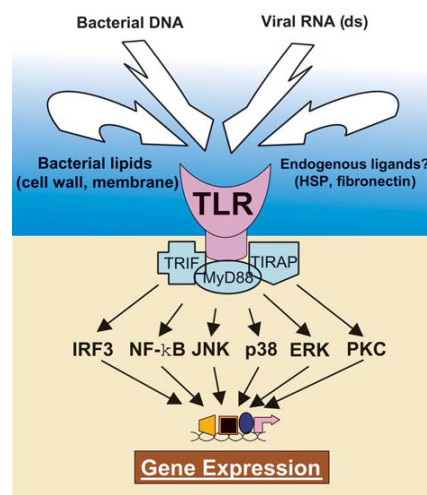


Fig. 1: Recognition of PAMPs through TLRs and their downstream signaling in mammalian cells [Dempsey et al., 2003]^[5]

The upregulation of respective genes lead to various cellular responses like the production of cytokines and chemokines, which induces cell trafficking and DC maturation by upregulation of MHC complexes and costimulatory molecules, which finally lead to the activation of T-cells.^[5]

1.1.1 Dendritic Cells

Dendritic cells, as afore mentioned, are mediators between the innate and the adaptive immunity. Dendritic cells migrate from the bone marrow *via* the blood stream to peripheral organs (Fig. 2). They are able to take up macromolecules and soluble antigens *via* different ways of engulfment, namely receptor mediated endocytosis, which includes the use of coated pits, and phagocytosis, which is accomplished by membrane ruffling through actin reorganization, which leads to the formation of large intracellular vacuoles.^[14, 15]

Maturation of dendritic cells can occur through various receptor signals: (i) Toll-like receptors on the cell surface, (ii) cytokine receptors, (iii) Fc receptors and (iv) cell death receptors.^[14]

Toll-like receptor mediated maturation plays a pivotal role during infections, meaning that a dendritic cell is matured when it recognizes bacterial cell wall components through their TLRs. [16]

Some critical molecules leading to the maturation of dendritic cells include $\text{TNF-}\alpha$, $\text{IL1-}\beta$ and PGE-2 , which are especially excreted when the body encounters a bacterial infection. [17]

Two factors, influencing dendritic cell maturation, are Fc receptors on dendritic cell surfaces, which can recognize immune complexes of antibodies and cell death receptors, leading to maturation through a danger signal of necrotic cells. [18-20]

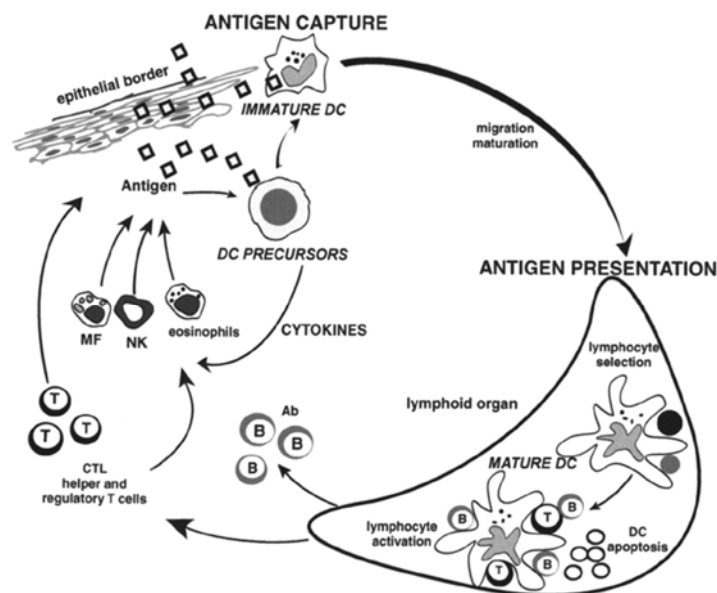


Fig. 2: Life cycle of a dendritic cell [Banchereau *et al.*, 2000]^[17]

A – INTRODUCTION

1.2 Adaptive Immunity

Dendritic cells are kept in an immature state upon challenge with a pathogen, which leads to the maturation and concurrent expression of costimulatory molecules on the surface, inducing the migration to a draining lymph node. There, the dendritic cell primes the naïve T-cells through association *via* the immunological synapse (Fig. 3), comprising two main ligands: (i) T-cell receptor - MHC interaction concurrently with CD4/CD8 molecules, (ii) and costimulatory molecules CD28-CD80/CD86 interaction. Finally, this leads to the expansion and proliferation of T-cells, which accounts for the adaptive immune response. [5]

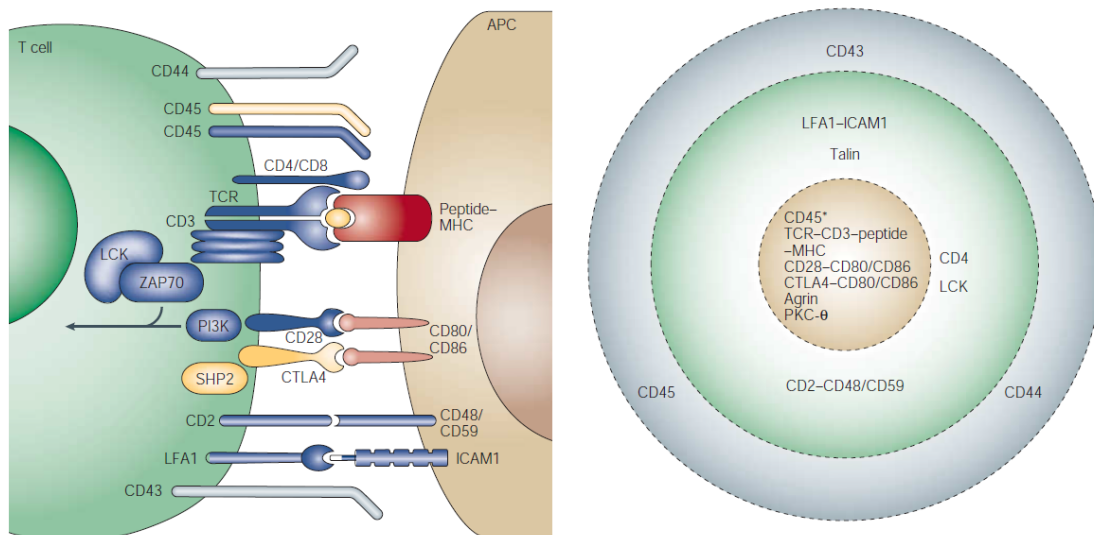


Fig. 3: Formation of the immunological synapse between an APC and a T-lymphocyte depicting the main interacting ligands [Huppa and Davis, 2003]^[21]

1.2.1 T-cells

Priming of T-cells in the lymphnode through a dendritic cell, which presents the appropriate antigen in the compatible MHC complex, is achieved through three distinct signals (Fig. 4).^[22]

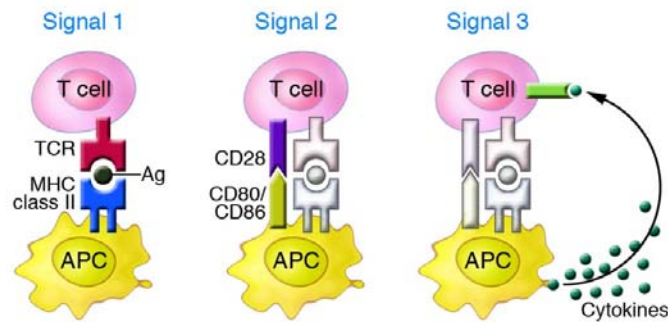


Fig. 4: Three distinct signals in T-cell priming [Gutcher and Becher, 2007]^[22]

Signal 1 represents the binding of the T-cell receptor to the matched MHC complex with the presented antigen epitope on the antigen presenting cell (APC). However, this signal does not fulfill the requirements for the activation of a T-cell, since two other crucial signals are needed for full activation.

Signal 2 is characterized by the association of several costimulation molecules, amongst others CD80 (B7.1) and CD86 (B7.2) on the dendritic cell and CD28 on the T-cell. This joining, which is called costimulation, markedly affects clonal T-cell proliferation. Another costimulation molecule affecting the priming of T-cells is CTLA-4 (CD152), which as well joins up with CD80/86 on dendritic cells, but acts as inhibitory signal to the T-lymphocytes.

Signal 3, required for full T-cell priming, is the cytokine excretion. It has been shown that IL-12 and IL-18 are key players during the differentiation of T-cells into a Th1 phenotype, whereas expression of IL-4 by T-cells favors the differentiation into a Th2 phenotype (Fig. 5).^[23, 24]

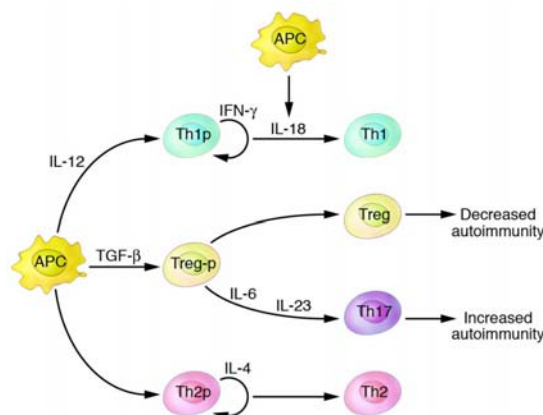


Fig. 5: T-cell polarization through various effector cytokines [Gutcher and Becher, 2007]^[22]

A – INTRODUCTION

1.2.1.1 CD4 T-cells

In 1986, two distinct subsets of CD4 T-cells have been originally described by Mosmann and Coffman. [25] Generally, CD4 T-cells play a pivotal role during infections in shaping immune reactions. These cells exert the ability of activating macrophages, aid B-cells for producing antibodies and recruit eosinophiles, neutrophils and basophiles to the site of infection.

Up to now at least 4 different T-cell subsets are known, each possessing different effector functions (Fig. 6). [25, 26]

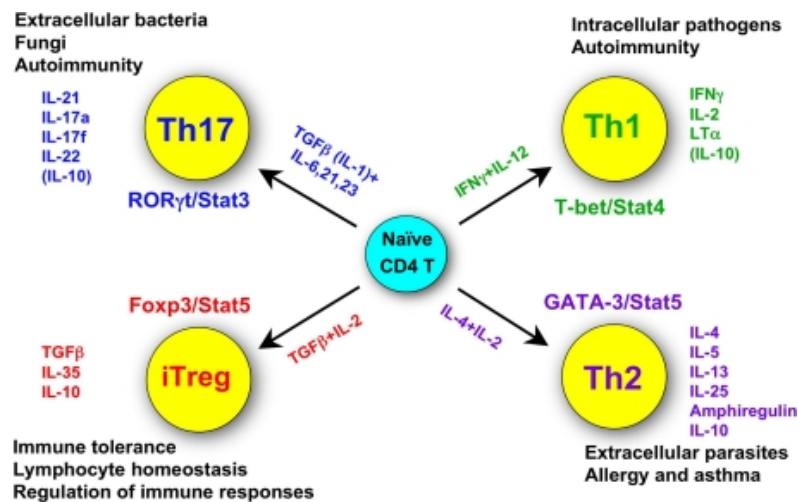


Fig. 6: CD4 T-cell subsets with expressed cytokine profiles and their immunologic effects [Zhu and Paul, 2008]^[26]

Th1 subset

The Th1 T-cell subset plays a critical role during the immune reaction against intracellular pathogens like mycobacteria, but has also been shown to induce various kinds of autoimmune diseases. [27, 28] Their typical cytokine profile comprises IFN- γ , lymphotoxin α (LT α) and IL-2, which leads to the activation of macrophages and T-cell memory. [29, 30]

Th2 subset

Th2 T-cells are vitally important for the immunological defense against extracellular pathogens, but have also been suggested to contribute to asthmatic reactions and allergic diseases. [27, 28] Cytokines produced by Th2 cells include IL-4, IL-5, IL-9, IL-10, IL-13, IL-25

and amphiregulin, which possess a major role in class switching of B-cells and Th2 differentiation. [31-33]

Th17 subset

The Th17 T-cell subset was shown to be involved in the body's defense against extracellular bacteria and fungi, but also in the development of organ specific autoimmune diseases. [34] Cytokines expressed by Th17 cells include IL-17a, IL-17f, IL-21 and IL-22, which have been shown to be involved in the induction of inflammatory processes, activating neutrophils, CD8 T-cells, B-cells, natural killer cells and dendritic cells. [35, 36]

T_{reg} subset

Regulatory T-cells, as afore mentioned, play a pivotal role during the induction of self-tolerance and prevention of graft rejection *via* mediating immunosuppressive effects and are defined by the expression of the cell surface markers CD4 and CD25 and the intracellular transcription factor Foxp3. [37] T_{regs} are able to express TGF- β , IL-10 and IL-35, which act on the differentiation of CD4 T-cells into iT_{regs}, and suppression of inflammatory diseases. [26]

1.2.1.2 CD8⁺ T-cells

Cytotoxic CD8⁺ T-cells are central mediators in the defense of intracellular pathogens. CD8⁺ T-lymphocytes are able to recognize antigens, which are presented in an MHC-I dependent context. Cytotoxic T-lymphocytes are able to directly kill target cells by cytolysis through perforin and the cell surface receptor Fas, but were also shown to express a range of inflammatory cytokines such as TNF and IFN- γ . Additionally, CD8⁺ T-cells are as well able to secrete chemokines, which act as an attractant for inflammatory cells. [38, 39]

A – INTRODUCTION

2 Hematopoietic Stem Cell Transplantation

Over fifty years ago, in the year 1959, the first successful hematopoietic stem cell transplantation (HSCT) was performed (Fig. 7).^[40] To date more than 25 000 HSCTs per year are conducted all over the world to treat diseases like lymphomas, leukemias, immune-deficiency illnesses, congenital metabolic defects, hemoglobinopathies and myelodysplastic and myeloproliferative syndroms.^[41]

Originally, HSCT was applied for two main reasons: to treat patients with inherited anaemias or immune deficiencies with high myeloablative chemotherapeutic or radiation doses^[40]

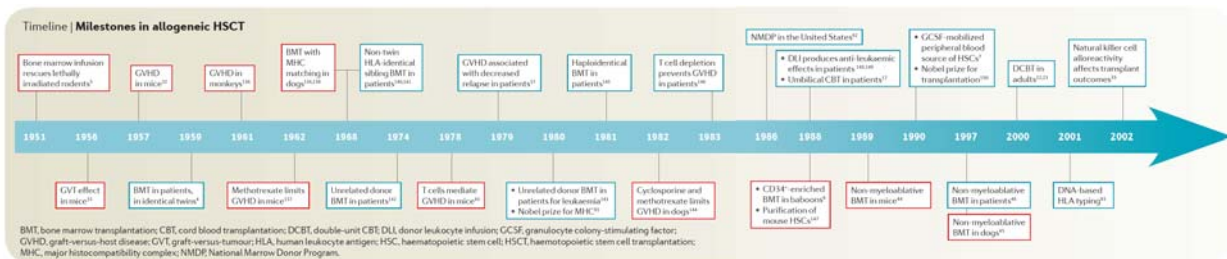


Fig. 7: Timeline of hematopoietic stem cell transplantation innovations over more than 50 years [Jenq and van den Brink, 2010]^[40]

In the beginning of exploring the features of HSCT, infused bone marrow was found to rescue rodents after receiving lethal doses of radiation.^[42]

2.1 Hematopoietic Stem Cells

Hematopoietic stem cells (HSC) are capable of the ability of indefinite self renewal and differentiation into all mature blood lineages.^[43, 44]

So far, hematopoietic stem cells are defined by the cell surface marker CD34 and can be derived from peripheral blood, bone marrow or umbilical cord blood.^[45] The selection of the stem cell source for HSCT, which can derive from autologous, syngeneic or allogeneic donors, is influenced by various factors like donor availability or transplantation indication.^[41] The stem cell graft can be derived from different types of donors. HSC donors can be grouped into 3 classes: (i) matched related donors, represented by siblings, (ii) matched

unrelated donors, constituted by MHC matched foreign donors and (iii) haploidentical donors, represented by parents. ^[46]

2.1.1 Bone Marrow derived Stem Cells

Bone marrow is usually harvested from the posterior iliac crest, filtered and afterwards either directly infused into the recipient or stored at 4°C up to 24h without cell loss, which makes the intercontinental transfer easy and applicable. Usually a cell dose of 2×10^8 cells/kg body weight is infused in the patient, meaning at least 700-1500 ml bone marrow have to be harvested. ^[41]

2.1.2 Peripheral Blood Stem Cells

The second source of hematopoietic stem cells is peripheral blood. The method of harvesting peripheral blood stem cells is much easier, less inconvenient and leads to a faster recovery of neutrophils and platelets after transfusion (Fig. 8). ^[47, 48] Since peripheral blood stem cells are naturally present in low numbers in the peripheral blood, donors are pretreated with granulocyte-colony stimulating factor (G-CSF), which triggers the proliferation and accumulation of the stem cells in the peripheral blood. ^[41]

Unfortunately the administration of G-CSF can elicit some adverse side effects like bone pain, headache, fatigue and nausea. ^[49] Another severe disadvantage of using peripheral blood stem cells is that patients may evolve a slightly higher risk of developing a graft-versus-host disease (GvHD; see section 2.3). ^[50, 51]

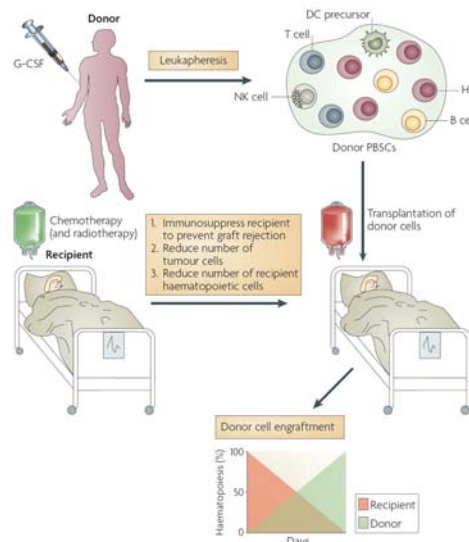


Fig. 8: Peripheral blood hematopoietic stem cell transplantation procedure [Shlomchik, 2007] ^[52]

A – INTRODUCTION

2.1.3 Umbilical Cord Blood Stem Cells

The transplantation of stem cells from umbilical cord blood is a safe and easy available procedure, which alleviates the risk of carryover viruses and developing severe side effect like GvHD due to the higher content of immature and naïve cells. ^[53] A match of 3-4 of the 6 HLA-A, HLA-B and HLA-DRB1 available antigens is acceptable with this kind of transplantation method. Due to the fact, that a lower quantity of cells is present in umbilical cord blood, the patient's immune system needs more time for recovery and regeneration, which in turn increases the risk of acquiring infections. ^[54]

2.2 Transplantation process

The transplantation process of a HSCT actually starts one week before (day -7 to day 0) transfusion of the stem cells. In this week, the patient is undergoing a conditioning regimen, meaning he is receiving high dose chemotherapy (cyclophosphamide is widely used) and often additionally radiotherapy (total body irradiation) to make sure that the preceding malignant disease is completely eradicated. ^[55] These harsh regimen doses destroy the recipient's immune system, which elicits on the one hand negative effects since the patient's body is not able to react to infections, but on the other hand evokes positive effects due to the fact, that cancer cells are eradicated and the patient's immune system as well can't initiate an immunological reaction against the infused donor stem cells. Unfortunately patients can develop side effects upon the administered conditioning regimen like oropharyngeal mucositis (Fig. 9) ^[56]



Fig. 9: Indications for oropharyngeal mucositis after conditioning regimen [Léger and Nevill, 2004] ^[56]

On day 0 of HSCT the donor stem cells are transfused into the patient. From this timepoint on, the patient is susceptible to bacterial, viral and fungal infections, especially due to

breaks in the natural mucosa and skin barriers, since the patient's immune system was destroyed and the new donor immune system isn't fully reconstituted yet (Fig. 10). This susceptibility to infections and the risk of developing a Graft-versus-Host Disease ranges from day 0 of transplantation up to day 180 post-transplantation. ^[56]

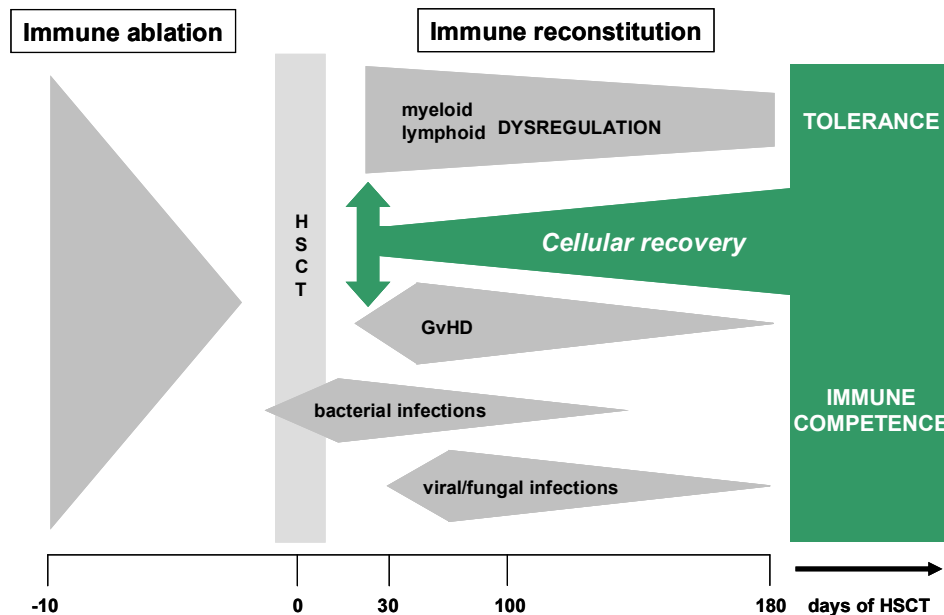


Fig. 10: Immune reconstitution after HSCT
[kindly provided by Birgit Jürgens, PhD]

2.3 Graft-versus-Host Disease

Billingham defined the requirements for developing GvHD in the year 1966 by following statements: (i) there have to be a sufficient number of immunologically competent cells in the graft; (ii) the host has to hold major antigens, which are missing at the graft's side; (iii) the host's immune repertoire has to be in a aggrieved condition, so that it is not able to initiate a immune response against graft cells. ^[57]

GvHD can be divided into two subgroups: (i) acute GvHD (aGvHD), which emerges within 100 days post-transplantation, and (ii) chronic GvHD (cGvHD) after 100 days post-transplantation. ^[56] The severity of GvHD can also be classified into different grades, namely clinically insignificant with the grades 0 and I and clinically significant with the grades II to IV. ^[41]

A – INTRODUCTION

Despite better HLA-typing procedures and immune suppression, Graft-versus-Host Disease (GvHD), which is frequently linked with high morbidity and mortality, remains a major problem after HSCTs. The risk of developing an acute GvHD after HSCT is about 40%, but can range from 10-80%, depending on various risk factors (see sections 2.3.1-0). [41]

2.3.1 Genetic impact factors for the development of GvHD

The most prominent factor influencing the development of GvHD is the Human Leukocyte Antigen (HLA), which is located on the short arm of chromosome 6 and presented by the Major Histocompatibility Complex (MHC), which plays a pivotal role in the humoral and cell-mediated immune response (Fig. 11).

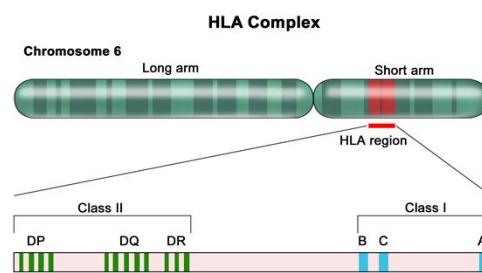


Fig. 11: Chromosome 6 with encoded HLA I and II complexes [Winslow, 2012]^[58]

The class I cell surface molecules (HLA-A, B- and C) are expressed on all nucleated cells in the human body, whereas the majority of class II molecules (DR, DQ and DP) are expressed on cells of the immune system and in particular found in the skin and the GI tract in high numbers. ^[59]

Another factor which influences the prediction of GvHD after HSCT is the Minor Histocompatibility Antigen (miHA). miHA are small peptides processed from intracellular proteins, which can vary due to genetic polymorphisms (Fig. 12). This variation affects the outcome of HSCT even between MHC matched siblings. ^[60, 61]

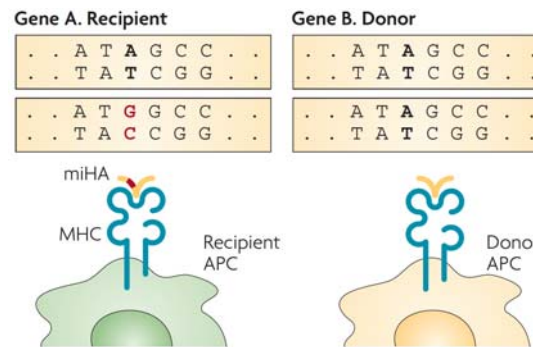


Fig. 12: Recognition of the miHA by recipient and donor APCs [Shlomchik, 2007] ^[52]

2.3.2 Non-HLA dependent factors for GvHD

Cytokine gene polymorphism was suggested to be one non-genetic mechanism contributing to the development of GvHD. So far, various kinds of cytokines are suggested to enhance the symptoms of GvHD. *Inter alia*, gene polymorphisms in TNF- α , IL-10, IL-6, IFN- γ , IL-1, TGF- β , IL-2, IL-13 and IL-4 are linked to an increased susceptibility for developing GvHD. ^[62]

Another non-HLA dependent factor affecting the progression of GvHD is the NOD2/CARD15 gene, which plays a central role in the innate immune response when responding to bacterial infections in the GI tract *via* the initiation of nuclear factor- κ B (NF- κ B). If this gene experiences a single nucleotide polymorphism (three are known up to now), NF- κ B levels are alleviated, and the risk for developing Crohn's disease and GvHD rises. ^[59, 63]

Other non-genetic risk factors for developing an acute GvHD are donor and recipient gender differences, alloimmunized donors (women, who gave birth to at least three children), increased age of the host, donor graft type, donor and host cytomegalovirus (CMV) status and preceding conditioning regimen. ^[41, 64, 65]

2.3.3 Course of acute GvHD

aGvHD can be divided into three phases: (i) the afferent phase, (ii) the induction and expansion phase (iii) and the effector phase (Fig. 13). ^[66] Due to preceding conditioning regimens, damages and thus activation of the intestinal flora take place, which enables bacterial lipopolysaccharides to transverse the intestinal lumen and manage to enter the

A – INTRODUCTION

circulation system during the afferent phase. These bacterial cell wall products thereby trigger the release of inflammatory cytokines (the so-called “cytokine-storm” like IL-1, TNF- α , IL-6 and IFN- γ) which lead to the activation of innate immune cells *via* Toll-like or NOD-like receptors. [40] These activated innate immune cells then in turn again secrete inflammatory cytokines which initiate the enhanced expression of MHC, co-stimulatory and adhesion molecules. [40, 67-69] This upregulation of APC cell surface molecules induces the second phase of GvHD. Donor T-cells receive a proliferation and expansion stimulus *via* contact-dependent activation by APCs and indirectly *via* the effects of cytokines. Donor T-lymphocytes react by producing IL-2 and IFN- γ , which in turn activate other T-cells and natural killer cells and induce macrophages to produce TNF- α thereby injuring the GI tract and skin mucosal surfaces. [25, 70, 71]

In the third and last phase, cytotoxic T-lymphocytes initiate damage through Fas-Fas ligand interaction, perforin-granzyme combination and TNF- α expression. TNF- α in turn triggers the release of IL-1, IL-6, IL-10, IL-12 and again TNF- α . [72-74]

The use of T-cell depleted HSC transfusions was shown to reduce the risk of developing GvHD and abrogates the use of immunosuppressive agents, but increases the risk of a malignant relapse. [75, 76]

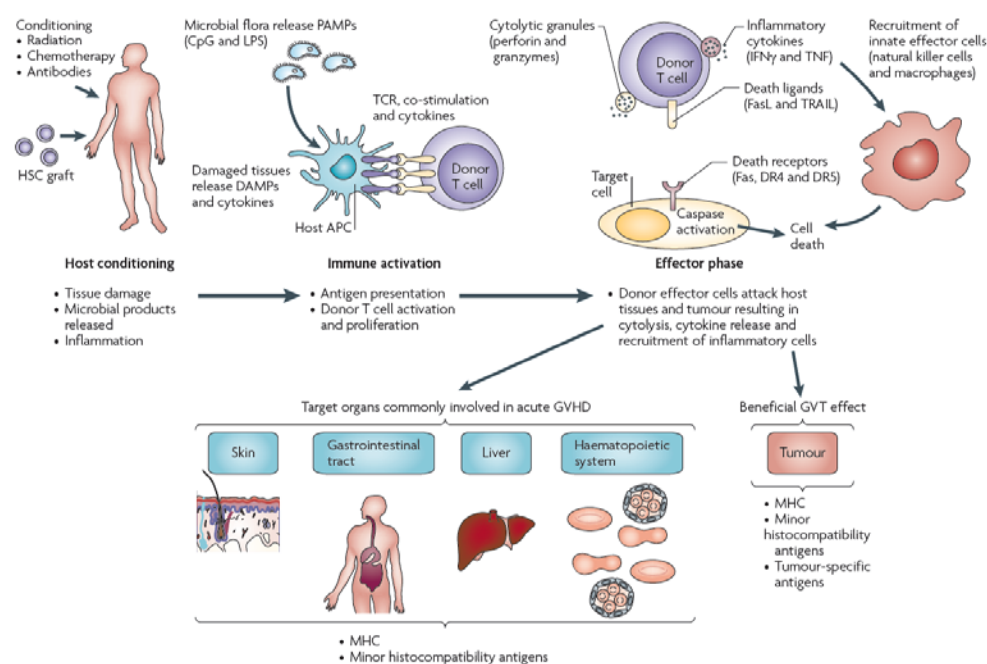


Fig. 13: Course of acute GvHD with interacting immune cells and affected body damage sites [Jenq and van den Brink, 2010] [40]

2.3.4 Symptoms of acute GvHD

Acute GvHD primarily affects the skin, upper and lower GI tract and the liver, but can sometimes also manifest as eye and oral mucosal damage. The classical symptom of acute skin GvHD is a skin rash, which covers hand palms, foot soles and later distributes over the face, neck, upper chest and trunk. In very severe forms the progress could lead to erythroderma including bullae formation and desquamation (Fig. 14).



Fig. 14: Erythroderma formation in stage IV aGvHD [Ball and Egeler, 2008]^[59]

During an acute liver GvHD, increased levels of alkaline phosphatase and an isolated bilirubinemia could be observed.

In an acute GI GvHD, diarrhea, anorexia and nausea emerge. In later stages abdominal pain, GI bleeding and ileus arise as prevalent syndroms.^[59]

2.4 Cells involved in GvHD

2.4.1 APCs

Antigen presenting cells (APCs) constitute a major cell population in the context of developing aGvHD. Professional APCs, especially dendritic cells (DCs), possess the ability of digesting proteins and loading peptide antigens on MHC complexes, transferring them to the cell surface and presenting them in combination with costimulatory and leukocyte antigen molecules *via* formation of the immunological synapse to T-cells.^[17] Two possible pathways for loading dendritic cells exist: the endogenous and the exogenous pathway. In the endogenous loading pathway, intracellular proteins are degraded, passed on to the endoplasmic reticulum where they are loaded on MHC I molecules. During the exogenous

A – INTRODUCTION

loading pathway, antigens are phagocytosed, hydrolyzed by peptidases and then loaded onto MHC II molecules. [77] APCs possess the ability of presenting extracellular antigens in an MHC I context. This process is called crosspresentation and vitally important for the development of immunity to infectious agents or tumors. [78]

In other words, in an MHC matched setting, proteins from the cytosol are degraded by a recipient APC, loaded onto a MHC I system and finally recognized by a donor CD8⁺ T-cells as foreign antigen. When an antigen is exogenously uptaken *via* endocytosis by a recipient or donor APC, it is processed and presented in the context of MHC class II, which is able to activate CD4⁺ donor T-lymphocytes (Fig. 15).

Concerning the situation after a HSCT this means that directly after transplantation host alloantigens are directly presented by host APCs or presented by donor APCs to donor T-cells.

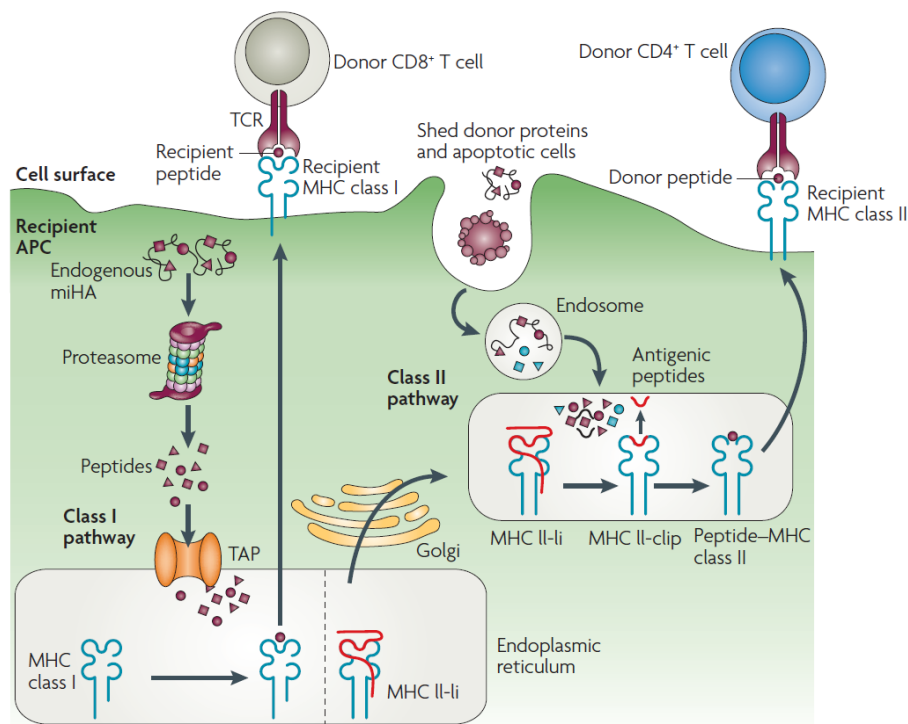


Fig. 15: MHC class I and II presentation by recipient APCs to donor CD8⁺ and CD4⁺ T-lymphocytes [Shlomchik, 2007]^[52]

2.4.2 T-cell populations

Overall, particularly CD8⁺ T-cells, upon interplay with APCs, is the central effector cell population in GvHD. [79, 80] CD4⁺ T-cells were also shown to play an essential role in the expansion of CD8⁺ T-lymphocytes, but recently were also suggested to initiate GvHD after depletion of CD8⁺ T-cells. [81]

Regulatory T-cells (T_{regs}) were shown to play a vital role in the development of GvHD. Regulatory cells were shown to prevent GvHD when infused into mice. [82] In line with these observations is, that if CD25⁺ T-cells are depleted from the graft, development of acute and chronic GvHD is enhanced. [83]

Another inhibitory cell population is the Natural Killer T-cell (NKT), which expresses NK- and T-cell surface markers. Increased numbers of these cells can be found in peripheral blood stem cell apheresis products, when donors were pretreated with G-CSF, and show a decreased potential to develop GvHD when infused. [84, 85]

2.4.3 NK-cell population

Natural killer (NK) cells play a pivotal role during immune responses. This cell population is able to distinguish healthy, leaving them unaffected, *versus* diseased and allogeneic cells, initiating lysis for removing them from the system. NK cells possess the ability to express cell surface receptors, so called killer-immunoglobulin receptors (KIRs), which predicts the alloreactivity e.g. after a stem cell transplantation. Published data state that GvHD *inter alia* evolves, if the recipient is lacking killer-immunoglobulin receptor ligands, leading to the lysis of allogeneic donor cells. [86]

2.4.4 B-cell population

The specific role of B-cells, central players in the immune system and as well involved in the development of aGvHD, is still not completely unraveled. On the one hand, B-lymphocytes are able to induce antibody-dependent complement activation and cell-mediated cytotoxicity. Moreover, B-cells secrete proinflammatory cytokines like IL-2, TNF- α , IL-6, IL-12 and IFN- γ , which trigger the activation of T-cells, NK-cells and macrophages. A decreased incidence of developing GvHD was shown upon B-cell

A – INTRODUCTION

depletion in mice. ^[87] These observations are consistent with published results in humans which show that high numbers of B-lymphocytes in the donor apheresis graft result in an increased incidence of developing aGvHD. ^[88] However, on the other hand B-cells are also able to exert immunoregulatory effects, for example by IL-10 production. ^[89]

3 Tolerance

Tolerance, which is defined as the unresponsiveness to (self-) antigens, can be classified into two categories: (i) the central tolerance induction and (ii) the peripheral tolerance induction. [90, 91]

3.1 Central Tolerance

Central tolerance takes place in the central primary lymphoid organ, the thymus. The thymus consists of three distinct areas: (i) the outer subcapsular zone, (ii) the cortex and (iii) the inner medulla. Generally spoken, the thymus represents the location, where naïve T-cells are matured by confronting them with self-antigens. [92]

In the subcapsular zone mostly completely immature T-lymphocytes, which recently migrated from the bone marrow to the thymus, can be found, expressing neither a CD4 nor CD8 cell surface marker. After the cells arrived in the subcapsular region, they migrate deeper into the thymus to enter the cortex area, where the actual selection and maturation procedure starts. CD4⁻ CD8⁻ T-lymphocytes start to rearrange their T-cell receptor (TCR) chains ($\alpha+\beta$) and begin to express both CD4 and CD8 cell surface markers. During this stage of maturation, specific epithelial cells start to express MHC class I and II molecules and present self-antigens in this context. If the semi-mature T-lymphocytes show a strong avidity to self-peptide, which means that they are potentially autoreactive, or are not even able to recognize self antigen at all, they are deleted by apoptotic mechanisms (Fig. 16). [93] However, if the semi-mature T-lymphocytes recognize the self-antigens with low, but measurable affinity, they are positively selected and differentiated (after travelling to the central region of the thymus) either into CD4⁺ CD8⁻, later on able to recognize antigens presented in an MHC class II context, or CD4⁻ CD8⁺ mature T-cells with the ability to recognize MHC class I molecules. After this positive selection, the mature T-cells travel to their operational area, the secondary lymphoid organs. [92, 94, 95]

A – INTRODUCTION

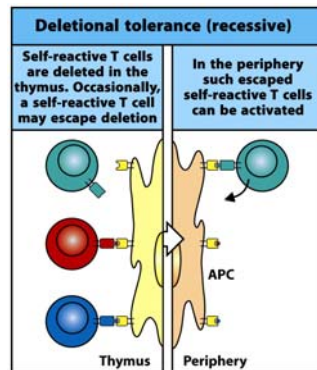


Fig. 16: Deletion of self reactive T-cells in the Thymus [Janeway's Immunobiology, 2008]^[96]

3.2 Peripheral Tolerance

Due to the fact that not all self-antigens can be produced by these specialized epithelial cells in the thymus, some T-cells escape this clonal selection process and would be able to induce an autoimmune reaction in the periphery. So far, there are three ways known, to inhibit any potential autoimmune reactivity of T-cells (Fig. 17).^[96]

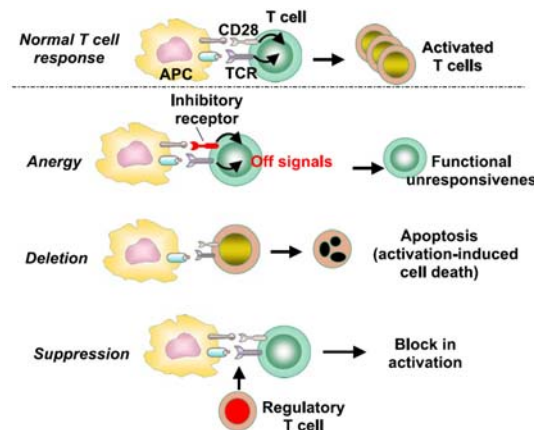


Fig. 17: Three ways of inhibiting the autoreactive action of T-lymphocytes [Abbas *et al.*, 2004]^[90]

One mechanism is that the autoreactive T-cell is silenced through a process called anergy. In this case, the T-cell forms an immunological synapse with an APC which lacks the additional costimulatory molecules on its surface. (Fig. 18) This leads to the shutdown of the cell rendering it unable, to react to any environmental signals, meaning to enter a stage of functional unresponsiveness. Anergy may also be induced upon expression of inhibitory molecules on the surface of T-cells. This inhibitory receptor (CTLA-4) interacts with the

CD80/86 molecule complex on the APC and drives the T-lymphocyte into an anergic state, unable to respond.^[90]

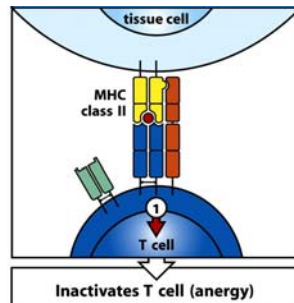


Fig. 18: Induction of anergy due to missing costimulatory molecules [Janeway's Immunobiology, 2008]^[96]

The second prominent way of inhibiting autoreactive T-cells is to induce apoptosis. If a T-lymphocyte was recently activated by an antigen and is shortly thereon restimulated by the same antigen, the T-cell starts to coexpress death receptors on its surface, namely Fas receptor (FasR or CD95) and Fas-ligand (FasL or CD95L). The interaction with these receptors leads to an intracellular activation of caspase cascades ultimately driving the cell into apoptosis.^[90]

The last alternative of inhibiting autoreactive T-cells is *via* the action of regulatory T-cells (T_{regs}). During the clonal selection process in the thymus, T_{regs} recognize self-antigen with low affinity, therefore neither be negatively deleted by too strong recognition nor simply positively selected. These regulatory T-cells then migrate to the periphery, where they encounter their self-antigens presented by an APC, inducing the secretion of anti-inflammatory cytokines as IL-10 and TGF- β , thus acting in an inhibitory fashion on self-reactive T-cells (Fig. 19).^[90, 97]

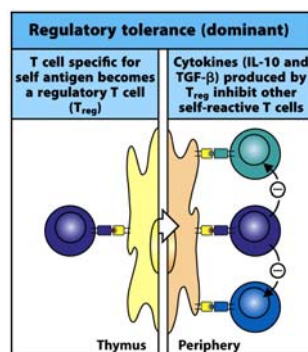


Fig. 19: Clonal selection of T_{regs} in the thymus and action of even those on self-reactive T-cells in the periphery [Janeway's Immunobiology, 2008]^[96]

4 Indoleamine 2,3-dioxygenase

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme (no secreted or extracellular form is known until now) which was first identified in rabbit intestines and attributed to contribute to the antimicrobial defense in the body. ^[98-100] The protein is encoded by one gene located on chromosome 8 with a size of around 15 kbp which comprises 10 exons. The gene is highly conserved amongst various species but was evolutionary adapted for different applications. ^[101] The promoter region of IDO comprises various sequence elements, *inter alia* sequences for responding to type I (α - and β) and more effectively type II interferons (γ). ^[102-105] Upon preceding activation of the IDO gene promoter through e.g. IFN- γ *via* the JAK/STAT signaling pathway, which act on interferon stimulatory response elements (ISREs) and γ -activating sequences (GAS), the IDO gene is transcribed/translated, resulting in an inactive around 45 kDa big apoenzyme. Subsequently, the incorporation of the haeme prosthetic group takes place, which converts the apoenzyme in an inactive IDO holoenzyme. To end up in a fully functional IDO enzyme, potential posttranslational modifications (PTMs) are initiated. Although some of the most important IDO PTMs, like oxidation, nitration, N-terminal acetylation and phosphorylation are known, most of IDO's PMTs remain unclear so far. (Fig. 20) ^[106]

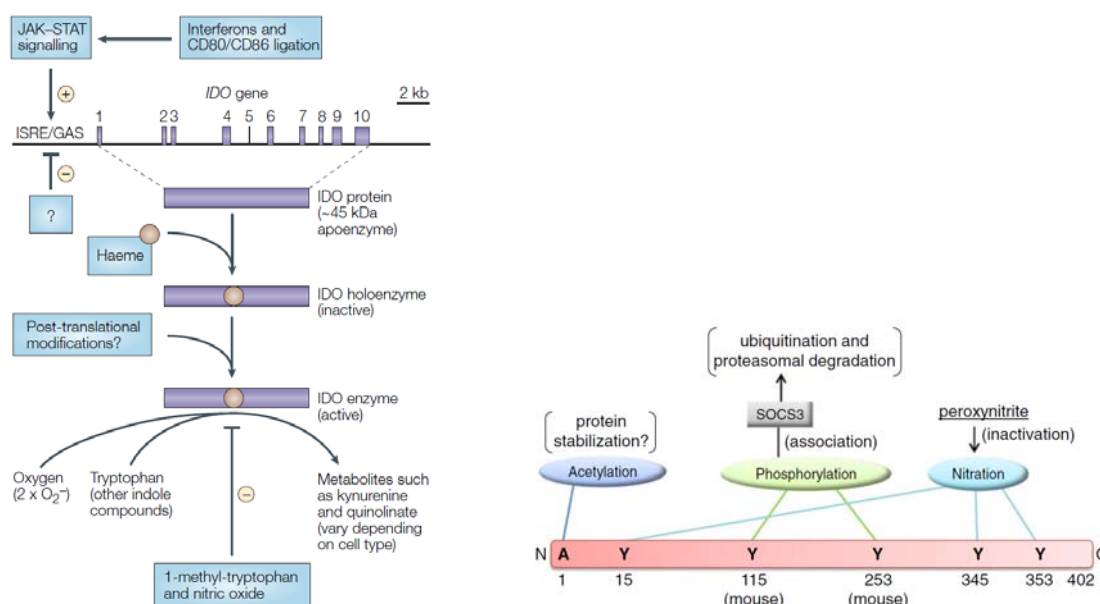


Fig. 20: Molecular Genetics of the IDO gene and protein [Mellor and Munn, 2004] ^[2] and post translational modifications [Fujigaki, 2012] ^[106]

IFN- γ was shown to be essential for IDO induction *in vitro*, and potentiated in the interplay with lipopolysaccharide (LPS), interleukin-1 (IL-1) and tumor necrosis factor (TNF).^[107-109]

This enzyme holds the ability to initiate the breakdown of the essential amino acid tryptophan to tryptophan metabolites.^[110] IDO is primarily present in immune cells, e.g. antigen presenting cells (APCs) and in a vast amount of various other tissues comprising endothelial cells^[111], astrocytes^[112], eosinophilic granulocytes^[113], bone marrow stromal cells^[114] and tumor cells^[115].

An innovative finding around the group Munn *et al.* revolutionized the whole thinking about IDO and its characteristics. As aforementioned, IDO was thought to contribute to the antimicrobial defense mechanism by depleting tryptophan in the inflammatory microenvironment, Munn *et al.* showed that tryptophan depletion plays a pivotal role in preventing T-cell driven fetal rejection, especially during pregnancy in mammals.^[116] Moreover IDO is suspected to contribute to the immunological escape of tumors *via* depletion of tryptophan (Fig. 21).^[117]

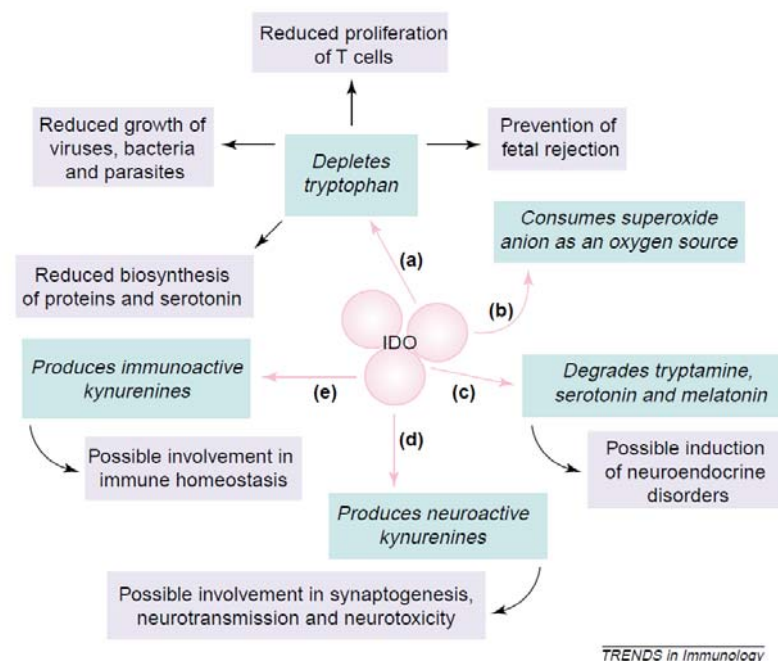


Fig. 21: Function of IDO [Terness *et al.*, 2002]^[11]

A – INTRODUCTION

4.1 Theories about IDO

Up to now two main theories about the function of IDO exist.

4.1.1 Tryptophan Starvation Theory

The first theory deals with tryptophan starvation, where IDO is supposed to act in a similar fashion on T-cells as on bacteria, viruses and parasites. ^[1, 118] This theory signifies that through the action of IDO local tryptophan storages are used up, which makes T-lymphocytes more prone to apoptosis. If T-cells are depleted from the essential amino acid tryptophan, they use to initiate cell cycle arrest and anergy in the first phase of amino acid starvation. ^[4, 118, 119] Nevertheless this state can easily be reversed by supplying cells with excess tryptophan thus reversing the IDO induced anergy. ^[118, 120-123]

The group around Munn *et al.* claimed that the local tryptophan concentration in surrounding T-cells has range between 0,5 μM and 1 μM to inhibit T-cell proliferation. ^[118] Interestingly, Frumento and colleagues affirmed that even in medium completely deplete of tryptophan, they could not observe a decreased T-cell proliferation. ^[124]

However, this theory should be regarded with suspicion, since local tryptophan concentrations *in vivo* range from 50 μM to 100 μM and can easily be restored by diffusion from the surrounding environment. ^[125]

4.1.2 Tryptophan Metabolite Theory

The second theory around IDO is the so called “tryptophan metabolite theory”. This theory states that upon tryptophan consumption tryptophan metabolites, so called kynurenines (especially 3-hydroxykynurenine and 3-hydroxyanthranilic acid), accumulate and force T-cells into cell cycle arrest or an apoptotic course due to their toxic effects. ^[124, 126, 127] Furthermore kynurenines are also thought to induce differentiation of CD4^+ T-cells into an immunosuppressive regulatory phenotype. ^[128] Up to now it seems that both mechanisms are contributing to the effects of IDO.

4.2 Tryptophan Degradation Pathway

In the course of tryptophan degradation, dietary tryptophan (around 99%), which is not used for protein synthesis, is metabolized along the tryptophan degradation pathway (Fig. 22). The residual 1% are converted into serotonin in the nervous system or gut or metabolized to melatonin in the pineal gland. ^[129] In the rate limiting step the 2,3 double bond of the indole ring of tryptophan is converted to kynurenine by oxidative cleavage mediated through either tryptophan 2,3 dioxygenase (TDO) or indoleamine 2,3 dioxigenase (IDO). ^[2] TDO is exclusively present in the liver where it catalyzes the conversion of tryptophan under steady-state conditions. ^[130, 131] Under inflammatory conditions enhanced IDO activity can be observed for the purpose of decreasing the local tryptophan environment.

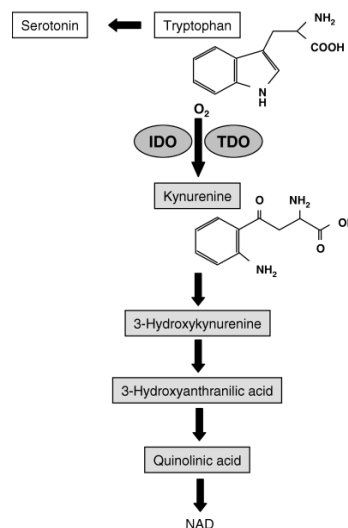


Fig. 22: Tryptophan degradation pathway [Iweala and Nagler, 2006]^[129]

4.3 IDO and Tolerance

IDO is assumed to play central role in immunological tolerance. During the late course of inflammation, antigen specific T-cells were described to release the potent immunological effector molecule interferon γ (IFN- γ), which acts in a paracrine dependent manner on dendritic cells (DCs). This action implicates that DCs initiate the activation induced cell death to control T-cell homeostasis. ^[132]

A – INTRODUCTION

As afore mentioned, Munn *et al.* discovered in 1998, that IDO contributes to the inhibition of fetal rejection at the maternal-fetal interface during pregnancy in mice. The group claims that IDO expression is restricted to primary trophoblast giant cells (TGCs) of fetal origin (entirely maternally inherited) in the perinuclear region. Interestingly, after placentation no IDO expression could be observed. Astonishingly, IDO deficient mice were able to produce normal healthy offspring, which points out that there has to be a compensatory or redundant immunosuppressive effect at the maternal-fetal interface preventing the rejection of the fetus. ^[133]

Moreover, IDO was shown to be constitutively expressed by extravillous trophoblast cells at the maternal-fetal interface in the human system. ^[134-137]

Another tolerance phenomenon can be observed in the case of autoimmunity. Primarily it was assumed that potential autoreactive T-cells are sent into apoptosis by dendritic cells activated through IFN- γ for maintaining peripheral tolerance and preventing autoimmunity.

^[1] Now it is known that IDO doesn't solely regulate the constitutive maintenance of selftolerance, since IDO depleted mice don't develop any lethal autoimmune diseases. Therefore IDO is rather categorized to be involved in acquired peripheral tolerance. ^[138]

Acquired tolerance could be harmful in the context of cancer development (Fig. 23), but can also have a beneficial impact regarding the tolerance induction against foreign antigens from the fetus or antigens at mucosal surfaces. ^[139]

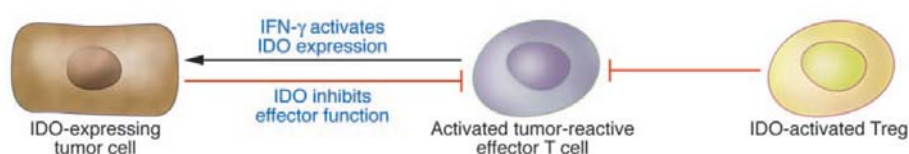


Fig. 23: Negative effects of IDO expression for the immunological reaction against tumor cells [Munn and Mellor, 2007]^[138]

In the context of defective self tolerance in the case of autoimmune diseases, IDO had been shown to worsen the effects and symptoms when inhibited in an EAE mouse model system. ^[140] On the other hand, if IDO is overexpressed it seems that immune responses are suppressed to a certain extent. This was astonishingly shown *via* the engraftment of IDO transfected MHC-mismatched murine lungs, preventing rejection without any immunosuppression. ^[141, 142]

More and more evidences accumulate that the downstream signaling of IDO, which result in tryptophan metabolites, accounts for the tolerogenic effect of T-lymphocytes, meaning inhibition of T-cell proliferation, and impairing NK-cell function. ^[124, 126, 143-145]

The working mechanism of kynurenines is not clear until now, however the G-protein coupled receptor GPR35 was supposed to bind the tryptophan metabolite kynurenic acid. However, the exact biological function of this orphan receptor is still unclear, since the correlation came about the finding that this receptor is highly expressed at specific sites in the gut where IDO is known to be as well highly expressed. ^[146] The induced expression of IDO in destined cells makes clear, that IDO, since it would be toxic if constitutively expressed through consumption of tryptophan, has to be tightly regulated in each system. ^[2]

5 Translation Process

5.1 Ribosome Structure

The structure of the eukaryotic ribosomal complex was resolved by cryo-electron microscopy (Fig. 24) ^[147]. These data showed a high structural homology between the pro- and eukaryotic ribosomal complex, allowing to model the interactions in the eukaryotic complex with the help of high resolution prokaryotic crystal structures and eukaryotic biochemical data. ^[148]

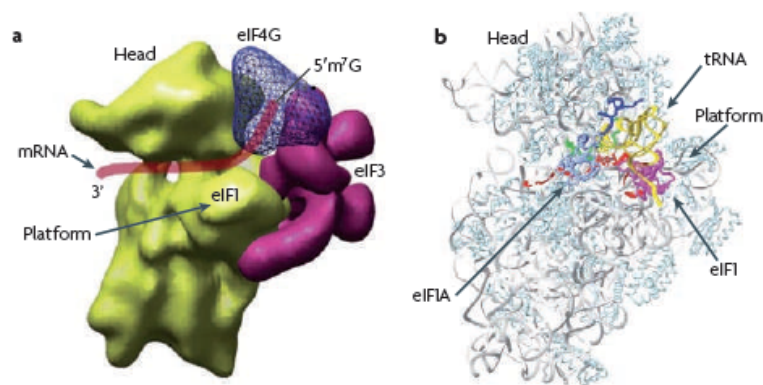


Fig. 24: (a) Cryoelectron microscopy analysis of the 40S subunit and (b) modeling of initiation factor recruitment [Jackson *et al.*, 2010] ^[148]

The ribosomal complex comprises three binding sites for tRNA (transfer RNA): (i) aminoacyl (A) site which has a high binding affinity for amino-acyl tRNAs (aa-tRNA), (ii) peptidyl (P) site which has a high binding affinity for peptidyl tRNAs and (iii) the exit (E) site, which has a high binding affinity for deacetylated tRNAs ^[149].

5.2 Translational Mechanism

The process of protein synthesis comprises three phases: (i) initiation, (ii) elongation and (iii) termination (with subsequent recycling). ^[150, 151]

5.2.1 Translation Initiation

Simplistically spoken, the phase of translation initiation comprises the steps of (i) codon-anticodon basepairing, (ii) selection of the initiator tRNA, (iii) scanning for the appropriate starting site and (iv) joining of the ribosomal subunits (Fig. 25).

In other words the eukaryotic initiation factor 2 (eIF2), which is GTP bound, and the initiator tRNA (Met-tRNA) assemble and build a complex, also known as the ternary complex (see Fig. 25/2). GTP is higher in its energetic status, since three phosphate residues are bound, than GDP, which is why GTP is bound to eIF2 to initiate translation. Subsequently after ternary complex formation, the 40S ribosomal subunit and various initiation factors (eIF1, eIF1A, eIF3, eIF5 and eIF2-GTP-Met-tRNA) assemble, building together the so called 43S preinitiation complex (see Fig. 25/3). ^[148, 152]

After assembly, the eukaryotic initiation factor 4E binds the 7-methylguanosine at the 5' cap of the mRNA and concurrently eIF4F and eIF4B are starting to unwind the mRNA at the cap-proximal region, thereby activating it (see Fig. 25/4). ^[153]

Thereafter the 43S preinitiation complex binds to the mRNA and scans for the appropriate start codon (see Fig. 25/5+6). Right after this process, the 48S initiation complex formation takes place (see Fig. 25/7) which switches the entire system to a closed conformation, leading to a displacement of the factor eIF1 to allow GTP hydrolysis by eIF5 (GDP-bound eIF2 was shown to have a 10-fold lower affinity to bind Met-tRNA_i^{Met} ^[154]). Mediated by eIF5B, eIF2-GDP and various other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) dissociate after attachment of the 60S ribosomal subunit to the complex (Fig. 25/8). Finally GTP is hydrolysed to GDP, which is mediated by eIF5B, and the factor eIF1A is released (Fig. 25/9). ^[148]

A – INTRODUCTION

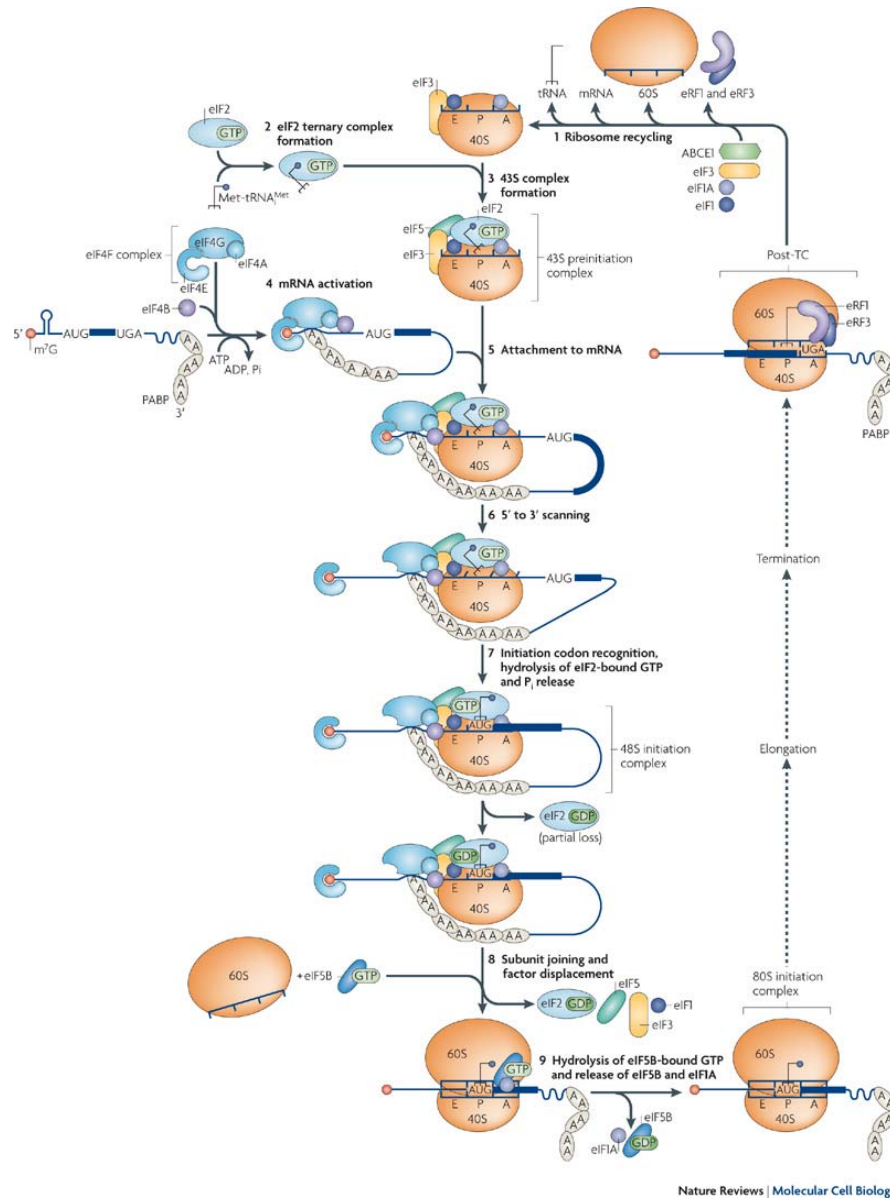


Fig. 25: Process of Eukaryotic Translation Initiation with subsequent recycling of the ribosomal subunits [Jackson *et al.*, 2010] ^[148]

5.2.2 Elongation

During the process of elongation, an aa-tRNA is selected and introduced into the A-channel of the ribosome. This phase can only be achieved when there is a perfect match of the codon at the ribosomal subunit and anticodon on the tRNA. Subsequently a peptide bond is formed between the incoming tRNA in the A-site and the tRNA in the P-site. The peptidyl tRNA is displaced from the A-site to the P-site and the deacetylated tRNA from the P-site to the E-site

5.2.3 Termination

The process of translational termination comprises the recognition of a stop codon, the release of the processed polypeptide chain and the dissociation of the ribosomal subunits. ^[148]

In other words, eRF1, possessing the ability of recognizing all three stop codons, namely UAG, UAA and UGA, mediates the binding of eRF3 to the ribosome. Subsequently GDP bound to eRF3 is exchanged by GTP simultaneously with the dissociation of eRF1. Thereafter the process of GTP hydrolysis takes place which leads to the dissociation of eRF3. Finally, the ribosomal complex dissociation is mediated by the multifarious factor eRF3 and the ribosomal subunits are recycled and fed again into the translational cycle. ^[149]

5.3 Translation Inhibition

When cells are confronted with different types of stress, one way to overcome this situation is to shut down global protein synthesis. ^[155] The general shut-down mechanism of a cell is to limit the availability of ternary complexes. Therefore the α -subunit of the eukaryotic initiation factor 2 is phosphorylated under stress conditions and inhibits the exchange of GDP to GTP, mediated by eIF2B. ^[156, 157] Due to the fact that eIF2B has a higher binding affinity to the phosphorylated than to the unphosphorylated form of eIF2 α , it acts like a competitive inhibitor for eIF2B, which results in the persistent binding of eIF2B to the α -subunit. ^[158] Finally, this leads on the one hand to a global shut down of protein synthesis and on the other hand to a concurrent translation of stress related proteins.

6 The ISR and their main players

Upon sensing various kinds of stress, the routine mechanism in mammalian cells is always the same: shut down of the whole protein synthesis machinery (Fig. 26). Since protein translation is a very energy demanding process, the cell tries to save the amount of energy used for translation and instead reusing it for inducing stress related programs, which aids for the cellular surviving. ^[159]

The integration of various stress signals, which modulate the protein biosynthesis, is called the “Integrated Stress Response” (ISR). This mode of action is conserved from yeast to mammals. ^[159] The causes for stressed cells to initiate the ISR can be very different and the recognition mechanisms are well designed. Four different kinases exist in the mammalian cell, which all share a homologous kinase domain but differ in the regulatory domains for initiating the integrated stress response (Fig. 27). ^[160, 161]

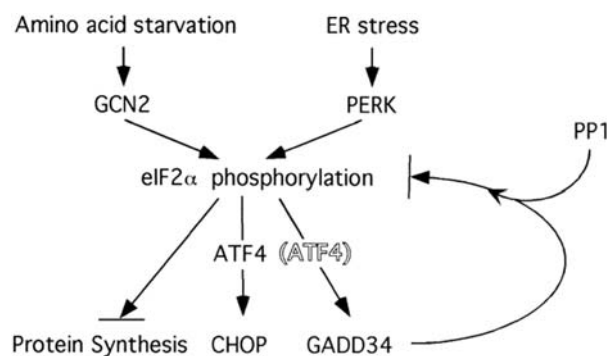


Fig. 26: The integrated stress response and its downstream signaling molecules [Novoa *et al.*, 2001]^[162]

Upon sensing double-stranded RNA, especially during a viral infection, protein kinase R (PKR) is activated and initiates the intracellular stress program. ^[163]

The endoplasmic reticulum (ER) resident transmembrane eIF2α kinase (PERK) possesses the ability to sense cellular stress triggered through malformed proteins. In this situation the load of newly synthesized proteins is bigger than the capacity of the ER to process the correct folding, therefore the global shutdown of protein synthesis assists the organelle to overcome protein load and decrease ER stress. ^[164-167] Amongst the three other eIF2α kinases, which all are cytosolic, PERK is the only kinase which is located at the ER membrane. ^[168]

In the erythroid lineage the stress sensor HRI (Heme-regulated eIF2 α kinase) triggers cellular reaction upon stress. It possesses the ability to sense heme deficiency, particularly during the development of cancerous tissue. ^[169, 170]

The last, and for this work most important eIF2 α kinase, is the general control non-derepressible 2 (GCN2). Its activation is triggered upon sensing nutritional stress, or more specific, amino acid deprivation. ^[171, 172]

Generally, the initiation of the ISR does not necessarily mean that the cell has to undergo apoptotic cell death. The phosphorylation of eIF2 α upon ER stress sensing and activation of PERK is rapidly reversible within minutes when the cellular stress declines. Only if the stress situation persists, proapoptotic signal molecules are translated and directs the whole system towards an apoptotic course. This means that cells have to hold a sensitive balance between un- and phosphorylated eIF2 α . ^[173, 174]

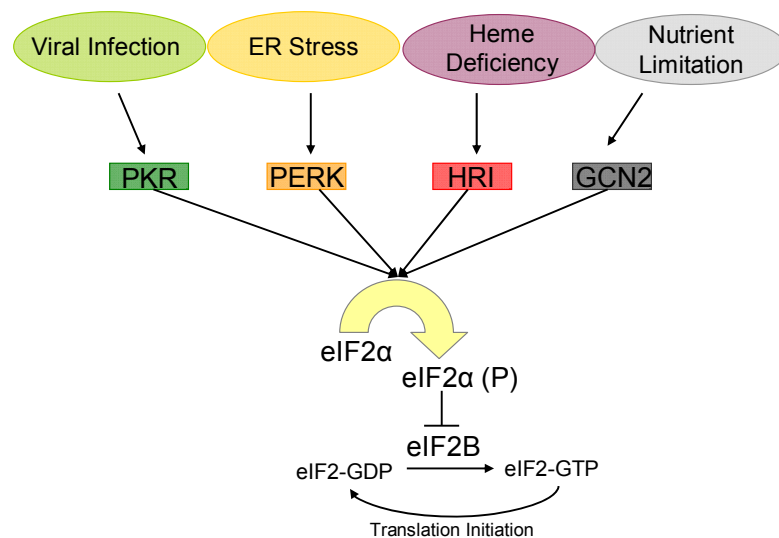


Fig. 27: Stress inducers and their sensor mechanisms [modified from Sikalidis *et al.*, 2011] ^[175]

A – INTRODUCTION

6.1 General Control Non-derepressible 2

The general control non-derepressible 2 (GCN2) is a serine/threonine protein kinase and belongs to the eIF2 α kinase superfamily. The around 180 kDa big GCN2 comprises various conserved structures like the histidyl-tRNA synthetase (HisRS)-related domain, a protein kinase domain (KD) and a pseudo-protein kinase domain (ψ KD) of unknown function. Additionally the structure also contains an N-terminal binding site for the GCN1/GCN20 complex, which plays a pivotal role in recognition of a starvation signal, which finally acts on the adaption of cell growth and differentiation. (Fig. 28) ^[176-178]



Fig. 28: Protein structure of human GCN2 [Rostovskaia *et al.*, 2003] ^[179]

GCN2 was first identified as an inducer of GCN4 in budding yeast, though it holds a major role in the mammalian system concerning the stress response upon amino acid deprivation. ^[179] *Saccharomyces cerevisiae* is known to hold the capacity of synthesizing all 20 essential amino acids, especially upon starvation conditions. The sensor for lacking essential amino acids was shown to be GCN2, binding uncharged tRNA to its histidyl-tRNA synthetase (HisRS)-like domain, as in the mammalian system. ^[176, 180-182]

An essential amino acid, of which nine exist in the mammalian system, is, per definition, an amino acid which is required for a healthy diet and can't be stored and synthesized *de novo* in the human body, meaning they must be supplied by ingested diet. ^[183] It has been shown that lacking essential amino acids elicits defense mechanisms in e.g. omnivorous, namely developing a taste aversion or regulating fatty acid homeostasis in the liver. ^[184, 185]

Upon diverse forms of stress, e.g. amino acid deprivation, uncharged tRNAs bind to the (HisRS)-related domain of GCN2 leading to the activation of the proximate KD domain. Upon this change in activation status, GCN2 is able to initiate the phosphorylation of eIF2 α , thus inhibiting eIF2B, which leads to increased translation of GCN4 in yeast and ATF4 in the mammalian system (Fig. 29). ^[176, 178]

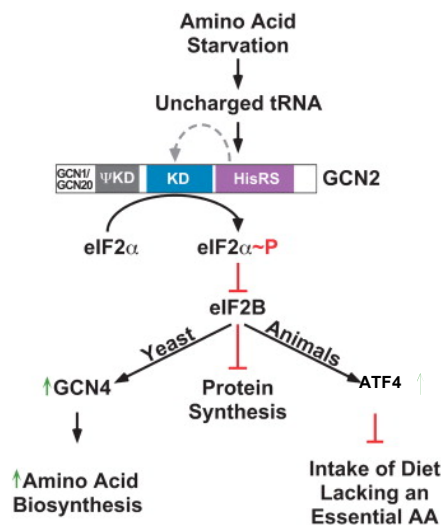


Fig. 29: GCN2 as a mediator in response to amino acid starvation (Modified from Dever and Hinnebusch, 2005) ^[176]

Up to now it is clear that in the mammalian system the GCN2 pathway is essential for a response to amino acid starvation, but not solely being responsible for the adaption to nutrient deprivation. It is speculated that the mTORC1 pathway could also be involved in a GCN2 independent manner during the response to amino acid deprivation, at least in the murine system. ^[183]

6.2 Eukaryotic initiation factor 2

The eukaryotic initiation factor 2 (eIF2) is the effector molecule, at which all stress responsive pathways converge. Hence, it marks the rate limiting step in the maintenance of protein translation upon several different stimuli. ^[186] In other words, the phosphorylated form of eIF2 is able to inhibit protein synthesis under stress situations, thus holding a crucial role in cellular energy conservation and. ^[155, 168]

A – INTRODUCTION

The eukaryotic initiation factor 2 was first discovered over 35 years ago as a GTP-bound protein associated with the initiator t-RNA. [187]

The protein consists of three subunits, which increase in molecular mass: the α -, the β - and the γ -subunit (Fig. 30).

The α -subunit of eIF2 holds a major role in the translational mechanism. It is the only subunit, which can be phosphorylated at the Ser⁵¹ position, which acts as a phosphate acceptor. As afore mentioned, various stress sensors (PKR, PERK, HRI, GCN2) are able to phosphorylate and therefore initiate a stress related program, which leads the cell either into adaptive or apoptotic programs. [168, 188]

The β -subunit of the eIF2 comprises several phosphorylation sites. [189] Moreover it contains guanine nucleotide binding domains, a binding site for eIF5 on the N-terminal portion and a binding site for the nucleotide exchange factor eIF5B on the C-terminal portion. [190, 191] However since the nucleotide binding domains on the N-terminus of the protein are located more distant from each other than usual, it seems that the β -subunit doesn't seem to be that important for guanine nucleotide binding. [192]

The primary amino acid sequence of the γ -subunit of eIF2 comprises three consensus guanine nucleotide domains. This implicates that the γ -subunit is more involved in guanine nucleotide binding than the β -subunit. [186, 192]

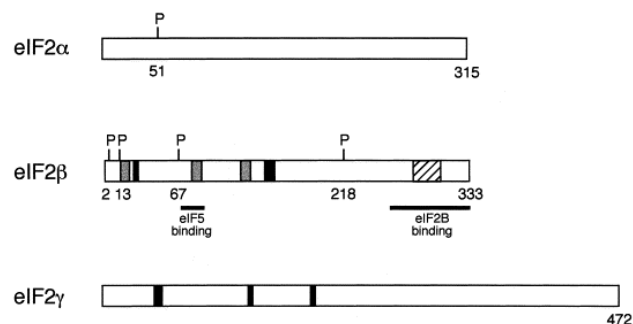


Fig. 30: Domain structure of α -, β - and γ -subunit of eIF2: The number of amino acids is depicted on the right side, the boxes represent the polypeptide chains, black bars indicate nucleotide binding domains [Kimball, 1999] [186]

Structural analysis revealed that the amino acid sequences of eIF2 are highly conserved among different species. The eukaryotic initiation factor 2 in humans and *Saccharomyces cerevisiae* share a homology of 58%, 47% and 72% for the α -, β - and γ -subunit, respectively. ^[193] This sequence homology again underscores the importance of this factor for cell viability. ^[186]

6.2.1 Biological function of eIF2

The primary function of eIF2 is to mediate the transfer of the initiator t-RNA (Met-rRNA_i) to the 40S ribosomal subunit to assemble with various other initiation factors for building the preinitiation complex (Fig. 31). Simplistically spoken, eIF5 binds to the complex and starts the hydrolysis of GTP. Finally eIF2 bound to GDP is released ^[187, 194]

After this process, GDP bound to eIF2 has to be exchanged to GTP, which is mediated *via* eIF2B, such that eIF2 can be implemented and reused in another initiation cycle. ^[186, 187]

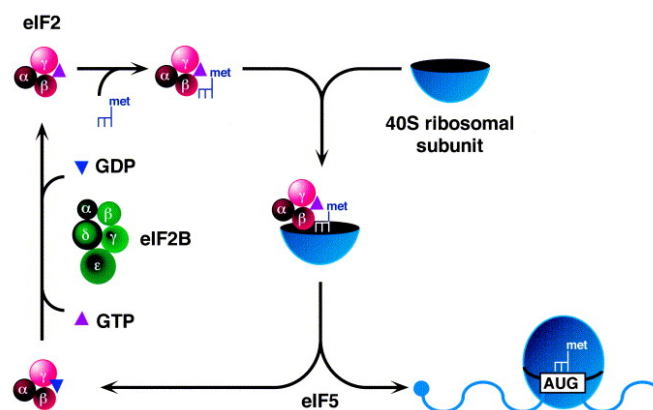


Fig. 31: Biological function of eIF2 [Kimball, 1999] ^[186]

6.3 Activating Transcription Factor 4

Activating transcription factor-4 (ATF-4) is a 39 kDA big leucine zipper transcription factor which belongs to the family of the basic-region leucine zippers. ATF-4 is also known as cAMP-response element binding protein-2 (CREB-2) or Tax-responsive enhancer element-binding protein 67. Up to now two alternative splicing variants of ATF-4 are known. ^[195] The ATF-4 gene is located on chromosome 22, which is translated into the

A – INTRODUCTION

ATF-4 mRNA. This mRNA comprises three short open reading frames (uORFs) in the 5' region and an adjacent coding sequence (Fig. 32). The uORFs were shown to play a pivotal role in the response to ER stress and hypoxia. [196]

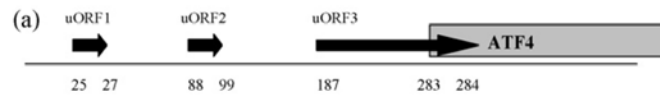


Fig. 32: Human ATF-4 mRNA sequence [Ameri and Harris, 2007] [197]

The ATF-4 protein comprises 351 amino acids, which form motifs especially influencing homo/heterodimerization, DNA binding and regulation of ATF4 protein stability. Additionally a transcriptional activation domain is included in the N-terminus of the protein (Fig. 33). [198, 199]

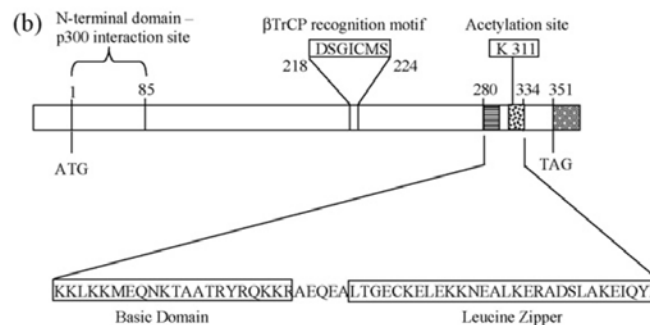


Fig. 33: Protein structure of human ATF-4 [Ameri and Harris, 2007] [197]

ATF-4 is able to interact and form homo- and heterodimers with various binding partners, such as members of the AP-1, C/EBP- and C/EBP-family proteins, *inter alia* CHOP. [200-203]

ATF-4 can additionally be posttranslationally modified, since ATF-4 comprises a recognition motif for β TrCP, which binds to this region, when phosphorylated, and leads to proteasomal degradation of ATF-4. Contrary to this transaction, the histone acetyltransferase p300 is able to inhibit the degradation process by stabilizing ATF-4. [204, 205]

ATF-4, which acts as a stress responsive gene, plays a pivotal role in amino acid deprivation, ER stress response and oxidative stress. [174, 206, 207]

Under stressed conditions the eukaryotic initiation factor 2 α is phosphorylated which leads to a global inhibition of translation, while inducing specifically the translation of ATF-4 mRNA (Fig. 26). [196]

What exactly happens in a nonstressed and stressed condition in the murine system is depicted in Fig. 34. In unstressed conditions eIF2-GTP-Met-tRNA_i ternary complexes are

A – INTRODUCTION

abundant, meaning that the 40S ribosome catches a ternary complex for the translation of uORF1 and resumes scanning to uORF2. Given that there are enough complexes available, the 40S ribosome can acquire a new ternary complex in time for the translation of uORF2.

When the cell encounters a stress situation, e.g. after thapsigargin treatment, the whole protein translation machinery will be shut down, resulting in a ternary complex deficiency. Consequently the 40S ribosome is able to acquire a ternary complex when translating uORF1 and resumes scanning for uORF2. Due to the lack of ternary complexes, a new complex will not be available in time when the ribosome reaches uORF2. The ribosome then passes over uORF2, but will acquire a new ternary complex when reaching the ORF of ATF-4, thus translating the stress protein. [148]

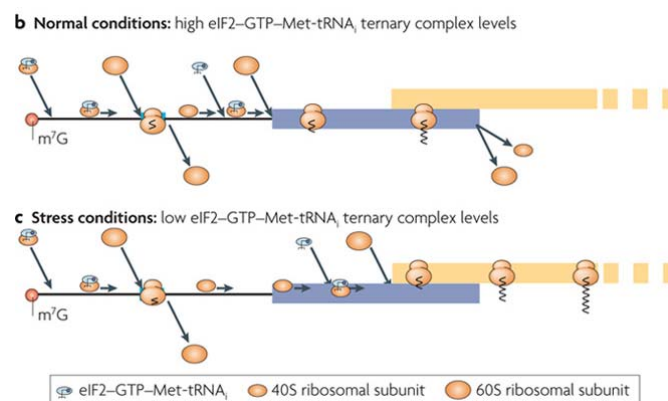


Fig. 34: Translational control of ATF-4 under nonstressed and stressed conditions [Jackson *et al.*, 2010] [148]

Overall ATF-4 seems to hold a crucial role, at least in the murine system, in various biological processes like eye development, cellular proliferation, hematopoiesis, bone development, fertility, long term memory storage and synaptic plasticity, revealed through knockout mouse studies. [197, 208-212] Interestingly ATF-4 seems to contribute to the development of cancer, since it induces VEGF and E-selectin expression, which is correlated with increased metastasis. [206, 213]

6.4 C/EBP Homologous Protein

The CHOP gene encodes a C/EBP homologous protein, also known as CHOP10, GADD153 (growth-arrest- and DNA-damage-inducible gene 153), DDIT3 (DNA-damage-inducible transcript 3) or C/EBP ζ which is tightly regulated by various stress factors in human and mouse cells. ^[214] CHOP, a 29 kDa protein which comprises 169 amino acid residues in the human system and 168 amino-acid residues in rodents, holds a pivotal role in immune functions, cell differentiation and proliferation. ^[215, 216] This nuclear protein was originally identified to be induced upon DNA damage and is capable to form heterodimers with various members of the C/EBP family ^[215], which are then able to identify target DNA sequences. ^[214, 217]

CHOP is composed of two functional domains, the N-terminal transcriptional activation domain and the C-terminal basic-leucine zipper (bZIP) domain, comprising an amino acid rich area for DNA binding and a leucine zipper dimerization motif (Fig. 35).

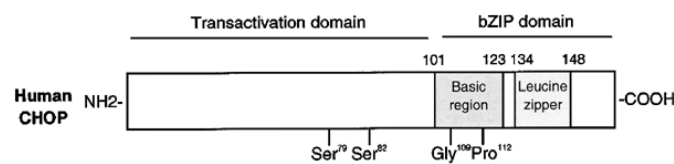


Fig. 35: Domain structures of CHOP [Oyadomari and Mori, 2004] ^[216]

Interestingly, CHOP is not solely controlled on transcriptional and translational level, but also by post-translational phosphorylation on serine 78 and 81 *via* p38 MAPK. ^[218]

Some years ago it was discovered that the CHOP promoter contains an amino acid response element (AARE), which displays an important binding motif for the transcription factor ATF4 in the amino acid response, which highlights the importance of CHOP as an ATF4 downstream target (Fig. 26). ^[218, 219] Additionally the promoter also includes two ER stress response elements (ERSE-1, ERSE-2), which are located in opposite directions and mediate the cellular response upon ER stress (Fig. 36). ^[220, 221] The ERSE elements of the CHOP promoter show a high sequence homology to the ERSE elements on the promoter regions of BiP, GRP94, PDI and CRT (Calreticulin). A weak homology is found for the CHOP AARE sequence when comparing with C/EBP- and ATF-binding sites. ^[222]

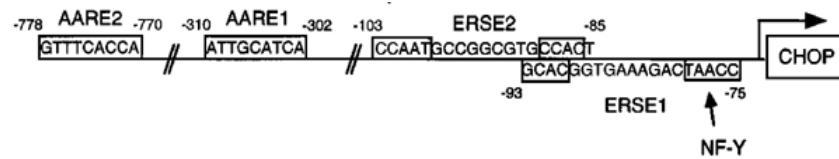


Fig. 36: Structure of the CHOP promoter with amino acid response elements (AARE) and ER stress response elements (ERSE)

[Oyadomari and Mori, 2004] ^[216]

CHOP is concomitantly expressed with the ER chaperone BiP ^[223-225] and can either be induced by direct ^[226-228] or indirect ^[229-231] stress response agents, which lead to an impairment in folding capacity in the ER. Some examples of ER stress inducing drugs are tunicamycin (glycosylation blocker), thapsigargin (calcium ATPase inhibitor specific for the ER), A23187 (calcium ionophore) and dithiothreitol (DTT; disruption of disulfide bonds). ^[228] The group around Zinszer *et al.* identified in 2003 that CHOP holds a major role in programmed cell death since CHOP^{-/-} MEFs show increased cellular viability compared to wt cells when challenged with ER stress inducing toxin. ^[214] The contribution to programmed cell death is, *inter alia*, achieved through downregulating BCL2 and upregulating GADD34, ERO1 α and TRB3. ^[218]

CHOP is endogenously expressed in the cytosol at extremely low levels under nonstressed conditions. However, if stress is induced in the cell, CHOP translocates from the cytosol to the nucleus and accumulates there. ^[215]

Overall CHOP is considered to be involved in the development of several diseases like diabetes, brain ischemia and neurodegenerative diseases as a result of aggregated proteins in the ER. In diabetic conditions, prolonged ER-stress leads to apoptosis of β -cells *via* CHOP, CHOP mediates ER-stress in vulnerable neurons which leads to delayed cell death and in the case of neurodegenerative diseases like Parkinson's, Alzheimer's or Huntington's disease, CHOP mediates apoptosis upon malformed proteins. ^[216, 232]

7 Alternative Pathways for the Adaption to Nutrient Deprivation

7.1 Autophagy

Autophagy has been described as the process of degradation of intracellular components. [233, 234] Generally three types of autophagy exist: (i) macroautophagy (ii) microautophagy and (iii) chaperone-mediated autophagy. In this thesis, autophagy is used for the term of macroautophagy. Autophagy can additionally be divided into two distinct systems: (i) the non-selective degradation system and (ii) the selective degradation system, which recognizes ubiquitinated proteins. [235]

Autophagy has several important roles comprising adaption to starvation, degradation of intracellular proteins and organelles, development, anti-aging, clearance of microorganisms, cell death mediation, tumor suppression and antigen presentation. [235, 236]

Mizushima described in 2005 that the extent of autophagy is tightly regulated and can range from “basal autophagy”, which includes the turnover of intracellular components, and “induced autophagy”, which is initiated upon stress induction for gaining new energy supply. [236]

Autophagy is highly conserved upon yeast, plants and animals and consists of several consecutive steps (Fig. 37).

The first step in the induction of autophagy is the building of a phagophore (also called isolation membrane), which looks like a Golgi cisternae. Elongation of this isolation membrane leads to a double membraned autophagosome, where the fusion of the two extremities is mediated via SNARE (soluble N-ethylmaleimide-sensitive-factor attachment receptor) proteins. How exactly these isolation membranes are built up is, up to now, not clearly understood. It is believed that a so called “pre-autophagosomal structure” (PAS) could give rise to this complex, but no evidence was found up to now to prove this hypothesis. [237] After fusion with an endosome, the autophagosome associates with a lysosome, which enables the now called autolysosome to degrade enclosed particles.

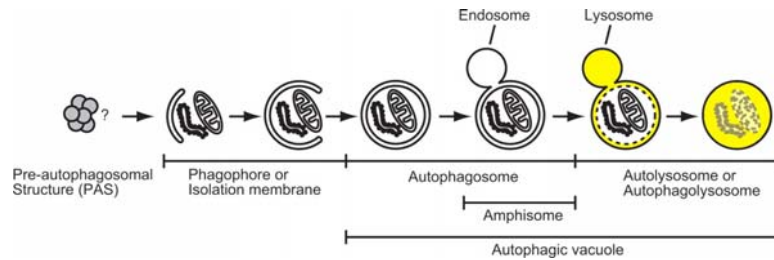


Fig. 37: Formation of an autophagosome via defined steps [Mizushima, 2007]^[235]

The autophagic pathway can be used for several purposes: (i) simple transport tool from the cytoplasm to a lysosome or endosome, (ii) degradation of malformed proteins and organelles and (iii) breakdown of cellular products for energy supply.^[236]

The latter point constitutes the most important function of autophagy. If a cell experiences stress, e.g. nutrient limitation, autophagy is induced within minutes and cellular products are fed into the autophagic loop to gain basic amino acids, helping the cell to overcome stress situations and, thus, survive.^[238]

One mammalian specific factor important for the regulation of autophagy is Beclin-1 with its interaction partner Bcl-2 (B-cell lymphoma 2).^[239] This interaction is reduced upon starvation conditions, resulting in the dissociation of Beclin-1 from Bcl-2, leading to the initiation of autophagy.^[240, 241]

Another important autophagic protein is LC3, which was identified being located in the autophagosomal inner membrane.^[242] LC3 is thought to act as receptor for p62/SQSTM1 (sequestosome 1), which accumulates in autophagic cells and seems to have the ability to recruit ubiquitinated proteins to the autophagosomal membrane.^[243, 244] Generally, two forms of LC3 exist: (i) LC3-I and (ii) LC3-II. Upon induction of autophagy, the cytosolic form (LC3-I) is cleaved and recruited to the autophagosomal membrane, where it is lipidated, resulting in the smaller LC3-II form. Thus, especially the processed form LC3-II, is a powerful marker for autophagy.^[236]

A – INTRODUCTION

7.2 PI3K/mTOR Pathway

mTOR (mammalian target of rapamycin), well known since the discovery of rapamycin in the 1970s, which acts as specific inhibitor for mTOR, has first been described in the yeast system as TOR1 and TOR2, and later the mammalian homologue was identified. [245, 246] mTOR, which constitutes a 1,5-2,0 MDa multiprotein complex, is a serine/threonine protein kinase which belongs to the family of phosphoinositide (PI)-3 kinase-related kinases (PIKKs). [247-249]

mTOR plays a pivotal role in cell cycle progression, translation control, ribosomal biogenesis, transcription control and autophagy. [250, 251] If the cell encounters nutrient deprivation, e.g. through amino acid withdrawal, mTOR senses this insufficiency, which rapidly leads to the dephosphorylation of 4E-BP1 and S6K1 and interferes with the translational machinery. This means, like during the integrated stress response, global protein translation is shut down, whereas stress specific mRNAs are upregulated (Fig. 38). [252-254]

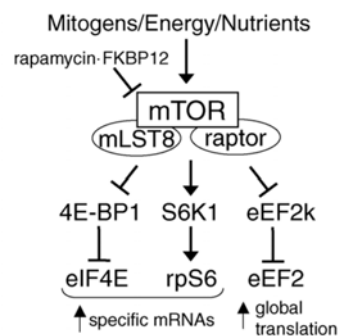


Fig. 38: Stress sensor mTOR and its downstream signaling pathway [Tee and Blenis, 2005]^[247]

eIF4E (eukaryotic initiation factor 4E) plays a crucial role in the initiation of translation. Generally, eIF4E interacts with a translational protein called 4E-BP1, which normally blocks the formation of the translation complex. In the case of abundant nutrient supply, 4E-BP1 is highly phosphorylated, which leads to the dissociation from eIF4E leading to the completion of complex formation. If the cell experiences stress, e.g. upon nutrient deprivation, 4E-BP1 is rapidly dephosphorylated and the interaction between 4E-BP1 and eIF4E can not be broken up, thus leading to a translational stop. [252]

Another downstream target in the PI3K/mTOR pathway is the S6K1 (protein S6 kinase 1), which is as well dephosphorylated and thus inactivated upon amino acid starvation. Blockage through dephosphorylation of S6K1 leads to the inhibition of its substrate rpS6 (ribosomal protein S6), which is found in the 40S ribosomal complex, affecting the translational process. ^[253]

The third most important protein, which is affected through mTOR, is eEF2 (eukaryotic elongation factor 2). mTOR regulates the activity of eEF2 kinase, which phosphorylates eEF2, leading to the impairment of translation, resulting in reduced elongation rates. ^[254]

B – HYPOTHESIS

B - HYPOTHESIS

The knowledge described was the basis for this project. IDO competent dendritic cells are known to exert immunosuppressive action on surrounding T-lymphocytes in the course of an allogeneic mixed lymphocyte reaction. However, this immunosuppressive effect is short-lived, due to the fact that upon withdrawal of IDO competent DCs and reintroducing these T-lymphocytes into a 2nd round allogeneic mixed lymphocyte reaction, T-cells are able to react against foreign lymphocytes and regain the ability of their full proliferative capacity.

The immunosuppressive effect of IDO *via* tryptophan depletion and kynurenine accumulation was described to trigger the integrated stress response, and thus, being easily reversible upon tryptophan repletion.

These preceding data incited our group to reveal the mechanism of the integrated stress response induced through tryptophan starvation and fathom the effects in the human system.

Our hypothesis states that the integrated stress response, initiated *via* tryptophan starvation, proves also true for the human system and hence is the cause for the reversibility of tolerance induction through dendritic cells.

C - MATERIALS AND METHODS

1 Isolation and purification of human peripheral blood mononuclear cells with subsequent enrichment of T-lymphocytes

1.1 Isolation and purification of human PBMCs

Venous blood was drawn by a medical specialist using the Vacuette[®] blood collection set (Greiner Bio One) comprising a collection set with luer adapter (#45006), heparinized collection tubes (#455084) and standard tube holders (#450201). After collection, 100 µl of whole blood were withdrawn under sterile conditions and used for analyzing the blood count (Sysmex KX-21N) and to ascertain any leukocyte number abnormalities. All following steps were conducted under sterile conditions in a laminar flow hood (Ehret, Esco Infinity Class II BSC). The amount of whole blood was then collected in falcon tubes and diluted 1:1 with 1x PBS (PAA, H15-002). Up to 36 ml of diluted whole blood were then carefully overlaid on 14 ml Lymphoprep[™] (Axis-Shield, #LYS 3773) and centrifuged (Thermo-Scientific, Heraeus Multifuge 1S-R) at 1000 g, at 21°C for 20 min without breaks. After centrifugation, the interphase (Fig. 39), containing human peripheral blood mononuclear cells (PBMCs), was carefully transferred to a 50 ml falcon tube. PBMCs were then washed with 1x PBS and centrifuged at 460 g, at 4°C for 7 min.

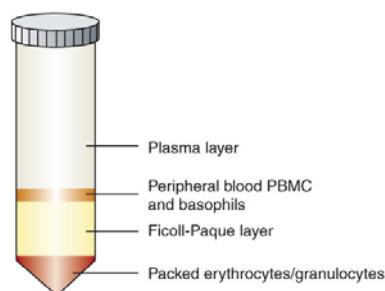


Fig. 39: Interphase of human PBMCs after Lymphoprep[™] centrifugation [Munoz and Leff, 2006]^[255]

C – MATERIALS AND METHODS

1.2 Enrichment of T-lymphocytes via MACS (Magnetic Cell Separation)

The supernatant was removed and the cell pellet was resuspended in 40 ml MACS separation buffer (see Table 1) and again centrifuged at 460 g, at 4°C for 7 min. The supernatant was carefully removed and the cell pellet was resuspended with 5 ml MACS Buffer. 100 µl were used to perform a cell count (Sysmex), whereas the total cell suspension was again centrifuged at 460 g, at 4°C for 7 min. Afterwards the supernatant was removed and the appropriate amount of MACS Buffer and Biotin labeled antibody was added according to the manufacturers protocol (Pan T-cell Isolation Kit II, Miltenyi Biotec, #130-091-156) and incubated for 10 min at 4°C. Thereupon, again an appropriate amount of MACS buffer and anti-biotin microbeads were added to the mixture and incubated for 15 min at 4°C. Then 20 x volume of MACS buffer were added to the tube and centrifuged at 460 g, at 4°C for 7 min. Meanwhile, the MACS separation stand including a magnetic unit and a separation column (25 MS, MACS Miltenyi, #130-042-201) were adjusted and equilibrated with 3 ml of MACS buffer. The supernatant was carefully removed and the cells were resuspended in 500 µl up to 1×10^8 cells. The cell suspension then was loaded onto the column and separated through magnetic and gravitational forces. All non-T-cells were retained in the column through magnetic labeling, whereas all T-lymphocytes were able to pass through and were collected in a separate tube (indirect separation technique) (Fig. 40). The tube was washed with 9 ml MACS buffer, which was as well loaded onto the column for separation.

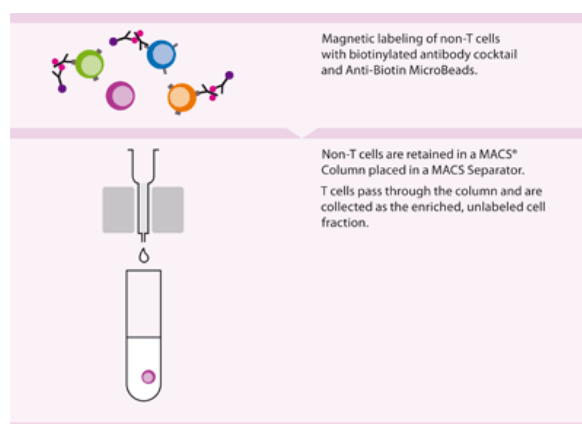


Fig. 40: MACS T-cell enrichment technique with indirect separation [Miltenyi Biotec, 2012] ^[256]

C – MATERIALS AND METHODS

After the separation step, T-cells were counted with the Sysmex, centrifuged at 460 g, at 4°C for 7 min and the supernatant was removed. Finally the cell number was adjusted for the optimal working concentration with the appropriate medium.

Buffer Compositions										
	MACS Buffer	Running Buffer (10x)	Blotting Buffer (10x)	Washing Buffer	Antibody Dilution Buffer	Stripping Solution (pH 2)	Annexin V Buffer	Erylysis Buffer (pH 7,2-7,4)	Company	Product #
Glycin		144 g	140 g			0,938 g			Roth	3908.2
Tris Base		30 g	30 g						AppliChem	A1086,1000
SDS		10 g				5 g				
dH ₂ O		ad 1000 ml	ad 1000 ml			ad 500 ml	ad 500 ml	ad 1000 ml	Sigma	L3771
1x PBS	500 ml			500 ml	100 ml				PAA	H15-002
HSA (20 %)	12,5 ml								CSL Behring	0053-7695-34
EDTA (500 mM)	2 ml								Promega	V4231
Tween 20				1 ml	0,2 ml				Sigma	P1379
10x Roche Western Blocking Reagent					5 ml				Roche	11921673001
HEPES							10 ml		Affymetrix	16928
NaCl							7,56 g		Roth	3957.1
CaCl ₂							0,37 g		Roth	CN93.1
NH ₄ Cl								8,024 g	Roth	5470.1
KHCO ₃								1 g	Roth	P748.2
Na ₂ EDTA								37,2 mg	Sigma	E5134

Table 1: Buffer compositions

1.3 CFSE labeling

For proliferation studies, cells were labeled with CFSE (carboxyfluorescein succinimidyl ester, Sigma, # 21888-25MG-F). CFSE staining is used to assess cellular proliferation. When a cell divides, CFSE intensity is decreased by half and decreases with further cell divisions, thus indicating cell proliferation. Cells were centrifuged at 460 g, at 4°C for 7 min, the supernatant was removed and the cells were adjusted to 1×10^7 /ml with CFSE-PBS (1x PBS, 0,1 % HSA). CFSE was added (1,5 μ M) to the suspension and incubated for 15 min at 37°C. 1 ml FBS was added to 1×10^7 cells (Invitrogen, #10500-064) to quench the reaction and the cell suspension was incubated for 2 min on ice. Afterwards tryptophan depleted medium was added to the suspension (10 ml per 1×10^7 cells) and cells were

C – MATERIALS AND METHODS

centrifuged at 460 g, at 4°C for 7 min. The supernatant was removed and the washing step was repeated. Finally, the cell number was adjusted to the required concentration with either tryptophan deplete or tryptophan replete medium.

1.4 T-cell culture conditions

After adjusting the cell number, cells were plated in either 6-well (IWAKI, #3810-006), 12-well (IWAKI, #3815-048), 24-well (IWAKI, #3820-024) or 96-well (Nunc, #163320) plates with Tryptophan deficient medium (PAA, #T1144,2500) or Tryptophan sufficient medium (PAA, #E15-840) both supplemented with 1% Glutamine (PAA, #M11-004), 1 % Penicillin/Streptomycin (PAA, #P11-010) and either 1 %, 5 % or 10 % tryptophan deplete FBS (PAA, #A11-107) to ascertain cell culture conditions fully deplete of tryptophan.

Cells were simulated either with 50 ng/ml PMA (*phorbol*-12-myristate-13-acetate, Sigma, #P1585-1MG) and 500 ng/ml Ionomycin (Sigma, 10634-1MG) or a monoclonal anti-CD3 (Sigma, #C7048-100TST) and anti-CD28 (Sigma, #C7831-100TST) antibody after plate precoating with anti-Mouse IgG1 (Sigma, #M8770).

To increase stress conditions *in vitro*, cells were stimulated with thapsigargin (Sigma, #T9033). Thapsigargin is a non-competitive calcium pump inhibitor in the endoplasmic reticulum, thereby depleting the ER from calcium and leading to the raise in cytosolic calcium concentration.

The Jurkat T-cell line was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, #ACC282) and maintained in Gibco® RPMI 1640 medium (Invitrogen, 21875-034) supplemented with 1 % Glutamine, 1 % Pen/Strep and 10 % FBS before use. Since Jurkat cells have a doubling time of 25-35 hours, cells were split at an optimal ratio of 1:2 to 1:3 every 2 to 3 days.

2 Isolation and purification of murine splenocytes

Spleens were harvested from C57BL/6 or BALB/c mice and subsequently singularized using a 70 μ M cell strainer (Becton Dickinson, #352350), while flushing the single cells with RPMI 1610, supplemented with 1 % Glutamine, 1 % Pen/Step and 10 % dialyzed FBS, into a falcon tube. The cells were then centrifuged at 460 g, at 4°C for 7 min, the supernatant was removed and 1 ml erylisis buffer (see Table 1) per spleen was added, mixed well and incubated on ice for 1 min. The lysis process was stopped by adding 40 ml MACS Buffer (see section 1.2) and cells were again centrifuged at 460 g, at 4°C for 7 min. The supernatant was again removed, the pellet was resuspended in 5 ml MACS Buffer and a cell count was performed with the Z2 Coulter Counter (Beckman Coulter).

2.1 Enrichment of murine T-cells via MACS separation

T-cells were enriched as human T-cells (see section 1.2) with a mouse Pan T Cell Isolation Kit II (Miltenyi, #130-095-130) according to the manufacturer's protocol.

3 Sample Preparation

3.1 Protein samples

Cells were harvested by transferring the cell suspension in a tube and flushing the wells with 1x PBS, which was also transferred to the tubes. The cells were then centrifuged at 460 g, at 4°C for 7 min, the supernatant was removed, and the pellet was resuspended 1:1 with 1x PBS and 2x Novex[®] Tris-Glycine SDS Sample Buffer (Invitrogen, #LC2676). Before, the sample buffer was supplemented with 3% Dithiothreitol (Sigma, #43815-1G). The samples were then heated (Eppendorf, Thermomixer Compact) to 99°C for 10 min and stored until usage at -80°C.

3.2 RNA samples

3.2.1 RNA isolation

RNA was isolated with a QIAshredder[®] (QIAGEN, #79654) and the RNeasy[®] Mini Kit (QIAGEN, #74104) according to the manufacturer's instructions (RNeasy[®] Mini Handbook, "Purification of Total RNA from Animal Cells Using Spin Technology"). RNA concentration was determined by measurement of 1 µl with Nanodrop (Thermo Scientific, ND-1000). Finally all samples were adjusted to equal concentrations (1 µg/20 µl) with nuclease free H₂O (Fermentas, #R0581) and stored at -80°C until being used.

3.2.2 Reverse transcription

1 µg of isolated RNA was transferred into an RNase and DNase free tube and heated at 65°C for 15 min. Then 4 µl M-MLV RT-5X Buffer (Promega, #M531A), 2 µl 100mM DTT (Invitrogen, #Y00147), 1 µl Random Primers (Promega, #C118A), 1 µl 10mM dNTPs (Promega, #U120A, #U121A, #U122A, #U123A), 1 µl nuclease free H₂O and 1 µl M-MLV Reverse Transcriptase (Promega, #M170B) were added and the samples were incubated at 37°C for 90 min. Finally the cDNA (complementary DNA) was stored at -80°C until usage.

3.3 Flow cytometric samples

100 µl of cell suspension were transferred to plastic tubes, diluted with 100 µl Annexin V Buffer (see Table 1) and the antibody cocktail was added (Table 2). The suspension was then incubated for 20 min in the dark at 4°C and analyzed by flow cytometry (see section 0)

Antibody	Fluorochrome	Specificity	Company	Catalog number	Dilutions
CD25	PE-Cy7	Mouse-anti-human	Becton Dickinson	#557741	1:500
CD3	PE-TxRed	Mouse-anti-human	Becton Dickinson	#624004	1:500
CD4	FITC	Mouse-anti-human	Dako	#F0766	1:200
CD8	RPE	Mouse-anti-human	Dako	#R0806	1:2000
Annexin V	APC	Unspecific	Becton Dickinson	#550474	1:200
Dapi	Dapi	Unspecific	Sigma Aldrich	# 9542-1MG	1:100 (of 0,03 µg/ml stock)

Table 2: Flow cytometric antibodies and respective dilutions

4 Analyses Methods

4.1 Protein analyses

4.1.1 SDS PAGE

Samples were thawed and heated to 99°C for 10 min. In the meantime, either 10 % SDS PAGE gels were prepared (Table 3) or 4–20% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, #456-1094) were arranged in the Mini PROTEAN® Tetra Cell chamber (Bio-Rad, #165-8000). The gels were run constantly at 80 V for 20 min (for running buffer see Table 1), until the loading dye band has passed the border between stacking and separating gel. The protein size was estimated using the PageRuler® Plus Prestained Protein Ladder 10-250 kDa (Thermo Scientific, #26619) and CHOP expression was monitored *via* a CHOP Lysate (Santa Cruz Biotechnology, #sc-120383). Then the voltage was increased to 150 V and run for 90 min. Finally the gel pack was disassembled and the gel was blotted.

Separating Gel		Stacking Gel	
dH ₂ O	4,9 ml	dH ₂ O	3,4 ml
30 % Acrylamide/ 0,8% Bis	4 ml	30% Acrylamide/ 0,8% Bis	830 µl
1,5 M Tris pH 8,8	3 ml	1 M Tris pH 6,8	630 µl
20% SDS	60 µl	20% SDS	25 µl
APS	120 µl	APS	50 µl
TEMED	14,4 µl	TEMED	5 µl

Table 3: Composition of SDS PAGE separating and stacking gel

4.1.2 Western blotting

The SDS PAGE gel was blotted (for blotting buffer see Table 1) onto a PVDF membrane (Bio-Rad, #162-0177) at 300 mA for 60 min. If mTOR (see section 7.2) was going to be analyzed, the gel was cut between 100-130 kDa before blotting and blotted at 300 mA for 120 min. After the blotting procedure, the membrane was cut to the area of interest and blocked with 1x (diluted with 1x PBS) Western Blocking Reagent® (Roche, #11921673001) for 60 min with shaking on RT. Then the blot was incubated in primary antibody solution

C – MATERIALS AND METHODS

(Table 4) at 4°C with shaking o/n. On the next day the blot was washed three times in washing buffer (see Table 1) for 5 min and then incubated in secondary antibody solution (Table 4) for 1 h. The developing analysis was performed with the ODYSSEY® CLx imager (Li-cor Bioscience) and analyzed with the image quantification system ImageJ (NIH Image).

Antibody	Company	Product Number	Dilution/ Concentration
Primary Antibodies			
pelF2α	Cell Signaling Technology	#9721S	1:500
eIF2α	Cell Signaling Technology	#9722S	1:500
CHOP	Santa Cruz Biotechnology	#sc-7351	1:100
LC3	nanoTools	#0231-100/LC3-5F10	1,5 µg/ml
Beclin-1	Santa Cruz Biotechnology	#sc-11427	1:400
SQSTM1/p62	Cell Signaling Technology	#8025	1:500
pmTOR	Cell Signaling Technology	#2971	1:600
mTOR	Cell Signaling Technology	#2972	1:850
prpS6	Cell Signaling Technology	#2215	1:700
α-Tubulin	Calbiochem	#CP06	1:1000
GAPDH	Ambion Life Technologies	#AM4300	1:10 000
Secondary Antibodies			
Goat anti-Mouse IgG (H+L) (DyLight 800), Polyclonal	Thermo Scientific	#35521	1:10 000
Goat anti-Rabbit IgG (H+L) (DyLight 800), Polyclonal	Thermo Scientific	#35571	1:10 000

Table 4: Primary and secondary antibodies with respective working dilutions

C – MATERIALS AND METHODS

4.2 RNA analyses

4.2.1 Real time PCR

Primers were designed (Table 5) and ordered at Eurofins MWG Biotech with HPSF purification and 0,01 µmol synthesis scale.

Gene	Gene Bank Accession #	Species	Fwd Sequence (5'-3')	Rev Sequence (5'-3')
GAPDH	NM_002046	Human	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA
eIF2α	NM_004094	Human	TGGTGAATGTCAGATCCATTGC	TAGAACGGATACGCCTTCTGG
ATF4	NM_182810	Human	CTCCGGGACAGATTGGATGTT	GGCTGCTTATTAGTCTCCTGGAC
CHOP	NM_001195055	Human	GAACGGCTCAAGCAGGAAATC	AGTCGCCTCTACTTCCCTGG

Table 5: Investigated genes with respective forward (fwd) and reverse (rev) primer sequences

The mastermix was prepared by adding 10 µl Maxima™ SYBR Green/ROX 2X qPCR Master Mix (Fermentas, #K0221), 3 µl nuclease free H₂O and 2 µl of a 10 pmol primer mix (forward and reverse). 10 µl of this mastermix were added to a MicroAmp® Fast Optical 96-Well Reaction Plate 0.1 ml (Applied Biosystems, #4346906), while cooling the plate with ice. cDNA was thawed and diluted with nuclease free H₂O to a concentration of 1,5 ng/ml. 5 µl were added to the reaction plate which was finally sealed with a MicroAmp® Optical Adhesive Film (Applied Biosystems, #4311971). The reaction included two holding stages (50°C and 95°C for 2 min and 10 min respectively), 40 cycles (95°C heating for 15 sec, 60°C cooling for 1 min) and a melting curve stage (95°C for 15 sec, 60°C for 1 min, 95°C for 30 sec) with a total duration time of approximately 120 min. Obtained results were then analyzed and calculated, in consideration of the primer efficiency (Formula 1), using the “efficiency corrected calculation model” (Formula 2).^[257]

$$E = 10^{-1/\text{slope}}$$

Formula 1: Efficiency calculation according to Pfaffl, 2006^[257]

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_P \text{ target (MEAN control - MEAN sample)}}}{(E_{\text{Ref}})^{\Delta C_P \text{ Ref (MEAN control - MEAN sample)}}}$$

Formula 2: Efficiency corrected calculation model [Pfaffl, 2006]^[257]

4.3 Flow cytometric analyses

Expressed cell surface markers were measured by flow cytometry (Becton Dickinson, LSR2) and analyzed with FlowJo (Tree Star).

D - RESULTS

1 The Integrated Stress Response and Autophagy Induction in the murine system

1.1 *Optimal culture conditions for murine splenocytes*

The background for this study was to investigate whether previous findings by Munn *et al.* in 2005, which state that the induction of the integrated stress response is strongly correlated with tryptophan starvation in mice^[4], are also valid for the human system. Prior to the investigation of the ISR in the human system, the aim was to reproduce these published results in murine cells.

In order to ascertain that a high apoptotic decline does not interfere with the detectability of ISR induction, the rate of apoptosis was assessed in different culture conditions.

For this purpose, BALB/c or C57BL/6 splenocytes (3×10^6 and $4,5 \times 10^6$) were activated with PMA/Ionomycin and cultured in TRP deplete or TRP replete medium, supplemented with 1 % or 10% TRP free serum, for 24 h. We reasoned that to detect the initiation of the ISR, cells should have limited access also to other nutrients. This means that sufficient, but not abundant nutrient should be supplied in the culture. This was the rational to probe increasing serum concentrations for medium supplementation. Additionally, cells were stimulated with 1 μ M thapsigargin (TG) for up to 4h. [159, 167, 174, 196, 214, 258]

As depicted in Fig. 42, the susceptibility to apoptosis in murine splenocytes, cultured in 1 % TRP free serum, was elevated indicated by the high proportion of 70-80 % apoptotic cells. Cellular survival was markedly increased to approximately 90 % upon supplementation of 10 % TRP free serum (Fig. 43). In this cell culture condition, neither stimulation with 1 μ M TG nor activation with PMA/Ionomycin increased apoptosis in murine splenocytes even for 4 h.

Thus for further experiments, cell cultures were maintained in TRP deplete or replete medium supplemented with 10 % TRP free serum.

D – RESULTS

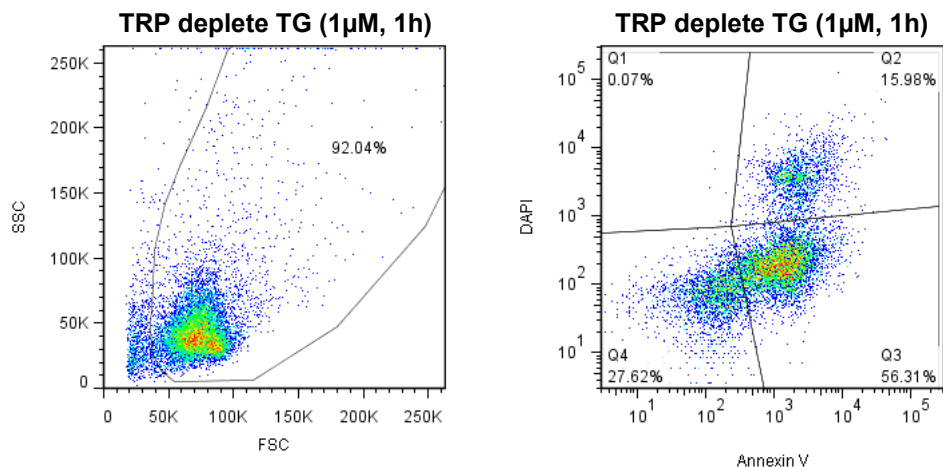


Fig. 41: Dotplot of murine splenocytes maintained under suboptimal culture conditions
Gating strategy for Annexin V / DAPI staining for detection of apoptosis in resting murine splenocytes (3×10^6), cultured in TRP free medium supplemented with 1 % TRP free serum and stimulated with TG ($1 \mu\text{M}$) for 1 h

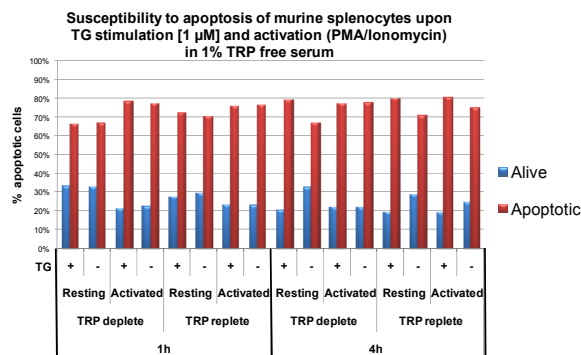


Fig. 42: Susceptibility to apoptosis of murine splenocytes maintained under suboptimal culture conditions
Apoptotic rate of murine splenocytes (3×10^6) cultured for 24 h in TRP free or TRP containing medium supplemented with 1 % TRP free serum and challenged with thapsigargin (TG, $1 \mu\text{M}$) for up to 4h

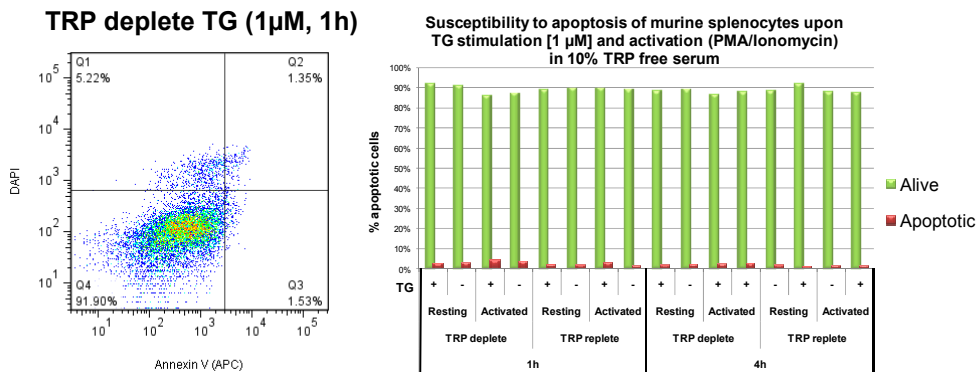


Fig. 43: Susceptibility to apoptosis of murine splenocytes maintained under optimal culture conditions
Apoptotic rate of resting murine splenocytes (4.5×10^6) cultured in TRP free medium supplemented with 10 % TRP free serum and stimulated with TG ($1 \mu\text{M}$) for up to 4h

1.2 Initiation of the integrated stress response in murine splenocytes upon tryptophan starvation

Munn *et al.* showed in 2005 that the ISR stress protein CHOP was expressed by tryptophan withdrawal in PMA/Ionomycin activated murine splenocytes.^[4] This experiment aimed for reproducing these findings.

Murine splenocytes (7×10^6) were activated with PMA/Ionomycin and cultured in either TRP deplete or TRP replete medium, supplemented with 10 % TRP free serum, for 24 h. The integrated stress response was monitored *via* phosphorylation of eIF2 α and CHOP expression.

Our results show (Fig. 44 and Fig. 45) that CHOP expression was not detected in murine splenocytes. Since no CHOP band was detectable in the positive CHOP lysate control, we suggest that the antibody was not appropriate for detection.

However, activation through PMA/Ionomycin was shown to trigger enhanced expression of phosphorylated eIF2 α (Fig. 44). The quantification by densitometric analysis revealed a 1,28-fold increase in activated cultures maintained in TRP deplete or TRP replete culture medium (Fig. 45 and Table 6). Remarkably, no difference in phosphorylation of eIF2 α was noted, neither in resting nor in activated cells cultured in TRP deplete compared to TRP replete medium (Fig. 45 and Table 6). Likewise, endogenous eIF2 α levels increased 1,2 to 1,3-fold upon activation, but again no difference was observed comparing TRP deplete to TRP replete conditions. These findings suggest a non specific upregulation of protein expression upon activation with PMA/Ionomycin. These observations are in marked contrast to published data by Munn *et al.* and indicate that the ISR is not initiated in murine splenocytes.

However, increased protein expression through stimulation with PMA/Ionomycin was though specific for stress related proteins, since marker protein expression for autophagy was not affected upon stimulation (see section 1.3).

D – RESULTS

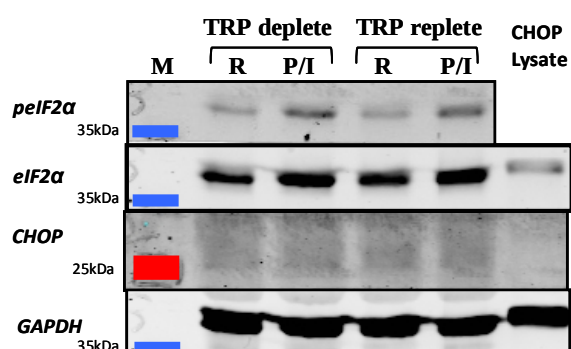


Fig. 44: ISR marker protein expression in murine splenocytes

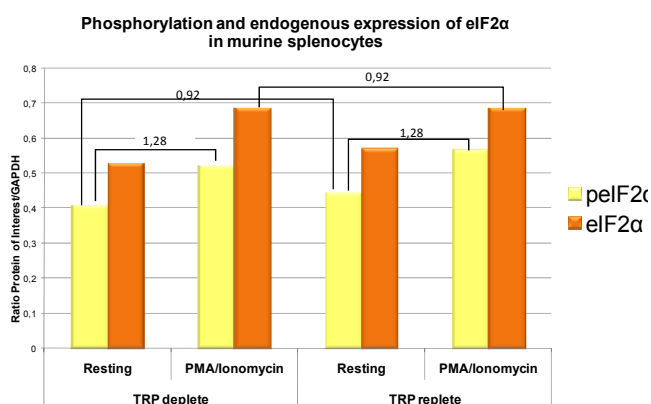


Fig. 45: Densitometric analysis of ISR associated marker proteins

Numbers indicate the fold increase of activated versus resting and TRP deplete versus TRP replete ratios

<u>pelf2α</u>	<i>Resting</i>	<i>Activated</i>	<i>Fold increase</i>
	trp deplete	trp deplete	1,28
	trp replete	trp replete	1,28
Fold increase	0,92	0,92	

<u>eIF2α</u>	<i>Resting</i>	<i>Activated</i>	<i>Fold increase</i>
	trp deplete	trp deplete	1,30
	trp replete	trp replete	1,20
Fold increase	0,93	1,00	

Table 6: Fold changes of ISR protein expression upon maintenance in TRP deplete or TRP replete culture medium and activation with PMA/Ionomycin

Fold changes were calculated by dividing TRP deplete by TRP replete ratios

1.3 Assessment of autophagy in murine splenocytes upon tryptophan starvation

Because our investigations did not find ISR induction by tryptophan starvation in murine cells, as alternative pathway involved in cellular adaption to nutrient deprivation, autophagy, was analyzed. Like before, murine splenocytes (7×10^6) were activated with PMA/Ionomycin and cultured in either TRP deplete or TRP replete medium, supplemented with 10 % TRP free serum, for 24 h. Autophagy induction was analyzed by Beclin-1 and p62/SQSTM1 protein expression.

Levels of expression of Beclin-1 and p62/SQSTM1 (Fig. 46 and Fig. 47) were not affected by activation, which is illustrated by only minimal fold changes as shown in Table 7. Different from proteins involved in the ISR, activation with PMA/Ionomycin did not enhance Beclin-1 or p62/SQSTM1 expression in cells. Moreover, levels of Beclin-1 and p62/SQSTM1 were not increased in TRP free culture conditions (Table 7). These data suggest that autophagy is not triggered upon TRP starvation in murine splenocytes.

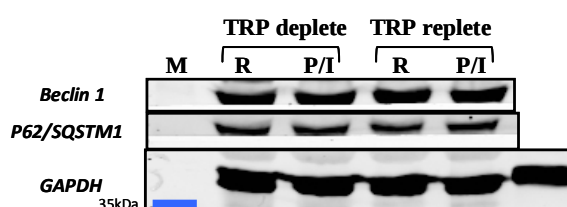


Fig. 46: Autophagy in murine BALB/c splenocytes

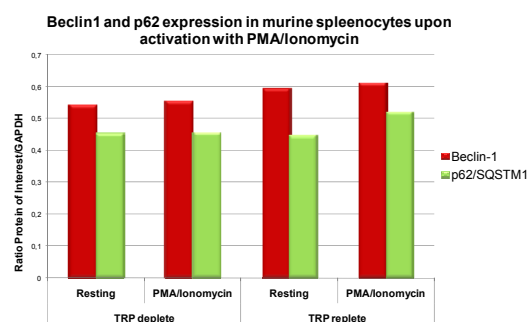


Fig. 47: Densitometric analysis of autophagy associated marker proteins

<i>Beclin-1</i>	<i>Resting</i>	<i>Activated</i>	<i>Fold increase</i>
	TRP deplete	TRP deplete	1,02
	TRP replete	TRP replete	1,03
<i>Fold increase</i>	0,91	0,90	

<i>p62/SQSTM1</i>	<i>Resting</i>	<i>Activated</i>	<i>Fold increase</i>
	TRP deplete	TRP deplete	1,01
	TRP replete	TRP replete	1,16
<i>Fold increase</i>	1,01	0,88	

Table 7: Fold changes of autophagy marker protein expression in murine splenocytes maintained in TRP deplete or TRP replete culture medium and activated with PMA/Ionomycin

Fold changes were calculated by dividing TRP deplete by TRP replete or activated by resting ratios

2 The Integrated Stress Response and Autophagy Induction in the Jurkat T-lymphocyte cell line

2.1 *Characterization of Jurkat T-lymphocytes*

The Jurkat T-cell line has been widely used as a model system deciphering the induction of the ISR. ^[259, 260] In our study, this cell line was used initially to explore a potential effect of TRP starvation on the induction of the ISR in human cells. First, for characterization of Jurkat cells, susceptibility to apoptosis and activation status was investigated *via* monitoring Annexin V / DAPI and CD25 surface expression, respectively. Jurkat T-lymphocytes (1×10^6) were activated with PMA/Ionomycin and cultured either in TRP deplete or TRP replete medium, supplemented with 1 % or 10 % TRP free serum, for 24 h. Afterwards, cells were stimulated with either 1 μ M or 10 μ M TG for up to 4 h, to determine the maximal dose and time of TG, which is tolerated by cells.

As Fig. 48 shows, apoptosis was slightly increased by up to 18 % upon maintenance in TRP deplete culture medium supplemented with only 1 %, in comparison to cultures supplemented with 10 % TRP free serum (Fig. 49), represented by only 8 % apoptotic cells. Even prolonged exposure to high (10 μ M) concentrations of TG did not further enhance susceptibility to apoptosis (Fig. 48). Similarly, the survival of these cells was not decreased in TRP free cell culture conditions.

Overall, Jurkat T-cells appeared very robust when cultured under suboptimal culture conditions (low serum concentrations, TRP deprivation, prolonged high concentrated TG stimulation).

Further, the cell activation marker CD25 was shown to be permanently expressed on Jurkat T-cells, suggesting that they are constitutively activated (Fig. 50).

D – RESULTS

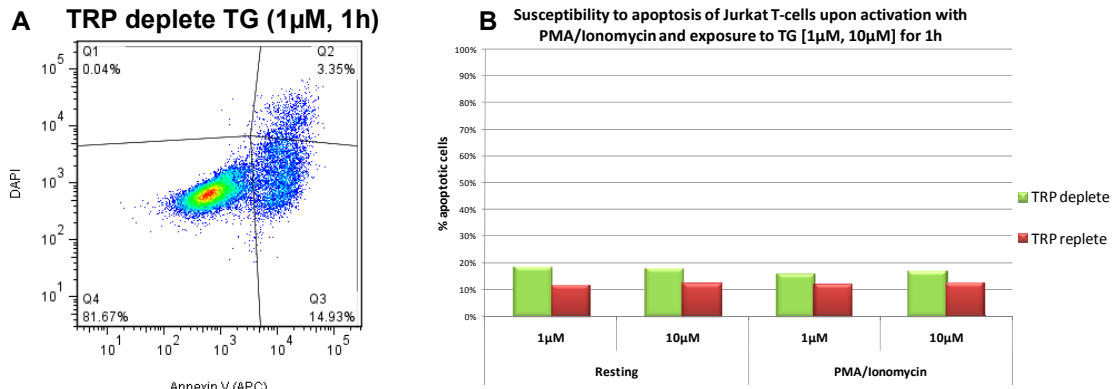


Fig. 48: Apoptotic response of Jurkat T-lymphocytes maintained in culture medium supplemented with 1 % TRP free serum
(A) Representative Annexin V / DAPI staining dotplot of resting human Jurkat T-cells cultured in TRP free medium and stimulated with 1 μ M TG for 1 h; **(B)** Susceptibility to apoptosis of human Jurkat T-cells cultured in TRP deplete and TRP replete medium and stimulated with TG (1 μ M, 10 μ M, 1h)

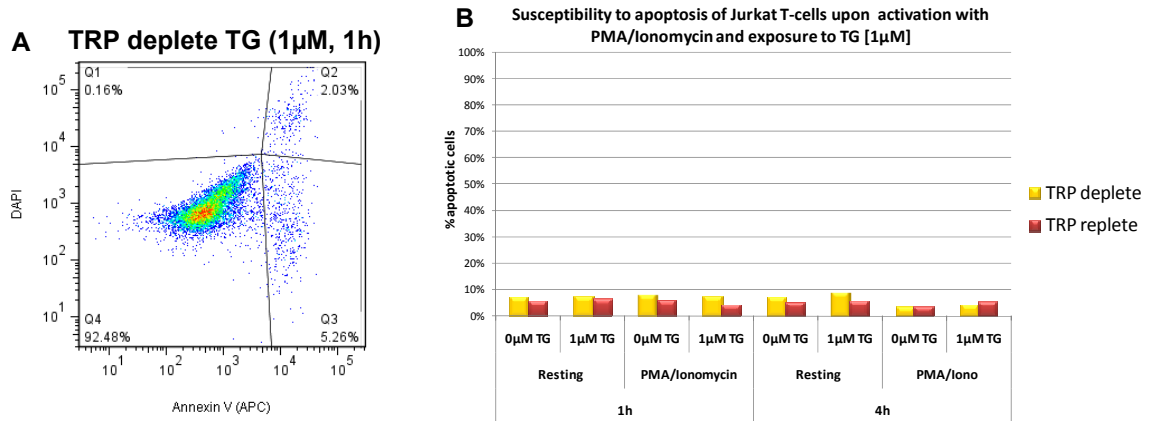


Fig. 49: Apoptotic response of Jurkat T-lymphocytes maintained in culture medium supplemented with 10 % TRP free serum
(A) Representative Annexin V / DAPI staining dotplot of resting human Jurkat T-cells cultured in TRP free medium and stimulated with 1 μ M TG for 1 h; **(B)** Susceptibility to apoptosis of human Jurkat T-cells cultured in TRP deplete and TRP replete medium and stimulated with TG (1 μ M, 1h, 4h)

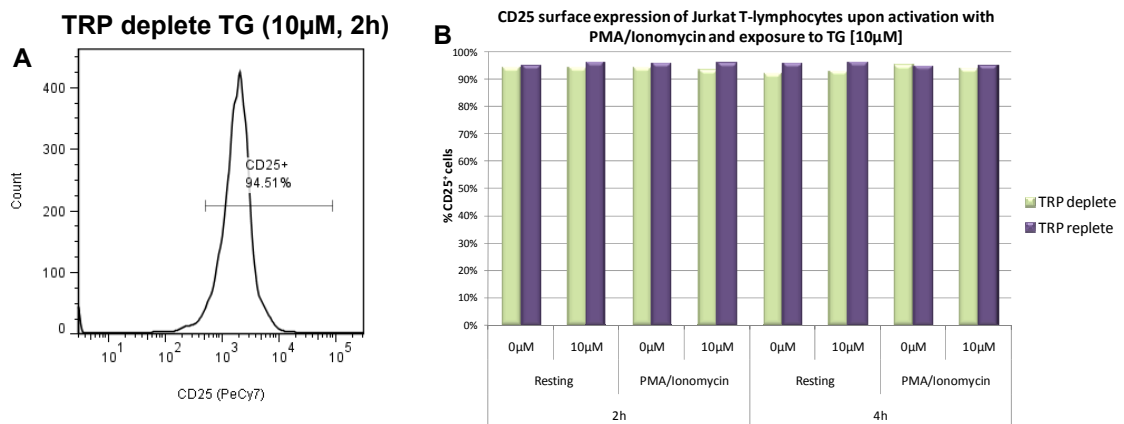


Fig. 50: CD25 surface expression on Jurkat T-cells
(A) CD25 surface expression on Jurkat T-cells upon activation with PMA/Ionomycin and stimulation with TG (10 μ M, 2 h, 4 h)
(B) Histogram of CD25 surface expression of resting Jurkat T-cells cultured in TRP free medium and stimulated with TG [10 μ M] for 2 h

D – RESULTS

2.2 Initiation of the integrated stress response in human Jurkat T-lymphocytes

Having ascertained that in cell cultures supplemented with even low TRP free serum concentrations and exposure to TG did not induce overt apoptosis in Jurkat cells, the effects of TG on Jurkat T-cells were analyzed for its ability to induce stress response proteins to serve as a positive control.

Jurkat cells (1×10^6) were cultured in TRP replete medium, supplemented with 1 % TRP free serum, and exposed to TG (1 μ M, 10 μ M) for up to 1h.

Compared to untreated cells (0 μ M), exposure to 1 μ M and 10 μ M TG induced an up to 1,24 and 1,61-fold increase in phosphorylated eIF2 α , respectively (Fig. 51 and Fig. 52), peaking after 60 min of exposure. Induction of stress related proteins through exposure to TG was a rapid process, detectable within 15 minutes, which is in line with published data [159, 167, 174, 196, 214, 258]. In contrast, constitutively expressed eIF2 α was not affected by TG treatment and relatively steadily expressed, except for the 60 min timepoint, illustrated by an approximately 20 % decrease in eIF2 α phosphorylation.

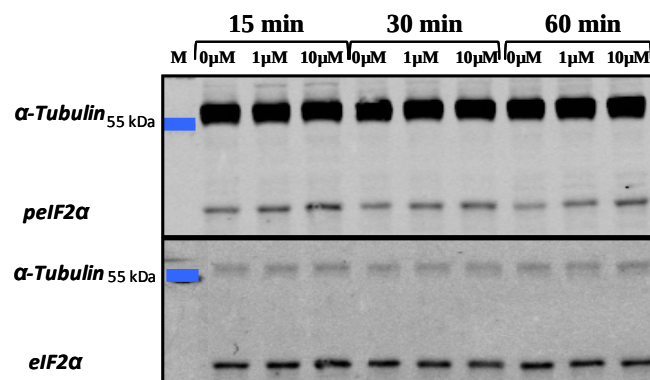


Fig. 51: Effects of TG stimulation on Jurkat T-cells

Phosphorylation of eIF2 α in Jurkat T-cells upon TG treatment [1 μ M, 10 μ M] for different time points (15 min, 30 min, 60 min)

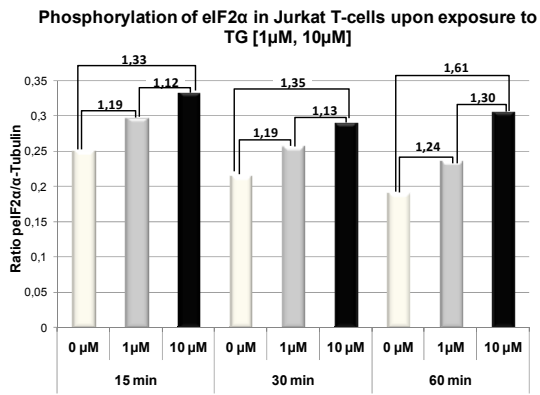


Fig. 52: Densitometric analysis of eIF2 α phosphorylation in Jurkat cells upon challenge with TG (1 μ M, 10 μ M) for up to 60 min

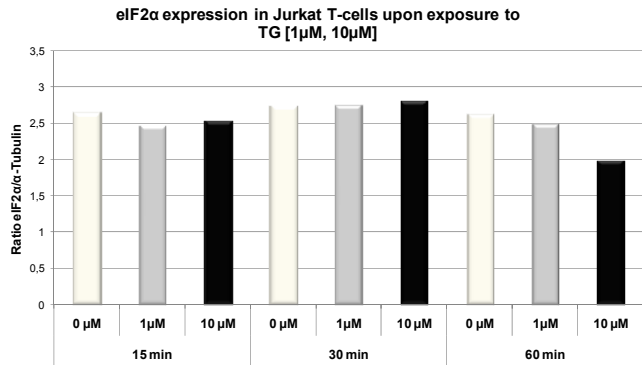


Fig. 53: Densitometric analysis of constitutive eIF2 α expression in Jurkat cells upon challenge with TG (1 μ M, 10 μ M) for up to 60 min

D – RESULTS

To investigate the cellular response of Jurkat cells to TRP deprivation, Jurkat T-cells (1×10^6) were activated with PMA/Ionomycin and either cultured in TRP deplete or TRP replete medium, both supplemented with 10 % TRP free serum, for 24 h. Thereafter, cells were challenged with TG (1 μ M, 10 μ M) for up to 4 h.

A decrease in eIF2 α phosphorylation upon activation with PMA/Ionomycin was observed in cells maintained in TRP replete culture medium which resulted in an increase of the fold change between TRP deplete and TRP replete culture conditions (Fig. 54). Addressing the molecular background of this observation is beyond the scope of this thesis.

In contrast to the findings with phosphorylation of eIF2 α , the levels of CHOP expression remained constant upon activation in cells maintained in TRP replete medium, whereas CHOP expression was enhanced in cells cultured in TRP deplete medium and even further increased upon challenge with TG compatible with activating the GCN2 pathway.

Cellular stress, indicated by increased levels of p-eIF2 α expression, was additionally increased upon exposure to TG, as represented by a fold increase of 1,16 and 1,73 in resting and activated cells, respectively. However, no elevated levels of eIF2 α phosphorylation were observed under TRP replete culture conditions.

In contrast to p-eIF2 α , CHOP expression levels were elevated in cells maintained in TRP deplete as well as TRP replete culture medium, as shown by a 2,18- and 3,37-fold induction in resting and in activated cells. Levels of CHOP expression were particularly enhanced in TRP deplete versus TRP replete medium indicated by a 4,22-fold increase in resting and a 9,04-fold increase in activated cells, respectively. Unphosphorylated eIF2 α was stably expressed upon challenge with TG, whether cells were maintained in TRP deplete or TRP replete medium.

Thus, Jurkat cells represented a model system, in which stress response proteins were inducible by exposing cells to TRP deplete culture conditions as well as to the ER stress inducing compound TG.

D – RESULTS

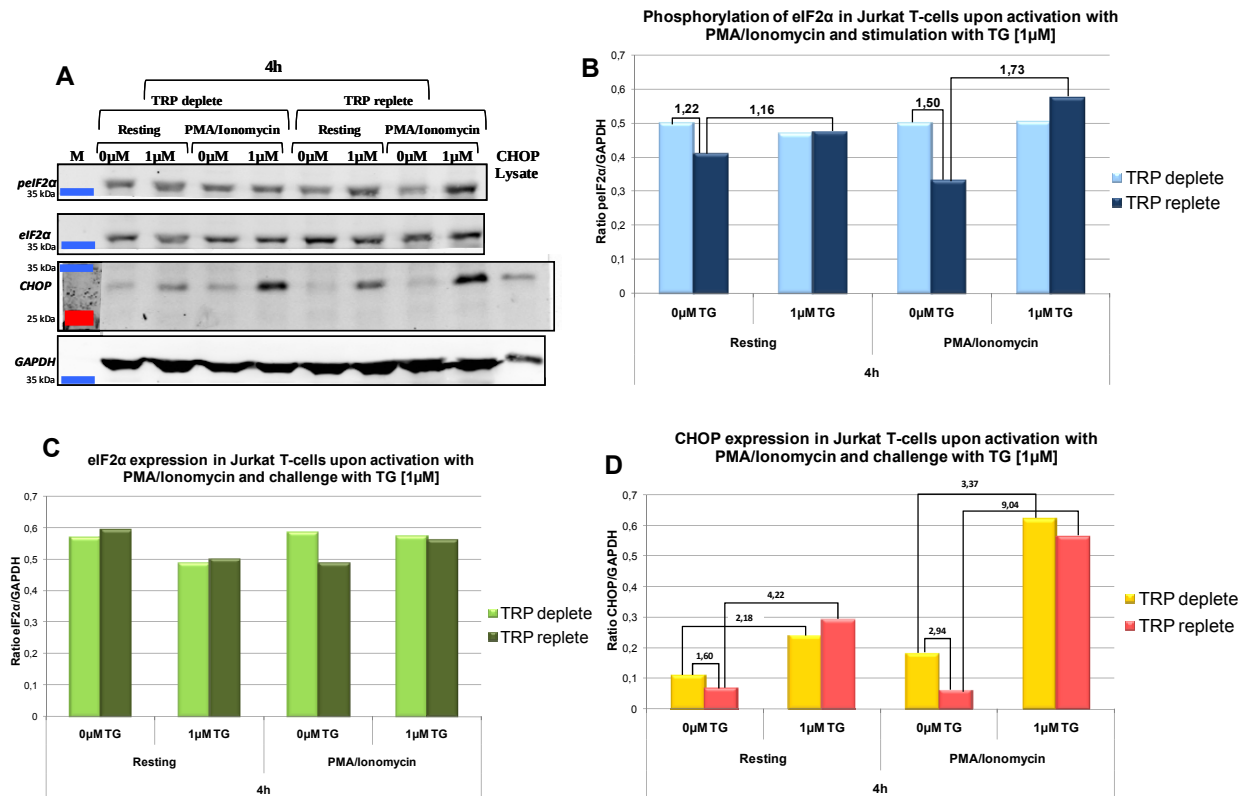


Fig. 54: Induction of the ISR in the Jurkat T-cell line

(A) ISR marker protein expression; (B) Densitometric analysis of eIF2α phosphorylation; (C) Densitometric analysis of endogenous eIF2α expression; (D) Densitometric analysis of CHOP expression. Fold increase was calculated by dividing ratios of TRP deplete by TRP replete medium.

	Without TG			
	Resting		Activated	
	TRP deplete	TRP replete	TRP deplete	TRP replete
eIF2α	0,96		1,20	
pelf2α	1,22		1,50	
CHOP	1,60		2,94	

Table 8: Fold increase of ISR marker protein expression in Jurkat cells

Fold increase was calculated by dividing the ratio values of TRP deplete by TRP replete conditions
Numbers of pelf2α and eIF2α indicate mean values of 2 experiments (4 h timepoint).

Overall these data suggest that the integrated stress response was clearly detectable in Jurkat T-cells upon TRP starvation, which was indicated by increased expression of two marker molecules, pelf2α and CHOP (Table 8).

D – RESULTS

2.3 Autophagy in Jurkat T-lymphocytes upon TRP deprivation

In order to investigate the effects of TRP deprivation on the induction of autophagy, Jurkat T-cells (1×10^6) were activated with PMA/Ionomycin, cultured for 24 h in either TRP deplete or TRP replete medium, both supplemented with 10 % TRP free serum, and challenged with TG ($1 \mu\text{M}$, 1 h).

The results show that, in contrast to ISR related proteins, autophagy was not induced in Jurkat cells, as depicted in Fig. 55 and Fig. 56. Beclin-1 expression was not enhanced in cells cultured under TRP starvation compared to cells cultured in TRP replete medium. Autophagy induction was as well monitored by the autophagosomal form of LC3. However, the antibody used did not work out for proper detection (data not shown).

These data indicate that autophagy is not induced during the cellular response to TRP deprivation but rather that Jurkat T-cells sensing TRP deficiency respond by a specific induction of the ISR.

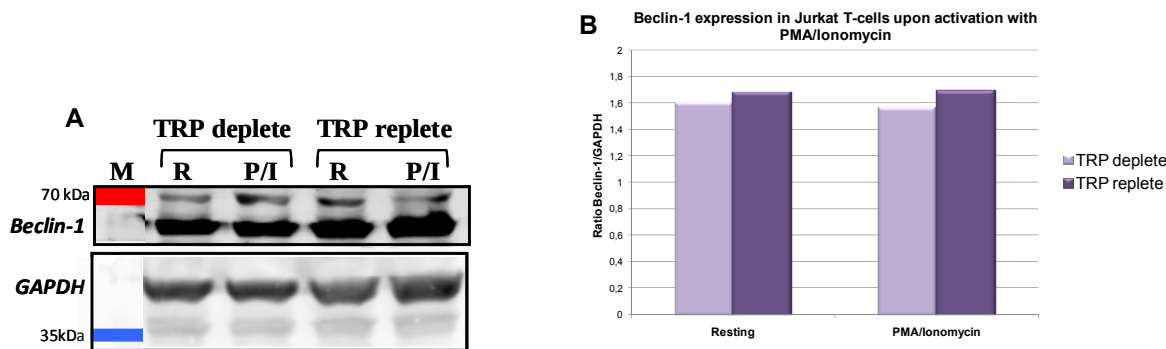


Fig. 55: Autophagy induction in Jurkat T-cells
(A) Autophagy induction monitored via Beclin-1 protein expression and (B) respective densitometric analysis

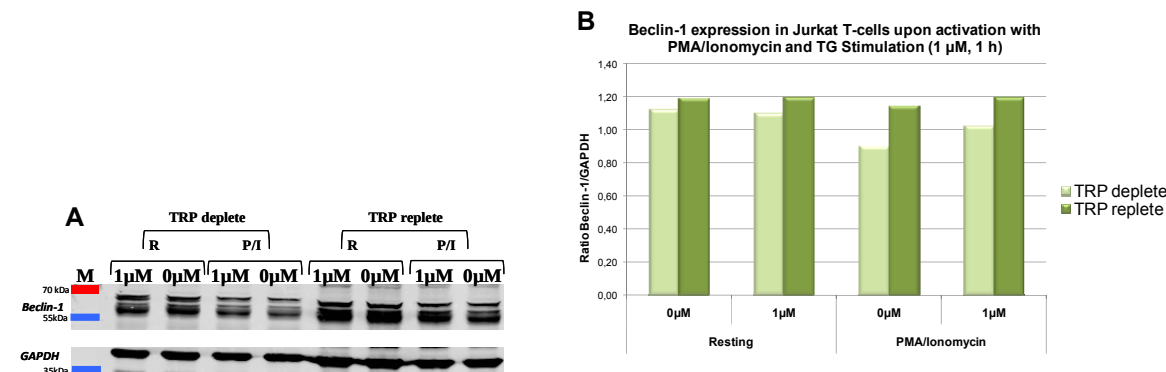


Fig. 56: Autophagy induction in human Jurkat T-cells
(A) Beclin-1 protein expression in Jurkat cells upon TRP deprivation and (B) respective densitometric analysis

3 The Integrated Stress Response and alternative pathways for the adaption to nutrient deprivation in primary human T-lymphocytes

3.1.1 Apoptotic response of primary human T-lymphocytes to TG treatment

To define optimal concentrations for TG exposure on primary human T-cells, susceptibility of apoptosis was determined by Annexin V / DAPI staining. Therefore human primary T-lymphocytes (2×10^6) were activated with PMA/Ionomycin and cultured in either TRP deplete or TRP replete medium and stimulated with TG (1 μ M, 10 μ M) for 1 h.

As Fig. 57 shows, an increase in apoptotic response was observed when cells were stimulated with TG concentrations higher than 1 μ M. Thus in contrast to Jurkat T-cells, primary human T-lymphocytes do not tolerate TG doses greater than 1 μ M.

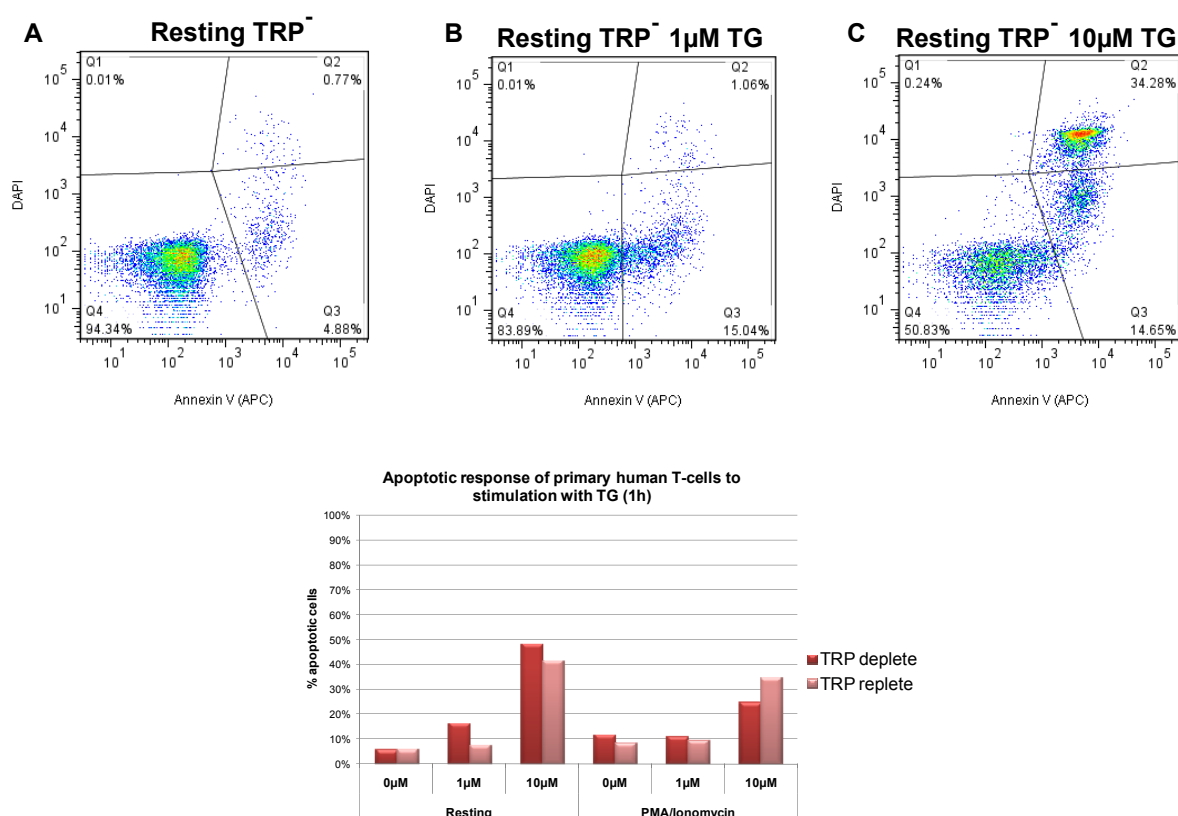


Fig. 57: Apoptosis rate of human T-cells upon TG stimulation

Annexin V / DAPI staining of flow cytometric measurement (A) Resting primary human T-cells cultured in TRP deplete medium (B) Resting primary human T-cells cultured in TRP deplete medium and stimulated with TG [1 μ M] (C) Resting primary human T-cells cultured in TRP deplete medium and stimulated with TG [10 μ M]

D – RESULTS

3.2 Effects of TRP deprivation on the proliferative response of primary human T-lymphocytes

To characterize functional effects of TRP deprivation on primary human T-cells, CD25 cell surface expression, proliferation, absolute cell number and apoptotic decline were determined in parallel. For this purpose, human primary T-lymphocytes (1×10^5) were activated with α CD3/CD28 and cultured in either TRP deplete or TRP replete medium for up to 7 days. 10 % TRP free serum was determined to represent optimal serum concentration for medium supplementation. This concentration was used throughout the experimental investigations in primary human T-cells.

Cells cultured in TRP deplete as well as TRP replete medium showed similar levels of expressed CD25 on their surface, induced by α CD3/CD28 stimulation, indicating cellular activation (Fig. 58). The level of CD25 expression slightly decreases over time in cells cultured under TRP starvation.

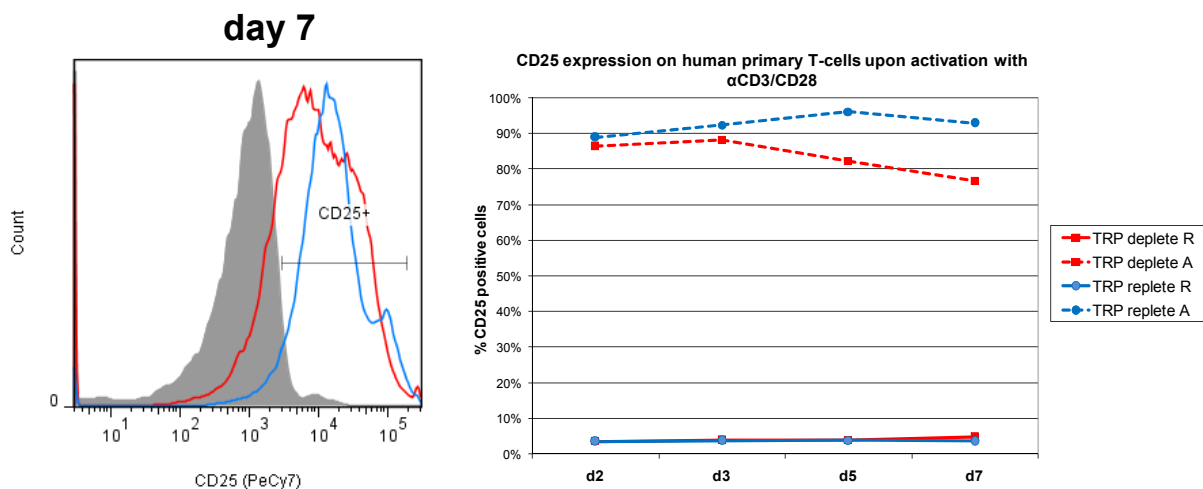
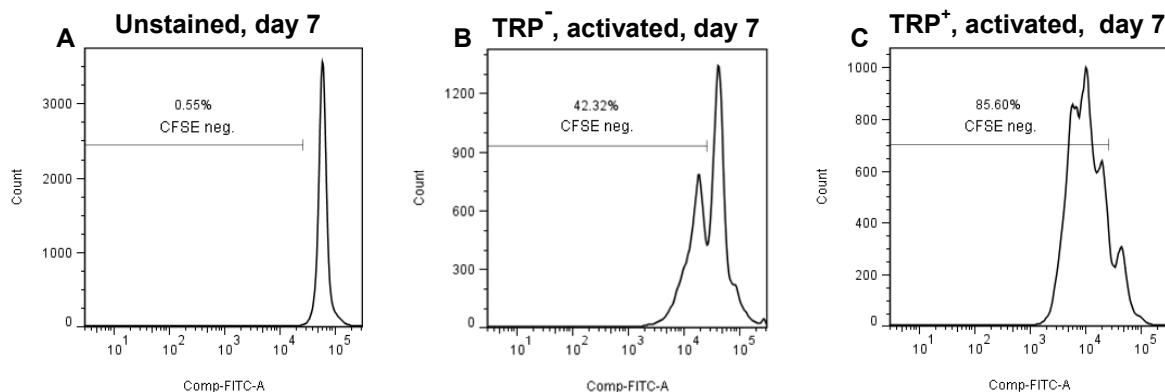


Fig. 58: CD25 cell surface expression of primary human T-cells upon activation with α CD3/CD28
CD25 expression on primary human T-cells after activation on day 7: Resting (grey filled line), activated T-cells cultured in TRP deplete medium (red line) and activated T-cells cultured in TRP replete medium (blue line)

D – RESULTS

The proliferative response of T-cells stimulated in TRP deplete and TRP replete cell culture medium was assessed by CFSE dilution and the division index as given by the FlowJo program. The percent CFSE negative cells depicts the total proportion of cells that have undergone cell division by dilution of the dye. The division index depicts the average number of cell divisions the responding cell population underwent, meaning it ignores the peak of undivided cells and has a particular focus on the performance of the cells, which are dividing. Our results show that primary human T-lymphocytes, activated with α CD3/CD28 and cultured under TRP replete conditions, showed abundant proliferation, indicated by 90 % CFSE negative cells (Fig. 59). In contrast, cells cultured in TRP deplete conditions showed a significantly impaired proliferative response to α CD3/CD28 stimulation. This was indicated by a 50 % reduction of CFSE negative cells. Likewise, the division index was reduced by 60% in cells stimulated in TRP deplete conditions, which is compatible with an inability of cells responding to α CD3/CD28 stimulation to undergo further cell divisions. These observations suggest that a small proportion of cells is able to divide, but 60 % of the total cell population is arrested in cell cycle, as claimed by Munn *et al.* in 1999. ^[118]



D – RESULTS

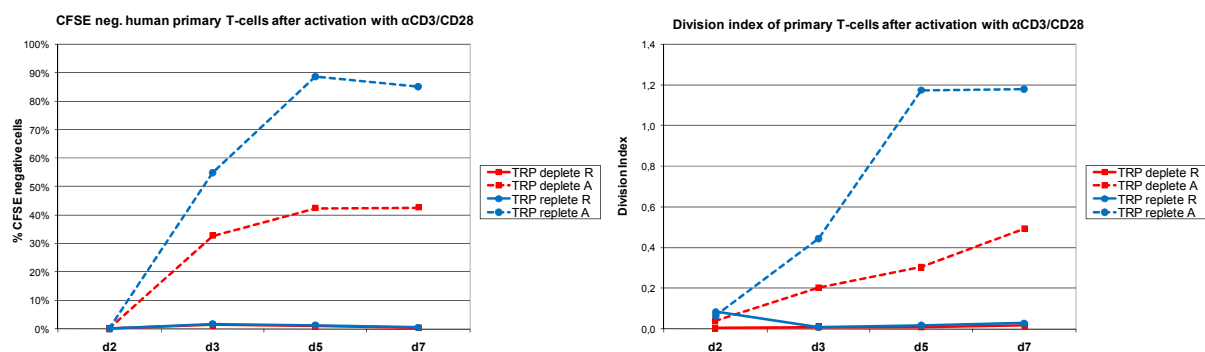


Fig. 59: Proliferative capacity of human primary T-cells

CFSE dilution and division index in TRP deplete and TRP replete medium supplemented with 10 % TRP free serum on day 7: (A) Unstained Control; (B) Activated primary human T-cells cultured in TRP deplete medium; (C) Activated primary human T-cells cultured in TRP replete medium

		Division Index			
		day 2	day 3	day 5	day 7
Activated	TRP deplete	0,04	0,21	0,31	0,50
	TRP replete	0,07	0,45	1,18	1,18

Table 9: Division index of primary human T-cells activated with α CD3/CD28

These data are consistent with previous published literature ^[118] which shows that primary human T-lymphocytes cultured under TRP starvation were not inhibited to be activated by α CD3/CD28, but were markedly impaired to proliferate.

D – RESULTS

The effect of TRP depletion became even clearer when absolute cell numbers were analyzed. The absolute count of T-cells cultured in TRP deplete conditions remained at the same level, represented by 1×10^5 cells, even after 7 days of activation with α CD3/CD28, whereas cells cultured in TRP replete culture conditions were able to expand in total cell number of up to three fold, represented by $3,2 \times 10^5$ cells (Fig. 60). No differences in absolute cell numbers were denoted in resting T-cells cultured in TRP deplete or TRP replete medium.

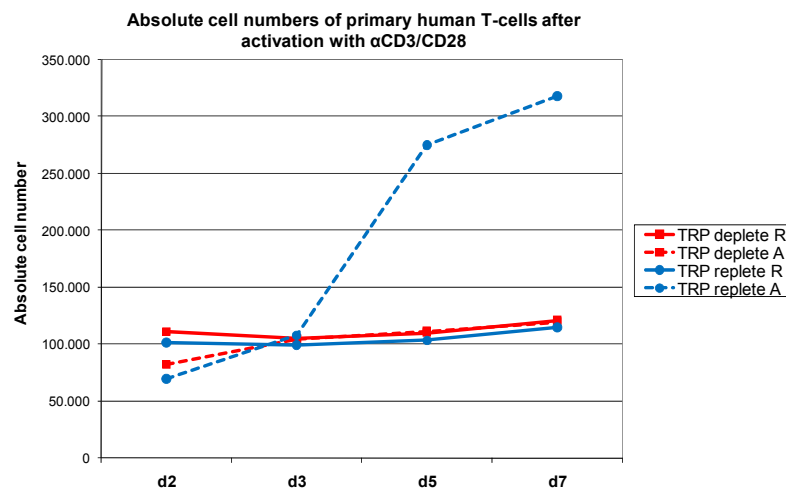


Fig. 60: Expansion of primary human T-cells
Absolute cell number of primary human T-cells cultured in either TRP deplete or TRP replete medium

D – RESULTS

Since CFSE dilution showed 40 % of proliferating cells in TRP deplete medium but in parallel absolute cell count remained constant, we speculated whether altered apoptosis would explain this paradox finding. Indeed, as depicted in Fig. 61, cells cultured in TRP deplete medium are more susceptible to undergo apoptotic cell death (50 % Annexin V positive cells by day 5) as compared to TRP replete conditions (12 % Annexin V positive cells by day 5).

Thus, the increased apoptotic response of T-lymphocytes cultured under tryptophan starvation may represent the correlation between proliferation (Fig. 59) and constant cell number (Fig. 60).

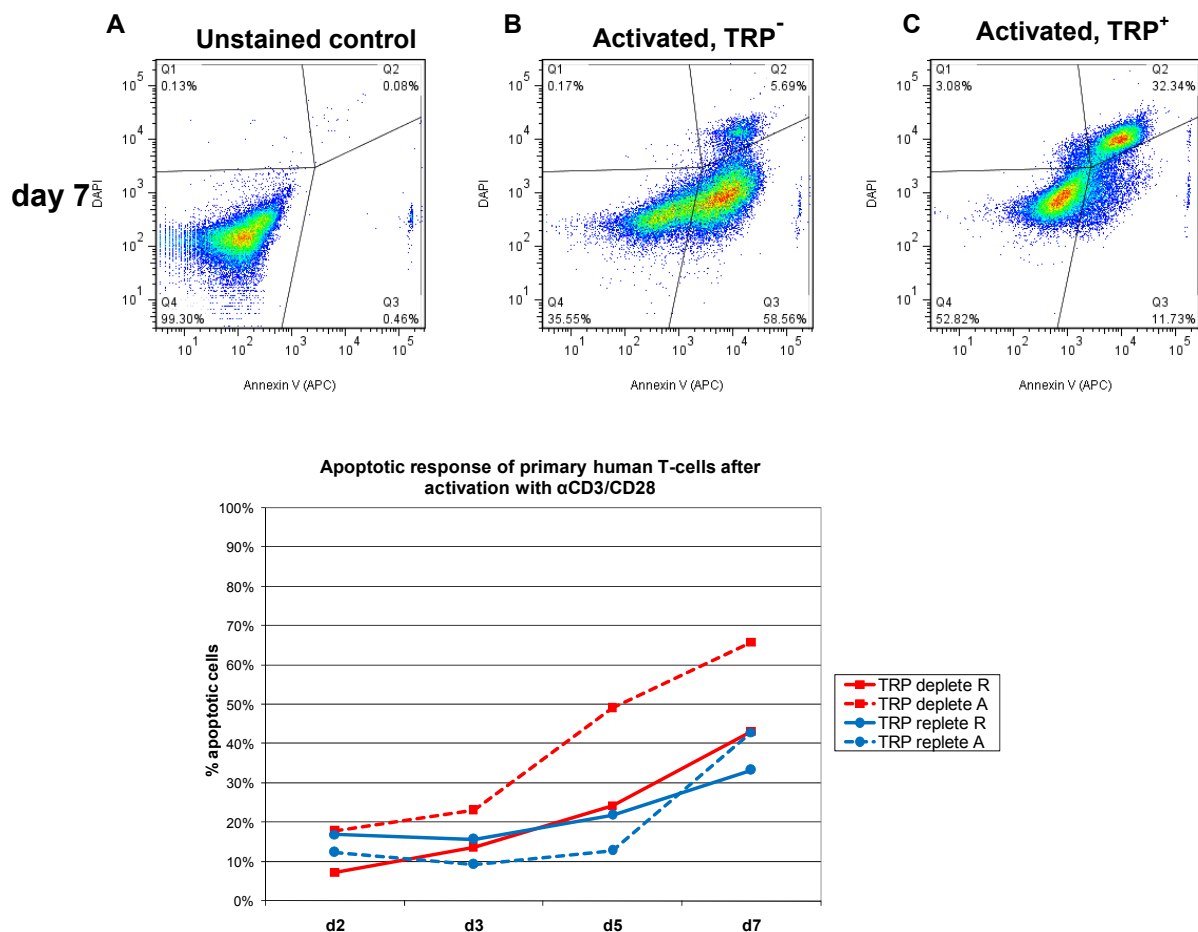


Fig. 61: Apoptotic susceptibility of human primary T-cells

Annexin V / DAPI staining of primary human T-cells (A) Unstained Control; (B) Activated human primary T-cells cultured in TRP deplete medium; (C) Activated human primary T-cells cultured in TRP replete medium

3.3 Induction of the ISR in primary human T-lymphocytes

Optimal cell numbers were determined to range between 6×10^6 and 8×10^6 , to detect ISR responsive proteins by western blot. In our effort to reproduce published data by Munn *et al.*, first, primary human T-cells (7×10^6) were activated with PMA/Ionomycin and cultured in either TRP deplete or TRP replete medium for 24 h.

The CHOP transfected lysate shows a positive signal in donor 2, though no CHOP expression was observed in primary human T-cells, which means that CHOP was not expressed in primary human T-cells (Fig. 62).

As Fig. 63 illustrates, no increase in eIF2 α phosphorylation was denoted in resting or activated cells cultured in TRP deplete medium, which was represented by a fold change of 1,07 and 1,04 (Table 10). Additionally, activation with PMA/Ionomycin did not lead to an increased phosphorylation of eIF2 α , shown by a fold change of only 0,94 and 0,95.

These results indicated that primary human T-lymphocytes did not respond to TRP deprivation by the initiation of an integrated stress response neither in resting conditions nor upon activation with PMA/Ionomycin.

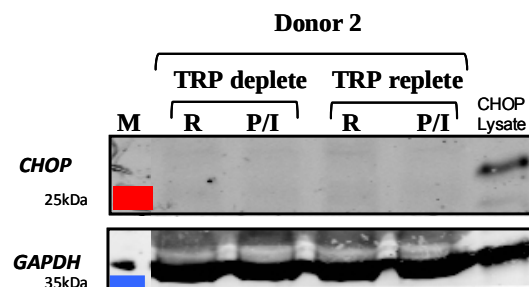


Fig. 62: Induction of ISR in human primary T-cells
CHOP expression upon activation with PMA/Ionomycin

D – RESULTS

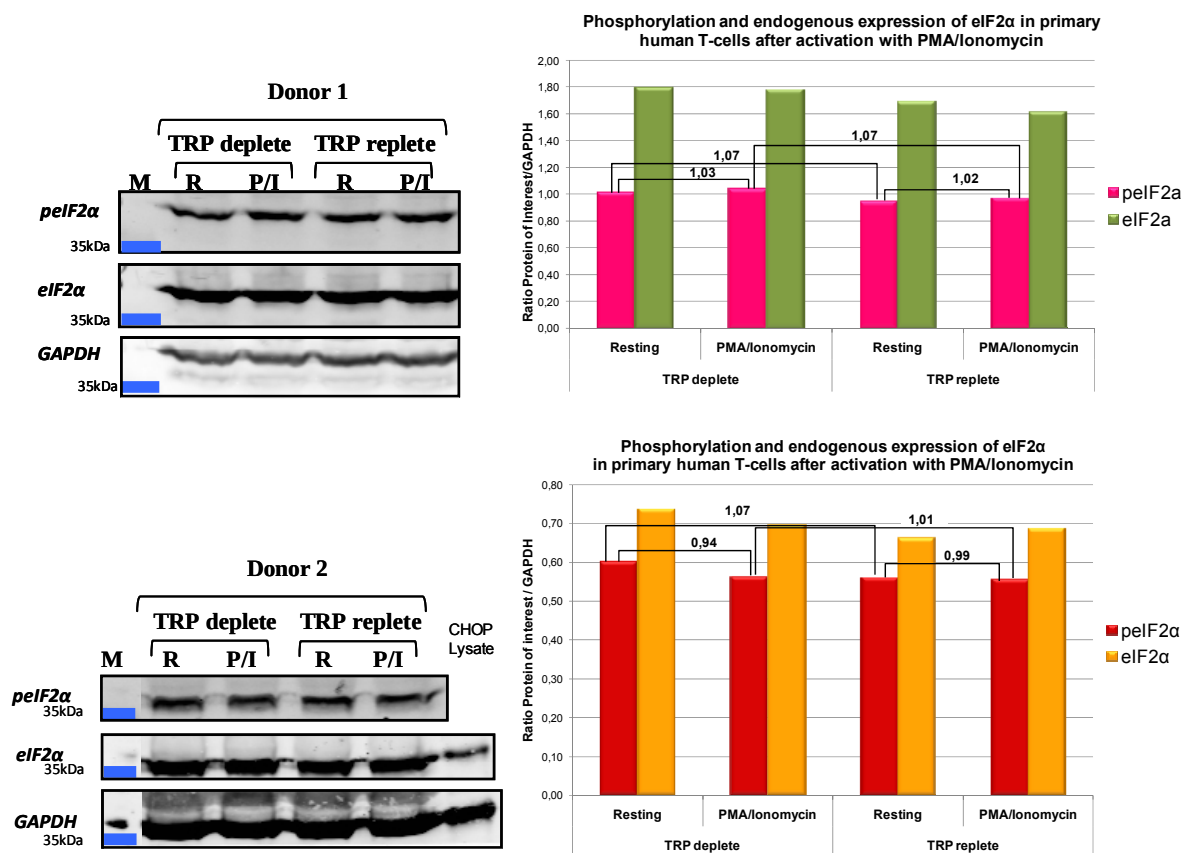


Fig. 63: Initiation of the ISR in human primary T-cells

Phosphorylation status and endogenous expression of eIFα upon activation with PMA/Ionomycin. Numbers depict fold changes. Fold changes were calculated by dividing TRP deplete by TRP replete or activated by resting ratios.

		Fold Change	
		TRP deplete	TRP replete
<u>pelf2α</u>	Resting	1,07	
	Activated	1,04	

		Fold Change	
		Activated	Resting
<u>pelf2α</u>	TRP deplete	0,99	
	TRP replete	1,00	

Table 10: Fold increase of stress response marker proteins
Numbers indicate the mean of the fold increase of 2 experiments

We found that cellular stimulation with α CD3/CD28, compared to PMA/Ionomycin, induced more pronounced activation, indicated by the induction of the cell surface marker CD25. Therefore, as next step, human primary T-lymphocytes (6×10^6) were activated with α CD3/CD28 and cultured in either TRP deplete or TRP replete medium for up to 7 days. In addition, cells were also challenged with TG (1 μ M) for 1h, in order to increase intracellular stress.

Remarkably we found that activation with α CD3/CD28, in contrast to PMA/Ionomycin in murine splenocytes, induced higher phosphorylation of eIF2 α and increased expression of CHOP (Fig. 64) in activated as compared to resting cells, irrespective of whether the cells were cultured in TRP deplete or TRP replete medium.

Of note, no elevated levels of p-eIF2 α or CHOP were observed in cells cultured in TRP deplete or TRP replete medium, whether or not the cells were resting or activated, indicating that in fresh human T-cells the ISR is not inducible by tryptophan starvation.

The ER stress inducer TG was shown to enhance expression levels of p-eIF2 α and CHOP in resting cells maintained in TRP deplete conditions on day 2, but had only a marginal effect whether or not cells were activated (Table 11).

These data are in contrast to results obtained from Jurkat T-cells, which show that upon TG challenge, increased phosphorylation of eIF2 α is solely present by culturing cells in TRP replete medium. Furthermore the data partially coincide with results obtained from murine cells. No increased eIF2 α phosphorylation was observed in mouse splenocytes in TRP deplete cell culture conditions.

Overall, the induction of stress related proteins in primary human T-lymphocytes is rather dependent on the type of activation stimulus than on withdrawal of TRP from the environment.

D – RESULTS

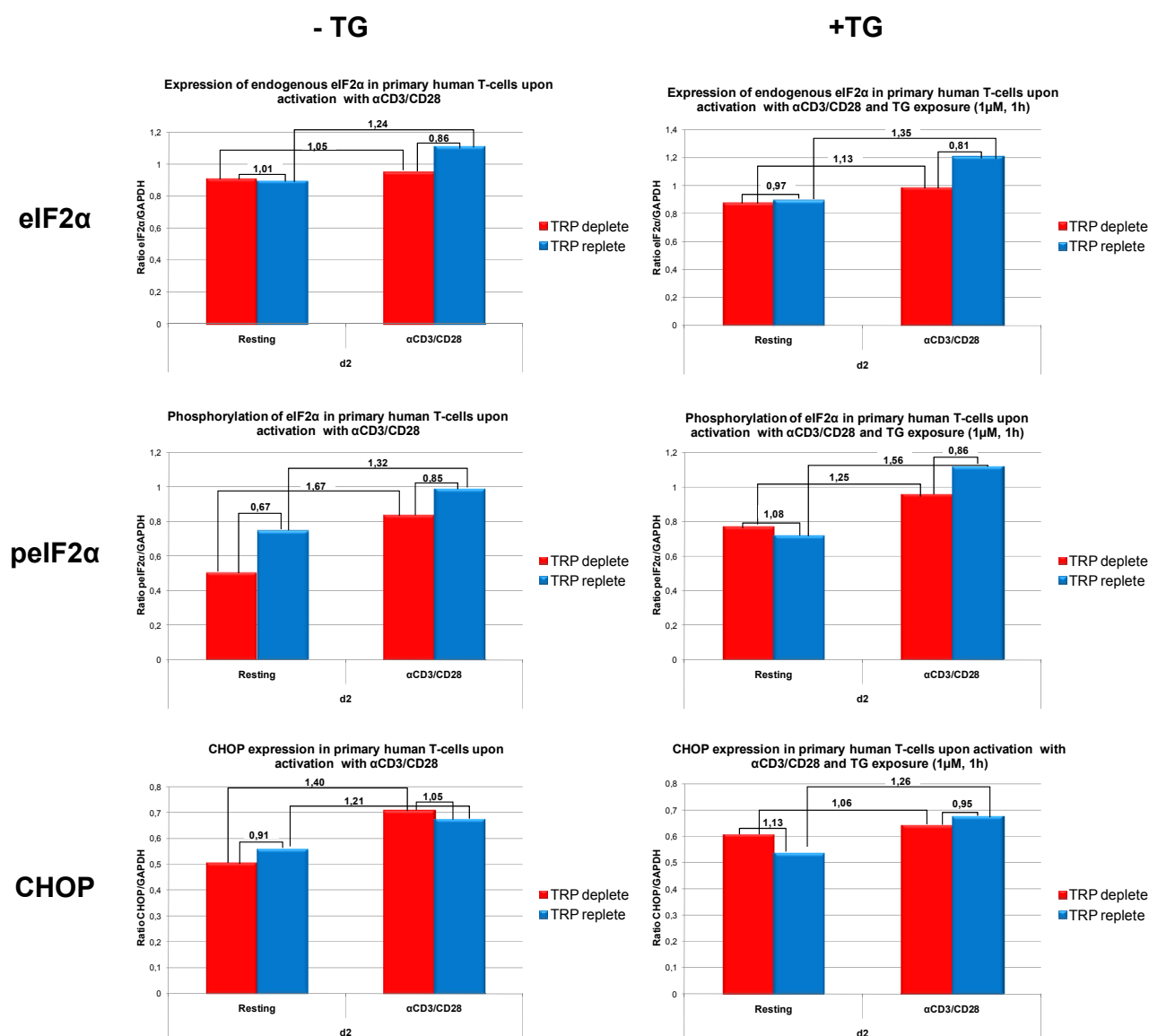


Fig. 64: Phosphorylation and endogenous expression of eIF2α and CHOP in αCD3/CD28 activated primary human T-cells
The fold increase was calculated by dividing TRP deplete by TRP replete or activated by resting ratios

		Fold Increase by TG	
		TRP deplete	TRP replete
eIF2α	<i>Resting</i>	0,96	1,00
	<i>Activated</i>	1,04	1,09
peIF2α	<i>Resting</i>	<u>1,53</u>	0,96
	<i>Activated</i>	1,14	1,13
CHOP	<i>Resting</i>	<u>1,20</u>	0,95
	<i>Activated</i>	0,90	1,00

Table 11: Effects of TG stimulation on αCD3/CD28 activated primary human T-cells
The fold increase was calculated by dividing +TG by -TG ratios

Because no induction of the ISR was observed in cells harvested after 2 days of TRP starvation, we speculated that in order to detect ISR induction, primary human T-cells need to be exhausted from intracellular tryptophan stores. Therefore, human primary T-cells (6×10^6) were activated with α CD3/CD28 on day 0 and cultured in either TRP deplete or TRP replete medium, both supplemented with 10 % TRP free serum, for up to 7 days. In order to increase intracellular stress, cells were additionally exposed to TG (1 μ M) for 1 h.

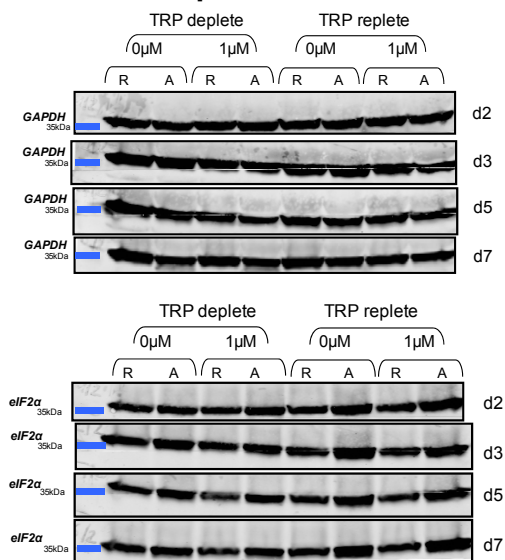
Activation with α CD3/CD28 induced increased p $\text{eIF2}\alpha$ and endogenously expressed $\text{eIF2}\alpha$ and CHOP on d2 to a similar extent in cells either cultured in TRP deplete and TRP replete medium (Table 12). Increased expression of endogenous $\text{eIF2}\alpha$ and CHOP was retained for up to 7 days post activation. However, as shown before, whole protein levels are increased in the primary phase of activation through stimulation with α CD3/CD28, which suggests that activation slightly increases total protein expression, which is not ISR specific.

Remarkably, no increased $\text{eIF2}\alpha$ phosphorylation or CHOP expression was observed even after 7 days of culture in cells maintained in TRP deplete medium versus TRP replete culture medium.

However, a slight enhancement, represented by a 1,3-fold increase, in p $\text{eIF2}\alpha$ and CHOP was denoted at day 5 post activation, indicating that enforced cellular stress (prolonged activation, TG exposure and TRP exhaustion) may elicit the induction of the ISR (Fig. 67 and Table 12).

D – RESULTS

Endogenous expressed control proteins



ISR induced stress proteins

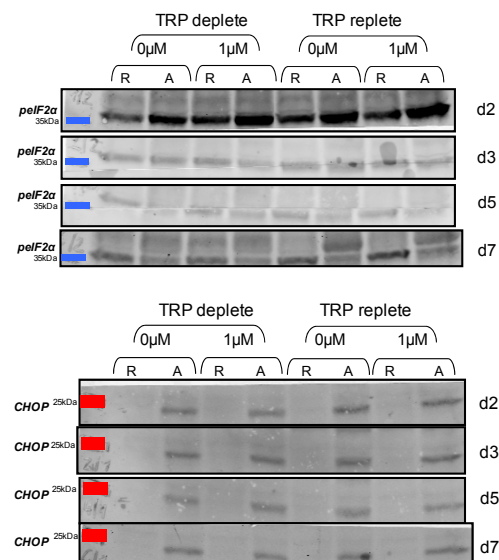
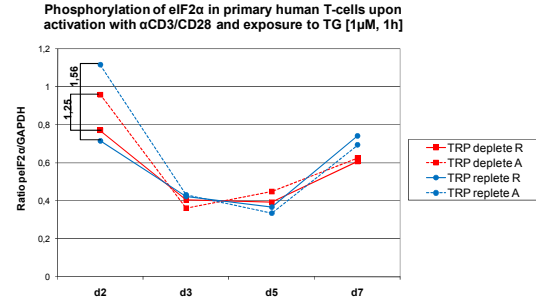
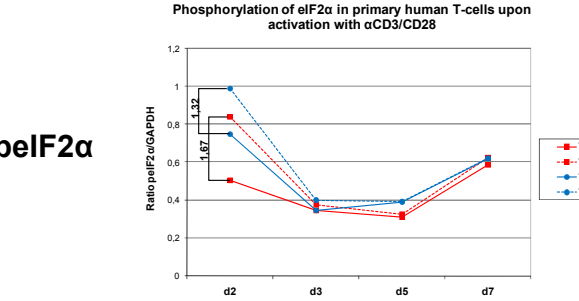
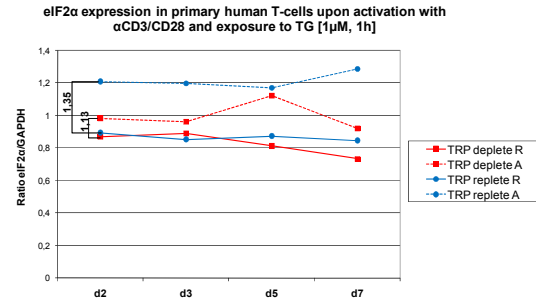
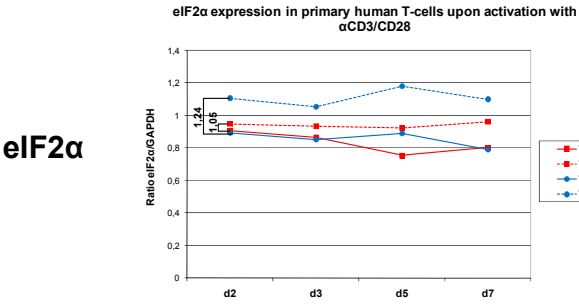


Fig. 65: ISR marker protein expression upon activation with αCD3/CD28 and exposure to TG [1 μM, 1 h]

- TG

+ TG



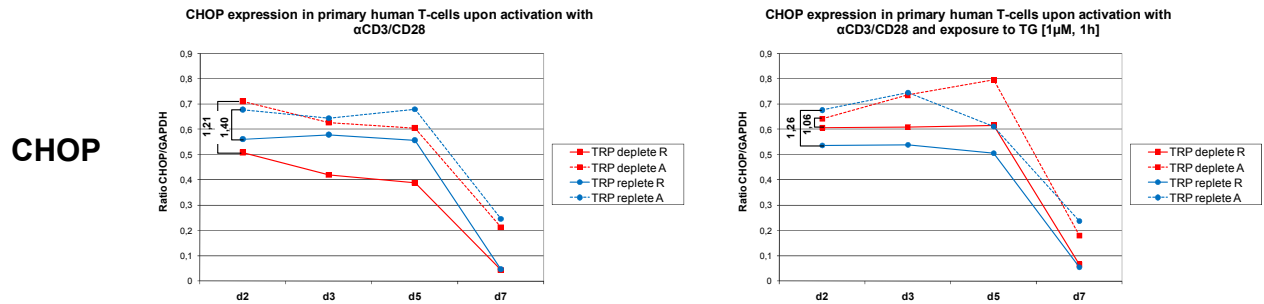


Fig. 66: Densitometric measurement of ISR marker protein expression after stimulation with α CD3/CD28 and TG [1 μ M, 1 h]

			-TG				+TG			
			d2	d3	d5	d7	d2	d3	d5	d7
eIF2α	Resting	trp deplete	1,01	1,02	0,85	1,01	0,97	1,04	0,93	0,87
		trp replete								
	Activated	trp deplete	0,86	0,89	0,78	0,87	0,81	0,80	<u>0,96</u>	0,72
		trp replete								
p-eIF2α	Resting	trp deplete	0,67	1,00	0,80	0,94	1,08	0,96	1,07	0,82
		trp replete								
	Activated	trp deplete	0,85	0,94	0,83	1,01	0,86	0,84	<u>1,34</u>	0,90
		trp replete								
CHOP	Resting	trp deplete	0,91	0,72	0,70	0,93	1,13	1,13	1,22	1,18
		trp replete								
	Activated	trp deplete	1,05	0,97	0,89	0,87	0,95	0,99	<u>1,30</u>	0,76
		trp replete								

Table 12: Fold changes in phosphorylation and endogenous expression of eIF2 α and CHOP in human primary T-cells over 7 days

Fold changes were calculated by dividing TRP deplete by TRP replete ratios

D – RESULTS

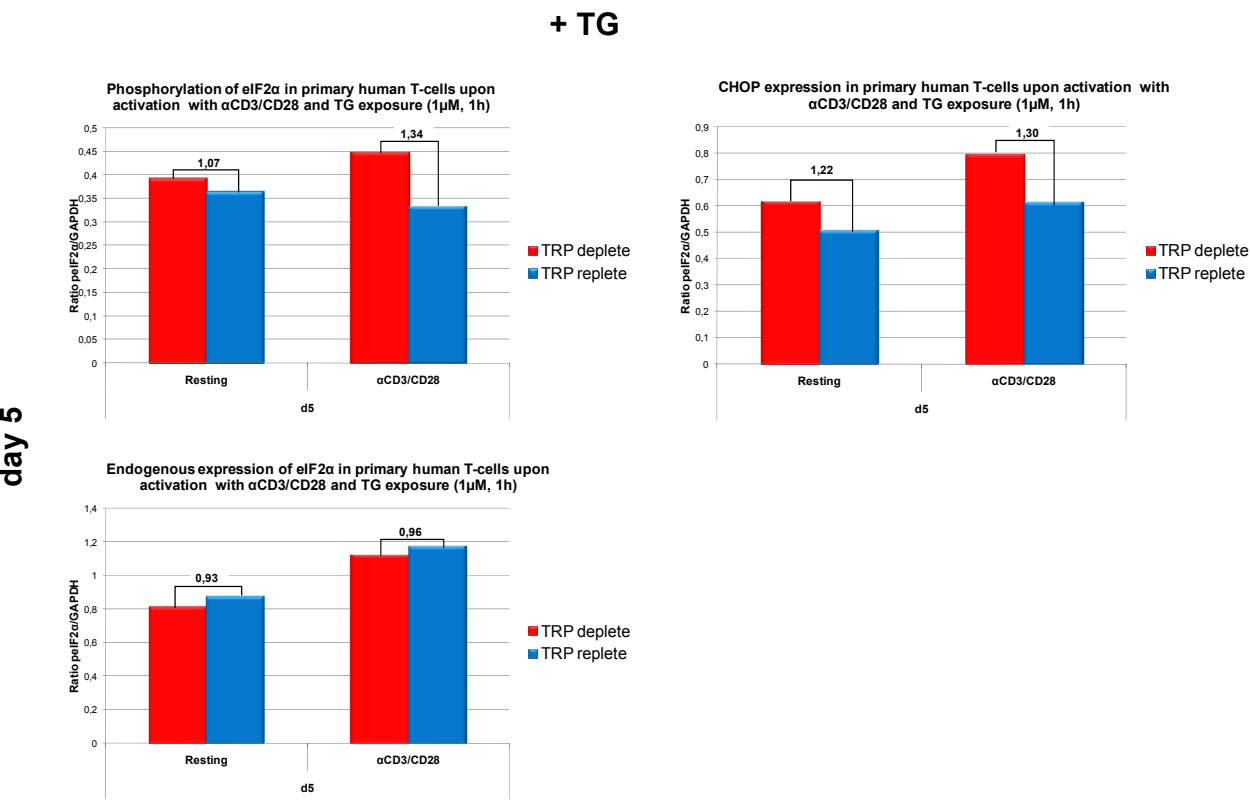


Fig. 67: Expression of stress related proteins in primary human T-cells on day 5 of maintenance in TRP deplete or TRP replete culture medium with additional TG exposure

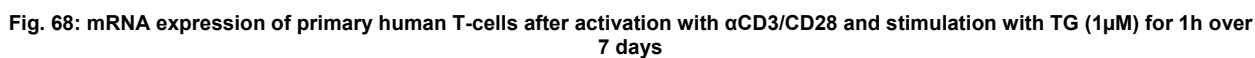
In parallel to investigating the ISR on protein level, the RNA expression profile during ISR induction was monitored.

mRNA expression of ISR specific genes was shown to be apparently different compared to protein expression of ISR responsive molecules. Activation through α CD3/CD28 did not increase the expression level of CHOP or ATF4 in contrast to observations made on translational level.

TG is able to increase CHOP and ATF4 mRNA levels in resting human T-cells in the initial phase of activation, represented by day 2 and day 3. Overall, resting cells cultured in TRP deplete conditions show slightly higher expression in stress responsive genes compared to cells cultured in TRP replete medium, whereas similar levels of mRNA expression was observed in activated cell cultures.

mRNA levels of constitutively expressed eIF2 α were comparable in resting cells cultured in TRP deplete and TRP replete as well as in activated cells, cultured either in TRP deplete or replete conditions. In contrast to CHOP and ATF4 mRNA, challenge with TG does not change expression levels of eIF2 α , neither in resting nor in activated cells.

These observations suggest that the induction of the ISR upon tryptophan deprivation is initiated on transcriptional, but not on translational level in primary human T-lymphocytes.



3.4 Alternative pathways for the adaption to nutrient deprivation in primary human T-lymphocytes

3.4.1 Induction of autophagy in primary human T-lymphocytes

Since autophagy is also regulated in the cellular adaption to stress, the next step was to investigate the involvement of autophagy upon tryptophan starvation in primary human T-cells. For this reason, human primary T-lymphocytes (6×10^6 , 7×10^6) were activated with PMA/Ionomycin or α CD3/CD28 and either cultured in TRP deplete or TRP replete medium, both supplemented with 10 % TRP free serum.

Autophagy induction was monitored by the expression of the autophagy specific protein Beclin-1. As shown in Fig. 69, Beclin-1 expression is not increased in cells cultured in TRP deplete medium.

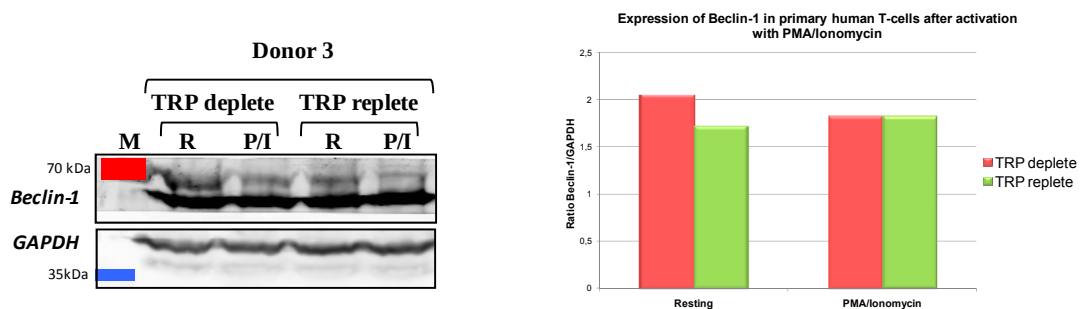


Fig. 69: Autophagy induction in primary human T-cell
 7×10^6 human primary T-lymphocytes stimulated with PMA/Ionomycin and cultured in TRP deplete or TRP replete medium for 24 h

D – RESULTS

To ascertain that intracellular TRP storages were fully emptied, human primary T-lymphocytes were activated through stimulation with α CD3/CD28 on d0 and maintained in culture for up to 7 days. Additionally, for increasing cellular stress, TG (1 μ M) was added for 1 h before the analysis of autophagy responsive proteins.

As outlined in Fig. 70, no enhanced Beclin-1 expression was observed when cells were starved of TRP, even in the presence of TG. However, as for the expression of stress related proteins, activation with α CD3/CD28 slightly enhances Beclin-1 expression levels, retaining for up to 7 days.

Summarizing, these results indicate that autophagy is not initiated in human primary T-lymphocytes upon TRP starvation.

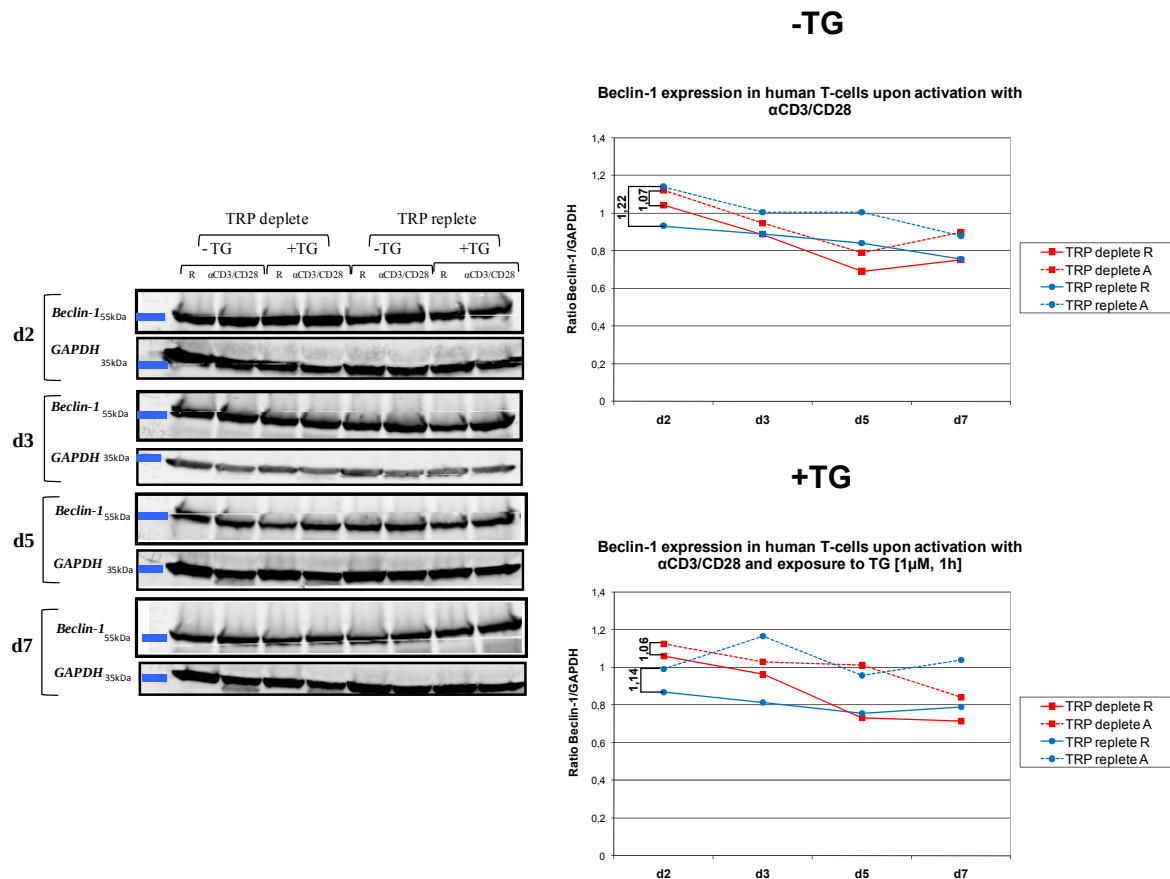


Fig. 70: Beclin-1 expression in primary human T-cells upon activation on day
 6×10^6 human primary T-lymphocytes were activated with α CD3/CD28, cultured in TRP deplete or TRP replete medium for 8 days and exposed to TG [1 μ M, 1 h]

3.4.2 Initiation of the mTOR pathway in primary human T-lymphocytes

Another key downstream signaling pathway, mTOR, was investigated in the context of TRP deprivation. Differently from ISR and autophagy induction, phosphorylation of mTOR and its downstream signaling molecule rpS6K decreases upon cellular stress.

Thus, in parallel to examining the ISR and autophagy, the involvement of mTOR in response to TRP withdrawal was investigated.

Therefore, human primary T-lymphocytes (6×10^6) were activated with α CD3/CD28 stimulation and cultured in TRP deplete or TRP replete medium, supplemented with 10 % TRP free serum, for up to 7 days. In order to increase intracellular stress, cells were additionally stimulated with TG (1 μ M) for 1 h.

As Fig. 71 shows, cellular activation by α CD3/CD28 resulted in an increase of mTOR and rpS6K phosphorylation as well as endogenous mTOR expression during the initial phase, which was in line with ISR responsive proteins. This increase is retained for 7 days peaking at a maximum on day 5.

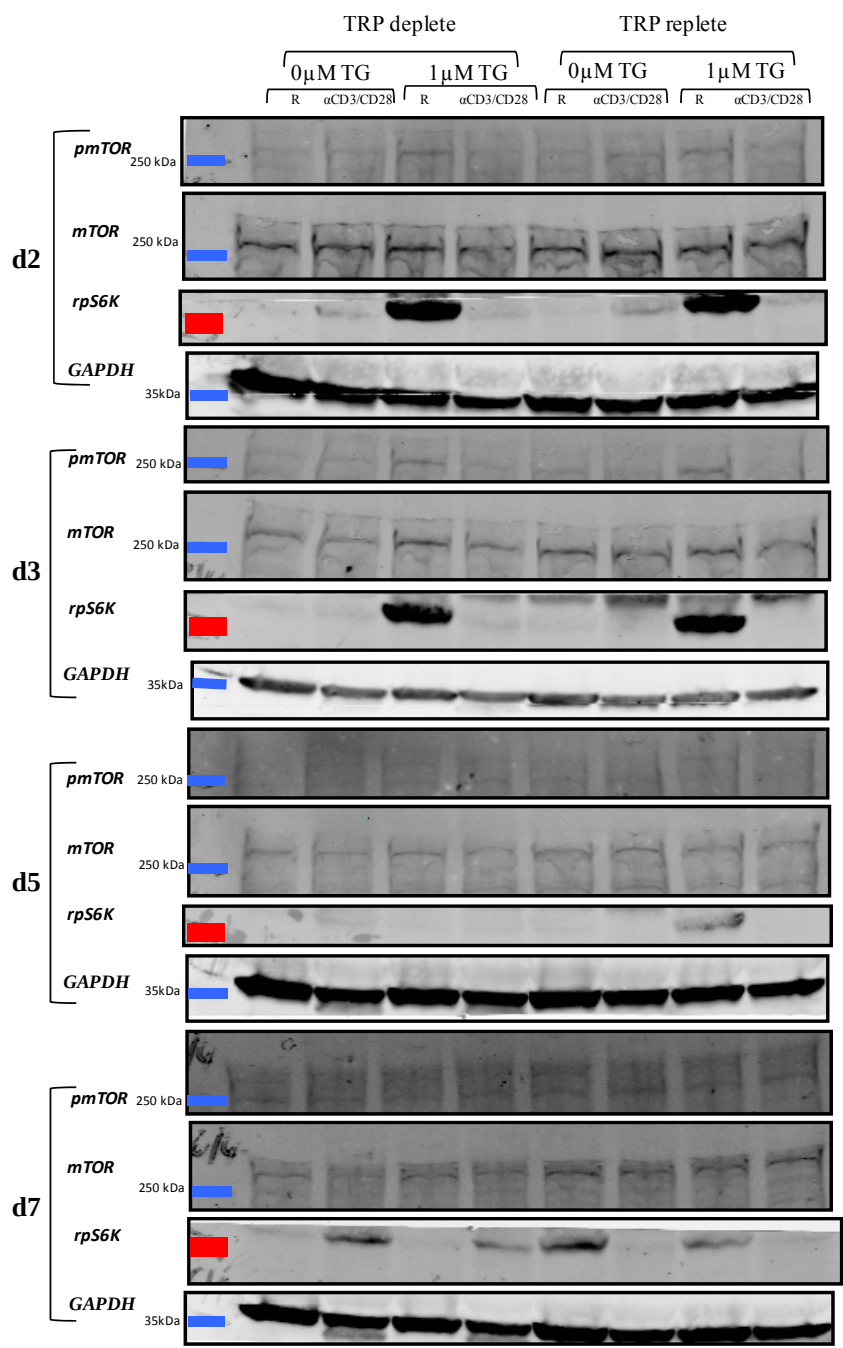
Phosphorylation of mTOR was slightly decreased in cells cultured in TRP deplete medium compared to TRP replete medium during the initial stress phase (day 2 and day 3), indicating that the mTOR pathway was affected upon TRP deprivation in primary human T-cells.

The role of the mTOR pathway upon TRP starvation became even more evident, when the phosphorylation status of the mTOR downstream molecule rpS6K was assessed. Phosphorylation of rpS6K decreased in resting cells maintained in TRP free cell cultures compared to cells cultured in TRP replete medium peaking at day 7, represented by an up to 72 % reduction in rpS6K phosphorylation (Fig. 72 and Table 13). The additional challenge with TG resulted in a significant increase in rpS6K phosphorylation in resting cells, but did not additionally affect phosphorylation levels in activated cells.

Remarkably, phosphorylation levels of rpS6K were markedly decreased at day 7 after stimulation with α CD3/CD28. Challenge with TG abrogates the effect observed between cells cultured in TRP deplete *versus* TRP replete conditions.

D – RESULTS

Overall these data indicate that the mTOR pathway is involved in the cellular response to TRP deprivation of human primary T-lymphocytes, which is shown by decreased phosphorylation of mTOR and its downstream marker rpS6K.



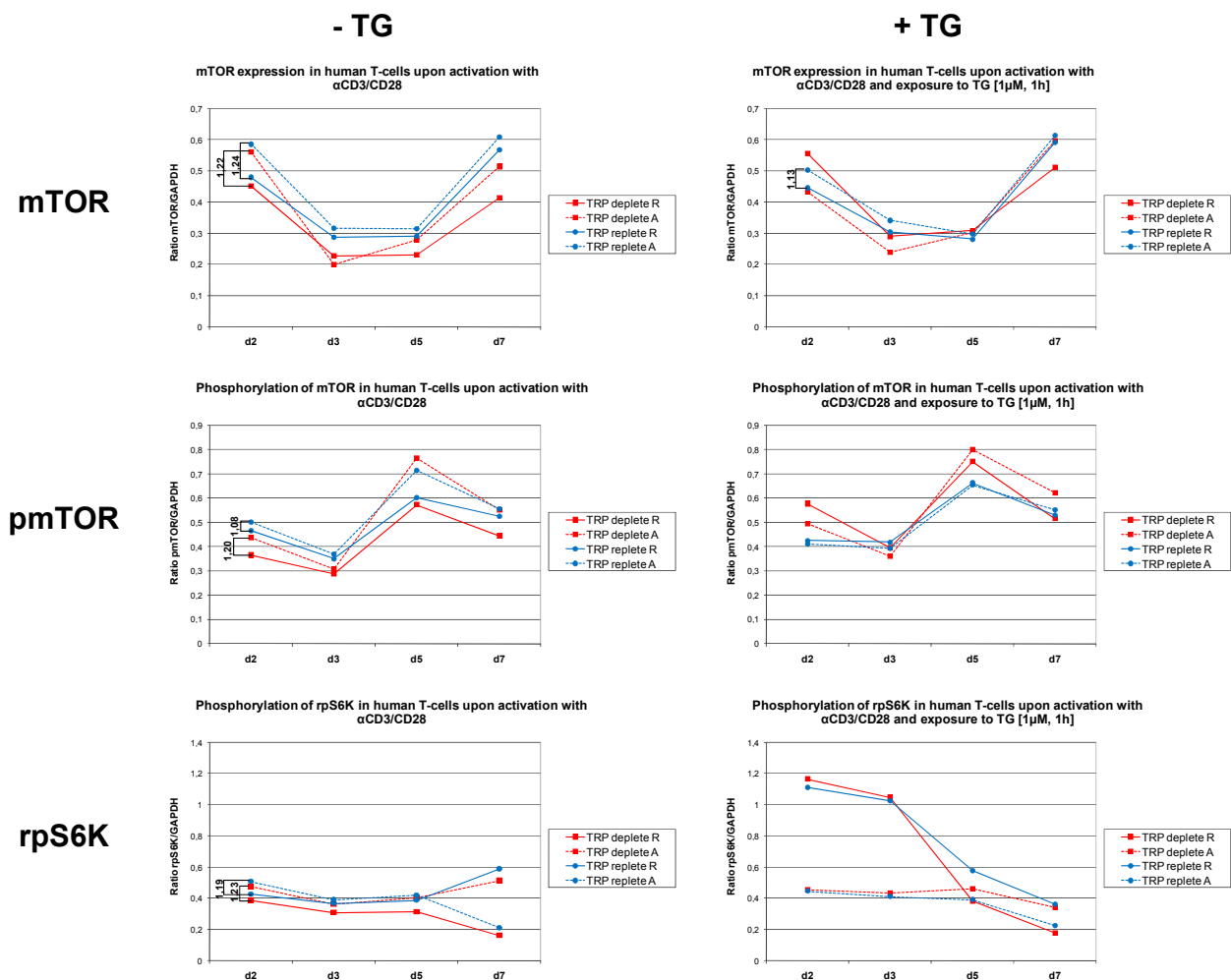


Fig. 71: Initiation of the mTOR pathway in human primary T-cells upon TRP deprivation
Endogenous expression and phosphorylation status of mTOR and rpS6K upon activation with α CD3/CD28 and TG stimulation (1 μ M, 1 h). Fold changes were calculated by dividing activated by resting values

DAY 7

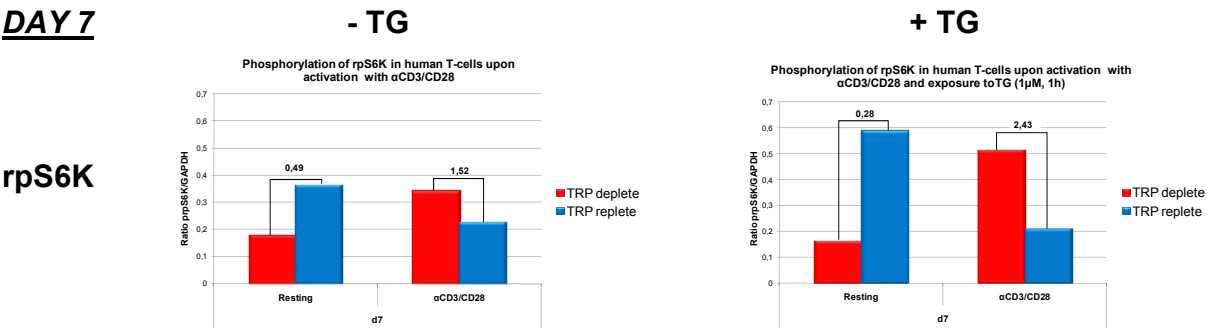


Fig. 72: Fold changes in rpS6K phosphorylation on day 7 after activation with α CD3/CD28
Fold decrease was calculated by dividing TRP replete by TRP deplete conditions

D – RESULTS

			-TG				+TG			
			d2	d3	d5	d7	d2	d3	d5	d7
pmTOR	<i>Resting</i>	<i>TRP replete</i>								
		<i>TRP deplete</i>	<u>0,78</u>	<u>0,82</u>	0,95	<u>0,84</u>	1,36	0,95	1,13	0,97
	<i>Activated</i>	<i>TRP replete</i>								
		<i>TRP deplete</i>	<u>0,87</u>	<u>0,83</u>	1,07	0,99	1,20	0,92	1,22	1,13
rpS6K	<i>Resting</i>	<i>TRP replete</i>								
		<i>TRP deplete</i>	0,91	<u>0,84</u>	<u>0,81</u>	<u>0,28</u>	1,05	1,02	<u>0,66</u>	<u>0,49</u>
	<i>Activated</i>	<i>TRP replete</i>								
		<i>TRP deplete</i>	0,94	0,93	0,96	2,43	1,02	1,05	1,18	1,52

Table 13: Fold decrease of phosphorylated mTOR pathway marker proteins

Numbers indicate the fold decrease. The fold decrease was calculated by dividing TRP replete by TRP deplete ratios.

3.5 Summary

In the course of this project, following observations were made:

o **Murine system**

- Activation with PMA/Ionomycin induced increased expression of stress related proteins in cells maintained in TRP deplete and TRP replete medium
- ISR was not induced in cells starved of TRP
- No induction of autophagy upon TRP deprivation was observed in murine cells

These observations made in the murine system suggest that the expression of stress related proteins in murine cells is dependent on cellular activation and stand in contrast to published results by Munn *et al.*

o **Jurkat T-cells**

- Activation with PMA/Ionomycin showed no effect on the expression of stress related proteins in TRP deplete and TRP replete medium
- Challenge with thapsigargin increased the expression of stress related proteins in cells cultured in TRP replete, but not in TRP deplete medium
- ISR induction was observed in cells cultured under TRP starvation
- No induction of autophagy was detected upon TRP starvation

These data propose that Jurkat cells, do not respond to activation stimuli, in contrast to murine and primary human cells. This may be related to the anyhow constant activation of this cell line. However, Jurkat cells induce the ISR program upon tryptophan deprivation, while autophagy is not affected during cellular adaption upon tryptophan withdrawal.

D – RESULTS

o *Primary human T-cells*

- Activation with α CD3/CD28 induced CD25 cell surface marker expression similarly in TRP deplete and TRP replete conditions
- TRP starvation inhibited cellular proliferation and increased the susceptibility to apoptosis
- Activation with PMA/Ionomycin did not lead to the increased expression of stress related proteins
- Activation with α CD3/CD28, in contrast to PMA/Ionomycin, enhanced the expression of stress related proteins. However, the extent of increase of stress related proteins was similar in TRP deplete and TRP replete cell culture conditions
- Stimulation with TG caused an upregulation of stress related proteins in cells cultured in TRP deplete and TRP replete conditions, which stays in contrast to observations made in Jurkat cells
- Upon TRP starvation no ISR induction was observed on protein level
- However, stress related genes were upregulated on transcriptional level
- Autophagy was not involved in the cellular adaption to TRP deprivation
- The involvement of the mTOR pathway was noted upon TRP starvation at later timepoints, suggesting that internal TRP storages had to be used up before cellular adaption could be initiated

These findings suggest that the type of activation stimulus plays a role in primary human T-cells with respect to ISR marker protein expression, which is also observed in different expression of CD25 cell surface marker (data not shown). Stimulation with α CD3/CD28 represents a T-cell receptor mediated activation stimulus, whereas stimulation with PMA/Ionomycin is achieved through non physiological activation of the protein kinase C, which could probably affect the outcome in these cells. Moreover, the ISR and autophagy is not responsible for the inhibition of allogeneic T-cell proliferation. However, the PI3K/mTOR pathway may play a role in the cellular response to tryptophan deprivation in human primary T-cells upon depletion of intracellular tryptophan storages thus accounting for the inhibition of proliferation.

E - DISCUSSION AND CONCLUSION

IDO, an intracellular enzyme is the rate limiting enzyme of tryptophan metabolism in human organisms and is able to deplete tryptophan from the local microenvironment and accumulate tryptophan metabolites. Both these effects have been suggested to be involved in immunoregulation and contribute to the induction of immune tolerance.^[110] Tryptophan is one of nine essential amino acids which can neither be stored nor synthesized *de novo* by the body and thereby has to be supplemented by diet. The perception of IDO mediating fundamental immunoregulation was initiated when in 1998 the group around Munn *et al.*^[116] claimed that IDO is able to exert profound immunosuppressive effects in mammalian cells, especially in semi-allogeneic pregnancy in mice.

Several working mechanisms were suggested for the action of tolerance induction by IDO. The tryptophan starvation theory states that tryptophan deprivation in the cellular microenvironment leads to the inhibition of T-cell proliferation, which was also proposed as the cause for the observations made by Munn in 1998^[116]. Furthermore, it was demonstrated that primary human T-cells, cultured within a milieu devoid of the essential amino acid tryptophan, were not able to proliferate, compared to cells cultured under tryptophan abundant conditions. This inhibition could be reversed by the addition of tryptophan.^[118] Proliferative inhibition upon tryptophan starvation was also observed in human peripheral blood mononuclear cells. Moreover it was found that the proliferative inhibition was related to missing tryptophan and not to an inhibitory effect of tryptophan metabolites.^[134]

Another working model suggested for the tolerogenic effect exerted by IDO, was the accumulation of tryptophan metabolites, so called kynurenines in the cellular microenvironment. Kynurenines, especially 3-hydroxykynurenine and 3-hydroxyanthranilic acid were suggested to trigger apoptosis thus leading to a suppression of allogeneic T-cell proliferation.^[124, 126, 127]

The proliferative decline of murine T-cells and the subsequent recovery by the addition of tryptophan in the course of cellular response to tryptophan deprivation was explained by a stable cell cycle arrest for at least 12 h by colleagues of Lee *et al.* They suggested that the

E – DISCUSSION AND CONCLUSION

inability to proliferate is not due to an inability of synthesizing proteins but rather to an arrest at a specific cell cycle checkpoint. ^[119]

However Fallarino *et al.* observed in 2006 that both, tryptophan deprivation and kynurenine accumulation in the local environment are necessary for tolerance induction. ^[261]

In contrast to these findings, Frumento *et al.* claimed in 2002 that the slight decrease in proliferation could not be traced back to deprived tryptophan in the culture medium but rather to unknown factors missing in the tryptophan free serum, which seem to be vitally important for cellular expansion. ^[124] To this point, the possibly absent unknown factors normally contained in the serum, could be excluded as a source of influencing cellular proliferation, since cells grown in standard medium supplemented with the same tryptophan free serum, showed no decline in proliferation in our model system.

If a cell is lacking an essential amino acid, several cellular adaption processes can be initiated to overcome this stress situation. In 2005, Munn *et al.* suggested a novel mechanism of T-cell regulation by tryptophan starvation. He reported that mouse splenocytes and splenic T-cells are capable of initiating the integrated stress response (ISR), upon sensing tryptophan deprivation *via* the GCN2 pathway. ^[4] The ISR leads to the inhibition of global protein translation on the one hand and to the expression and phosphorylation of stress related proteins, i.e. eIF2 α and CHOP, on the other hand. The involvement of the ISR has since then only been scarcely addressed in human cells.

Alternative cellular pathways also suggested to be initiated upon the cellular response to nutrient deprivation include autophagy and the PI3K/mTOR pathway.

To uncover whether any type of cellular stress response represents a molecular mechanisms of proliferative inhibition by tryptophan starvation in primary human T-cells, we analyzed marker molecules of the ISR, autophagy and the PI3K/mTOR pathway in resting and activated human T-cells upon culture in tryptophan free medium.

First, we ensured that primary human T-cells activated by α CD3/CD28 and maintained in TRP free medium were inhibited in proliferation. We made the observations that T-cells showed inhibited proliferation while maintaining stable cell numbers, corroborating that tryptophan deprivation prevents T-cells from a full response to polyclonal stimulation.

E – DISCUSSION AND CONCLUSION

Furthermore, our observations showed an increased apoptotic response in α CD3/CD28 activated primary human T-lymphocytes upon maintenance in tryptophan deplete culture conditions, which is in line with previous published data.^[119]

These data suggest that the proliferative inhibition may be related to increased apoptotic susceptibility of T-cells under tryptophan starvation.

For analysis of the ISR we first investigated phosphorylation of the key molecule eIF2 α , at which diverse stress related pathways converge. Some groups revealed that phosphorylation of the α -subunit of the eukaryotic initiation factor 2 was increased upon withdrawal of the essential amino acid leucine in mouse and hamster cells.^[162, 174, 262]

In 2010, Zaborske *et al.* stated that even in lower eukaryotes, for instance yeast, the phosphorylation of the eukaryotic initiation factor 2 α is detected upon tryptophan withdrawal.^[263]

In our study, we observed that ISR responsive marker proteins were expressed in Jurkat T-cells upon tryptophan starvation, indicating that the ISR was detectable in response to tryptophan deprivation in our model system. We found a 1,34-fold increase of pEIF2 α and a 2,94-fold increase of CHOP by 4 hours of culture of Jurkat cells in TRP free medium.

However, in human primary T-cells stress related proteins were only somewhat enhanced upon cellular activation with α CD3/CD28, in contrast to activation with PMA/Ionomycin, but no difference was observed whether or not cells were maintained in TRP deplete or TRP replete cell culture medium. Cellular activation appeared to generally enhance protein translation since the cellular content of unphosphorylated eIF2 was as well increased. These data indicate that the ISR is not involved in the cellular adaption of human primary T-cells to tryptophan deprivation in their environment.

As second marker, the expression of the downstream molecule CHOP was as well examined for the induction of the ISR. Munn *et al.* showed the induction of the GCN2 pathway indirectly *via* CHOP expression in PMA/Ionomycin activated murine splenocytes and splenic T-cells deprived of tryptophan.^[4] Other publications claim that CHOP protein and mRNA expression is enhanced in various types of mouse and hamster cells, if either depleted of glucose or another essential amino acid, leucine.^[162, 183, 196, 264] In 2009,

E – DISCUSSION AND CONCLUSION

Barnes *et al.* postulated that they were able to detect CHOP mRNA after depriving human monocytes from tryptophan. ^[265]

Like with pelf2 α and different from observations by Munn *et al.* in 2005, no CHOP signal was noted by western blotting in murine cells. While we cannot finally exclude that discrepancies in technical procedures were the cause for this difference, our findings in Jurkat cells demonstrating a significant effect of tryptophan starvation on CHOP expression, validated our approach in human cells.

As well, we detected CHOP protein expression in human T-lymphocytes upon activation with α CD3/CD28. However, no increased CHOP expression was observed in cells cultured in tryptophan deplete compared to tryptophan replete conditions.

Forouzandeh *et al.* described in 2008 that CHOP expression is induced in α CD3/CD28 activated human primary CD8⁺ T-cells upon culturing the cells in either tryptophan free medium or upon co-culture with IDO expressing fibroblasts. Furthermore, CHOP expression was also increased upon addition of tryptophan metabolites. Unfortunately, the group did not include a negative control of human T-cells alone cultured under tryptophan replete conditions, which implies that they cannot exclude any interferences of the fibroblast co-culture with the cellular reaction of these T-cells. ^[3] With respect to our work this means that CHOP is expressed upon activation, which is in line with published data by Forouzandeh. However, our observations showed that CHOP was as well expressed in cells cultured in tryptophan replete conditions. Furthermore, it was observed that HeLa cells deprived from leucine, showed a 10-20 fold increase in CHOP mRNA expression. The latest timepoint of analysis in this study was 8 h after starvation induction. ^[219] Nevertheless, our experimental setup aimed for depleting intracellular tryptophan storages. Therefore later timepoints were examined for a differential mRNA expression. The fold increase of mRNA expression in our primary T-cells ranged between 0,2 and 3-fold which could be explained by lower total protein expression in primary cells compared to immortalized cell lines.

Since no differences in ISR marker genes were observed under tryptophan deprivation, we added the chemical ER stress inducer TG to our cultures in order to increase the cellular stress in primary human T-cells. Thapsigargin is an enzyme isolated from the plant

E – DISCUSSION AND CONCLUSION

Thapsia garganica and acts as a competitive calcium pump inhibitor in the ER, thereby depleting the ER from calcium while increasing cytosolic calcium concentrations.

The treatment with TG slightly enhanced the expression of ISR marker proteins in primary human T-cells, which is in line with published data in mouse cells. [159, 167, 174, 196, 214, 258] In contrast to primary human T-cells, thapsigargin increases the expression of stress related proteins only in Jurkat cells cultured under tryptophan abundant conditions.

Taken together, these observations suggest that human primary T-cells are very robust to initiate the ISR evoked by nutrient deprivation. Even additional chemical stress signals induce only a slight increase in the expression of stress related proteins. T-cells seem to avoid to initiate an integrated stress program, but rather to undergo apoptosis, if stress persists. In contrast, the Jurkat T-cell line appears to respond to “non-physiological” thapsigargin treatments and show higher resistance to apoptosis to even higher stimulatory concentrations. To conclude, these findings clearly indicate that in contrast to the common view, the GCN2 pathway is not a predominant mechanism of tryptophan deprivation in primary human T-cells.

As several pathways interplay during the cellular adaption to amino acid deprivation, alternative nutrient sensing pathways were investigated in the course of cellular response to amino acid withdrawal.

We were able to show that the alternative pathway, as the induction of autophagy, is not involved during cellular adaption to nutrient deprivation, especially during tryptophan starvation. Indeed, the expression level of the marker protein Beclin-1 remained unchanged whether or not cells were cultured in TRP deplete or replete conditions. These observations are in line with published data showing that autophagy is not induced, even upon total amino acid starvation, in a human colorectal cancer and HeLa cell line. [266, 267]

Finally, our findings suggest that the mTOR pathway is indeed affected in primary human T-lymphocytes upon tryptophan starvation. In general, phosphorylation of mTOR marker proteins is decreased upon cellular stress, in contrast to the ISR and autophagy. Indeed, phosphorylation of mTOR and the downstream molecule rpS6K decreased in cells maintained in TRP deplete conditions by day 7 after polyclonal activation. We speculate

E – DISCUSSION AND CONCLUSION

that this delayed effect is compatible with the presence of intracellular TRP stores, which have to be exhausted before stress responses are initiated. These observations are in line with published data stating that the GCN2 pathway is not sufficient for sensing amino acid deprivation in mammalian cells, but rather the mTOR pathway coincides, leading to a functional combined activity of the GCN2 and the mTOR pathway in the murine system. [178]

Interestingly, in other cell systems, such as murine hepatocytes, the mTOR pathway was not involved in amino acid deprivation induced autophagy, which suggests that the effects of amino acid removal is more a coincidental phenomenon rather than a causal reason for the initiation of mTOR and autophagy in murine cells. [268]

Previously published data indicated that single amino acid starvation, *inter alia* tryptophan withdrawal, does neither intervene with the induction of the integrated stress response nor with the activation of the mTOR pathway in human hepatoma cells. Only if these cells undergo total amino acid deprivation, phosphorylation of the downstream molecule 4E-BP1 is decreased, leading to the inhibition of the mTOR pathway. [269, 270] With regards to our observations these data suggest that single amino acid deprivation is sufficient in primary human T-cells to induce the PI3K/mTOR pathway if intracellular tryptophan storages are entirely depleted of tryptophan.

As previously described in literature, indoleamine-2,3-dioxygenase is expressed and functional active, meaning able to catabolize tryptophan, in murine granulocytes. [271] As recently demonstrated by our group, IDO is expressed in murine dendritic cells, but the enzyme is not catalytically active in this system (unpublished data). Moreover, our group also evidenced that human DCs, stimulated *via* IFN- γ and LPS, are able to produce functional IDO. [272]

Even though similarities in the secondary structure of IDO in the murine and the human system can be observed, murine IDO (mIDO) has a threefold higher substrate efficiency ratio than human IDO (hIDO). [273] The authors claim that the reason for this increased substrate efficiency could be explained by an increased level of tryptophan in the serum, requiring a higher tryptophan conversion rate.

E – DISCUSSION AND CONCLUSION

The similarities and discrepancies between human and mouse IDO show that although the secondary structure is similar in both systems, the activity could be entirely different. The same could hold true for the stress sensor general control-nonderepressible 2. Even though the structural homology of GCN2 between yeast, mouse and human is similar, the functional activity could differ strongly.

This study clearly found that murine cells, human cell lines and primary human cells react totally different upon diverse stress situations. One explanation for this disparity may be the fact that cell lines acquired several mutations. As our data show, Jurkat cells are less prone to apoptotic cell death compared to primary human T-lymphocytes which as well may result from genetic aberrations. Furthermore, Jurkat T-cells are in a state of constant activation rendering them unable to react to activation signals, in contrast to murine or primary human cells. Overall, these observations are compatible with a concept that human cell lines and fresh human cells use different strategies to overcome stress situations. Upon sensing amino acid starvation, cell lines as being immortalized, are inclined to initiate a stress related program, while fresh human T cells display a higher sensitivity to undergo apoptosis.

Summarizing, our observations confirm that tryptophan deprivation in the microenvironment causes the inhibition of proliferation of primary human T-lymphocytes. We propose that cells lacking access to the essential amino acid tryptophan are not able to proliferate and expand in cell number but display increased apoptotic susceptibility.

As a mechanism, neither the integrated stress response nor the autophagy pathway is initiated upon tryptophan starvation. From these findings we conclude that these two stress responsive pathways do not account for the reversible effect of tolerance induction through tryptophan depletion.

However, the PI3K/mTOR pathway may play a role during the cellular adaption to tryptophan deprivation in primary human T-cells. However, the initiation of this pathway occurred only late after activation. Since the effect of tryptophan depletion on cell proliferation was detectable early after stimulation, our findings rather suggest that an increased apoptotic decline and cell cycle arrest may account for the inhibitory effect of tryptophan starvation on T-cell proliferation.

E – DISCUSSION AND CONCLUSION

Further investigations on this project will include first the replenishment of tryptophan to cultures for showing if cellular proliferation is resumed and the inhibition of the PI3K/mTOR pathway could be compensated. Furthermore, the effect of kynurenines on primary human T-cells with regards to cellular proliferation, apoptotic response and inhibition of the PI3K/mTOR pathway will be investigated. Moreover, cell cycle studies have to be performed by monitoring cyclins (cyclin D), cyclin dependent kinases (CDK4) and cyclin dependent kinase inhibitors (p21, p27, p16^{INK4a}) in order to identify the role of tryptophan depletion on cell cycle arrest, as shown by Munn *et al.* in 1999 ^[118], and apoptotic response. In addition, by inhibition of apoptosis i.e. by the caspase inhibitor Z-VAD, the effect of tryptophan withdrawal will be in depth analyzed with regards to proliferation and PI3K/mTOR pathway induction in primary human T-cells.

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G - INDEX OF FIGURES AND TABLES

1 Table of Figures

Fig. 1: Recognition of PAMPs through TLRs and their downstream signaling in mammalian cells.....	- 10 -
Fig. 2: Life cycle of a dendritic cell	- 11 -
Fig. 3: Formation of the immunological synapse between an APC and a T-lymphocyte depicting the main interacting ligands	- 12 -
Fig. 4: Three distinct signals in T-cell priming	- 13 -
Fig. 5: T-cell polarization through various effector cytokines	- 13 -
Fig. 6: CD4 T-cell subsets with expressed cytokine profiles and their immunologic effects... ..	- 14 -
Fig. 7: Timeline of hematopoietic stem cell transplantation innovations over more than 50 years	- 16 -
Fig. 8: Peripheral blood hematopoietic stem cell transplantation procedure	- 17 -
Fig. 9: Indications for oropharyngeal mucositis after conditioning regimen.....	- 18 -
Fig. 10: Immune reconstitution after HSCT	- 19 -
Fig. 11: Chromosome 6 with encoded HLA I and II complexes	- 20 -
Fig. 12: Recognition of the miHA by recipient and donor APCs.....	- 21 -
Fig. 13: Course of acute GvHD with interacting immune cells and affected body damage sites.....	- 22 -
Fig. 14: Erythroderma formation in stage IV aGvHD.....	- 23 -
Fig. 15: MHC class I and II presentation by recipient APCs to donor CD8 ⁺ and CD4 ⁺ T-lymphocytes	- 24 -
Fig. 16: Deletion of self reactive T-cells in the Thymus.....	- 28 -
Fig. 17: Three ways of inhibiting the autoreactive action of T-lymphocytes	- 28 -
Fig. 18: Induction of anergy due to missing costimulatory molecules	- 29 -
Fig. 19: Clonal selection of T _{regs} in the thymus and action of even those on self-reactive T-cells in the periphery	- 29 -
Fig. 20: Molecular Genetics of the IDO gene and protein and post translational modifications	- 30 -

G – INDEX OF FIGURES AND TABLES

Fig. 21: Function of IDO	- 31 -
Fig. 22: Tryptophan degradation pathway	- 33 -
Fig. 23: Negative effects of IDO expression for the immunological reaction against tumor cells	- 34 -
Fig. 24: (a) Cryoelectron microscopy analysis of the 40S subunit and (b) modeling of initiation factor recruitment.....	- 36 -
Fig. 25: Process of Eukaryotic Translation Initiation with subsequent recycling of the ribosomal subunits.....	- 38 -
Fig. 26: The integrated stress response and its downstream signaling molecules	- 40 -
Fig. 27: Stress inducers and their sensor mechanisms	- 41 -
Fig. 28: Protein structure of human GCN2	- 42 -
Fig. 29: GCN2 as a mediator in response to amino acid starvation.....	- 43 -
Fig. 30: Domain structure of α -, β - and γ -subunit of eIF2	- 44 -
Fig. 31: Biological function of eIF2.....	- 45 -
Fig. 32: Human ATF-4 mRNA sequence	- 46 -
Fig. 33: Protein structure of human ATF-4	- 46 -
Fig. 34: Translational control of ATF-4 under nonstressed and stressed conditions	- 47 -
Fig. 35: Domain structures of CHOP	- 48 -
Fig. 36: Structure of the CHOP promoter with amino acid response elements (AARE) and ER stress response elements (ERSE).....	- 49 -
Fig. 37: Formation of an autophagosome <i>via</i> defined steps	- 51 -
Fig. 38: Stress sensor mTOR and its downstream signaling pathway.....	- 52 -
Fig. 39: Interphase of human PBMCs after Lymphoprep TM centrifugation.....	- 55 -
Fig. 40: MACS T-cell enrichment technique with indirect separation.....	- 56 -
Fig. 41: Dotplot of murine splenocytes maintained under suboptimal culture conditions-	66 -
Fig. 42: Susceptibility to apoptosis of murine splenocytes maintained under suboptimal culture conditions.....	- 66 -
Fig. 43: Susceptibility to apoptosis of murine splenocytes maintained under optimal culture conditions.....	- 66 -
Fig. 44: ISR marker protein expression in murine splenocytes.....	- 68 -
Fig. 45: Densitometric analysis of ISR associated marker proteins	- 68 -

G – INDEX OF FIGURES AND TABLES

Fig. 46: Autophagy in murine BALB/c splenocytes	- 69 -
Fig. 47: Densitometric analysis of autophagy associated marker proteins.....	- 69 -
Fig. 48: Apoptotic response of Jurkat T-lymphocytes maintained in culture medium supplemented with 1 % TRP free serum.....	- 71 -
Fig. 49: Apoptotic response of Jurkat T-lymphocytes maintained in culture medium supplemented with 10 % TRP free serum.....	71
Fig. 50: CD25 surface expression on Jurkat T-cells	71
Fig. 51: Effects of TG stimulation on Jurkat T-cells.....	- 72 -
Fig. 52: Densitometric analysis of eIF2 α phosphorylation in Jurkat cells upon challenge with TG (1 μ M, 10 μ M) for up to 60 min	- 73 -
Fig. 53: Densitometric analysis of constitutive eIF2 α expression in Jurkat cells upon challenge with TG (1 μ M, 10 μ M) for up to 60 min	- 73 -
Fig. 54: Induction of the ISR in the Jurkat T-cell line.....	- 75 -
Fig. 55: Autophagy induction in Jurkat T-cells	- 76 -
Fig. 56: Autophagy induction in human Jurkat T-cells	76
Fig. 57: Apoptosis rate of human T-cells upon TG stimulation.....	- 77 -
Fig. 58: CD25 cell surface expression of primary human T-cells upon activation with α CD3/CD28.....	- 78 -
Fig. 59: Proliferative capacity of human primary T-cells	- 80 -
Fig. 60: Expansion of primary human T-cells.....	- 81 -
Fig. 61: Apoptotic susceptibility of human primary T-cells	- 82 -
Fig. 62: Induction of ISR in human primary T-cells	- 83 -
Fig. 63: Initiation of the ISR in human primary T-cells	- 84 -
Fig. 64: Phosphorylation and endogenous expression of eIF2 α and CHOP in α CD3/CD28 activated primary human T-cells	- 86 -
Fig. 65: ISR marker protein expression upon activation with α CD3/CD28 and exposure to TG [1 μ M, 1 h].....	- 88 -
Fig. 66: Densitometric measurement of ISR marker protein expression after stimulation with α CD3/CD28 and TG [1 μ M, 1 h]	- 89 -

G – INDEX OF FIGURES AND TABLES

Fig. 67: Expression of stress related proteins in primary human T-cells on day 5 of maintenance in TRP deplete or TRP replete culture medium with additional TG exposure	- 90 -
Fig. 68: mRNA expression of primary human T-cells after activation with α CD3/CD28 and stimulation with TG (1 μ M) for 1h over 7 days	- 92 -
Fig. 69: Autophagy induction in primary human T-cell.....	- 93 -
Fig. 70: Beclin-1 expression in primary human T-cells upon activation on day.....	- 94 -
Fig. 71: Initiation of the mTOR pathway in human primary T-cells upon TRP deprivation-	97
-	
Fig. 72: Fold changes in rpS6K phosphorylation on day 7 after activation with α CD3/CD28 -	97 -

2 List of Tables

Table 1: Buffer compositions	- 57 -
Table 2: Flow cytometric antibodies and respective dilutions	- 61 -
Table 3: Composition of SDS PAGE separating and stacking gel	- 62 -
Table 4: Primary and secondary antibodies with respective working dilutions.....	- 63 -
Table 5: Investigated genes with respective forward (fwd) and reverse (rev) primer sequences.....	- 64 -
Table 6: Fold changes of ISR protein expression upon maintenance in TRP deplete or TRP replete culture medium and activation with PMA/Ionomycin	68
Table 7: Fold changes of autophagy marker protein expression in murine splenocytes maintained in TRP deplete or TRP replete culture medium and activated with PMA/Ionomycin.....	- 69 -
Table 8: Fold increase of ISR marker protein expression in Jurkat cells	75
Table 9: Division index of primary human T-cells activated with α CD3/CD28	- 80 -
Table 10: Fold increase of stress response marker proteins	84
Table 11: Effects of TG stimulation on α CD3/CD28 activated primary human T-cells.....	86
Table 12: Fold changes in phosphorylation and endogenous expression of eIF2 α and CHOP in human primary T-cells over 7 days.....	- 89 -
Table 13: Fold decrease of phosphorylated mTOR pathway marker proteins	- 98 -

H - LIST OF ABBREVIATIONS

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AARE	amino acid response element
aa-tRNA	amino-acyl tRNA
aGvHD	acute graft versus host disease
AP-1	activator protein 1
APC	antigen presenting cell
APS	ammonium persulfate
(A) site	aminoacyl site
ATF4	activating transcription factor 4
BCL2	B-cell lymphoma 2
BiP	binding immunoglobulin protein
bZIP	basic-leucine zipper
C/EBP	CCAAT-enhancer-binding protein
CaCl ₂	calcium chloride
cAMP	cyclic adenosine monophosphate
CD4	cluster of differentiation 4
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
cGvHD	chronic graft versus host disease
CHOP	CCAAT/enhancer binding protein (C/EBP) homologous protein
CREB-2	cAMP Responsive Element Binding-2
CRT	calreticulin
C-terminal	carboxyl-terminal
CTLA-4	cytotoxic T-Lymphocyte Antigen 4
DCs	dendritic cell
DDIT3	DNA damage-inducible transcript 3
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides

H – LIST OF ABBREVIATIONS

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
eIF2	eukaryotic initiation factor 2
ER	endoplasmic reticulum
eRF	eukaryotic release factor 1
ERSE	ER stress response elements
(E) site	exit site
FBS	fetal bovine serum
Fc Receptor	fragment crystallizable
Foxp3	forkhead box P3
g	gravitational force
GADD153	growth-arrest- and DNA-damage-inducible gene 153
GAS	γ -activating sequences
GCN2	general control non-derepressible 2
G-CSF	granulocyte colony-stimulating factor
GPR35	G protein-coupled receptor 35
GvHD	graft versus host disease
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HisRS	histidyl-tRNA synthetase
HLA	human leukocyte antigen
HRI	heme-regulated eIF2 α kinase
HSA	human serum albumin
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IDO	indoleamine-2,3-dioxygenase
IFN- γ	interferon- γ
IgG	immunoglobulin G
IL-1	interleukin-1

H – LIST OF ABBREVIATIONS

ISR	integrated stress response
ISREs	interferon stimulatory response elements
iT _{regs}	inducible regulatory T-cells
JAK/STAT	janus kinase/ signal transducer and activator of transcription
KD	kinase domain
kDa	kilo dalton
KHCO ₃	potassium bicarbonate
KIRs	killer-immunoglobulin receptors
LC3	light chain 3
LPS	lipopolysaccharide
LT α	lymphotoxin α
mA	milliampere
MACS	magnetic cell separation
MAPK	mitogen-activated protein kinase
MEFs	mouse embryonic fibroblasts
Met-tRNA	methionine-tRNA
MHC	major histocompatibility complex
miHA	minor histocompatibility anigen
min	minute
ml	milliliter
M-MLV RT	moloney murine leukemia virus reverse transcriptase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
NA ₂ EDTA	disodium ethylenediaminetetraacetate
NaCl	sodium chloride
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NH ₄ Cl	ammonium chloride
NK cell	natural killer cell
NKT	natural killer T-cell

H – LIST OF ABBREVIATIONS

N-terminal	amino-terminus
o/n	over night
p62/SQSTM1	sequestosome 1
PAMPs	pathogen-associated molecular patterns
PAS	pre-autophagosomal structure
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PERK	protein kinase RNA-like <i>endoplasmic</i> reticulum kinase
PGE-2	prostaglandin E2
PI	phosphoinositide
PIKKs	phosphoinositide 3 kinase-related kinases
PKR	protein kinase R
PMA	<i>phorbol</i> -12-myristate-13-acetate
PRR	pattern recognition receptors
(P) site	peptidyl site
PTMs	posttranslational modifications
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpS6	ribosomal protein S6
RT	room temperature
S6K1	ribosomal S6 kinase
SDS	sodium dodecyl sulfate
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
TCR	T-cell receptor
TDO	tryptophan 2,3 dioxygenase
TEMED	tetramethylethylenediamine
TG	thapsigargin
TGCs	trophoblast giant cells
TGF- β	transforming growth factor-beta

H – LIST OF ABBREVIATIONS

TH1	T helper cell 1
TLR	toll-like receptor
TNF	tumor necrosis factor
TOR	target of rapamycin
TRB3	tribbles homolog 3 protein
T _{regs}	regulatory T cells
tRNA	transfer RNA
TRP	tryptophan
μl	microliter
μM	micromolar
uORF	upstream open reading frame
V	volt
VEGF	vascular endothelial growth factor
wt	wild type

SUPPLEMENTAL

ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is responsible for the catalysis of tryptophan to tryptophan metabolites, which triggers a depletion of this essential amino acid and kynurenine accumulation in the microenvironment of T-cells.

Tryptophan depletion through IDO was suggested as a molecular mechanism of IDO mediated immunosuppression and was also shown to activate the integrated stress response (ISR) via the GCN2 pathway in mice. This thesis aims to explore whether the effects of IDO on the GCN2 pathway are valid also in primary human T-cells, and whether the activation of the GCN2 pathway is associated with the impaired proliferative capacity in primary human T-cells upon sensing tryptophan deprivation.

Our findings first confirmed that tryptophan deprivation accounts for the inhibition of proliferation in human T-lymphocytes. Although human primary T-cells are able to respond to an activation stimulus by highly expressed CD25 cell surface molecules, proliferation is inhibited. T-cell inhibition associated with tryptophan deprivation was accompanied by an increased susceptibility to apoptosis.

Furthermore our data clearly indicated that, in contrast to the murine system, the integrated stress response was not induced upon tryptophan deprivation in primary human T-cells. Moreover, autophagy, an alternative stress related pathway, was not induced upon tryptophan withdrawal in the primary human system. Yet, the mammalian target of rapamycin (mTOR) pathway was inducible upon exposing primary human T-lymphocytes to enforced stress (tryptophan exhaustion, prolonged cell culture).

In conclusion, in primary human T-cells, the initiation of the ISR and autophagy pathway apparently do not contribute to the cellular adaption to tryptophan deprivation. However, the mTOR pathway and increased apoptotic susceptibility may account for the T-cell inhibitory effects evoked by IDO activity.

KURZFASSUNG

KURZFASSUNG

Indoleamine 2,3-dioxygenase (IDO) katalysiert die Umsetzung von Tryptophan zu Tryptophanmetaboliten. Dieser Prozess führt zur Erschöpfung dieser essentiellen Aminosäure in der direkten Umgebung der T-Zellen.

In Mäusen wurde gezeigt, dass Tryptophan Abbau durch IDO den „Integrated Stress Response“ auslöst und somit als molekularer Mechanismus für die IDO vermittelte Immunsuppression fungiert.

Ziel dieser Arbeit war, die Effekte von IDO im besonderen Bezug auf den GCN2 Signalweg zu erforschen und infolgedessen auch den Effekt von Tryptophan Erschöpfung infolge von Aktivierung des GCN2 Signalweges auf die Proliferation von humanen T-Zellen zu ergründen.

Unsere Resultate haben bestätigt, dass die Ursache für eine inhibierte T-Zellproliferation im humanen System durch Tryptophanmangel hervorgerufen wird. Obwohl primäre humane T-Zellen durch hohe CD25 Zelloberflächen Expression im Stande sind, auf Aktivierung zu reagieren, sind sie in ihrer Proliferation gehemmt. Neben dieser Inhibierung durch Tryptophan Mangel wurde zusätzlich noch ein erhöhter Anteil an apoptotischen Zellen verzeichnet.

Weiters haben unsere Daten deutlich gezeigt, dass der „Integrated Stress Response“, im Vergleich zum murinen System, in primären humanen T-Zellen nicht durch Tryptophanmangel hervorgerufen wird.

Darüber hinaus wurde Autophagie, ein alternativer Signalweg der durch Stress induziert wird, nicht durch Tryptophanentzug induziert.

Dahingegen wurde der mTOR Signalweg durch erhöhten Stress, wie zum Beispiel Tryptophanmangel und längere Kultivierung im Medium, in primären humanen T-Lymphocyten aktiviert

Zusammenfassend haben die Ergebnisse dieses Projektes gezeigt, dass der „Integrated Stress Response“ und Autophagie Signalweg in primären humanen T-Zellen offenbar keine Rolle in Bezug auf die zelluläre Antwort, bedingt durch Tryptophanmangel, spielt. Nichtsdestotrotz könnten der mTOR Signalweg und erhöhte Apoptose, hervorgerufen durch IDO spezifischen Tryptophanentzug, eine wichtige Bedeutung hinsichtlich der T-Zellinhibierung darstellen.

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