**"Zinc Supplementation Induces Regulatory T Cells *in vitro* and *in vivo*"**

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

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# I Introduction

## 1.1 The Immune System

The human body is constantly challenged by the exposure to foreign organisms or substances. Those can be inhaled, swallowed, or inhabit the skin and mucous membranes. Whether these organisms cause diseases is a result of either the pathogenicity of the organism or the integrity of host defense mechanisms. For protection, the human body has evolved different defense mechanisms comprising natural barriers, a nonspecific (innate) immune response, and a specific (adaptive) immune response. Both the innate and adaptive immune responses are each responsible for distinct tasks but are linked closely for cross-communication to defend pathogens efficiently. This complex interactive network of cells, humoral factors, and cytokines ensures appropriate host protection, since an inadequate immune response can result in severe infections and tumor development, as well as allergic or autoimmune diseases highlighting the importance of proper immune function. Further, the immune responses are distinguished by reaction-speed and specificity.

The innate immunity includes physical, chemical, and microbiological barriers, and comprises cellular elements. Those are immune cells as granulocytes, monocytes, macrophages, mast cells, natural killer (NK) cells, and additionally complement factors, cytokines, and acute phase proteins that together provide immediate host defense. The highly conserved nature of the response, that can be found in even the simplest animals, underlines its importance for survival [1].

On the other hand, the adaptive immunity develops over the lifespan and stands out by its memory function. Moreover, it represents the immune system of higher vertebrates and includes antigen-specific reactions through B and T cells. Whereas the innate response appears rapidly and is partially accompanied with collateral damages to normal tissues through lack of specificity, the adaptive response takes several days or weeks to develop but is highly precise. Moreover, the adaptive response leads to a stronger and quicker response in case of repeated exposure, to the same pathogen due to its memory function [2].

In adaptive immunity, B cells are the major cells involved in humoral immunity. They are characterized by the creation of antibodies that circulate in blood plasma and the lymphatic system, where they specifically bind to foreign antigens. On the contrary, T cells play a central role in cell-mediated immunity. Naïve T cells can differentiate into virtually all different types of effector and memory cells. Hence, heterogeneity of T cells is generated upon

priming. Mature T cells are subdivided into functionally two different subpopulations according to their co-receptor expression: $CD4^+$ helper T (Th) cells and $CD8^+$ cytotoxic T cells (CTL).

Th cells form a very heterogeneous population basically due to their differentiation state depending on the type of invading pathogens, signals, and strength of stimulation. Thus, they belong to a progressively increasing number of different subsets with specialized functions. Distinguishing different T cell subsets, the characteristic cytokine profiles and the expression of lineage-defining transcription factors can be used (see figure 1) [3].

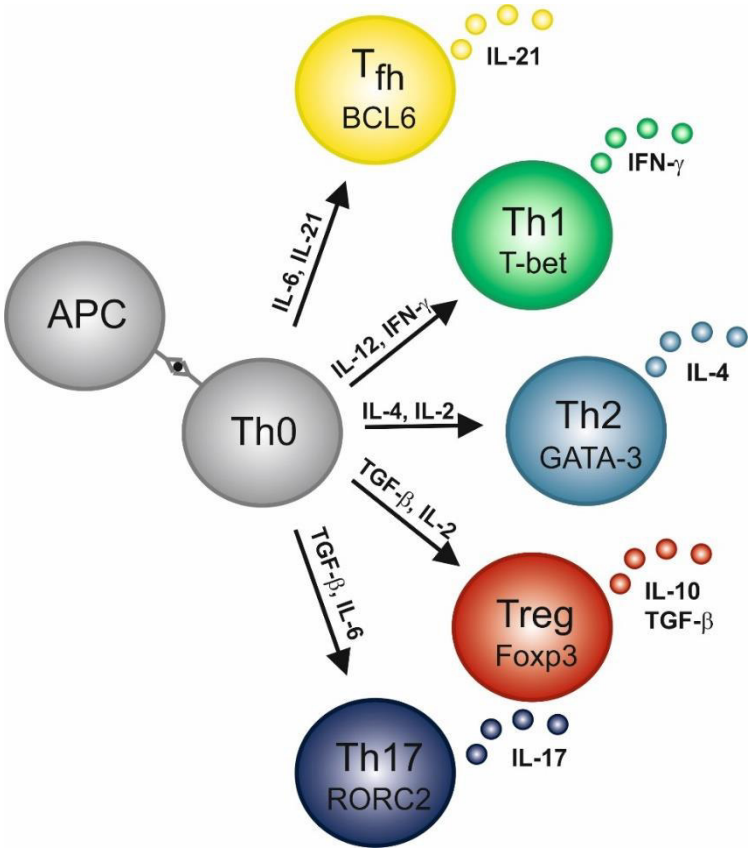


**Fig. 1 Th cell subpopulations.**
Th cells can differentiate of naïve T cells (Th0 cells) into different specific subpopulations. When Th0 cells are activated by antigen presenting cells (APC) the cytokine milieu in particular is essential for the differentiation into distinct Th subsets. These include Th1, Th2, Th17, Treg, and $T_{FH}$ cells that can be discriminated by their pattern of cytokine production and the expression of lineage-defining transcription factors.

Five principal subsets of Th cells have been identified: Th1, Th2, Th17, follicular helper T cells ($T_{FH}$), and regulatory T cells (Treg). Each comply with specific functions in the immune system [4]. Th1 cells trigger macrophages and CTL cells by secreting both interleukin (IL)-2 and interferon (IFN)-γ [5, 6], whereas Th2 cells promote the humoral immune response and eosinophil activation by producing IL-4, IL-5, IL-9, and IL-13 [7]. Th17 cells

are involved in host defense against bacterial and fungal infections by secreting IL-17A, IL-17F, IL-21 and IL-22 [8]. Treg cells are required to maintain self-tolerance [9], and $T_{FH}$ cells provide help to B cells for antibody production [10].

Nowadays, especially Treg cells are in focus of interest, since their dysfunction is often related to inadequate, excessive, or absent immune responses, leading to immune disorders [11, 12]. Disturbed immune responses manifest in allergic diseases, autoimmune diseases, graft-versus-host disease (GVHD), an increased risk of transplant rejections, and immunodeficiency. Consequently, several control mechanisms exist to ensure immune homeostasis and to discriminate between self or non-self [1]. To date elimination of autoreactive immune cells (clonal deletion), or functional inactivation (anergy) are well-known mechanisms for self tolerance [13]. Additionally, Treg cells are highly important for immune regulation since exhibiting the capacity to suppress effector cells such as $CD4^+$ and $CD8^+$ T cells, antigen presenting cells (APC), and NK cells by cell-mediated suppression and cytokine production [9].

## 1.2 Regulatory T Cells

Induction of tolerance by active suppression of inappropriate immune responses is critical to prevent autoimmunity and to maintain immune homeostasis. In this regard, Treg cells play a major role [14], although the mechanisms controlling Treg cell development and suppressor function are not yet fully elucidated. Treg cells are highly important for the regulation of immune responses to self-antigens, allergens, and commensal microbiota as well as for immune responses to infectious agents and tumors. They consist of several subsets, including naturally occurring Treg cells (nTreg) and inducible Treg cells (iTreg). nTreg arise during thymic development and comprise the majority of the peripheral Treg cell pool in naïve mice and in human cord blood with about 5-10% of $CD4^+$ Th cells [9, 15]. In addition, iTreg cells can be induced peripherally at sites of inflammation and at environmental interfaces in mice and men [16-19].

In general, Treg cells are characterized by constitutive high expression of CD25, also known as interleukin-2 receptor α-chain, which is a component of the high affinity IL-2R, and by their cell surface and cytoplasmic expression of the co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) [20]. Additionally, they express the forkhead/wingedhelix family transcriptional repressor Foxp3 [20, 21] and can be identified by $CD127^{low}$ (IL-7 receptor α-chain) expression. The use of Helios as nTreg cell marker is controversially discussed, especially in humans [22-24].

High CD25 ($CD25^{hi}$) expression is required for Treg cell survival and is used as part of Treg cell-mediated suppression. CD25 has a strong affinity to IL-2 leading to diminished availability for effector cells. CTLA-4 is involved in the suppressive function of Treg cells by downregulating CD80 and CD86 expression on APC. Foxp3 is essential for Treg cell development and suppressive activity. Already described in mice and humans mutations of Foxp3 result in severe diseases like scurfy or immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, which leads to the lack of functional Treg cells. This results in systemic autoimmunity, highlighting the vital role of Treg cells in maintaining immune homeostasis [21, 25, 26].

Furthermore, it was shown that Treg cells can also be induced *in vivo* or *in vitro* from $CD4^+CD25^-$ naïve T cells following antigen exposure and T cell receptor (TCR) stimulation in adequate environments, in both mice [16, 27, 28] and humans [18, 19, 29, 30]. Those are referred to as iTreg cells. To induce iTreg cells numerous different factors, including transforming growth factor (TGF)-β, IL-2, retinoic acid and leukemia inhibitory factor (LIF) can be used [16, 31, 32]. However, the mechanisms of Treg cell mediated suppression are likely multiple and still not fully understood. For suppression of effector cells, Treg cells use diverse mechanisms as secretion of suppressor cytokines (e.g., IL-10, TGF-ß, IL-35), local consumption of activating cytokines (e.g., IL-2), signaling via cell-surface molecules (e.g., Galectin-1), and direct cell-mediated killing (via the granzyme complex) [33]. Moreover, they are capable of altering cell surface marker expression by trans-endocytosing CD86 and CD80 co-stimulatory ligands on target APC [34]. This is achieved by CTLA-4, which recognizes those molecules, causes internalization and digestion by Treg cells.

However, mainly antigen-specific Treg cells are generated in i*n vitro* models. Studies demonstrate that antigen-specific Treg cells are much more potent as suppressors of the induction of autoimmune disease, as compared to polyclonal Treg cells [35, 36]. Therefore, antigen-specific activated Treg cells can be generated for potent suppression of the induction of organ-specific and systemic autoimmune diseases [35, 37]. Interestingly, proceeding inflammatory diseases, such as allergic inflammations, have been shown to induce the generation of iTreg cells, which in turn hamper exacerbated immune responses thereby preventing fatal acute inflammation [38]. Another Treg cell feature of particular interest is that they exhibit anti-inflammatory and anti-tissue remodeling effects, including the inhibition of transplant vasculopathy, which accelerates the rejection of a donor organ [39]. The therapeutic potential of Treg cells for transplant recipients and patients suffering from autoim-

mune diseases has led to extensive studies aimed at understanding the molecular mechanisms that regulate Treg cell development, maintenance, and function [12, 40]. Therefore, induction and increase of iTreg cells is a promising step in the treatment of various adverse immune responses.

## 1.3 Molecular Mechanisms Controlling Regulatory T Cell Function

Treg cell development and survival depends on a high number of key factors and signals, including IL-2, TGF-β, co-stimulatory molecules like CD28, and transcription factors like Foxp3 (see figure 2). The latter is referred to as the master regulator for Treg cell generation and their suppressive functions, since it is required to manifest the Treg cell phenotype [41, 42]. Foxp3 influences gene expression directly by binding to the regulatory elements of *il-2* and *ifn-γ* genes and induces active deacetylation of histone H3. Thus, chromatin remodeling is inhibited and gene transcription is controlled. Additionally, Foxp3 binds to *gitr*, *cd25*, and *ctla-4* genes, which leads to an increased histone acetylation that contributes to elevated expression of respective proteins [43, 44]. Moreover, methylation and demethylation of the DNA plays an important role in Treg cell fate. Characteristic for the Treg cell lineage is the demethylation of CpG islands located at the conserved non-coding DNA sequence (CNS)2 of the *foxp3* gene locus [45, 46]. Thus, the transcriptional profile and the epigenetic landscape both give rise to a unique Treg cell signature.

Foxp3 interacts and collaborates with numerous transcription factors that are required to define the Treg cell phenotype as well as to establish the Treg cell-characteristic transcriptional program [47]. Those include nuclear factor of activated T cells (NFAT), cyclic AMP-responsive element-binding protein (CREB)-ATF, nuclear factor-kappa-light-chain-enhancer of activated B Cells (NF-κB), as well as Signal Transducer and Activator of Transcription 5 (STAT5) and different Smad-proteins activated by IL-2 and TGF-β receptor signaling [4]. Recent studies suggest an important role for the transcription factors interferon regulatory factor (IRF)-1 [48], and Krüppel-like factor (KLF)-10 [49, 50], which may directly regulate Foxp3 expression and subsequently influence Treg cell development. IRF-1 is referred to as negative regulator of Foxp3 expression, as *in vivo* IRF-1 deficiency results in selective and marked increase in highly differentiated and activated Treg cells [48]. Thus, IRF-1 plays a direct role in the generation and expansion of Treg cells specifically repressing Foxp3 transcriptional activity. In contrast to IRF-1, KLF-10 is mentioned as an essential

transcription factor for proper Treg cell function. Here, animals carrying a disruption in *klf-10* gene are impaired in Foxp3 activation. Furthermore, KLF-10-deficient Treg cells have impaired cell differentiation, enhanced expression of Th1, Th2, and Th17 cytokines, and a reduced capacity to suppress effector cells [49].

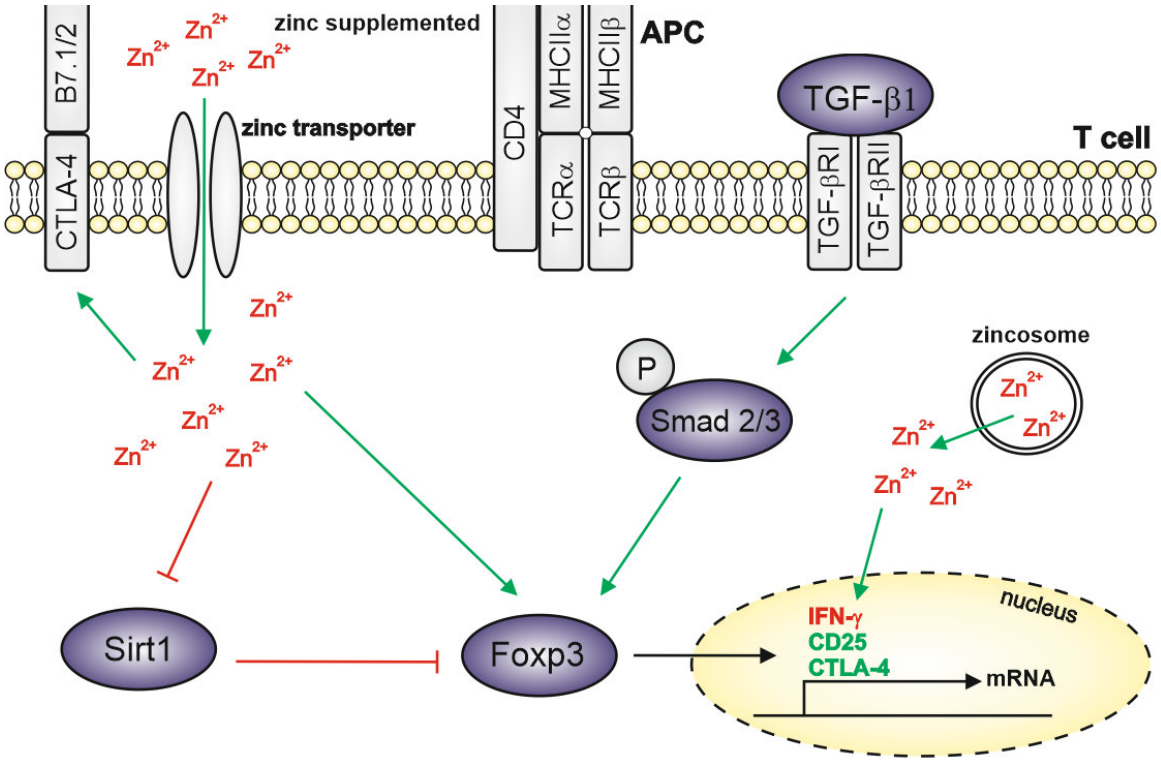


**Fig. 2 Molecular mechanisms controlling Treg cell function.**
Treg cell development is critically dependent on the expression of various proteins interacting with Foxp3 and on the cytokine/ trace element milieu. Treg cells can be induced (green arrows) by TGF-β1 signaling via Smad 2/3 as well as by zinc stimulation. Zinc induces Foxp3 expression, but represses (red arrows) IRF-1 and Sirt1, leading to elevated expression of Foxp3. Foxp3 as well as zinc dampen the IFN-γ production but elevate the expression of CD25 and CTLA-4 (APC, antigen presenting cell).

Besides transcription factors, cytokines play an indispensable role in T cell differentiation. Studies indicate a fundamental role of TGF-β1 establishing immunological tolerance by induction of Treg cells in mice [16, 51, 52] and humans [53, 54]. So far, TGF-β1-mediated Smad signaling has been revealed to be important in Foxp3 induction and cytokine suppression, since Smad-binding elements were found in the CNS 1 region of the Foxp3 promoter [55, 56]. However, Smad-independent pathways such as mitogen-activated protein (MAP)-kinase signaling are also mentioned to be important for Treg cell induction, indicating high complex interactions for the development of this part of the adaptive immune system [57].

## 1.4 Tolerance and Transplantation

The term “immunological tolerance” was first introduced in 1945 when Ray Owen observed placental interchange resulted in red cell chimerism between dizygotic bovine twins [58]. The understanding of tolerance mechanisms in immunology is of main interest in transplantation, since it is essential for effective control of the recipient’s complex response to donor tissue. A successful therapy demonstrates allospecific immunosuppression while minimizing side effects. Additionally, immune competence to infectious pathogens and during cancer drug administration should be preserved. Permanent graft survival after its withdrawal is desired. While transplant tolerance has been largely elusive in humans, it has been an achievable feat in rodents. Tolerance is only infrequently accomplished other than after liver transplantation in humans and remains one of the major obstacles in successful transplantation of solid organs. Nevertheless, transplantation remains the therapy-of-choice for end-stage organ failure [59]. In clinical practice, tolerance is defined as “a well-functioning graft lacking histological signs of rejection, in the absence of any immunosuppressive drugs (for at least one year), in an immunocompetent host” [60, 61]. Major improvements in transplantation medicine are achieved due to surgical techniques, major histocompatibility complex (MHC) matching, and administration of immunosuppressive drugs resulting in a one-year survival rate of over 90% for most solid organ grafts [62]. Long-term survival of transplanted organs currently depends on continuous exposure to combinations of immunosuppressive drugs. However, this therapy is associated with drug side effects and risks infection and cancer. Moreover, drug-related adverse effects like nephrotoxicity, hypertension, diabetes and hyperlipidemia are well-known [63]. To improve therapeutic immunosuppression, it is necessary to understand tolerance processes, to diminish rejection, and to minimize drug usage [64].

The process of graft rejection involves both the innate and the adaptive immune systems. The innate immune system predominates in the early phase of the allogeneic response, in which chemokines and cell adhesion play an essential role. The latter is not only essential for leukocyte migration into the graft but also for facilitating dendritic cell and T cell trafficking between lymph nodes and the transplant. This results in a specific and acquired alloimmune response mediated by T cells. Subsequently, T cells and cells of the innate immune system function synergistically to reject the allograft through different mechanisms, including contact-dependent T cell cytotoxicity, granulocyte activation by either Th1- or Th2-de-

rived cytokines, NK cell activation, alloantibody production, as well as complement activation. Blocking individual pathways generally does not prevent allograft rejection in general, and long-term allograft survival is achieved only after simultaneous blockade of several of them [65]. Thus, the success of clinical transplantation depends largely on efficient suppression of the unwanted immune response by immunosuppressive agents. Since T cells are the central players in graft rejection, most current immunosuppressive drugs like Rapamycin or Cyclosporine A (CsA) target T cell activation and clonal expansion [66]. Non-human primate studies have identified successful preclinical tolerogenic approaches, from T cell depletion and mixed chimerism to co-stimulation blockade and cellular therapies [67, 68]. Still, new treatment strategies for solid organ transplantation are needed, as currently available immunosuppressive drugs do not ultimately protect the patient against the loss of the organ graft during rejection. 15% of total organ grafts are rejected within the first six months after transplantation.

Here, the discovery of Treg cells offers a new paradigm for transplantation research because intra-graft Treg cell frequency seem to correlate with clinical graft acceptance, survival, and function [69, 70]. This is of prime importance since many state of the art post-transplant immunosuppressive treatments, like usage of Rapamycin or CsA, affect Treg cells generation, survival, and function in mice and humans.

Regarding this, Rapamycin and other mTOR inhibitors are known to induce anergy and induce Treg cell differentiation, whereas calcineurin inhibitors, as CsA, have often been associated with antagonistic effects on tolerance induction [71, 72]. CsA inhibits conventional T cells, but also Treg cell development *in vivo*. Studies suggest a dose-dependent CsA-impact on Treg cells: while the administration of CsA in high doses in rats have adverse effects on frequency and function of Treg cells, low doses are associated with pro-tolerogenic effects, resulting in graft tolerance [73, 74]. Thus, a modulation of the T cell response is an attractive approach for the induction and maintenance of allograft-specific tolerance, making long-term immunosuppression unnecessary.

## 1.5 Graft Versus Host Disease

Besides solid organ transplantation, also allogeneic hematopoietic stem cell transplantation (HSCT) is an important therapeutic tool for treating malignant and non-malignant diseases. However, the applicability of tis therapy remains limited due occurrence of graft versus host disease (GVHD) or host versus graft disease (HVGD) in case of solid organ transplantation, making the induction of tolerance highly important. In GVHD the donor's immune

system attacks recipient's tissues because of its recognition as non-self. The reaction can be characterized as acute or chronic depending on the time of onset and site of organ involvement and is defined by 3 phases [75]: (1) activation of host APC; (2) donor T cell activation; and (3) target organ damage by effector cells. The first phase is characterized by host APC activation, due to released pro-inflammatory cytokines (TNF-α, IL-1, IL-6) and pathogen-associated molecular patterns (PAMP) in response to recipient tissue damage. In the second phase, donor T cells proliferate and differentiate into effector cells due to presented allo-antigens and co-stimulatory molecules by APC. Activation of donor T cells against dissimilar antigens results in rapid production of cascades of cellular mediators and soluble inflammatory agents, which together promote inflammation and amplify local tissue damage in the third phase of GVHD [76].

The incidence of acute GVHD is directly related to the degree of mismatch between Human Leukocyte Antigen (HLA) proteins expressed by the HCT donor and recipient [77]. But even in patients that receive HLA-matched (HLA-A/B/C/DRB1) grafts, GVHD arises in approximately 40% of the patients due to differences in minor histocompatibility antigens, and requires systemic therapy [78].

Chronic GVHD is a complex, multisystem disorder with myriad manifestations that can involve any organ [79]. It is typically characterized by fibrosis, but also by erythematous rash, nausea, vomiting, diarrhea and liver dysfunction. The incidence ranges from 30% in recipients of fully HLA-matched HCT to 60-70% in recipients of mismatched or unrelated donor HCT. For chronic GVHD, recipient's age and the occurrence of acute GVHD are the most important risk factors [76]. Chronic GVHD can: (1) evolve from acute GVHD; (2) develop after resolution of acute GVHD; and (3) be acquired *de novo*. In some patients, clinical features of acute and chronic GVHD may be present simultaneously [76].

GVHD is characterized by Th1 and Th17 effector cell responses, whereas Treg cells are known to have a protective role. Treg cell infusion and administration of agents inducing Treg cells have been shown to inhibit activation and proliferation of alloreactive donor T cells, thereby reducing GVHD in mice [80] and men [81]. Recent explorations showed that zinc supplementation has beneficial effects in GVHD, by increasing stability of Foxp3 expression in Treg cells in humans [82, 83]. Thus, GVHD seems to be dampened by Treg cell induction and stabilization.

## 1.6 Zinc Homeostasis and the Human Body

The essential trace element zinc is indispensable for the immune function. Although the human body contains a total amount of 2 to 4 g zinc [84], no specialized storage system can be found making a daily zinc-intake necessary to achieve a steady state for proper immune function. Supplied zinc is absorbed throughout the small intestine and is distributed via the plasma, where zinc concentrations merely reach 90 µg/dl representing less than 1% of the total body content [85]. The plasma zinc pool is very small. Nevertheless, it is highly mobile and immunologically important. Zinc is a predominantly intracellular ion [86] and is distributed between the cell nucleus (30-40%), cytoplasm, organelles, and vesicles (50%) [87], including zinc-specific vesicles called "zincosomes" that can segregate high amounts of zinc upon stimulation [88, 89].

Like plasma zinc, the vast majority of cellular zinc is bound to proteins leaving only a minor loosely bound or unbound part of intracellular zinc, which is referred to as free zinc. Today, up to 4000 proteins and a comparable number of transcription factors are identified to contain zinc-binding motifs [90, 91]. Tightly protein-bound zinc is required for catalytic, co-catalytic and structural functions of enzymes [87]. Zinc is involved in the stabilization of structural domains, such as in zinc fingers and related structures, and enables interaction of proteins or nucleic acids, as for many transcription factors [92].

To avoid excessive accumulation of zinc, an efficient homeostatic control has been evolved during evolution: the Zip and ZnT transporters. These are two families of eukaryotic zinc transporters responsible for zinc homeostasis. The Zrt-like, Irt-like Protein (Zip) Family comprises of 14 genes named solute carrier family 39 (SLC39)A1 to A14. Zips transport zinc into the cytosol, whereas the zinc transporter (ZnT) family of 10 genes (SLC30A1-10) transport zinc in the opposite direction. The same transporter families also regulate the intracellular distribution of zinc into the endoplasmatic reticulum, mitochondria, and Golgi apparatus [93, 94]. In addition, zinc uptake can be mediated by diffusion via amino acids, calcium-conducting channels, and several receptors [95-99].

Changes in the intracellular free zinc concentration can affect signaling pathways leading to altered cellular responses [86, 100, 101]. Moreover, cellular activation and stimulation can result in intracellular zinc fluctuation [102], assuming of an interaction between zinc homeostasis and signal transduction, indicating zinc to possess similar functions as the second messenger calcium [103].

Free zinc is essential for appropriate development and function of the innate as well as the adaptive immunity. However, the importance of zinc for proper immune function is most obvious in zinc-deficient individuals. Whereas intoxication by excessive exposure is rare, zinc deficiency is widespread and known for a long time since the first report in 1961 [104]. Zinc deficiency manifests itself on different levels and can be subdivided into either severe or marginal zinc deficiency. Moreover, zinc deficiency can be either inherited or acquired. On the one hand severe zinc deficiency is caused by metabolic disorders regarding zinc uptake. This is observed in its most severe form in the zinc malabsorption syndrome acrodermatitis enteropathica, an autosomal recessive disorder that bases on a mutation of the intestinal zinc uptake protein Zip 4. Amongst other symptoms, acrodermatitis enteropathica is characterized by periorificial and acral dermatitis, mucocutaneous lesions, diarrhea, neuropsychological disturbances, weight loss, and frequent severe infections [105, 106].

On the other hand, acquired severe zinc deficiency has been observed in patients receiving total parental nutrition without zinc supplementation [107]. The arising symptoms are mostly similar to those in acrodermatitis enteropathica. Additionally, the patients develop abnormalities including lymphopenia, decreased ratios of $CD4^+$-to-$CD8^+$ T cells, decreased NK-cell activity, and increased monocyte cytotoxicity. Interestingly, it can be readily corrected by proper zinc supplementation [108]. In general, zinc deficiency leads to an increased susceptibility to infection and parasitic disease, and is furthermore the fifth leading cause of mortality and morbidity in developing countries.

Marginal zinc deficiency can be characterized by slight weight loss, oligospermia and hyperammonemia [107]. It results from nutritional zinc deficiency often observed in vegetarianism, due to high consumption levels of zinc-chelating agents as lignins and phytates, counteracting zinc absorption [109].

Moreover, parallels exist between the declined immune function in elderly and zinc deficiency even in industrialized countries [110, 111]. A significant percentage of the elderly show reduced serum zinc levels. Zinc supplementation studies indicate that this deficiency contributes to increased susceptibility to infectious diseases. Zinc deficiency also occurs frequently during pregnancy, lactation, phases of rapid growth, or is observed in individuals suffering from renal insufficiency [84, 112]. The overall frequency of zinc deficiency worldwide is expected to be higher than 17% [113, 114]. Thus, zinc deficiency occurs very widespread and because of clinical manifestations it has to be seen as a serious nutritional problem, having a great impact on human health.

## 1.7 Zinc and the Immune System

The innate immunity is the first line of defense in humans and consists of different cell types and numerous soluble proteins. Cells of the innate immune system are activated by conserved structures of pathogens, called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that are recognized by conserved receptors. These trigger processes like cytokine production, killing of target cells, or antigen presentation to cells of the adaptive immune system. In case of infection, polymorphonuclear neutrophils (PMN) are the first cells that actively enter the infected tissue, following a concentration gradient of chemical messengers, known as chemotaxis. It is reported that this and other cellular functions are disturbed by altered zinc levels. Zinc deficiency leads to reduced PMN chemotaxis [115, 116], whereas elevated zinc concentrations are reported to induce chemotactic activity directly *in vitro* [117]. PMN are essential to mortify pathogens by phagocytosis and generation of reactive oxygen species (ROS) during respiratory burst. Zinc deficiency leads to decreased phagocytosis and ROS production [118]. The latter are produced by NADPH oxidase, which is inhibited by zinc deficiency as well as by zinc excess [119-121]. Additionally, the capture of extracellular bacteria within neutrophil extracellular traps, called NETosis, is a zinc-dependent process, which can be abrogated by zinc chelation [122].

Besides PMN, macrophages take up pathogens or cellular debris by phagocytosis and kill pathogens by oxidative burst, which are both zinc dependent processes [123]. The differentiation of macrophages is calcitriol-dependent and can be enhanced by zinc sequestration [124].

Furthermore, NK cells play a major role in immunity, by screening cells for bound antibodies to perform antibody-dependent cell-mediated cytotoxicity (ADCC). Additionally, they examine immune cells for major histocompatibility complex class I (MHC-I) molecules and eliminate cells with altered and reduced MHC-I expression, as this absence can indicate an attempted evasion of T cell immune surveillance. In this regard, zinc is involved in the recognition of MHC-I on target cells, by p58 killer cell inhibitory receptors on NK cells for the inhibition of the killing activity [125, 126]. Interestingly, the NK cell number as well as their activity depends on the serum zinc level. The lytic activity of NK cells is decreased by zinc deficiency, probably due to diminished stimulation from T cells via IL-2 [127]. Zinc supplementation restores NK cell activity and increases differentiation of $CD34^+$ progenitors towards NK cells [128]. However, merely the inhibitory signal is zinc

dependent, whereas the MHC-I interaction and positive signals are zinc-independent. Thus, zinc is required to maintain proper NK cells function, since zinc deficiency results in functional loss and evokes nonspecific killing.

The adaptive immune system is also highly zinc-dependent, especially concerning the B- and T cell activation, development, as well as T cell differentiation [101, 127, 129]. Notably, proliferation and function of T cells are highly dependent on the organism's zinc status, whereas zinc seems to have no direct influence on the activity of B cells [130], but on pre-B cell development [131]. Thus, there are fewer naïve B cells during zinc deficiency that can react to neo-antigens. In line with that, zinc deficient patients, like elderly and hemodialysis patients, show a reduced response to vaccination [132], a disturbed antibody production [133], and a higher risk of parasitic infections [134]. This is due to a disturbed activation of early B cells, the immunoglobulin class switch towards IgE, and thereby the further antibody specification. In this context, zinc deficiency impairs IL-4-dependent STAT6 phosphorylation that is essential for T cell reactivity and IL-4-dependent early B cell activation. Additionally, many diseases, like rheumatoid arthritis and plasma cell neoplasias go along with reduced serum zinc levels. This is related to an IL-6 overproduction that is associated with autoantibody production, as IL-6 is responsible for the activation and differentiation of B cells into plasma cells. In this regard, an adequate zinc concentration is essential for IL-6 induced STAT3 phosphorylation since it is pathologically increased during zinc deficiency [135]. Thus, a strict regulation of zinc homeostasis is necessary to keep the immune system balanced.

Opposed to this, T cell differentiation and function are highly zinc-dependent, since various T cell defects are described due to zinc deficiency that manifest in thymus atrophy and lymphopenia [101, 136, 137]. Additionally, the activity of cytotoxic T cells [138] and the relative amount of $CD8^+$ $CD73^+$ T cells is decreased [127]. Zinc acts as an essential cofactor for thymulin, which is produced by the thymus and released by thymic epithelial cells [139]. It induces differentiation of immature T cells [140] and acts on mature T cells in the periphery. Herein, it modulates the cytokine release by peripheral blood mononuclear cells (PBMC), and proliferation of $CD8^+$ T cells in combination with IL-2.

Furthermore, zinc signaling induce blast transformation of human T cells [141] and influences the expression of the high-affinity receptor for IL-2 on mature T cells resulting in decreased T cell proliferation during zinc deficiency [142]. Moreover, high zinc concentrations are known to induce IL-2, the soluble IL-2 receptor (sIL-2R), and IFN-γ in PBMC [143, 144]. Consistently, both zinc deficiency and slightly elevated zinc levels influence

T cell function. Increasing zinc levels during T cell activation are postulated to calibrate TCR signaling leading to T cell responses following suboptimal stimuli [145]. In contrast, high zinc concentrations inhibit T cell activity in mixed lymphocyte cultures (MLC) as demonstrated by suppressed IFN-γ expression [82, 144]. However, physiological doses of zinc induce and stabilize Treg cells in MLC, leading to a reduced secretion of IFN-γ and a dampened allogeneic immune reaction [146]. Zinc deficiency provokes a decrease of T cell proliferation after mitogen stimulation [147], whereas zinc supplementation is able to reverse the zinc deficiency induced changes, which is also seen in patients with acquired immune deficiency syndrome [148]. Furthermore, the risk for infections and Th2-driven allergies are increased, as well as the development of autoimmune diseases in zinc deficiency [136].

Nearly all immune cells are directly affected by zinc, in particularly by zinc deficiency, indicating that zinc is a main regulator of cellular function and signal transduction in the immune system. However, the majority of these effects are characterized by investigations of isolated cell populations and signaling pathways *in vitro*, making further research necessary to assess zinc-related effects *in vivo*.

# II Aim of the Study

A well-functioning immune system is highly important, since a dysregulation is associated with adverse immune reactions. How fundamentally important a well-balanced zinc homeostasis is for a proper immune reaction, can be easily demonstrated by patients suffering of zinc deficiency, displaying a higher rate of infection. Moreover, zinc deficiency is often associated with a higher incidence of allergies, autoimmune diseases, or transplant rejections. Hence, a balanced immune system is crucial to defend invading pathogens and to avoid self-reactive and overactive immune responses by tolerance mechanisms respectively. In this regard, particularly Treg cells are important since their dysfunction is often related to inadequate, excessive, or absent immune responses leading to immune disorders. Thus, they are in the focus of interest of therapeutic use for modulating transplantation rejection or autoimmune diseases. Regarding this, in clinical therapy antigen-specific Treg cells are tried to be generated *in vitro* and *in vivo* because they are described to be more potent suppressors for adverse immune reactions than unrestricted Treg cells.

Thus, the first aim of this study was to analyze the capacity of physiological zinc supplementation to induce antigen-specific Treg cell in graft versus host disease using the mixed lymphocyte culture *in vitro*. For this purpose, the pro-inflammatory cytokine production and proliferation were analyzed, reflecting the severity of an allogeneic immune response respectively.

It is well-known that for adequate Treg cell function the expression of the master transcription factor Foxp3 is essential. Foxp3 can be induced by zinc supplementation, however only little information on the regulation of its expression itself is available so far. Therefore, secondly this study was conducted to gain insight into the molecular mechanisms responsible for Treg cell induction by zinc supplementation. In this context, the TGF-β1-induced Smad signaling pathway involved in Treg cell induction was investigated, as well as the expression of several transcription factors associated to Foxp3 regulation.

The third aim of this study was to uncover the capability of zinc supplementation for clinical use. Regarding this, the capacity of zinc supplementation to beneficially influence the immune reaction was studied. Therefore, Treg cell and Th17 cell differentiation in an *in vivo* kidney transplantation model was investigated. Modulation of Treg cells is still a major goal in transplantation to improve patients life's. The immunomodulatory capacity of zinc is highly relevant and needs to be studied since zinc application in physiological doses is non toxic and inexpensive making zinc a favorable candidate for the treatment of adverse immune reactions.

# III Materials and Methods

## 3.1 Materials

### 3.1.1 Equipment

- Analytical scales, 770/GS/GJ (Kern, Balingen-Frommern)
- Blot transfer chamber, Mini-trans (Bio-Rad, München)
- Cell counter, Casy 1 TT (Scharfe System, Reutlingen)
- $CO_2$ incubator, MCO-17AIC (Sanyo, Gunma, Japan)
- Electrophoresis chamber for polyacrylamide gels, Mini-Protean 3 Electrophoresis Module Assembly (Bio-Rad, München)
- Electrophoresis chamber for polyacrylamide gels, Protean II xi (Bio-Rad, München)
- Electrophoresis net, EPS 3500 XL (Amersham Pharmacia Biotech, Uppsala, Sweden)
- Electrophoresis power supply, Power Pac 300 (Bio-Rad, München)
- Electrophoresis power supply, Power Pac 1000 (Bio-Rad, München)
- ELISA reader, Magellan (Tecan, Crailsheim)
- ELISA washer, Atlantis (Asys Hitech, Eugendorf)
- ELISA washer, ELx50 (BioTek, Bad Friedrichshall)
- Flow cytometer, FACScan and FACSCalibur (Becton Dickinson, Heidelberg)
- Floor centrifuge, Varifuge 3.0 RS (Heraeus Christ, Osterode)
- Floor centrifuge, Z 400 K (Hermle, Wehingen)
- Freezer, -20°C (Bosch, München)
- Freezer, -80°C, MDF-U71V (Sanyo, Gunma, Japan)
- Heat sealer, Polystar 100 (Rische and Herfurth, Hamburg)
- IsoFlow vaporizer (Eickemeyer, Stratford, Ontario, Canada)
- Laboratory scales, 1265 MP (Sartorius, Göttingen)
- Liquid scintillation β-counter 1219 Rackbeta (LBK, Wallace, Freiburg)
- Luminescent image analyzer, LAS-3000 (Fujifilm, Düsseldorf)
- Magnet stirring bar retriever, MR3001 (Heidolph, Schwabach)
- Microscope, SM-LUX (Leitz, Wetzlar)
- Microscope, Leica Wild M650 (Leica, Heerbrugg, Switzerland)
- Mikrowave, NN-A850W (Panasonic Deutschland, Hamburg)
- Multipipetter, Multipette plus (Eppendorf, Hamburg)

- Multiplate reader, Ultra 384 (Tecan, Crailsheim)
- pH measuring device, HI 9321 (Hanna Instruments, Kehl am Rhein)
- Photometer, BioPhotometer (Eppendorf, Hamburg)
- Pipetter, pipetus-akku (Hirschmann, Eberstadt)
- Pipettes, 0.5-10 µl, 10-100 µl an 100-1000 µl, Research (Eppendorf, Hamburg)
- Refrigerator (Bosch, München)
- Shaker, HS 250 basic (IKA Labortechnik, Staufen)
- Sonicator, Vibra Cell (Sonics & Materials, Danbury, CT, USA)
- Sterile working station, KR-130 and KR-210 (Kojair, Vilppula, Finland)
- StepOnePlus™ Real-Times PCR System (Life Technologies Corporation, Carlsbad, CA, USA)
- Table top centrifuge, 5417 (Eppendorf, Hamburg)
- Table top centrifuge, Biofuge A (Heraeus, Osterode)
- Thermomix, comfort and compact (Eppendorf, Hamburg)
- Vortex, Reax (Heidolph, Schwabach)
- Weighing scales, 1265 MP (Sartorius, Göttingen)
- Western Blot chamber, Mini-Protean 3 Electrophoresis Module Assembly (Bio-Rad, München)

### 3.1.2 Laboratory Supplies

- Aneurysm-clip-system Yasargil, Titan Standard Clips (Aesculap, B Braun, Melsungen)
- Bürker counting chamber (Brand, Wertheim)
- Casy-Cups (Scharfe System, Reutlingen)
- Cell culture flasks, T25 and T75 (Nunc, Roskilde, Denmark)
- Cell culture 6-well, 12-well, 24-well, 96-well plates (VWR, Darmstadt)
- Centrifuge tubes, Sorvall GS3 (Kendro, Langenselbold)
- Centrifuge tubes, Sorvall SA-600 (Kendro, Langenselbold)
- Dental cotton role Gr.2, 300g (RWTH University Hospital, Aachen)
- Diadust micro needle holder 120mm (Aesculap, B Braun, Melsungen)
- Ethilon, black, monophil 11/0 (Ethicon, New Jersey, USA)
- Ethilon, black, monophil 9/0 (Ethicon, New Jersey, USA)
- Ethilon, black, monophil 7/0 (Ethicon, New Jersey, USA)

- FACS tubes (Sarstedt, Nümbrecht)
- Film cassette, 20 x 25 cm (Bio-Rad, München)
- Gauze 10cm x 20cm sterile (Fing & Walter GmbH, Merchweiler)
- Gel blotting paper GB003 (Schleicher and Schuell, Dassel)
- Gelastypt (Sanofi aventis, Paris, France)
- Latex gloves (Kimberly-Clark, Zaventem, Belgium)
- Microcentrifuge tubes, 1.5 ml (Sarstedt, Nümbrecht)
- Micro forceps Jeweler type 0.2 mm (Aesculap, B Braun, Melsungen)
- Micro forceps Jeweler type 0.3 mm (Aesculap, B Braun, Melsungen)
- Micro scissors 145 mm (Aesculap, B Braun, Melsungen)
- MiniSpike® (B Braun, Melsungen)
- MoliNea® (Paul Hartmann AG, Heidenheim)
- Nitrocellulose membrane Trans-blot transfer medium, pure (Bio-Rad, München)
- One-way pipettes, 5 ml, 10 ml, and 25 ml (Grenier, Nürtingen)
- Optical Tubes 8er Stripes ABI PRISM (Applied Biosystems, Foster City, USA)
- Optical Caps 8er Stripes ABI PRISM (Appield Biosystems, Foster City, USA)
- Pipette Combitips, 1 ml, 2.5 ml, 5 ml, and 10 ml (Eppendorf, Hamburg)
- Pipette tips, 1-10 µl and 10-1000 µl (Sarstedt, Nümbrecht)
- Pipette tips, 100-1000 µl (Eppendorf, Hamburg)
- Plastic tubes with tops, 15 ml and 50 ml (Falcon, Heidelberg)
- Plastic wrap (RUF, Bremen)
- Polystyrene tubes with tops, 13 ml (Sarstedt, Nümbrecht)
- Sponges, 8 x 11 cm (BioRad Laboratories, München)
- Scintillation tubes (PerkinElmer, Rodgau)
- Shavor Onyx (Albert Kerbl GmbH, Buchbach)
- Syringe, 1 ml, 2 ml, 5 ml, 10 ml, 20 ml (Braun, Melsungen)
- Tabotamp (Ethicon, New Jersey, USA)
- Tissue-Tek® Standard Cryomold® 25 mm x 20 mm x 5 mm (Sakura Finetek Germany GmbH, Staufen)
- UV-cuvette, UVette (Eppendorf Hamburg)
- Vacuum filter, Stericup 0.22 µm GP ExpressPlus (Millipore, Schwalbach)
- Vicryl, violett, braided 2/0 (Ethicon, New Jersey, USA)
- Vise micro suture forceps 3mm (Aesculap, B Braun, Melsungen)

- VWR® Superfrost® Plus Micro Slide (VWR International, Darmstadt)

### 3.1.3 Cell Culture Media and Additives

- Culture medium RPMI 1640 (Sigma-Aldrich, Steinheim)
- Culture medium DMEM (4.5% Glucose) (Sigma-Aldrich, Steinheim)
- Culture medium IMDM (Sigma-Aldrich, Steinheim)
- Fetal calf serum (Sigma-Aldrich, Steinheim)
- L-glutamine, 200 mM (Sigma-Aldrich, Steinheim)
- Non-essential amino acids (100x) (Sigma-Aldrich, Steinheim)
- Penicillin/Streptomycin, 10,000 U/ml + 10,0000 µg/ml (Sigma-Aldrich, Steinheim)
- Phophate Buffered Saline (PBS) without $Ca^{2+}$ and $Mg^{2+}$ (Sigma-Aldrich, Steinheim)
- Sodium pyruvate (Sigma-Aldrich, Steinheim)
- Ultra Doma PF (Lonza, Verviers, Belgium)

### 3.1.4 Immunological Reagents

#### 3.1.4.1 Immunoprecipitation and Western Blot Antibodies

- Goat anti-biotin conjugated HRP (Cell Signaling, Beverly, MA, USA)
- Goat anti-rabbit conjugated HRP (Cell Signaling, Beverly, MA, USA)
- Horse anti-mouse conjugated HRP (Cell Signaling, Beverly, MA, USA)
- Mouse anti-human Foxp3 (Abcam, Cambridge, UK)
- Rabbit anti-human β-Actin (Cell Signaling, Beverly, MA, USA)
- Rabbit anti-human IRF-1 (Cell Signaling, Beverly, MA, USA)
- Rabbit anti-human pSmad 2/3 (Cell Signaling, Beverly, MA, USA)
- Rabbit anti-human pSmad 1/5/8 (Cell Signaling, Beverly, MA, USA)

#### 3.1.4.2 Flow Cytometry Antibodies

- Mouse anti-human CD4 conjungated FITC (Becton Dickinson, Heidelberg)
- Mouse anti-human CD25 conjungated APC (Becton Dickinson, Heidelberg)
- Mouse anti-human CD152 conjungated PE-Cy5 (Becton Dickinson, Heidelberg)
- Mouse anti-human Foxp3 conjugated PE (Becton Dickinson, Heidelberg)
- Mouse anti-human RORC2 conjugated PE (R&D Systems, Minneapolis, USA)
- Mouse anti-rat CD4 conjungated FITC (eBiosciences, San Diego, CA, USA)

- Mouse anti-rat CD25 conjungated APC (eBiosciences, San Diego, CA, USA)
- Mouse anti-rat Foxp3 conjungated PE (eBiosciences, San Diego, CA, USA)
- Mouse anti-rat RORγt conjungated PE (Abcam, Cambridge, UK)

#### 3.1.4.3 Flow Cytometry Isotype Control Antibodies

- Mouse IgG1,κ conjugated FITC isotype (Becton Dickinson, Heidelberg)
- Mouse IgG1,κ conjugated APC isotype (Becton Dickinson, Heidelberg)
- Mouse IgG2a,κ conjugated PE-Cy5 isotype (Becton Dickinson, Heidelberg)
- Mouse IgG1,κ conjugated PE isotype (Becton Dickinson, Heidelberg)
- Mouse IgG2a,κ conjugated FITC isotype (Becton Dickinson, Heidelberg)

### 3.1.5 Miscellaneous Reagents

- Acetic acid, 100% (Merck, Darmstadt)
- Acylamide/bisacrylamide (37,5:1), 30% (Serva, Heidelberg)
- Agarose (Gibco, Karlsruhe)
- Aqua ad iniectabilia (Braun, Melsungen)
- Betaisodonna® (Mundi pharma GmbH, Limburg, Netherlands)
- Bepanthen® (Bayer AG, Leverkusen)
- Biocoll, Ficoll 1.077 g/ml (Biochrom, Berlin)
- Power SYBRGreen PCR Master Mix (Applied Biosystems, Warrington, UK)
- qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems, Nippon Genetics Europe, Düren)
- Bromphenol blue (Riedel-de-Haën, Hannover)
- Bovine serum albumin (Fluka, Buchs, Switzerland)
- Casy-Ton (Scharfe System, Reutlingen)
- Chloroform (RWTH University Hospital, Aachen)
- Custodiol (Köhler Chemie GmbH, Bensheim)
- Cyclosporin A (Alexis Corporation, Lausen, Switzerland)
- Poly-Alcohol Haut Antiseptikum (Antiseptica chem. pharm. Produkte GmbH, Pull-heim)
- Dimethylsulfoxide (DMSO) (Carl-Roth, Karsruhe)
- Di-Natriumhydrogenphosphat anhydrous (Carl-Roth, Karlsruhe)
- Ethanol, absolut (RWTH University Hospital, Aachen)
- Ethanol, 70%, denatured (RWTH University Hospital, Aachen)

- Fat-free milk powder Sucofin (Trade Service International, Zeven)
- Fluozin-3A (Life-Technologies, Darmstadt)
- Fluozin-3AM (Life-Technologies, Darmstadt)
- Glucose (5%) (B Braun, Melsungen)
- Glycerin (Merck, Darmstadt)
- Glycine (Sigma-Aldrich, Steinheim)
- Heparin sodium, 5000 (Ratiopharm, Ulm)
- Hydrochloric acid, 32 % (Merck, Darmstadt)
- IL-10, recombinant, human (Peprotech Inc., NY, USA)
- IL-2, recombinant, human (Peprotech Inc., NY, USA)
- Isloflurane (Forene®, 100% v/v) (AbbVie Deutschland GmbH & Co. KG, Wiesbaden)
- Ketamin 10% (Medistar Arzneimittelvertrieb GmbH, Ascheberg)
- Lipoluma Plus (Lumac LSC B.V., Groningen, Niederlande)
- Loading dye solution, O'Range 6x (MBI Fermentas, St. Leon-Rot)
- LumiGLO reagent and peroxide (Cell Signaling, Beverly, MA, USA)
- $Na_3VO_4$ Protease Inhibtor Cocktail (Sigma-Aldrich, Steinheim)
- Nitric acid ($HNO_3$; 69%) (Sigma-Aldrich, Steinheim)
- β-Mercaptoethanol (Merck, Darmstadt)
- Methanol (RWTH University Hospital, Aachen)
- Methyl-[$^3$H]-thymidine (Amersham Biosciences, Buckinghamshire, UK)
- Natriumdihydrogenphosphate-monohydrate anhydrous (Carl-Roth, Karlsruhe)
- Paraformaldehyde (Sigma-Aldrich, Steinheim)
- Phytohemagglutinin (PHA) (Becton Dickinson, Heidelberg)
- Ponceau S (Fluka, Buchs, Schweiz)
- Propidium Iodide, Minimum 95% (HPLC) (Sigma-Aldrich, Steinheim)
- Protein marker, biotinylated (Cell Signaling, Beverly, MA, USA)
- Pyrithione (Merck, Darmstadt)
- Recombinant human IL-2 (Peprotech Inc., NY, USA)
- Sodium azide (Merck, Darmstadt)
- Sodium chloride (AppliChem, Darmstadt)
- Sodium chloride 0.9% solution (Braun, Melsungen)
- Sodium dodecyl sulfate (Merck, Darmstadt)
- Temgesic (Medistar Arzneimittelvertrieb GmbH, Ascheberg)

- Methyl-[$^{3}$H]-thymidine (Amersham Biosciences, Buckinghamshire, UK)
- Tissue-Tek® Uni-Cassette® TT III green and orange (Sakura Finetek Germany GmbH, Staufen)
- TGF-β1, recombinant, human (R&D Systems, Minneapolis, USA)
- Tris-(hydroxymethyl)-aminomethane (Tris Base) (Merck, Darmstadt)
- Trizol Reagent (Ambion, Austin, TX, USA)
- Tween-20 (Merck, Darmstadt)
- Xylazin 2% (Medistar Arzneimittelvertrieb GmbH, Ascheberg)
- Zinc apartate, solution (Unizink®) (Köhler Pharma GmbH, Alsbach-Hähnlein)
- ZinPyr-1 (NeuroBioTex, Galveston, USA)
- $ZnSO_4$ x 7 $H_2O$ (Merck, Darmstadt)

### 3.1.6 Commercial Available Kits

- Human ELISA Component-Set for IFN-γ detection (BD Biosciences Pharmingen, Heidelberg)
- Human Foxp3 buffer set (Becton Dickinson, Heidelberg)
- Mouse/rat Foxp3 buffer set (eBiosciences, San Diego, CA, USA)
- Q-Script cDNA Synthesis Kit (Quanta, Gaithersberg, MD, USA)

## 3.2 Methods

### 3.2.1 Preparation of Zinc Solutions

Zinc sulfate was dissolved in Aqua ad iniectabilia to a stock solution of 100 mM. The solution was sterile filtered with bacteria filters (pore size 0.2µm) and subsequently further diluted in protein-free medium (Ultradoma) providing a 2mM zinc sulfate solution.
Zinc-aspartate (Unizink®, 0.6mg/ml) and Cyclosporine A was diluted in 5% Glucose for animal experiments respectively.

### 3.2.2 Cell Cultures

Each cell lines listed in table 1 was cultured in $CO_2$-incubators maintained at 37°C, 5% $CO_2$, and saturated air humidity. The cells were examined under a microscope twice a week and transferred into fresh medium. Fetal calf serum (FCS) was inactivated at 56°C for 30 min. Freshly isolated primary cells were used depending on the respective experiment settings.

**Table 1. Cell lines and corresponding culture medium**

| Cell line | Media | Supplements | Subculture |
|---|---|---|---|
| **Hut78[1], Raji[1], BJAB[1]** | RPMI 1640 | 10% heat-inactivated FCS<br>1% L-Glutamin<br>1% Penicillin/Streptomycin | Hut78: $2x10^5$ cells/ml<br>Raji: 0.5 ml + 12 ml<br>BJAB: 0.5 ml + 9.5 ml |
| **Huh7[1]** | DMEM<br>(4.5 g glucose) | 10% heat-inactivated FCS<br>1% L-Glutamin<br>1% Penicillin/Streptomycin | 1:20 |
| **EBV** | IMDM | 18% heat-inactivated FCS<br>1% L-Glutamin<br>1% Penicillin/Streptomycin | 2,5 ml + 7,5 ml |
| **PBMC** | RPMI 1640 | 5% or 10% heat-inactivated FCS<br>1% L-Glutamin<br>1% Penicillin/Streptomycin | |

[1] from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen)

### 3.2.3 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) comprise blood leucocytes carrying a round shaped nucleus, including T and B cells, monocytes, and NK cells. Compared to granulocytes and erythrocytes they maintain a lower density, which simplifies their isolation by Ficoll-density-gradient. Ficoll is composed of high molecular polysaccharides and epichlorohydrin, a reactive organic compound. After centrifugation, erythrocytes and granulocytes settle to the bottom of the gradient, whereas low density cells such as thrombocytes remain in the upper phase. Due to their density, PBMC accumulate in an interphase above the Ficoll solution and can be removed selectively.

**Reagents:**

- Heparin (25,000 U/ml)
- PBS 1x
- Biocoll (densitiy 1.077 g/ml)
- Whole blood

**Procedure:**

1. Whole blood containing 2500 U heparin was diluted 1:1 with PBS
2. The blood/PBS mixture was slowly pipetted onto Ficoll
3. The Ficoll gradient was centrifuged (20 min, 600 g, RmT, acceleration/deceleration "5")
4. The PBMC-containing interphase was carefully removed and placed in a new 50 ml plastic tube, which was then filled to the top with PBS (5 min, 300 g, RmT)
5. The PBMC were washed twice with PBS (5 min, 300 g, RmT)

### 3.2.4 Lysis of Erythrocytes

Hypotonic lysis of erythrocytes is recommended for PBMC isolation, as some contaminating erythrocytes remain after isolation. Erythrocytes respond more sensitive to hypotonic solutions and can therefore be easily eliminated without affecting the PBMC. This procedure comprises the brief resuspension of PBMC in sterile water to induce erythrocyte lysis. Subsequently, the addition of the same volume of two-fold concentrated PBS recreates isotonic conditions.

**Reagents:**

- Aqua ad iniectabilia
- PBS (2x and 1x)

- RPMI medium containing 10 % heat-inactivated FCS, 1% L-glutamine and 1% penicillin/streptomycin

**Procedure:**

1. The cell pellet was briefly resuspended in 10 ml Aqua ad iniectabilia with rapidly addition of 10 ml 2x PBS, followed by centrifugation (5 min, 300 g, RmT)
2. The PBMC were washed with 1x PBS (5 min, 300 g, RmT)
3. The cell concentration was adjusted with RPMI medium

### 3.2.5 Paraformaldehyde-Fixation of PBMC

Paraformaldehyde is the smallest polyoxymethylene, which is often used as a disinfectant, fungicide, and fixative. For fixation, a 3% paraformaldehyde-solution can be used to prevent cytokine production and proliferation of cells used as foreign antigen-stimulus in One-way MLC.

**Reagents:**

- Paraformaldehyde
- PBS
- RPMI medium without and with additives (additives: 10 % heat-inactivated FCS, 1% L-glutamine and 1% penicillin/streptomycin)

**Procedure:**

1. 1 g paraformaldehyde was dissolved in 25 ml PBS
2. The solution was stirred for 1 h (50°C) and sterile-filtrated
3. The cells were adjusted to $2x10^6$/ml in RPMI 1640 (without additives)
4. Depending on the needed cell number x ml cell suspension was incubated with equal volumes of paraformaldehyde-solution (3%) for 3 min
5. The cells were washed tree times with PBS (5 min, 300 g)
6. The cells were finally resuspended in RPMI 1640 (with additives) to a concentration of $2x10^6$/ml

### 3.2.6 Generation and Stimulation of Mixed Lymphocyte Cultures

In Two-way MLC equal amounts of genetically diverse PBMC are mixed and incubated. In One-way MLC $2x10^6$ PBMC are incubated with one-fifth of paraformaldehyde-fixed cells. After incubation over an experiment-dependent period of time, various parameters like protein- or cytokine expression, and proliferation were analyzed.

**Reagents:**

- RPMI medium containing 10% or 5% heat-inactivated FCS, 1% L-glutamine and 1% penicillin/streptomycin
- $ZnSO_4$ (2 mM)
- CsA (1 mg/ml)

**Procedure:**

1. Isolated PBMC were adjusted to a concentration of $2x10^6$/ml
2. PBMC were incubated in 6-well plates with either RPMI medium, or $ZnSO_4$ (50 µM), or CsA (1 µg/ml), for 15 min at 37°C and 5% $CO_2$
3. The same volume of PBMC of two donors (with the same stimulation) were mixed and dispersed into a 24-well plate (final volume: 1 ml/well)
4. MLC were incubated at 37°C and 5% $CO_2$ for 5 days
5. Supernatants were collected and used for analysis of cytokine expression by ELISA. Cell pellets were used for FACS analysis, western blot, and real-time PCR

### 3.2.7 Allogeneic Kidney Transplantation in Rats

**Reagents:**

- Sterile NaCl (0.9%)
- Custodiol
- Poly-alcohol-antiseptic-solution

**Procedure:**

1. Donor kidney preparation
    a. Analgesia was performed by i.p. injection of Ketamin/Xylazin (4:1, 10 I.U./rat)
    b. The rat was laid down on a MoliNea® pad fixed on a heatable plate
    c. The rat was put in dorsal position, and the stomach was shaved and disinfected using poly-alcohol-antiseptic-solution
    d. Reflexes were tested (lid's blinking reflex, tail tip reflex, between toes reflex)
    e. Fixation was performed by tapes followed by laparotomy by a longitudinal incision
    f. Four retractors were used to spread the abdomen and the gut was relocated to the right side, protected by moistly gauze (NaCl 0.9%)
    g. The left kidney was exposed and bleeding was stopped by cauterization

h. The blood stream of the left kidney artery and vein was interrupted by clamping the main artery and vein using aneurysm clips (situated far proximal and far distal off renal vein and artery).
i. The left kidney vein was opened and the kidney was flushed retrograde via renal artery using Custodiol
j. The ureter was cut far distal and the main artery was cut below the proximal and above the distal clip
k. Kidney was stored in Custodiol on ice

2. Recipient kidney transplantation
    a. Analgesia was performed by subcutaneous injection of 0.0075 mg/kg 30 min before the rat was deeply anesthetized by Isoflurane
    b. Point b to g of "donor kidney preparation" was done equally for recipient rats
    c. The main artery and vein were dissected and aneurysm clips were used far distal and far proximal to interrupt the blood stream
    d. A small ventral cut was performed in both, followed by intense flushing of those vessels to avoid formation of blood clots
    e. The donor kidney was laid in the abdominal cavity and the anastomosis of the renal artery and the renal vein was done using 9/0 Ehtilon (continuous suture, end-to-side)
    f. The artery cuff was ligated by 7/0 Ethilon, the abdominal cavity was dried using cotton dental roles and Tabotamp was placed around the suture to avoid bleeding
    g. The clips were removed and the organ was monitored during reperfusion
    h. Anastomosis of the ureter was done by using 11/0 Ethilon (end-to-end, 4 nodes), and the ureter peristalsis was monitored
    i. Nephrectomy of the recipients' left kidney was performed using 2/0 Ethilon (ligation of kidney pole; the adrenal gland and the renal capsule remain). The contralateral kidney stays in recipient
    j. The abdominal wall was closed by double-layered continuous suture using 2/0 Vicryl
    k. Rats were single-housed for 48 h

### 3.2.8 Rat Splenocyte Isolation

**Reagents:**

- Sterile $H_2O$
- PBS
- PBS (2x)

**Procedure:**

3. Double-layered gauze was mounted on a 50 ml centrifuge tube
4. The spleen was removed by using sterile forceps and stored in 15 ml PBS in a 50 ml centrifuge tube on ice
5. The spleen was homogenized with the plunger end of the syringe
6. The cells were transferred through the gauze into the 50 ml tube (the gauze served as a cell strainer)
7. 10 ml PBS were used to wash the petri dish and transfer of the remaining splenocytes through the gauze into the tube
8. The cells were centrifuged at 300g for 5 min
9. The supernatant was discarded and the pellet was resuspended in 10 ml $H_2O$, followed by rapid administration of 10 ml PBS (2x) for lysis of erythrocytes
10. The tubes were filled up with PBS and centrifuged at 300g for 5 min
11. The supernatant was discarded and the pellet was resuspended in 25 ml RPMI medium. 10 µl were used for cell counting by using a Neubauer counting chamber
12. Splenocytes were adjusted to $1x10^6$ cells/ml

### 3.2.9 Tissue Sample Harvesting

After animal anesthesia, blood was taken from the left heart ventricle by using sterile syringe and injection needle. The blood was immediately filled into 15 ml plastic tube containing 10 µl heparin (25,000 U/ml) and stored on ice.

Subsequently, the spleen was removed and stored in a 50 ml plastic tube filled with 15 ml PBS on ice. Blood and spleen were processed for FACS analysis.

### 3.2.10 Enzymed-Linked Immunosorbent Assay

Sandwich enzyme-linked immunosorbent assays (ELISA) are commonly used for determining the amount of secreted molecules, like cytokines. Sandwich ELISA start with the immobilization of an antigen-specific antibody, named "capture antibody", in a 96-well plate. After that, the plate is blocked with BSA to prevent unspecific binding. Subsequently, titrated standard and diluted samples are added to the appropriate wells. After incubation of the samples/standards, excess cytokines are aspirated and a biotinylated antibody specific for a different epitope of the investigated cytokine ("detection antibody") is provided. Finally, the enzyme reagent consisting of avidin-HRP-conjugate is added. After further wash steps to remove excessive antibodies, the substrate (p-Nitrophenyl phosphate) is added. A chemical reaction proceeds inducing a change in color so that the adsorption can be measured by a well-plate reader.

**Reagents:**

- Carbonate buffer: 29 mM sodium carbonate, 22 mM sodium hydrogen carbonate (pH 9.6)
- Coating buffer: Carbonate buffer + 1 % BSA
- Assay diluent: PBS + 10 % FCS
- Wash buffer: 2000 mL PBS + 1 ml Tween 20
- Capture antibodies (monoclonal anti-human IFN-γ)
- Biotinylated detection antibodies (monoclonal anti-human IFN-γ)
- Streptavidin horseradish peroxidase conjugate (SAv-HRP)
- Standard solutions (Recombinant human IFN-γ)
- Substrate solution: Tetramethylbenzidine (TMB) and Hydrogen Peroxide (1:1)
- Stop solution (1 N $H_2SO_4$)

**Procedure:**

1. Coating of the micro-titer-plates and assay procedure were performed in accordance with the manufacturers' instructions.

### 3.2.11 Flow Cytometry

Flow cytometry is of increasing relevance for various research issues. Among other functions it allows the accession of cell surface marker expression, sorting of cells according to particular characteristics, performance of intracellular staining, visualizing of apoptotic

cells, and determination of cell cycle progression. Despite its name, not every FACS machine is capable of cell sorting and they are often used for cell analysis only.

The cell suspension flows through a nozzle into a stream of sheath fluid leading to cell scattering before entering into a laser path. At an interrogation point the cells are analyzed for reflection, diffraction, or refraction of light, or fluorescence. A photodiode measures forward-angle scatter (FSC) light whilst photodetectors sense emitted side scatter light (SSC). Combination of both detectors reports the size and granularity of the investigated cells. Fluorescence-labelled cells emit light, which is separated into characteristic wavelength and whose intensity is measured by photodetectors equipped with colored filters. As light passes through the photodetectors, the light signals are transformed into electrical signals. Each photodetector can be adjusted by changing its voltage and amplifier gain.

Specific binding of fluorochrome-conjugated antibodies is controlled by using isotype controls. Isotype controls are antibodies bearing the identical isotype but lacking specificity towards antigens in the investigated organism. Herewith, unspecific binding as, for example arising by antibody-binding to Fc-receptors, can be distinguished from specific binding to the desired antigen.

Flow-cytometry was applied for cell surface marker analysis (section 3.2.12), intracellular staining (section 3.2.13), and detection of the intracellular zinc level (section 3.2.23).

### 3.2.12 Cell-Surface Marker Analysis

Immune cells express cell-specific surface marker, which can be detected by specific antibodies. Thus, the molecule's expression level per cell can be analyzed as well as the total and relative amount of cells expressing the specific molecule.

**Reagents:**

- PBS 1x
- PBS/FCS (2 %)
- Lysing solution 10x: magnesium chloride; 1.5 M ammonium chloride, 100 mM sodium bicarbonate, $H_2O$ (pH 7.4)
- Antibodies

**Procedure:**

1. 100 µl whole blood or $1x10^6$ cells/100 µl PBC/FCS were transferred into FACS tubes
2. For erythrocyte-lysis (section 3.2.4) 2 ml lysing solution (1x) was added and incubated for 10 min at 4°C in the dark and washed twice in 2 ml PBS (5 min, 300 g)
3. The pellets were resuspended and 100 µl PBS/FCS was added to each tube

4. Direct fluorescent-labelled antibodies were added and incubated with cells for 20 min at RmT in the dark
5. Samples were washed in 2 ml PBS (5 min, 300 g)
6. The supernatants were discarded, and the pellets were resuspended in 300 µl PBS/FCS
7. The samples were analyzed by using FACS-Calibur or FACS-Scan. FACS analysis was performed with CellQuest software (Becton Dickinson, Heidelberg).

### 3.2.13 Intracellular Staining

Intracellular staining is applied if the desired antigen is present within the cell. This is the case for molecules such as transcription factors or cytokines. In order to stain intracellular antigens with the fluorescence-associated antibodies, cells need to be permeabilized and fixated.

**Reagents:**

- PBS 1x
- Stainig buffer: PBS 1x, heat-inactivated FCS (2%), sodium azide (0.1%)
- Fixation solution
- Permeabilization solution
- Antibodies

**Procedure:**

Steps 1-5 were performed analogous to cell surface marker analysis (section 3.2.12)
Intracellular staining was performed as instructed by the manufacturer and analyzed by using a FACS-Calibur or FACS-Scan.

### 3.2.14 Propidium Iodide Staining

The fluorochrome propidium iodide (PI) is a red-fluorescent nuclear and chromosome counterstain. Since propidium iodide is not permeant to live cells due to their intact plasma membrane, it is commonly used to detect dead cells in a population.

**Reagents:**

- PI (1 mg/ml)
- PBS/FCS (1%)

**Procedure:**

1. The cells were adjusted to $1x10^6$ in 100 µl PBS/FCS and transferred into FACS tubes
2. 1 µl PI was added and the samples were incubated for 10 min at 4°C in the dark
3. The samples were washed by adding 2 ml PBS/FCS (5 min, 300 g)

4. The supernatants were discarded, and the pellets were resuspended in 300 µl PBS/FCS
5. The samples were analyzed by using FACS-Calibur or FACS-Scan

### 3.2.15 Cell Lysate Preparation

For the detection of non-secreted intracellular proteins, the cells need to be disrupted prior to SDS-PAGE procedure and western blot analysis. Cell lysates are generated in sample buffer containing β-mercaptoethanol to reduce disulfide bonds, and sodium-orthovanadate to inhibit protein tyrosine phosphatases and alkaline phosphatases from removing phosphate groups. This is important when phosphorylated proteins are investigated.

**Reagents:**

- Sample buffer: 65 mM Tris-HCl, 25% glycerine, 10% SDS, 1 mM sodium orthovanadate, bromphenol blue, pH 6.8
- β-mercaptoethanol

**Procedure:**

1. β-mercaptoethanol was freshly added to the sample buffer before usage
2. Samples were centrifuged in microcentrifuge tubes (5 min, 600 g)
3. The supernatants were removed, and the pellets were resuspended in 100 µl sample buffer
4. The samples were sonificated on ice (intensity 3, output control 5, duty cycle 50%, 10 sec)
5. The sonicated samples were boiled at 95°C for 5 min, and finally stored at -20°C

### 3.2.16 Sodium Dodecyl Sulfate Discontinuous Polyacrylamid Gele-lectrophoresis

The sodium dodecyl sulfate discontinuous polyacrylamid gele-electrophoresis (SDS-PAGE) functions to separate proteins according to their electrophoretic mobility. The anionic detergent sodium dodecyl sulfate (SDS) covers protein charges, providing uniformly negatively charged proteins. Addition of β-mercaptoethanol-containing sample buffer disrupts disulfide bridges within polypeptides. Furthermore, secondary and tertiary structures are dislocated by 5 minutes of protein-boiling. Thus, the polypeptides are present in their primary, linear structure and negatively charged, which allows for fractionation by approximate size during electrophoresis.

By using discontinuous SDS-PAGEs more concentrated bands develop that simplify the identification of the investigated protein. For this purpose, two different buffer sets are used.

The stacking gel concentrates the proteins, which are then separated according to their molecular weight by the resolving gel.

**Reagents:**

- Sample buffer: 65 mM Tris-HCl, 25% glycerine, 10% SDS, 1 mM sodium orthovanadate, bromphenol blue (pH 6.8)
- Distilled water
- Ammonium persulfate (APS) (10%)
- Stacking gel buffer: Tris-HCl (1.5 M, pH 8.8)
- Resolving gel buffer: Tris-HCl (0.5 M, pH 6.8)
- Electrophoresis buffer (5x): 0.12 M Tris-Base, 0.96 M glycine, 30 ml 10% SDS, 600 ml (pH 8.3)
- Acrylamide/Bisacrylamide (30%)
- N,N,N',N'-tetramethylethylenediamine (TEMED)
- Biotinylated molecular marker

**Procedure:**

1. The glass gel plates were rinsed with 70% ethanol, wiped clean with tissues, and placed in the gel holders
2. The reagents for the 10% resolving gel were mixed according to Table 2, pipetted between the glass plates, layered with distilled water, and left for 10 min of polymerization
3. The water was removed, the 4% stacking gel was prepared as shown in Table 2, the combs were placed in the gel, and the stacking gel was allowed to polymerize for 30 min
4. The gel combs were removed and the glass plates containing the polymerized gel were placed into the electrophoresis chamber
5. The chamber was filled with 1x electrophoresis buffer
6. The molecular weight marker was diluted in sample buffer (1:10) and boiled at 95°C for 5 min before usage
7. The samples and the molecular weight marker were vortexed and 20 µl of each sample were pipetted into the gel slots
8. The electrophoresis was conducted at 170 V for 90 min on ice

**Table 2. Gel reagents for SDS-PAGE**

| Substances | 10% Resolving Gel | Substances | 4% Stacking Gel |
|---|---|---|---|
| $H_2O$ | 2.08 ml | $H_2O$ | 3.66 ml |
| 1.5 M Tris-HCl pH 8.8 | 910 µl | 0.5 M Tris-HCl pH 8.8 | 2.34 ml |
| Acrylamide/ bisacrylamide, 30% | 467 µl | Acrylamide/ bisacrylamide, 30% | 3 ml |
| APS, 10% | 35 µl | APS, 10% | 90 µl |
| TEMED | 7.9 µl | TEMED | 20 µl |

### 3.2.17 Western Blot

Western blot includes the transfer of by SDS-PAGE separated proteins from polyacrylamide matrices onto nitrocellulose membranes utilizing electrical fields. The protein pattern remains conserved during protein transfer. Protein-bound SDS is washed out in this procedure so that the proteins regain their original secondary and tertiary structure to some extent. Hence, epitope-directed antibodies, enabling the detection of specific target proteins within a homogenized sample, can be used. In order to prevent non-specific antibody-binding to the nitrocellulose membrane, potential binding sites are blocked with protein solutions containing fat-free milk powder. Detection of high-affinity antibodies bound to protein epitopes is achieved by using species-specific secondary antibodies conjugated to the horseradish peroxidase enzyme (HRP). This enzyme converts substrate (LumiGLO solution) into its oxidized form, which then emits light detected by a digital biomolecular imager.

**Reagents:**

- Transfer buffer: 25 mM Tris-Base, 0.2 M glycine, 20% methanol (pH 8.5)
- Ponceau S solution
- TBS buffer (10x): 0.2 M Tris-Base, 2.28 M NaCl, pH 7.6
- TBS-T buffer (1x): TBS, 0.1% Tween 20
- Blocking buffer: 2.5 g fat-free milk powder dissolved in 50 ml TBS-T buffer
- Primary antibody: 10 ml TBS-T, 5% fat-free milk powder, and primary antibody (diluted 1:1000)
- Secondary antibody: 10 ml TBS-T, 5% fat-free milk powder, secondary antibody (diluted 1:2000), and HRP-conjugated anti-biotin antibody (diluted 1:1000)

- Detection solution: 250 µl LumiGLO reagent (20 x), 250 µl LumiGLO peroxide (20 x), and 4.5 ml distilled water for each gel
- Stripping buffer: 62.5 mM Tris-Base, 2% SDS, 0.7% β-mercaptoethanol (pH 6.8)

**Western blotting and detection:**

1. The nitrocellulose membrane and gel blotting paper were cut to fit the size of the gel
2. The membrane, gel blotting paper, and sponges were equilibrated in transfer buffer
3. The blot sandwich was assembled and placed in the blot module with the gel facing the cathode side and the membrane facing the anode side
4. The cooling element was placed next to the blot module and the module was filled with transfer buffer
5. The blotting process was performed for about 60 min at 100 V
6. The proteins were visualized by placing the membrane in Ponceau S solution and then washed with distilled water to ensure proper blotting
7. The Ponceau S solution was completely removed by transfer buffer and the membrane was rinsed with distilled water
8. The membrane was incubated with 25 ml blocking buffer for 1 h at RmT followed by incubation with the primary antibody at 4°C overnight in a 50 ml plastic tube.
9. The membrane was washed tree times with TBS-T for 5 min
10. The membrane was incubated with the secondary antibody for 1 h at RmT
11. Step 9 was repeated
12. The membrane was covered with 5 ml detection solution for 1 min
13. The membrane was drained of excess developing solution and wrapped in plastic wrap
14. Chemiluminescence was detected by LAS-3000 imager

**Western blot reprobing:**

1. The membrane was washed four times with TBS-T buffer
2. The membrane was incubated with stripping buffer for 30 min at 50°C (slight agitation)
3. The membrane was washed six times with TBS-T buffer
4. The washed membrane entered the western blot procedure at step 8 (section 3.2.17)

### 3.2.18 RNA Isolation

TRIzol® Reagent is a ready-to-use reagent, designed to isolate high quality total RNA, DNA and proteins from cell and tissue samples. It is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components, which facilitate the isolation of a variety of RNA species of large or small molecular size, by maintaining the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization.

**Reagents:**

- Chloroform
- Isoproyl alcohol
- DEPC-water: 0.1% (v/v) DEPC in A. bidest, autoclaved

**Procedure:**

1. $4x10^6$ cells were resuspended in 1 ml Trizol® Reagent and incubated for 5 min at RmT
2. 0.2 mL of chloroform per 1 mL of TRIzol® Reagent was added and samples were shaked vigorously by hand for 15 sec
3. Samples were incubated for 10 min at RmT and then centrifuged (15 min, 12,000 g, 4°C)
4. Note: The mixture separates into a lower red phenolchloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume
5. The aqueous phase of the sample was removed by pipetting the solution out and placing into a new tube
6. 0.5 ml of 100% isopropanol was added to the aqueous phase 75% ethanol (in DEPC-water) Samples were vortexed (5 sec), incubated at RmT for 10 min and then centrifuged (10 min, 12,000 g, 4°C)
7. The supernatant was removed from the tube, leaving only the RNA pellet
8. 1 mL of 75% ethanol was added to the RNA pellet
9. Samples were vortexed briefly and then centrifuged (5 min, 12,000 g, 4°C)
10. The supernatant was discarded and the RNA was air dried for 5 min
11. The RNA pellet was dissolved in RNase-free water and incubated at 57°C for 10 min
12. Samples were stored at -80°C until use

### 3.2.19 Photometric Determination of RNA or DNA Concentration

Spectrophotometry is used for determination of nucleic acid concentration and purity. Absorbance at wavelength of 260 nm and 280 nm is detected. Whereas the absorption peak of proteins takes place at 280 nm, the absorption peak of nucleic acids is at 260 nm. The ratio $A_{260}/A_{280}$ provides information about the purity of isolated nucleic acid. Pure preparations have A260/A280 ratios of 1.8 to 2.0. Ratios significantly lower indicate protein or phenol contamination (high A280) while higher ratios indicate salt contamination (high A260).

**Reagents:**

- Aqua ad iniectabilia

**Procedure:**

1. RNA was diluted 1:50 and DNA was diluted 1:10 in Aqua ad iniectabilia
2. The photometer was blanked with aqua ad iniectabilia
3. The RNA concentration was calculated as follows:

   Concentration (µg/ml) = dilution factor x 40 x extinction at 260 nm

   The DNA concentration was calculated as follows:

   Concentration (µg/ml) = dilution factor x 50 x extinction at 260 nm

### 3.2.20 Reverse Transcription

The reverse transcription of isolated RNA into complementary DNA (cDNA) was performed with the qScript cDNA-Synthesis Kit. This kit provides oligo-dT primer (complementary to the poly-A-tail at the 3'end of the mRNA) and random hexamer primers. Primer annealing to mRNA results in double-strand DNA/RNA molecules serving as a starting point for DNA synthesis by reverse transcriptase enzyme.

**Reagents:**

- Aqua ad iniectabilia
- 1 µg RNA template
- qScript Reaction Mix (5x)
- qScript Reverse Transcriptase

**Procedure:**

1. 1 µg RNA was diluted with 15 µl Aqua ad iniectabilia in a 1.5 ml reaction tube
2. 4 µl Reaction Mix (5x) and 1 µl reverse transcriptase were added
3. Tubes were mixed gently and heated as described in Table 3

**Table 3. Temperature conditions**

| Time | Temperature |
|---|---|
| 5 min | 25°C |
| 60 min | 42°C |
| 5 min | 85°C |

### 3.2.21 Quantitative Real-Time Polymerase Chain Reaction

The quantitative real-time polymerase chain reaction (Q-PCR) is an effective molecular-biological technique developed for the amplification and simultaneously quantification of DNA. Therefore, primers complementary to the target region anneal to a template DNA. A key role in the process of amplification plays the DNA polymerase, which enzymatically assembles a new DNA strand after hybridizing to the template's 3' end. In the course of PCR progression the newly-synthetized DNA serves in turn as template leading to exponential amplification of initial DNA templates.

The real-time PCR measures the amount of DNA product after each round of amplification by detection of double-strand DNA-intercalating reporter dyes such as SYBR Green. The emitted light of the dye increases relative to the amount of PCR product. Measurement of the DNA-intercalated fluorescent signals after every cycle enables the detection of the exponential phase. The number of cycles needed to exceed a certain threshold for fluorescence detection is called the cycle threshold ($C_t$). For gene expression quantification, the $\Delta\Delta C_t$ method is applied. Herein, the $C_t$ for the DNA of a gene of interest is normalized to the $C_t$ of DNA of a housekeeping gene of the same sample ($\Delta C_t$). Subsequently, the expression disparity among reference sample (e.g. untreated control) and sample is obtained by subtraction of their Ct values ($\Delta\Delta C_t$). The relative expression difference between sample and reference sample normalized to a housekeeping gene is calculated as follows: ratio = $2^{-\Delta\Delta Ct}$

**Reagents:**

- Aqua ad iniectabilia
- SYBR Select Master Mix (2x)
- Primer (100 µM)
- cDNA

**Procedure:**

5. Separate master mixes were prepared for each, the gene of interest and the housekeeping gene (see
6. Table 6), in a sterile 1.5ml reaction tube on ice as summarized in Table 4

**Table 4. Reagents per cDNA sample for real-time PCR**

| Reagent | Volume [µl] |
|---|---|
| Aqua ad iniectabilia | 3 |
| SYBR Select Master Mix | 10 |
| Primer sense (4 µM) | 1 |
| Primer antisense (4 µM) | 1 |

7. cDNA was diluted in Aqua ad iniectabilia (2:3) and 5 µl were added to 15 µl master mix provided in a PCR reaction tube
8. Amplification was performed as shown in Table 5
9. Data analysis was performed with the program StepOnePlus

**Table 5. Real-time PCR program**

| Temperature | Duration | Cycles |
|---|---|---|
| 95°C | 2 min | 1x |
| 95°C | 5 sec | 40x |
| 60°C | 30 sec | |

**Table 6. Primer for mRNA analysis**

| | | |
|---|---|---|
| **hFoxp3** | sense 5'-3' CACCTGGCTGGGAAAATGG<br>antisense 5'-3' GGAGCCCTTGTCGGATGAT | 63 bp |
| **hKLF-10** | sense 5'-3' AAGGAGTCACATCTGTAGCC<br>antisense 5'-3' TCCAGCTACAGCTGAAAGGC | 113 bp |
| **hIRF-1** | sense 5'-3' GTACCGGATGCTTCCACCTC<br>antisense 5'-3' GGAATCCCCACATGACTTCCT | 106 bp |

| | | |
|---|---|---|
| **hT-bet** | sense 5'-3' AGGGACGGCGGATGTTCCCA<br>antisense 5'-3' GCTGCCCTCGGCCTTTCCAC | 155 bp |
| **hGATA-3** | sense 5'-3' GCCCGGTCCAGCACAGAAGG<br>antisense 5'-3' TGAGGGGCCGGTTCTGTCCG | 142 bp |
| **hRORC2** | sense 5'-3' CAGTCATGAGAACACAAATTGAAGTG<br>antisense 5'-3' CAGGTGATAACCCCGTAGTGGAT | 85 bp |
| **hPBGD** | sense 5'-3' ACGATCCCGAGACTCTGCTTC<br>antisense 5'-3' GCACGGCTACTGGCACACT | 87 bp |

### 3.2.22 [$^3$H] Thymidine Proliferation Assay

Proliferation of cells can be measured by determining their incorporation of [$^3$H]-thymidine into cellular nucleic acids. For this purpose cell cultures are pulsed with [$^3$H]-thymidine during the last 16 h before harvesting. The incorporated [$^3$H]-thymidine can be quantified after cell lysis, accumulation and drying of released DNA on filter papers, and, dissolving of filter papers in a liquid scintillation cocktail. The scintillation cocktail contains liquid chemical medium capable of converting the kinetic energy of nuclear emissions into light energy, which can be measured by a scintillation β-counter. Thus, the β-emission originating by [$^3$H] decay serves as a parameter for the amount of incorporated [$^3$H]-thymidine and, therefore, for DNA synthesis. The degree of proliferation is indicated as counts per minute (CPM).

**Reagents:**

- RPMI medium containing 10% heat-inactivated FCS, 1% L-glutamine and 1% penicillin/streptomycin
- [$^3$H]-thymidine solution
- Scintillation cocktail Lipoluma
- Aqua dest.
- Ethanol 70% (v/v)

The [$^3$H]-thymidine was diluted 1:20 in RPMI-medium to a concentration of 1850 kBq/ml. Every sample of a 96 well plate received 10 µl of this solution (18.5 kBq/well).

**Procedure:**

1. 200 µl/well of 2x$10^6$ PBMC/ml were stimulated according to the experiment and incubated at 37°C, 5% $CO_2$ and saturated air humidity in a 96 well plate
2. 10 µl [$^3$H]-thymidine solution was added to every well
3. Incubation for 16 h at 37°C, 5% $CO_2$ and saturated air humidity
4. The incubation was stopped (plate can be frozen -20°C for later analysis)
5. Cells were lysed with a cell harvester machine and DNA was accumulated on a filter paper
6. Filter papers were washed five times with Aqua dest. and one time with ethanol (70%)
7. Filter paper were transferred into scintillation tubes and dried for 5 min at 70°C in an incubator
8. Every filter paper was dissolved in 3 ml Lipoluma and the scintillation tubes were sealed
9. The CPM was detected by a liquid scintillation β-counter

### 3.2.23 Detection of Labile Zinc

Labile zinc levels are detectable by specific fluorescent zinc-sensors. Two zinc-probes, Fluozin-3 and ZinPyr-1, were used in this thesis.

The lipophilic Zinpyr-1 ($C_{46}H_{36}C_{l2}N_6O_5$) is a membrane-permeant fluorescent sensor with high specificity and affinity for zinc ($K_D$ = 0.7 nM). The excitation and emission wavelength are 490 nm and 530 nm, respectively. Binding of zinc ions induce altered ZinPyr-1 fluorescence properties, which can be detected by a fluorescence reader or by flow-cytometry.

In addition, the chelator Fluozin3-AM ($K_D$ = 8.9 nM) is a specific zinc-selective sensor. Following zinc binding, Fluozin3-AM exhibits a single excitation peak at 494 nm and an emission peak at 516 nm. Two forms of Fluozin-3 are available; Fluozin3-AM and Fluozin-3A. Fluozin3-AM is present as an acetoxymethyl (AM)-ester that is able to permeate cell membranes and therefore detect intracellular zinc. Fluozin-3A, on the other hand, is the free acid of Fluozin-3 and is used to detect free zinc levels in buffers or cell lysates. In T cells Fluozin-3AM is used for the detection of vesicular zinc levels, whereas ZinPyr-1 is used to detect intracellular free zinc levels [149].

For calculation of the free zinc concentration, 50 µM of the zinc chelator TPEN (N,N,N'N'-tetrakis(-)[2-pyridylmethyl]-ethylenediamine) (serving as minimal value) and 100 µM $ZnSO_4$ plus the ionophore pyrithione (50 µM) (serving as maximal value) are added, in

order to determine minimal and maximal zinc concentrations, respectively. The labile zinc concentration is then calculated by the formula:

$$[Zn] = K_D \frac{(F - F_{\min})}{(F_{max} - F)}$$

### 3.2.24 Detection of Labile Zinc in Buffers

**Reagents:**

- FluoZin3-A (1 mM in DMSO)
- $ZnSO_4$ (2 mM)

**Procedure:**

1. Different zinc concentrations were added to the buffer to be investigated
    a. Zinc concentrations: 5 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, 150 µM, 200 µM, 300 µM
2. The buffer was dispersed in a 96 well plate (100 µl/well)
3. All measurements were conducted as triplicates
4. 1 µl of diluted Fluozin-3A was added (1µM )
5. The plate was incubated for 30 min in the dark
6. The detection was performed with the fluorescence plate reader at 485 nm excitation- and 535 nm emission-wavelength
7. The zinc concentration was calculated as described in section 3.2.23

### 3.2.25 Detection of Intracellular Zinc

**Reagents:**

- FluoZin3-AM (1 mM in DMSO) or ZinPyr-1 (2.5 mM in DMSO)
- $ZnSO_4$ (2 mM)
- TPEN (1 mM)
- Pyrithione (5 mM)
- RPMI medium containing 1% L-glutamine and 1% penicillin/streptomycin
- PBS 1x

**Procedure:**

1. $2x10^6$ PBMCs were distributed in 250 µl in RPMI medium without FCS
2. Fluozin3-AM or ZinPyr-1 was added at a final concentration of 1 µM or 10 µM, respectively
3. The cells were incubated at 37°C for 30 min while shaking

4. The cells were centrifuged at 300 g for 5 min and the supernatant was discarded
5. The pellets were resuspended in 100 µl PBS
6. 5 µl of mouse anti-human CD4-PerCP/CD25-APC was added and the cells were incubated for 20 min at RmT in the dark
7. 1 ml PBS was added and the cells were centrifuged at 300 g for 5 min
8. Supernatant was discarded and the pellets were resuspended in 900 µl PBS
9. 300 µl of this cell suspension was added in each FACS tube:
    a. TPEN (8.0 µl) → minimal zinc concentration
    b. $ZnSO_4$ (3.0 µl) + pyrithione (3.0 µl) → maximal zinc concentration
    c. Cells only → detection of intracellular zinc level
10. The cells were incubated at 37°C for 30 min
11. Single cell fluorescence intensity was detected by FACS analysis (FL-1: zinc probe, FL-3: CD4-PerCP, FL-4: CD25-APC). Zinc concentration was calculated as described in section 3.2.23

### 3.2.26 Atomic Absorption Spectroscopy

**Reagents:**

- $HNO_3$ (69%)
- Aqua dest.

**Procedure:**

1. Samples were weight and 1 ml $HNO_3$ was added
2. Digestion was performed over 4 h at 95°C
3. Samples were diluted 1:3 in Aqua dest. before measurement

### 3.2.27 Statistical Significance

The results derive from series of experiments (number of experiments is shown as n=x) and are expressed as mean values; the standard error of the mean (SEM) is presented. The statistical significance was calculated using GraphPad Prism software (version 5.01). Statistical significances calculated by student's t-test or Wilcoxon signed rank test are represented as follows: *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Multiple comparisons involved ANOVA calculation with Tukey's post-hoc-test. Mean values with significant differences are indicated by diverse letters with a significance of $p<0.05$.

# IV Results

## 4.1 Zinc Alleviates the Allogeneic Immune Reaction in MLC by Induction of Regulatory T Cells

The co-cultivation of PBMC of genetically diverse individuals is a well-known model to assess allogeneic immune reactions, also known as mixed lymphocyte culture (MLC). These are commonly used for *ex vivo* compatibility analysis in GVHD [150, 151]. Herein, expression of divergent HLA haplotypes trigger the allogeneic immune reaction characterized by lymphocyte proliferation, formation of T cell blasts, and production of pro-inflammatory cytokines such as IFN-γ, reflecting its severity [151]. It has been discovered, that zinc supplementation inhibits IFN-γ production in MLC [152] and induces Treg cells that are responsible for immune regulation [82]. This study validates former results by showing that zinc supplementation reduced the severity of MLC compared to non-supplemented controls, by stabilization of Treg cells after 5 days of incubation (figure 3).

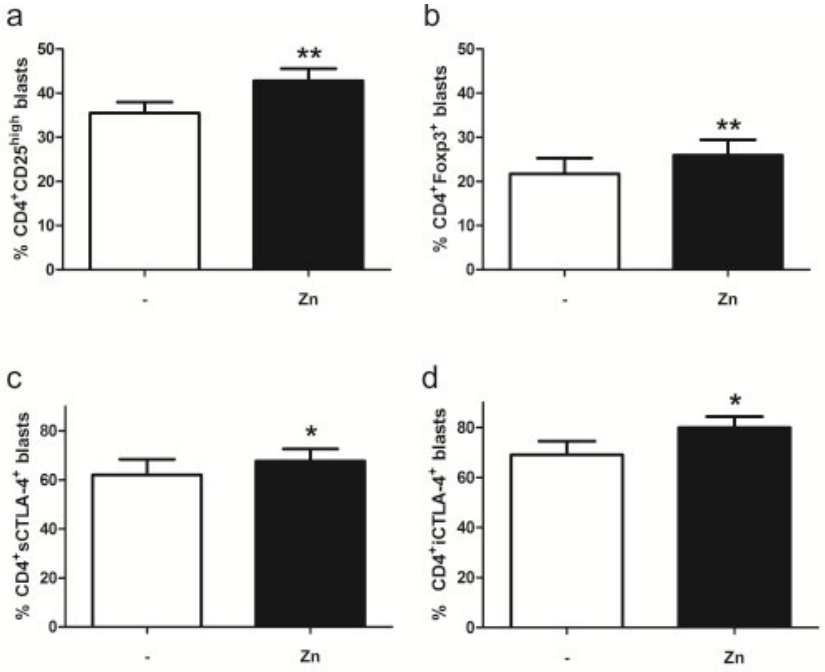


**Fig. 3 Zinc supplementation induces Treg cells in MLC.**
PBMC were adjusted to $2x10^6$ cells/ml and remained untreated (white bars) or were pre-treated with 50 µM zinc (black bars) for 15 min prior to MLC generation for 5 days. The percentage of Treg cells was analyzed by flow cytometry. Gating was performed on activated $CD4^+SSC^{hi}$ blasts as previously described [82] and the percentage of (**a**) $CD4^+CD25^{hi}$ (n=8) (**b**) $CD4^+Foxp3^+$ (n=7) (**c**) $CD4^+sCTLA\text{-}4^+$ (surface) (n=8) (**d**) $CD4^+iCTLA\text{-}4^+$ (intracellular) (n=7) cells is shown. * indicates a significance of $p<0.05$, ** of $p<0.01$ (student's t-test). Results are presented as mean values + SEM.

For Treg cell characterization surface marker expression was analyzed (figure 3a and c), as well as the intracellular Treg cell-specific transcription factor Foxp3 (figure 3b) and CTLA-4 (figure 3d). Zinc pre-treatment significantly increased the percentage of $CD4^+CD25^{hi}$ (figure 3a) and $CD4^+$ (surface) sCTLA-$4^+$ Treg cells (figure 3c), as well as $CD4^+Foxp3^+$ and $CD4^+$ (intracellular) iCTLA-$4^+$ Treg cells compared to untreated controls. Hence, these results confirm former findings showing a zinc-induced enrichment of inducible Treg cells during the allogeneic immune response.

## 4.2 Zinc Supplementation Specifically Influences Treg Cells in MLC

Our previous data illustrated that zinc supplementation induces Treg cells in MLC (figure 3) and reduces IFN-γ cytokine expression [82] resulting in an amelioration of the alloreaction. Adverse immune reactions are not only modulated by Treg cells, but also by effector T cells as Th1, Th2 or Th17 cells. Whether zinc administration in MLC specifically modulates Treg cells or also manipulates the other T cell populations remains to be elucidated. Therefore, the Th1 cell-specific (T-bet), Th2 cell-specific (GATA3) and Th17 cell-specific (RORC2) transcription factors were analyzed (figure 4a-c). As displayed, no significant difference in any T cell-specific transcription factor mRNA expression could be observed. Additionally, for non-activated T cells in PBMC experiments similar results were obtained (data not shown). This leads to the conclusion that zinc supplementation during MLC selectively affects Treg cell induction and stability.

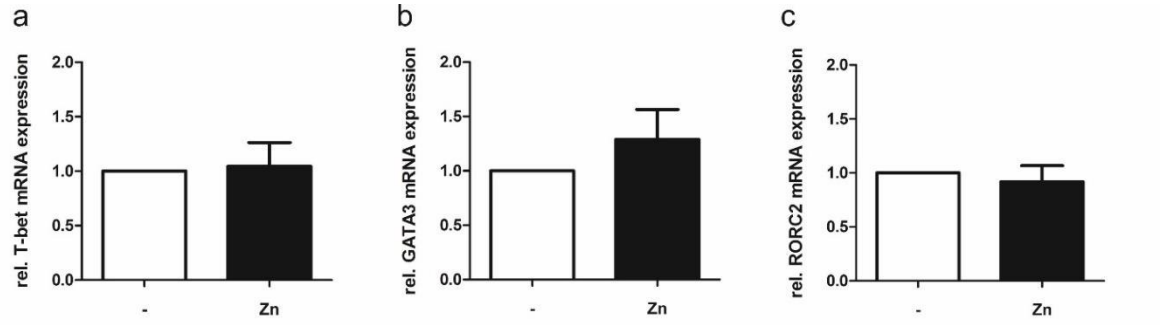


**Fig. 4. Th1, Th2, and Th17 cells remain unaffected by zinc supplementation.**
$2x10^6$ PBMC/ml remained untreated (white bars) or were pre-treated with 50 µM zinc (black bars) for 15 min prior to MLC generation for 5 days. The mRNA expression of the transcription factors (**a**) T-bet (**b**) GATA-3 and (**c**) RORC2 were measured. Results are normalized to the untreated control and represent mean values + SEM of at least n=6 independent experiments. No significant differences were detected by student's t-test.

## 4.3 Zinc Induces Treg Cells in MLC by Modulation of KLF-10 and IRF-1

Treg cell differentiation and function is highly dependent on a multitude of signaling pathways and on transcription factor activation or repression [57, 153]. The transcription factor KLF-10 is well known to be essential in Treg cell function, whereas the transcription factor IRF-1 has a negative impact on Treg cell differentiation [48, 49]. Hence, the KLF-10, IRF-1 and Foxp3 mRNA expression in zinc-supplemented samples and untreated controls after 5 days of MLC were analyzed (figure 5d-f). The same treatment was applied to the respective PBMC (figure 5a-c). Additionally, protein expression of Foxp3 and IRF-1 was determined in MLC (figure 5g-i).

The KLF-10 and Foxp3 mRNA expression in PBMC was not influenced by zinc supplementation compared to untreated controls, whereas a significant upregulation was observed in MLC. IRF-1 mRNA expression was significantly downregulated in zinc-supplemented MLC, whereas a significant increase was found in PBMC. Thus, an antithetic gene regulation in PBMC and MLC can be concluded.

In addition, the protein levels of both Foxp3 and IRF-1 confirm the regulation on transcription level of zinc-treated MLC in comparisons to the controls (figure 5g, h). Moreover, the IRF-1 protein expression is similarly reduced as its's mRNA expression in MLC (figure 5g, i). Furthermore, zinc deficiency has no significant impact on IRF-1 or Foxp3 expression (data not shown).

Due to statistical relationships, a correlation of Treg cell induction and pro-inflammatory cytokine production was performed (figure 5j). Here a negative correlation between KLF-10 mRNA expression and IFN-γ secretion was discovered; the higher the KLF-10 mRNA expression, the lower the IFN-γ secretion in MLC.

Summing up, zinc administration acts beneficial on Treg cell modulation in MLC via upregulation of the transcription factors KLF-10 and Foxp3, and inhibition of IRF-1.

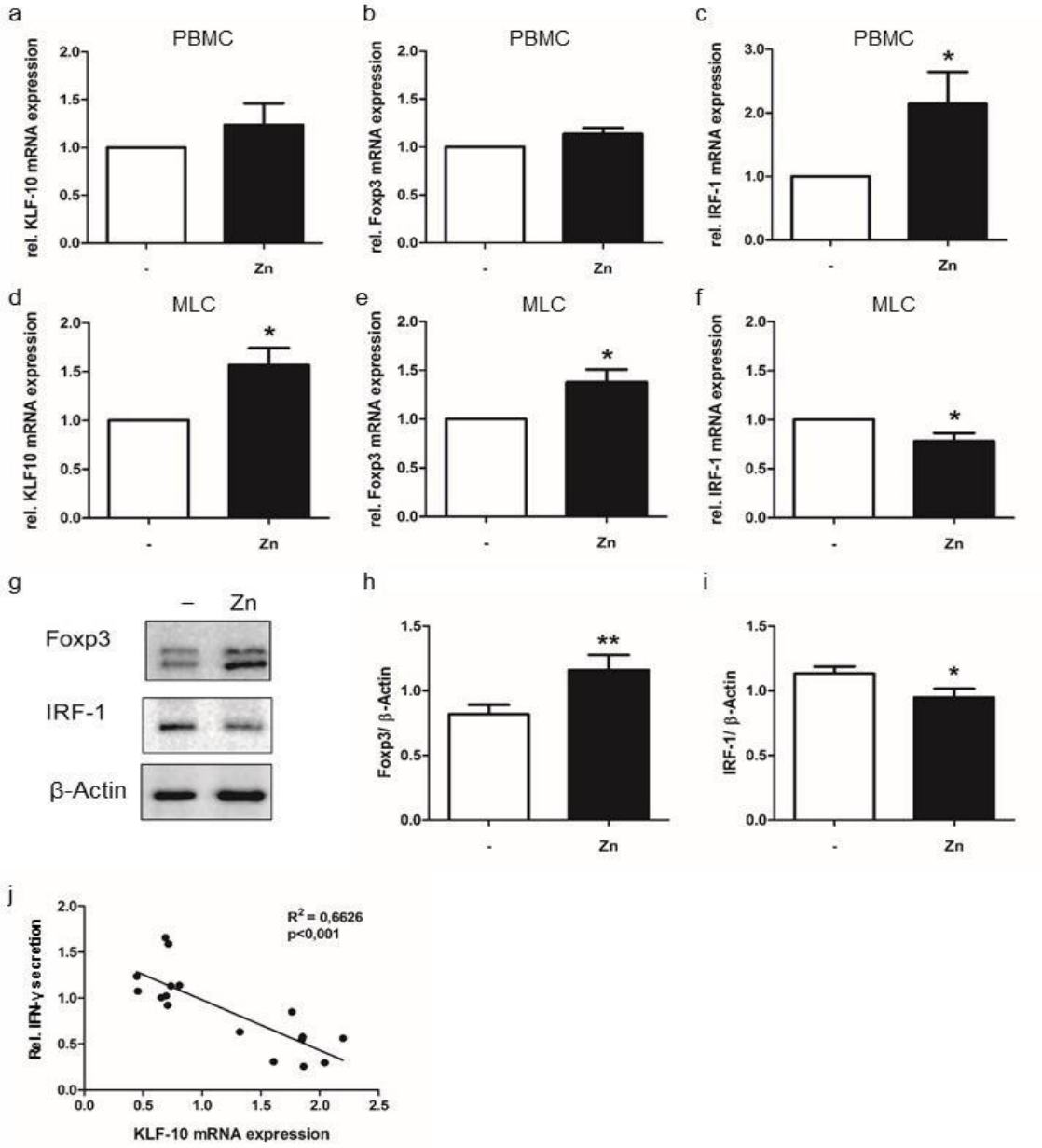


**Fig. 5. Zinc supplementation induces Treg cells by modulation of Foxp3, IRF-1 and KLF-10.**

$2x10^6$ PBMC/ml were pre-incubated with 50 µM zinc (black bars) or remained untreated (white bars) for 15 min prior to MLC generation. (**a-f**) mRNA expression of KLF-10, Foxp3, and IRF-1 was analyzed after 5 days in (**a-c**) PBMC and (**d-f**) MLC respectively. Results are normalized to the untreated control and represented as mean values + SEM of at least n=5 independent experiments. (**g-i**) Foxp3 and IRF-1 protein was analyzed by Western blotting after 5 days of untreated (white bars) or zinc-supplemented (50 µM) (black bars) MLC. (**g**) One representative experiment out of n=13 independent experiments is displayed. Results show mean values + SEM of (**h, i**) densitometric quantifications. (**j**) Correlation of KLF-10 mRNA expression and IFN-γ secretion was performed after 5 days of MLC (n=17). * indicates a significance of $p<0.05$, ** of $p<0.01$ (Wilcoxon signed rank test).

## 4.4 Zinc Supplementation Upregulates Antigen-Specific Treg Cells

Nowadays, the beneficial effects of antigen-specific T cells in clinical therapy has growing interest for treating diseases more specifically by reducing side effects of administered drugs. In the aforementioned figure 5 the beneficial effect of zinc administration on Treg cell in MLC was demonstrated. Whether antigen-specific Treg cells can be induced by zinc administration during priming, expansion or re-activation was analyzed by surface marker expression and intracellular transcription factor expression of Treg cells (figure 6). During priming and re-activation the intracellular Foxp3 expression (figure 6a), surface marker expression of CTLA-4 (figure 6b) as well as intracellular CTLA-4 expression (figure 6c) was significantly upregulated in T cells subjected to zinc compared to the untreated controls. In the phase of T cell expansion surface CTLA-4 expression was similar in control and zinc treated T cells.

These results indicate a zinc-mediated increase of antigen-specific induced Treg cells.

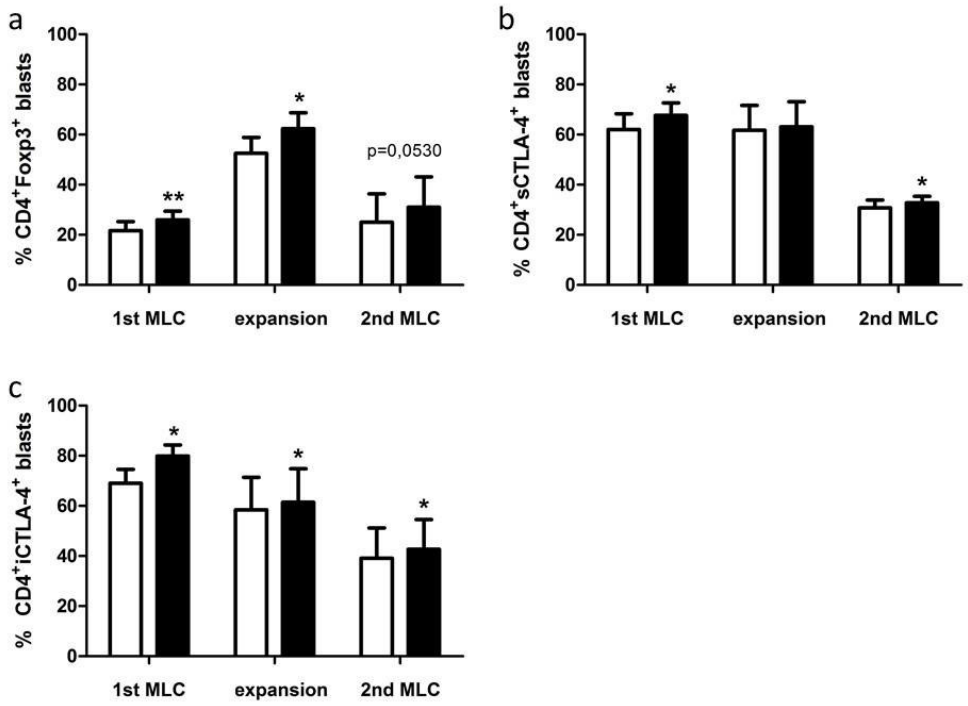


**Fig. 6. Antigen-specific T cells are upregulated by zinc treatment.**
Priming, expansion, and re-activation of antigen specific T cells was performed with zinc supplementation (50 µM; black bars) or without additives (white bars). Re-stimulation was performed by using the priming antigen and autologous PBMC. The percentage of Treg cells was analyzed by flow cytometry. Gating was performed on activated $CD4^+SSC^{hi}$ blasts and the percentage of (**a**) $CD4^+Foxp3^+$ (**b**) $CD4^+$(surface) $sCTLA\text{-}4^+$ (surface), and (**c**) $CD4^+$(intracellular) $iCTLA\text{-}4^+$ (intracellular) cells of at least n=7 independent experiments is shown. * indicates a significance of $p<0.05$, (student's t-test).

## 4.5 Influence of Zinc Supplementation on Transcription Factor Expression in Antigen-Specific T Cells

The transcription factor profile of T cells is highly important for their differentiation and function, since the different T cell subpopulations can be easily distinguished by their specific transcription factor expression. In this context, it is interesting to have a closer view on the expression profile during activation, expansion and re-activation of zinc-treated and untreated antigen-specific T cells (figure 7). Therefore, the mRNA expression of the transcription factors Foxp3 (for Treg cells), T-bet (for Th1 cells), GATA3 (for Th2 cells), and RORC2 (for Th17 cells) were investigated. Moreover, KLF-10 and IRF-1 were analyzed because they affect Foxp3 expression directly. In agreement to the previous results (figure 6) Foxp3 mRNA expression was significantly upregulated by zinc supplementation during priming and re-activation (figure 7a). All other lineage specific marker remained unaffected and were comparable to untreated controls (figure 7b-d). Correlating to Foxp3, the KLF-10 expression was elevated upon zinc administration. In contrast to that the negative regulator of Foxp3 expression, IRF-1, was not affected by zinc supplementation (figure 7f).

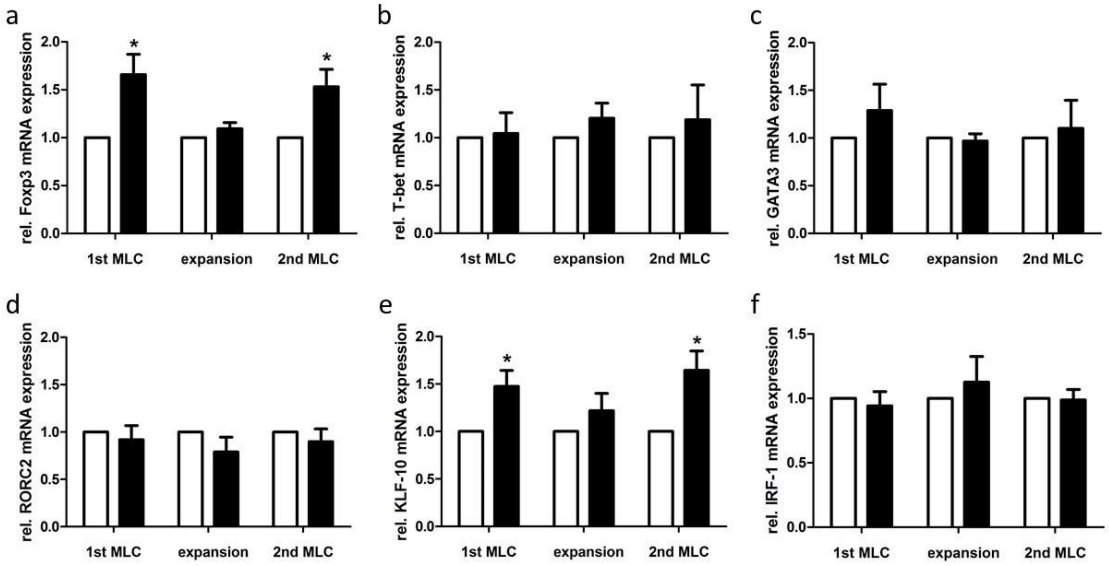


**Fig. 7. Analysis of transcription factor profile of antigen-specific T cells.**
Priming, expansion, and re-activation of antigen specific T cells was performed with zinc supplementation (50 µM; black bars) or without additives (white bars). Re-stimulation was performed by using the priming antigen and autologous PBMC. mRNA expression of (**a**) Foxp3, (**b**) T-bet, (**c**) GATA3, (**d**) RORC2, (**e**) KLF-10, and (**f**) IRF-1 was analyzed by real-time PCR. Results are normalized to the untreated control and represented as mean values + SEM of at least n=5 independent experiments. * indicates a significance of $p<0.05$ (student's t-test).

Hence, these results illustrate a beneficial effect of zinc administration regarding the induction of antigen-specific Treg cells by elevating the expression of Foxp3 and KLF-10 during priming and re-activation. During the phase of expansion no differences between untreated controls and zinc supplemented T cells were detectable.

## 4.6 Zinc Supplementation Provokes Diminished Reactivity of Antigen-Specific T Cells During Re-Stimulation

Zinc supplementation has an ameliorating effect on the severity of MLC alloreaction due to the induction of Treg cells as seen in figure 3 and 5. Antigen-specific Treg cells are discussed to be more efficient in the regulation of unwanted immune reactions regarding specific organs than non-antigen specific Treg cells [35, 37]. Therefore, the potential zinc effect on antigen-specific T cells was investigated by analyzing IFN-γ secretion (figure 8a) as well as cell proliferation (figure 8b) of primed and expanded T cells subjected to zinc treatment compared to untreated controls. Antigen priming was performed by cultivation with or without zinc supplementation over a period of 5 days in one way-MLC using fixed BJAB cells. Subsequently, T cells were expanded over 5 days and re-stimulated in a second one way-MLC by using BJAB cells or genetically distinct B cells. Here, it was uncovered that antigen-specific T cells subjected to zinc treatment secreted less IFN-γ and exhibit reduced cell proliferation compared to untreated T cells. In addition, re-activation of zinc-supplemented T cells by repeated stimulation with the priming B cells (BJAB) showed a significant lower responsiveness compared to expanded and re-stimulated T cells of control expansions. This effect was either seen when re-stimulation was solely performed with the priming antigen, or combined with autologous PBMC. However, T cells either derived from zinc-supplemented or control expansion showed no difference in cytokine secretion and proliferation, when re-stimulation was performed with a genetically different antigen (other B cells), as well as in combination with autologous PBMC (figure 8a, b).

Therefore, it can be assumed that zinc supplementation during immunological priming favors antigen-specific Treg cell induction resulting in a dampened immune reaction during cellular re-activation.

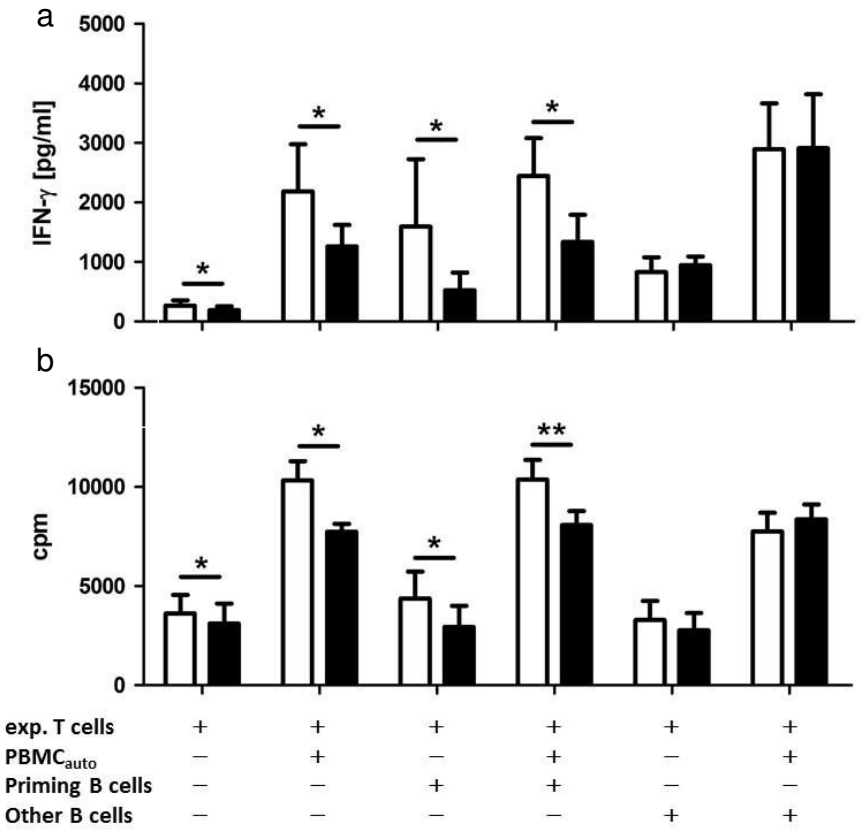


**Fig. 8. Antigen-specific T cells show diminished reactivity during re-activation due to zinc pre-treatment.**
Priming, expansion, and re-activation of antigen specific T cells was performed with zinc supplementation (50 µM; black bars) or were left untreated (white bars). Re-stimulation was performed by using autologous PBMC (PBMC$_{auto}$). (**a**) IFN-γ secretion was measured by ELISA. (**b**) Cell proliferation was determined by [$^3$H]-thymidine assay. Results are presented as mean values + SEM of n=6 independent experiments. * indicates a significance of $p<0.05$, ** of $p<0.01$ (student's t-test).

## 4.7 Zinc Supplemented Antigen-Specific T Cells Show no Cross-Reactive Potential

To test potential cross-reactivity of expanded T cells, allogeneic PBMC (PBMC$_{allo}$) were used during re-stimulation (figure 9a, b). Thus, we elucidated whether the ameliorating zinc effect seen in autologous PBMC re-stimulation experiments (figure 8) can be reproduced by foreign PBMC application. Therefore, priming, expansion and re-stimulation was performed as described before. After 5 days of re-stimulation IFN-γ secretion (figure 9a) and cell proliferation (figure 9b) was determined.

Again, re-stimulation of expanded T cells with the priming antigen showed a significantly reduced IFN-γ secretion (figure 9a) and cell proliferation (figure 9b) in zinc-sup-

plemented samples compared to untreated control samples. Instead, no difference between zinc-treated and control samples could be observed when experiments were done with allogeneic PBMC.

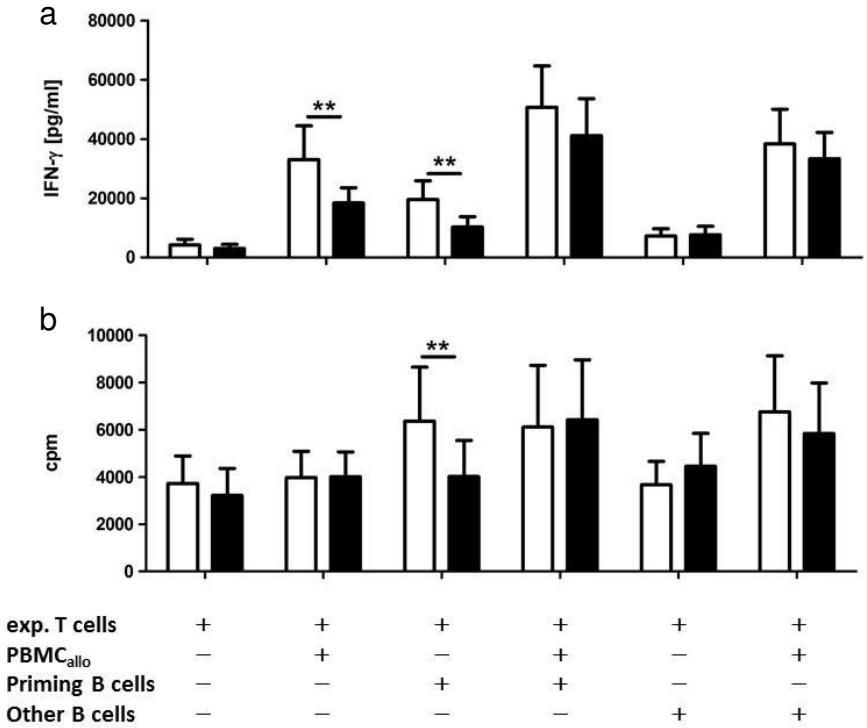


**Fig. 9. Zinc supplemented antigen-specific T cells show no cross-reactive potential.** Priming, expansion, and re-activation of antigen specific T cells was performed with zinc supplementation (50 µM; black bars) or were left untreated (white bars). Re-stimulation was performed by using allogeneic PBMC (PBMC$_{allo}$). (**a**) IFN-γ secretion was measured by ELISA. (**b**) Cell proliferation was determined by [$^3$H]-thymidine assay. Results are presented as mean values + SEM of n=8 independent experiments. ** indicates a significance of $p<0.01$ (student's t-test).

In conclusion, these results point to a zinc-mediated amelioration in re-activated antigen-specific T cells, but to no effect in cross-reactivity experiments. This leads to the presumption that zinc supplementation increases the stability of antigen-specific Treg cells, developed from activated and expanded T effector cells, but simultaneously permit immune responses towards neo antigens.

## 4.8 Zinc Supplementation Diminishes Allogeneic Reaction of Post-T Cell Activation

For treating diseases, it is of prime importance, whether already triggered immune reactions can still be modulated by late-phase additives and medication. Thus, the influence of zinc supplementation regarding the allogeneic T cell response post-exposure to a

priming antigen was investigated in the following experiment. Accordingly, the experimental setup described in section 4.4 was used, except zinc administration during T cell priming or expansion. Instead, zinc supplementation was performed for the first time 15 min before re-stimulation (second MLC) with the same antigen used for priming. Results are presented in figure 10a-d.

Zinc-induced downregulation of IFN-γ secretion was observed by re-stimulation with the priming antigen (figure 10a) as well as by combined re-stimulation of both priming antigen and autologous PBMC (figure 10b). Furthermore, cell proliferation within both experiments was significantly decreased (figure 10c, d).

These results indicate a positive modulation of the allogeneic T cell reaction by zinc supplementation, even when zinc treatment was initiated for the first time before re-stimulation.

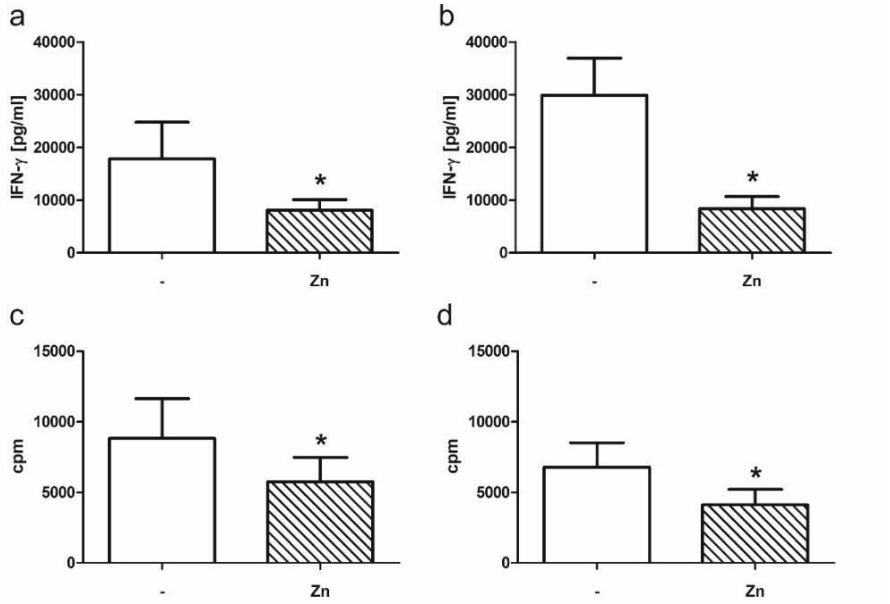


**Fig. 10. Diminished allogeneic reaction due to zinc-supply after T cell activation.**
Priming and expansion of antigen-specific T cells, as well as T cell re-stimulation was performed as described in materials and methods. Zinc supplementation (50 µM, striped bars) was performed shortly before re-stimulation (second MLC). (**a**, **c**) Re-stimulation of expanded T cells with priming antigen. (**b**, **d**) Re-stimulation of expanded T cells with priming antigen and autologous PBMC. (**a**, **b**) IFN-γ production was measured by ELISA. (**c**, **d**) Cell proliferation was determined by [$^3$H]-thymidine assay. Results are represented as mean values + SEM of at least n=6 independent experiments. * indicates a significance of $p<0.05$ (student's t-test).

## 4.9 Intracellular Free Zinc in Treg Cells

Besides a shift of the mRNA expression level, also altered intracellular free zinc concentrations influence numerous signaling pathways thus affecting cellular function and subsequently the immune response [101]. Particularly, T cell differentiation and function is

highly zinc dependent [101, 127, 154]. Therefore, the intracellular zinc content was investigated in resting (PBMC) and activated T cells (MLC). Additionally, we distinguished between activated T cells in MLC ($CD4^+CD25^{low}$ expressing T cells) and Treg cells ($CD4^+CD25^{hi}$ expressing T cells) (figure 11).

Zinc pre-treatment induced a slight increase in the intracellular free zinc concentration in $CD4^+CD25^{low}$ and $CD4^+CD25^{hi}$ expressing T cells in non-activated PBMC compared to untreated controls. Cellular activation in MLC (15 min and 60 min) provoked a slight elevation of the intracellular free zinc in $CD4^+CD25^{hi}$ Treg cells but not in $CD4^+CD25^{low}$ activated T cells compared to non-activated PBMC. Interestingly, zinc pre-treatment in MLC experiments reveal a significantly elevated intracellular zinc level in $CD4^+CD25^{hi}$ Treg cells, whereas the intracellular zinc level in $CD4^+CD25^{low}$ activated T cells remained unaffected. This effect was comparable for 15 min and 60 min MLC. However in 5 d MLC similar zinc level in Treg cells and activated T cells were observed and are comparable to the zinc concentration detected in zinc-supplemented PBMC.

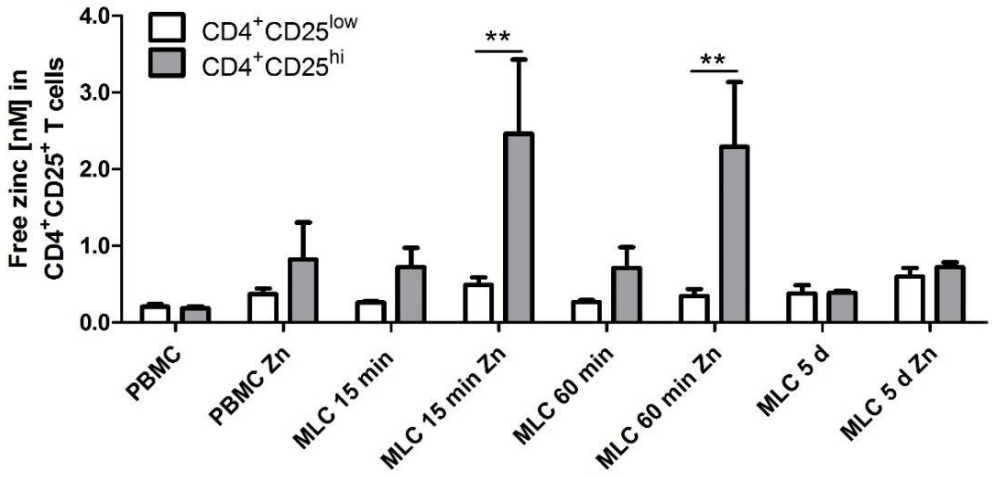


**Fig. 11. Intracellular free zinc in $CD25^{hi}$ and $CD25^{low}$ expressing T cells.**
$2x10^6$ PBMC/ml remained untreated or were pre-incubated with 50 µM zinc for 15 min. MLC were generated for 15 min, 60 min, and 5 d. The intracellular free zinc concentration was measured by Fluozin3-AM in $CD4^+CD25^{low}$ T cells (white bars) and $CD4^+CD25^{hi}$ T cells (grey bars). Results are represented as mean values + SEM of at least n=6 independent experiments.

Hence, the intracellular free zinc level is slightly elevated after T cell activation in Treg cells in MLC experiments compared to resting PBMC. Additionally, the intracellular zinc concentration is significantly increased in Treg cells compared to activated T cells in MLC during the early phase of activation (15 min, 60 min). Thus, the available zinc level right before cellular activation seem to be highly essential during the early stage of cellular activation whereas the late phase of T cell activation (5 d MLC) seem

to be non-relevant. Moreover, Treg cells can be characterized and easily distinguished from activated T cells by a substantially elevated intracellular free zinc content.

## 4.10 Zinc Pre-Conditioning is Important for Treg Cell Induction in MLC

Zinc signals are important in Treg cell function, as shown by a zinc-dependent induction and stabilization of Foxp3 expression in former studies [82] and in experiments shown in figures 3 and 5. To figure out whether the intracellular zinc status of T cells before activation is important for Treg cell induction, the zinc status of PBMC before MLC generation was manipulated (figure 12).

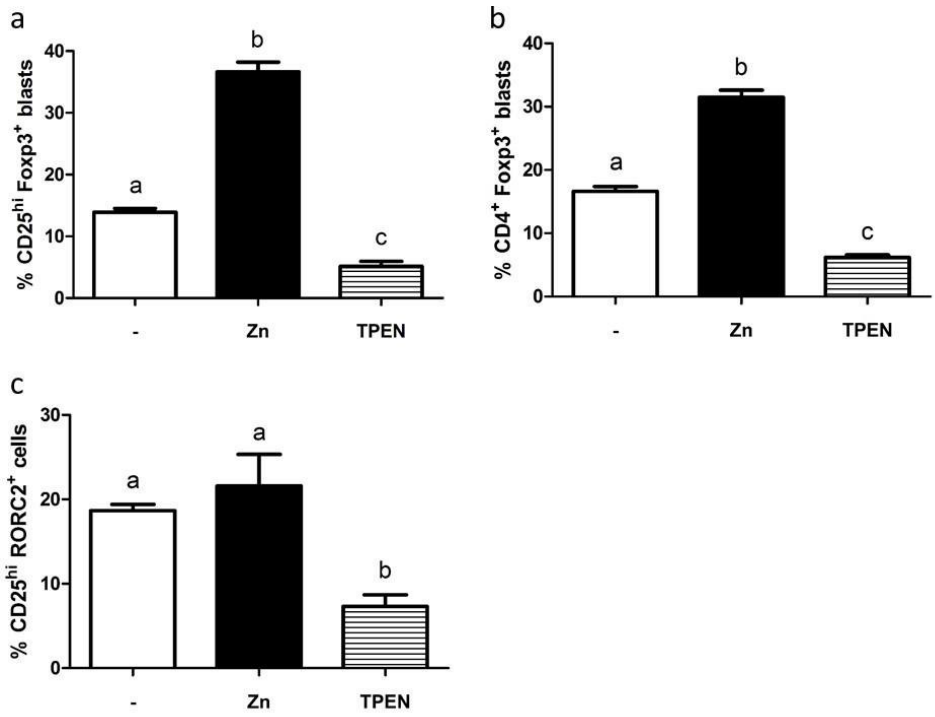


**Fig. 12. Zinc deficiency impairs Treg cell differentiation.**
$2x10^6$ PBMC/ml were pre-incubated with 50 µM zinc (black bars), 1.5 µM TPEN (striped bars) or remained untreated (white bars) for 15 min prior to MLC generation for 5 days. The percentage of Treg cells was analyzed by flow cytometry. Gating was performed on activated $CD4^+SSC^{hi}$ blasts. The percentage of (**a**) $CD25^{hi}Foxp3^+$ (n=6), (**b**) $CD4^+Foxp3^+$ (n=6), (**c**) $CD25^{hi}RORC2^+$ (n=6) is shown. Results show mean values + SEM. Data were analyzed by repeated-measures ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

PBMC were supplemented with 50 µM zinc or treated with 1.5 µM TPEN to induce zinc deficiency. The zinc chelator TPEN reduces the zinc concentration in the cytoplasm by

complexing the zinc ions [155]. Zinc supplementation significantly induced $CD4^+Foxp3^+$ and $CD25^{hi}Foxp3^+$ Treg cells in MLC (figure 12 a,b), whereas TPEN treatment significantly reduced Treg cell induction.

Moreover, zinc signals in general seem to be essential in T cell differentiation and activation, since zinc deficiency induced by TPEN also dampens Th17 differentiation. In contrast to that, zinc supplementation did not affect the Th17 population (figure 12c).

Thus, these results indicate that zinc deficiency adversely influences T cell differentiation whereas zinc supplementation selectively influences Treg cell differentiation. Thus, T cell-mediated immune reactions are highly dependent on the intracellular zinc status.

## 4.11 Zinc Treatment Augments the TGF-β1-Induced Smad Signaling Pathway

The pleiotropic cytokine TGF-β1 is known to play an essential role in immunology, since studies indicate an essential role in establishing immunological tolerance by induction of Treg cells in mice [16, 51] and men [53, 54]. Additionally, studies suggest an essential role for Smad 2/3 in Foxp3 induction and cytokine suppression [55, 56]. Moreover, the essential trace element zinc plays an important role in Treg cell induction [82, 156]. Therefore, the impact of TGF-β1 on Smad 2/3 phosphorylation as well as a combined stimulation of TGF-β1 and zinc in PBMC was analyzed (figure 13).

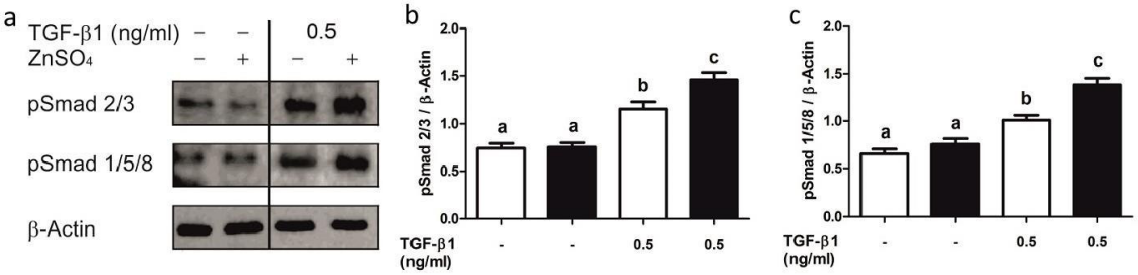


**Fig. 13. Influence of zinc supplementation on TGF-β1-induced Smad signaling in PBMC.** $2x10^6$ PBMC/ml remained untreated (white bars) or were pre-incubated with 50 µM zinc (black bars) for 15 min following TGF-β1 (0.5 ng/ml) stimulation for 1 h. (**a**) One representative experiment out of n=8-9 independent experiments is shown of (**b**) pSmad 2/3 (n=9) and (**c**) pSmad 1/5/8 (n=8). Protein content was analyzed by western blotting and results are shown as mean values + SEM of densitometric quantification. Data were analyzed by repeated-measured ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

We uncovered a significant upregulation of Smad 2/3 and Smad 1/5/8 phosphorylation by TGF-β1 stimulation, validating functionality of Smad signaling pathway induction.

Moreover, a statistically significant synergistic effect was found in samples pre-treated with zinc before TGF-β1 stimulation. Thus, an augmented Smad signaling in PBMC by usage of zinc pre-treatment followed by TGF-β1 stimulation could be demonstrated.

## 4.12 Modulation of Smad Signaling by TGF-β1 and Zinc in MLC

In general, anti-inflammatory cytokines like TGF-β1 or IL-10 are critical in suppression of the immune response [153, 157-159]. This is remarkably important due to the fact inappropriate immune regulation often results in autoimmune diseases. Thus, we first analyzed the effect of TGF-β1 and zinc administration on Treg cells in MLC (figure 14). As shown in figure 14b and figure 14c upregulation of phosphorylated Smad 2/3 was observed by combined zinc and TGF-β1 treatment comparable to the effect examined within PBMC (figure 13). This synergistic effect is more prominent when a concentration of rather 1.0 ng/ml TGF-β1 than 0.5 ng/ml is used (figure 14c). Unlike to PBMC, no effect was found regarding to phosphorylation levels of Smad 1/5/8 in MLC (figure 14d,e). However, simple zinc pre-treatment or TGF-β1 stimulation significantly induced Treg cells in MLC compared to untreated controls, indicated by elevated Foxp3 expression (figure 14f, g). Interestingly, combined zinc and TGF-β1 (1.0 ng/ml) treatment showed a statistically significant synergistic effect on Foxp3 expression in MLC (figure 14g). Furthermore, zinc and TGF-β1 respectively, as well as combined administration led to a significantly dampened IFN-γ secretion compared to the untreated control (figure 14h, i).

Summing up, these data indicate that zinc pre-treatment amplifies Treg cell amount in MLC, which can furthermore be increased by combined zinc and TGF-β1 treatment. Additionally, zinc raises TGF-β1-induced phosphorylation of Smad 2/3, illustrating a direct effect of zinc on the TGF-β1 signaling. Moreover, zinc and TGF-β1 are capable to suppress the pro-inflammatory IFN-γ cytokine secretion respectively. This effect was seen in treatment either with zinc or TGF-β1 as well as in combined treatment (figure 12h, i). This indicates that zinc and TGF-β1 both have the ability to dampen the allogeneic MLC reaction, as IFN-γ secretion represents MLC severity.

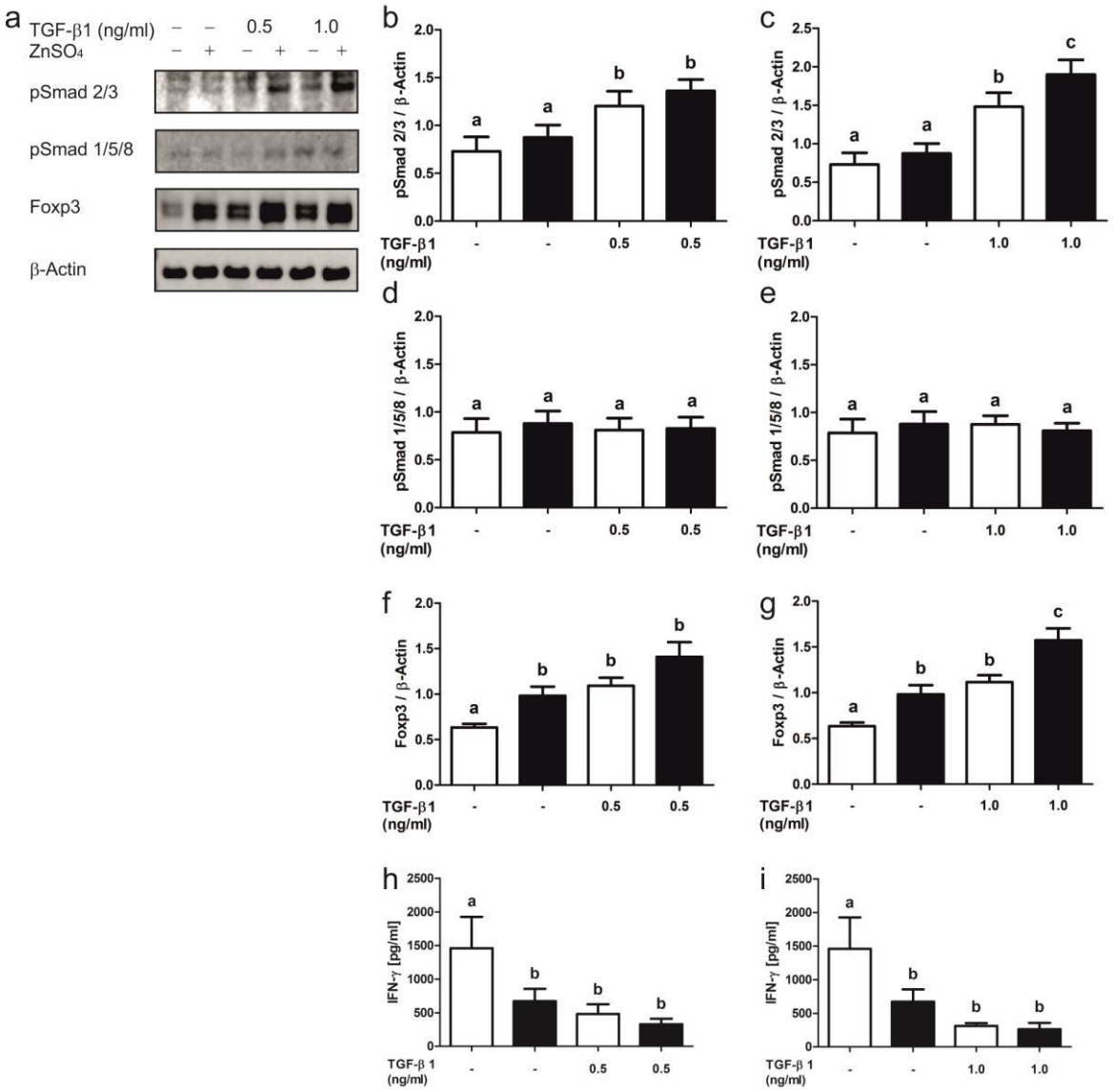


**Fig. 14. Impact of zinc pre-treatment and TGF-β1 stimulation in MLC.**
$2x10^6$ PBMC/ml remained untreated (white bars) or were pre-incubated with 50 µM zinc (black bars) for 15 min. MLC generation was performed, followed by TGF-β1 stimulation for 5 days with indicated concentrations. (**a**) One representative experiment out of n=8 independent experiments is shown. (**b**, **c**) pSmad 2/3, (**d**, **e**) pSmad 1/5/8 and (**f**, **g**) Foxp3 protein were measured by western blotting. Results show mean values + SEM of densitometric quantifications. (**h**, **i**) IFN-γ concentration was measured by ELISA (n=6). Data were analyzed by repeated-measured ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

## 4.13 Impact of IL-10 and Zinc Pre-Treatment on Treg Cells in MLC

IL-10 is necessary for Treg cell mediated immune suppression [36] but is still discussed whether it is required to maintain Foxp3 expression [38].

The following experiments indicate that IL-10 is not necessary for Foxp3 induction in MLC (figure 15a,b). Increased Foxp3 expression due to zinc treatment was abolished by IL-10 stimulation and returned to control level (figure 15b). Moreover, even the zinc-mediated increase of Foxp3 expression was diminished due to additional IL-10 stimulation. Nevertheless, the immune-suppressive activity of IL-10 was seen by analysis of IFN-γ secretion in MLC (figure 15c).

These results indicate that IL-10 plays an important role in Treg cell-mediated immune suppression by dampening the pro-inflammatory cytokine production in MLC, but has no direct effect on Foxp3 expression or stability in MLC as it was observed in TGF-β1 experiments. Thus, zinc pre-treatment favors the Smad signaling pathway activity and Treg cell induction in MLC but has no impact on IL-10 signaling.

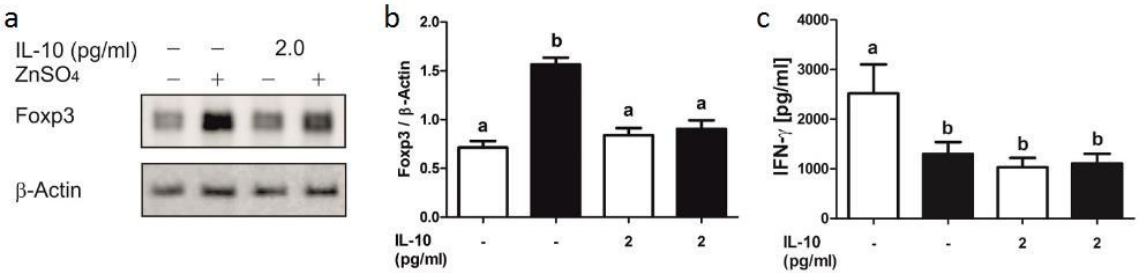


**Fig. 15. IL-10 does not influence Foxp3 but reduces IFN-γ production.**
$2x10^6$ PBMC/ml remained untreated (white bars) or were pre-incubated with 50 µM zinc (black bars) for 15 min. MLC generation was performed, followed by IL-10 stimulation (2 pg/ml) for 5 days. (**a**) One representative experiment out of n=5 independent experiments is shown. (**b**) Foxp3 protein was measured by western blotting. Results show mean values + SEM of densitometric quantifications. (**c**) IFN-γ concentration was measured in supernatants (n=9). Data were analyzed by repeated-measures ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

## 4.14 TGF-β1 Stimulation Elevates the Intracellular Free Zinc Content in T Cells

TGF-β1 and IL-10 play an important role in immune suppression as well as in Treg cell differentiation and function respectively. Nevertheless, different results were found in

MLC experiments regarding to Foxp3 induction by TGF-β1 and IL-10. Since the intracellular free zinc level in T cells is essential for development, maturation, differentiation, and function [13, 14, 19, 20, 30, 39], the intracellular zinc level in T cells during TGF-β1 and IL-10 stimulation was investigated in this study. In figure 16 the intracellular free zinc concentration is displayed in $CD4^+$ T cells measured with Fluozin-3 AM (figure 16a,c) and ZinPyr-1 (figure 16b, d). Stimulation of PBMC with 1.0 ng/ml TGF-β1 for 1 h significantly raised the intracellular zinc content compared to the untreated control. The cytoplasmic (figure 16a) as well as the lysosomal zinc (figure 16b) was elevated. In contrast, IL-10 stimulation did not affect the intracellular zinc level (figure 16c,d).

Accordingly, merely TGF-β1 stimulation triggers an intracellular zinc signal in $CD4^+$ T cells leading to an altered signal transduction and activation. Subsequently, the different results observed by TGF-β1 and IL-10 stimulation can be traced back to an altered intracellular zinc level.

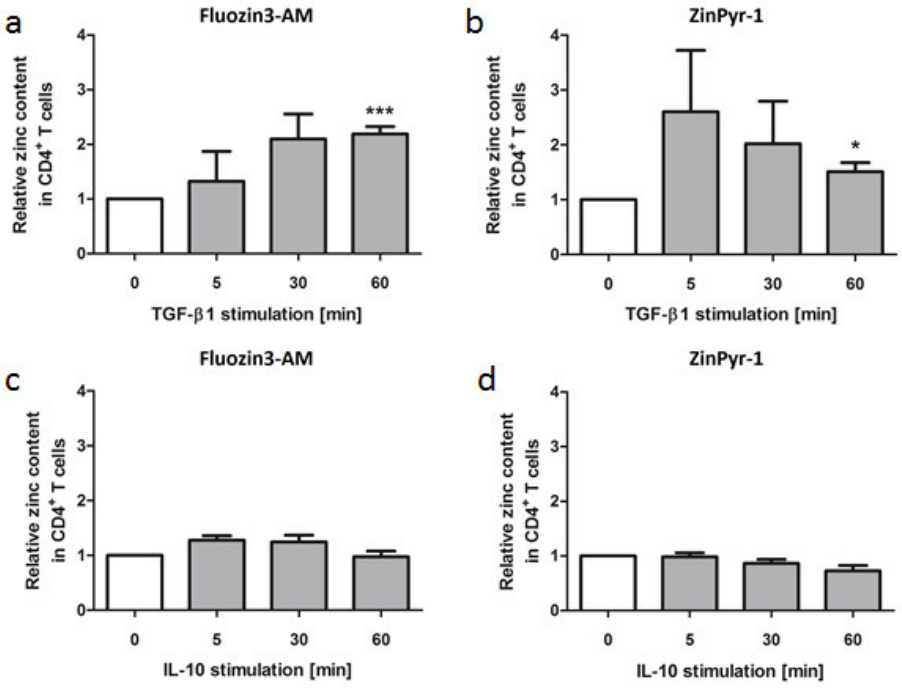


**Fig. 16. TGF-β1 elevates intracellular zinc content in $CD4^+$ T cells.**
$2x10^6$ PBMC/ml remained untreated (white bars) or were stimulated with 1.0 ng/ml TGF-β1 or 2.0 pg/ml IL-10 (grey bars) for 5 min, 30 min or 60 min. Intracellular free zinc was measured by (**a**, **c**) Fluozin-3 AM and (**b**, **d**) ZinPyr-1. * indicates a significance of $p<0.05$ compared to control, *** of $p<0.001$ compared to control (student's t-test). Mean values + SEM are shown.

## 4.15 Impact of TGF-β1 and Zinc Treatment on Hut78 T Cells

The impact of zinc pre-treatment on the phosphorylation of Smad 2/3 and Smad 1/5/8 was investigated in a human T cell line (Hut78). For Hut78 T cells the previous mentioned effect on Smad 2/3 phosphorylation in PBMC and MLC could be validated (figure 17a-c). Simple zinc supplementation for 15 min showed neither an effect on pSmad 2/3 nor on pSmad 1/5/8, whereas simple TGF-β1 stimulation lead to a significant increase of pSmad 2/3 compared to the unstimulated control. The combined zinc and 1.0 ng/ml TGF-β1 treatment resulted in a synergistic effect for Smad 2/3 signaling (figure 17c) but not for pSmad 1/5/8 (figure 17e) compared to simple TGF-β1 stimulation. Hence, zinc pre-treatment merely increases Smad 2/3 activity in Hut78 T cells and is therefore in line with our findings in MLC.

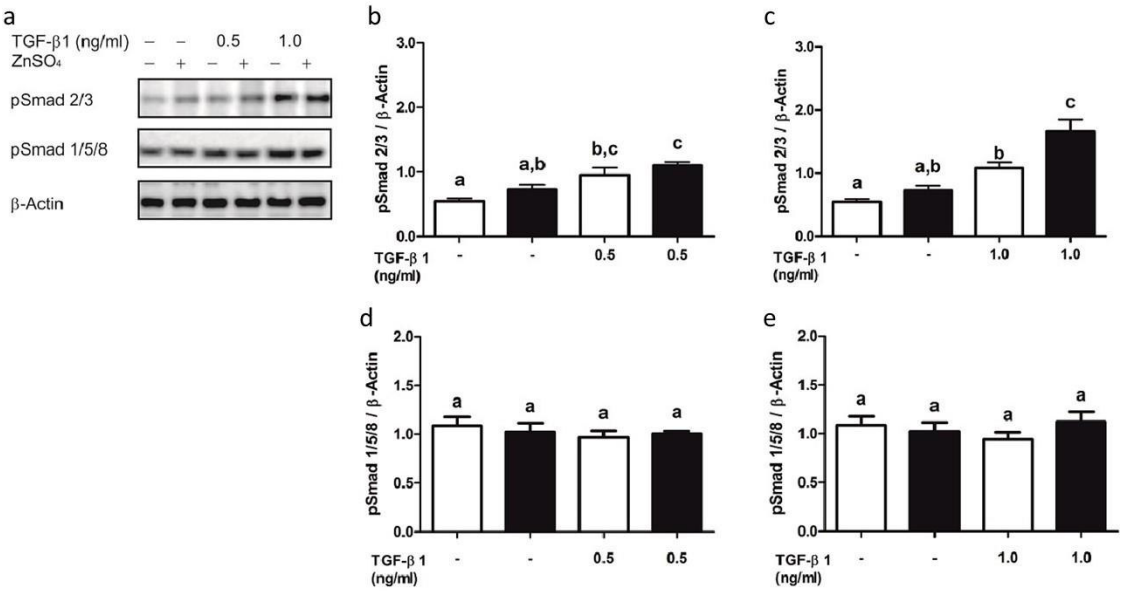


**Fig. 17. Zinc pre-treatment elevates TGF-β1-induced Smad signaling in Hut78 cells.**
$1x10^6$ Hut78 cells were left untreated (white bars) or were pre-incubated for 15 min with 50 µM zinc (black bars), followed by TGF-β1 stimulation for 1 h with 0.5 ng/ml or 1.0 ng/ml. (**a**) One representative experiment out of n=7 independent experiments in is shown. (**b**, **c**) pSmad 2/3 and (**d**, **e**) pSmad 1/5/8 (n=8) protein was measured by western blotting. Results are shown as mean values + SEM of densitometric quantifications. Data were analyzed by repeated-measures ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

## 4.16 Impact of TGF-β1 and Zinc Treatment on Hepatocytes, Monocytes and B cells

Zinc-dependent phosphorylation of Smad 2/3 and Smad 1/5/8 was additionally investigated in hepatocytes (Huh7) (figure 18a-d), B cells (Raji) (figure 18e-h), and monocytes (MonoMac1) (figure 18i-l) to exclude cell-line specific effects.

For both hepatocytes and monocytes, similar effects were uncovered as observed in PBMC (figure 13). Here, a synergistic effect due to combined zinc and TGF-β1 treatment was monitored for Smad 2/3 phosphorylation in hepatocytes (figure 18a, b) and monocytes (figure 18i, j), which was significant using 1.0 ng/ml TGF-β1 (figure 18b, j). Moreover, a comparable but not significant effect was found for Smad 1/5/8 phosphorylation in hepatocytes (figure 18c, d) whereas a significant upregulation of the Smad 1/5/8 phosphorylation by costimulation of zinc and TGF-β1 was uncovered in monocytes (figure 18l). On the contrary, phosphorylation of Smad 2/3 and Smad 1/5/8 is triggered by TGF-β1 stimulation in B cells (figure 18 e-h) but remained unaffected by zinc treatment.

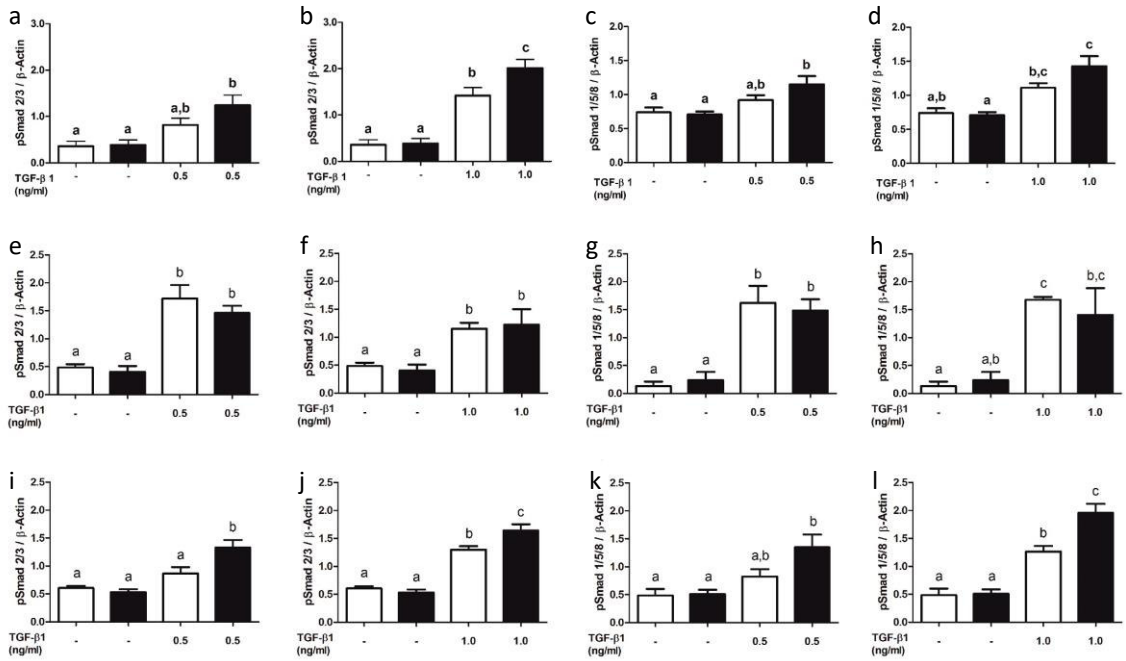


**Fig. 18. Zinc pre-treatment elevates TGF-β1-induced Smad signaling in hepatocytes.**
$1x10^6$ cells were left untreated (white bars) or were pre-incubated for 15 min with 50 µM zinc (black bars), followed by TGF-β1 stimulation for 1 h with indicated concentrations. Western blot analysis was performed for (**a**, **b**, **e**, **f**, **i**, **j**) pSmad 2/3 (n=8) and (**c**, **d**, **g**, **h**, **k**, **l**) pSmad 1/5/8 (n=8) in (**a**-**d**) in Huh7, (**e**-**h**) Raji, and (**i**-**j**) MonoMac1. Results are shown as mean values + SEM of densitometric quantifications. Data were analyzed by repeated-measures ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

Hence, zinc pre-treatment increases the TGF-β1-induced Smad 2/3 activity in T cells, hepatocytes, and monocytes and is therefore in line with our former findings. However, zinc-dependent Smad activation seems to be cell-type specific and dependent on experimental setups, since zinc pre-treatment upregulates Smad 2/3 phosphorylation in all experiments, but merely increases Smad 1/5/8 phosphorylation in monocytes and PBMC. In B cells Smad signaling seems to be zinc-independent.

## 4.17 Inhibition of the Smad Signaling Pathway Negatively Affects Treg Cells

The aforementioned investigations reveal a direct connection of pSmad 2/3 activation and Foxp3 expression. To analyze Smad-dependent Foxp3 expression Smad signaling was repressed by using SB 431542, an inhibitor of activin receptor-like kinase (ALK) 5 (the TGF-β type I receptor) and thus inhibiting TGF-βR signaling [40].

Simple zinc treatment significantly increased Foxp3 expression (figure 19b) whereas Smad 2/3 and Smad 1/5/8 phosphorylation remained unaffected (figure 19c, d). TGF-β1 stimulation triggered Foxp3 and pSmad 2/3 expression and additional zinc pre-treatment led to a synergistic effect. Pre-incubation of MLC with SB 431542 for 1 h resulted in a effectively reduced Smad 2/3 phosphorylation (figure 19c), whereas Smad 1/5/8 phosphorylation remained unaffected (figure 19d). Moreover, TGF-β1-induced Foxp3 expression as well as the aforementioned synergistic effect on Foxp3 expression of combined zinc and TGF-β1 treatment in PBMC and MLC was now diminished by SB 431542 pre-incubation and comparable to the untreated control (figure 19b).

Thus, inhibition of the Smad 2/3 signaling cascade simultaneously inhibits Foxp3 expression pointing to a correlation of Treg cell induction and Smad signaling in MLC.

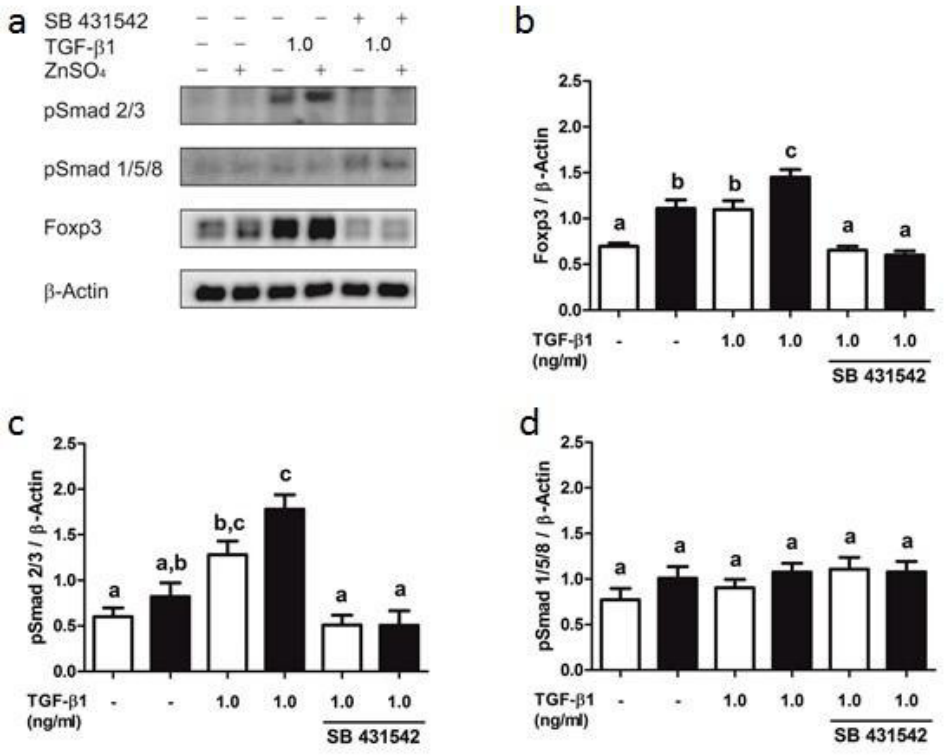


**Fig. 19. Inhibition of Smad signaling simultaneously inhibits Foxp3 expression in MLC.** $2x10^6$ PBMC/ml and remained untreated (white bars) or were pre-incubated with 50 µM zinc (black bars) for 15 min. MLC generation was performed, followed by SB 431542 incubation (1 h; 10 µM). Subsequently, samples were stimulated with TGF-β1 for 5 days (1.0 ng/ml). (**a**) One representative experiment out of n=5 independent experiments is shown. (**b**) Foxp3, (**c**) pSmad 2/3 and (**d**) pSmad 1/5/8 protein was measured by western blotting. Results are shown as mean values + SEM of densitometric quantifications. Data were analyzed by repeated-measures ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

## 4.18 Cyclosporine and MLC

Most current immunosuppressive drugs like Rapamycin or Cyclosporine A (CsA) target T cell activation and clonal expansion, since it is well known that T cells play a pivotal role in graft rejection [69, 70]. Calcineurin inhibitors, as CsA, have often been associated with antagonistic effects on tolerance induction because CsA inhibits conventional T cell development and Treg cell development respectively *in vivo* [72]. However, studies showed a dose-dependent influence of CsA on Treg cells. While the usage of higher doses have adverse effects on frequency and function of Treg cells, lower doses are associated with pro-tolerogenic effects resulting in graft tolerance [73, 74]. Hence, the

modulation of Treg cells seem to be an attractive approach for the induction and maintenance of allograft-specific tolerance enabling the avoidance of broad long-term immunosuppression.

Therefore, we investigated the influence of CsA as well as zinc-pre-treatment and CsA costimulation in MLC (figure 20). Zinc pre-incubation significantly increased the expression of Foxp3 (figure 20a, b). The inhibitory effect of CsA on Foxp3 expression is ameliorated in lower CsA concentrations (until 62.5 ng/ml) when zinc pre-incubation was used (figure 20a, b). In addition, zinc supported the CsA-mediated decrease of IFN-γ production (figure 20c).

Thus, zinc counteracts the inhibitory effect of CsA on Treg cell induction by elevated Foxp3 expression at lower CsA concentrations. Furthermore, combined zinc and CsA treatment significantly dampens the IFN-γ secretion compared to single treatment resulting in an ameliorated MLC severity.

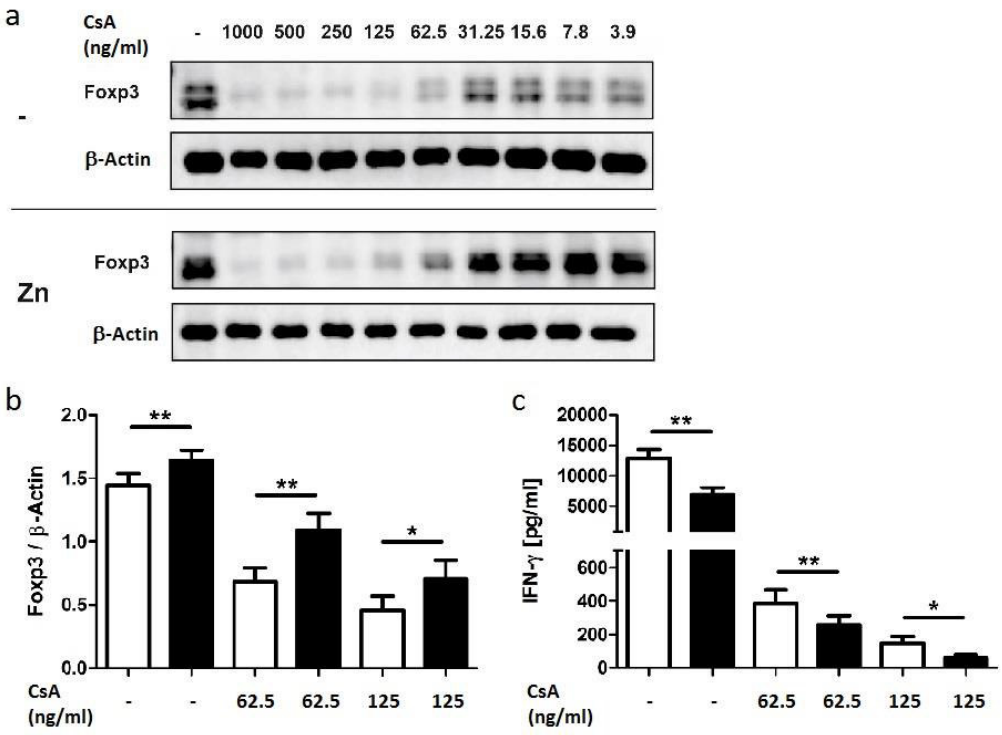


**Fig. 20. Zinc induces Treg cells and reduces IFN-γ secretion while CsA treatment.**
$2x10^6$ PBMC/ml and remained untreated (white bars) or were pre-incubated with 50 µM zinc (black bars) for 15 min. MLC generation was performed followed by CsA stimulation (3.9 ng/ml to 1 µg/ml) for 5 d. (**a**) One representative experiment out of n=6 independent experiments is shown. (**b**) Foxp3 protein expression was detected by western blotting and is displayed as densitometric quantification, (**c**) IFN-γ secretion was measured by ELISA. Results are shown as mean values + SEM. * indicates a significance of $p<0.05$ compared to control, ** of $p<0.01$ (student's t-test).

## 4.19 Zinc and Renal Transplantation

### 4.19.1 Zinc Buffer Capacity of Custodiol

Today, still a high number of patients are waiting anxiously for kidney transplants, but do not get a matched graft either due to the absence of an available donor or due to the short shelf life of donor kidneys. One feasible solution could be the extension and improvement of the cold storage conditions of the grafts to guarantee functionality [160]. Kidneys collected for transplantation are preserved in cold storage solutions to sustain the kidneys' function and to avoid cellular apoptosis. This is facilitated by the induction of vasoconstriction during storage. The metabolism is slowed down, which however still lead to an extenuated tubular and endothelial cell death [161]. Zinc as a pro-antioxidant is known to be involved in the inhibition of apoptotic enzymes such as caspases and acts tolerogenic [82, 162, 163]. Hence, it could be a favorable additive to organ protection solutions to improve organ preservation during cold storage. Therefore, the zinc buffer capacity of the storage solution Custodiol was determined, to guarantee free available zinc for cellular uptake (figure 21a). Free zinc in Custodiol was determined by adding different zinc concentrations ranging from 5 µM to 300 µM. As displayed, concentrations of 150 µM to 300 µM added zinc to Custodiol showed significantly increased free zinc concentrations compared to the untreated control. Therefore, at least 150 µM zinc has to be added to Custodiol to guarantee available zinc for cellular uptake.
In zinc-uptake experiments (figure 21b and c) PBMC were cultured in Custodiol (white bars) and RPMI 1640 medium (grey bars) respectively and the intracellular zinc concentration was detected by ZinPyr-1 (figure 21b) and Fluozin3-AM (figure 21c).

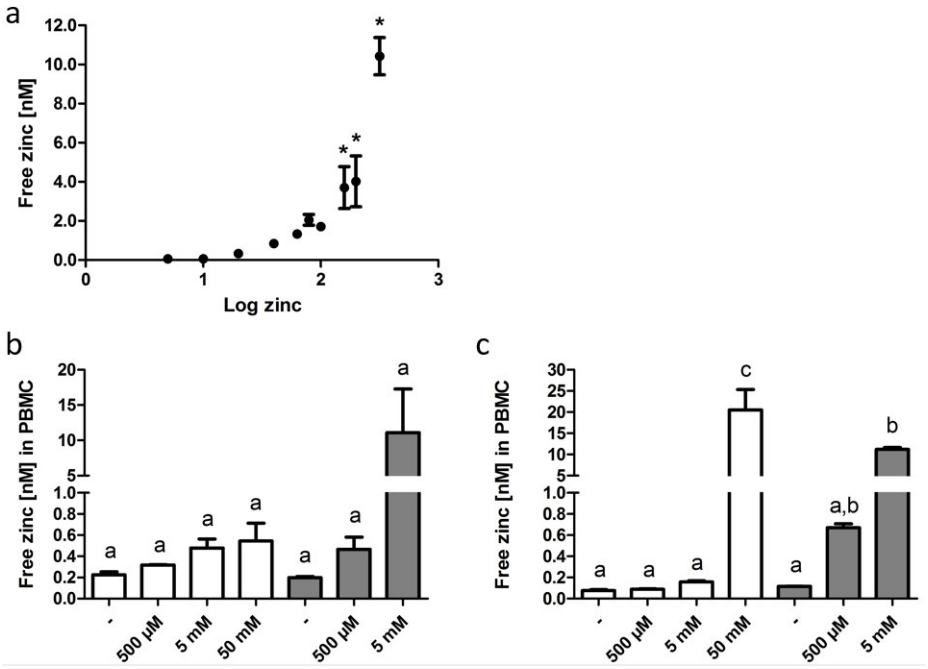


**Fig. 21. Zinc buffer-capacity of Custodiol and intracellular zinc uptake.**
Zinc concentrations ranging from 5 µM to 300 µM were added to Custodiol. (**a**) Free zinc was measured by using Fluozin3-A. (**b**, **c**) $2x10^6$ PBMC/ml were cultured in Custodiol (white bars) or in RPMI 1640 (grey bars) supplemented with indicated zinc concentrations. Intracellular free zinc was measured by (**b**) ZinPyr-1 and (**c**) Fluozin3-AM. Results are shown as mean values + SEM. Data in (**a**) were analyzed by repeated-measures ANOVA and Dunnett's post hoc test. * indicates a significance of $p<0.05$ compared to control. Data in (**b**, **c**) were analyzed by repeated-measures ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

Zinc supplementation of 5 mM in RPMI 1640 medium already provoked an elevation of the intracellular zinc level (figure 21b, c). Instead, 50 mM zinc need to be applied to Custodiol to elevate the intracellular free zinc content in PBMC significantly. Application of 50 mM zinc to PRMI 1640 medium led to apoptosis (data not shown) therefore excluding this concentration for further culture medium experiments.
Summing up, zinc administration of 150-300 µM guarantee available free zinc in Custodiol. To alter the intracellular zinc level in vital cells, at least 5 mM zinc needs to be supplemented to Custodiol.

### 4.19.2 Influence of Subcutaneous Administered Zinc, NaCl and CsA in Renal Transplantation in Rodents

The understanding of mechanisms promoting tolerance in solid organ transplantation is essential for successful graft acceptance. Thus, the recipients' immune response to the graft requires efficient controlling for successful long-term transplantation. To dampen the recipients' immune reaction immunosuppressive agents like CsA are administered.

CsA treatment inhibits not only conventional T cells, but also Treg cell development *in vivo*. Since the intra-graft Treg cell frequency seem to correlate with clinical graft acceptance, survival, and function, Treg cell induction might be a useful strategy for successful tolerance induction in transplantation [69, 70]. Zinc treatment *ex vivo* and *in vivo* favors the Treg cell induction, which leads to a dampened immune reaction and provokes tolerance. Therefore, zinc might be a useful additive to beneficially influence the immune reaction during transplantation.

Hence, we investigated the influence of subcutaneous administered zinc in low dose (6 µg/kg) dissolved in 0.9% NaCl (Zn6) and in 5% glucose (Zn6 G) as well as high dose zinc (30 µg/kg) dissolved in 0.9% NaCl (Zn30) and in 5% glucose (Zn30 G) compared to vehicle controls (0.9% NaCl and 5% glucose) and to CsA (15 mg/kg) in a kidney transplantation rat model *in vivo* (figure 22). During the whole duration (10 days) of the experiment, we could not observe any significant changes by each treatment regarding to peripheral expression of $CD4^+Foxp3^+$ Treg cells neither in blood (figure 22a), nor in the spleen at day 10 (figure 22b).

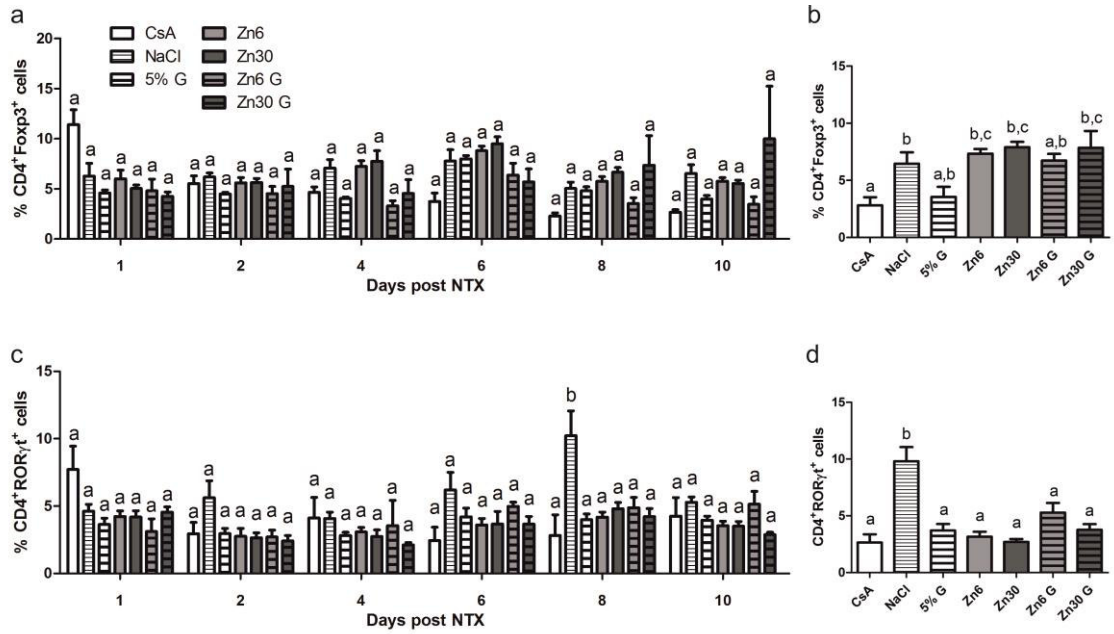


**Fig. 22. NaCl administration in physiological concentration induces Th17 cell incidence.** Rats were injected daily with 15 mg/kg CsA (n=5), 0.9% NaCl (n=5), 6 µg/kg zinc dissolved in NaCl (Zn6) (n=8), 30 µg/kg zinc dissolved in NaCl (Zn30), 6 µg/kg zinc dissolved in glucose (Zn6 G) (n=3), or 30 µg/kg zinc dissolved in glucose (Zn30 G) (n=3). (**a**) Peripheral $CD4^+Foxp3^+$, (**b**) $CD4^+Foxp3^+$ in the spleen, (**c**) peripheral $CD4^+ROR\gamma t^+$ cells, and (**d**) $CD4^+ROR\gamma t^+$ in the spleen were determined by FACS analysis. Results are shown as mean values + SEM. Data were analyzed by One-way ANOVA and Tukey post hoc test. Significantly different means do not share the same letters.

Significant upregulation of peripheral $CD4^+ROR\gamma t^+$ Th17 cells was found after 8 days of treatment in NaCl vehicle controls (figure 22c) as well as in the spleen (figure 22d). Hence, zinc administration showed no effect on Treg cell and Th17 cell differentiation. NaCl treatment negatively affect tolerance induction in this transplantation model by upregulating Th17 cells. The animals' body weight is a crucial parameter to classify the general state of health of animals and thus a useful indicator to assess the severity of the allogeneic immune reaction. Therefore, the body weight was measured daily (figure 23). The animals' body weight slightly decreased within the first three days after allogeneic kidney transplantation in all groups. During the following days, the mean body weight of all groups except those treated with the CsA increased continuously up to day 10. The zinc treated groups exhibited higher body weight compared to the placebo groups. Especially, the high zinc group (Zn30) showed a significant elevation at day 9 and 10.

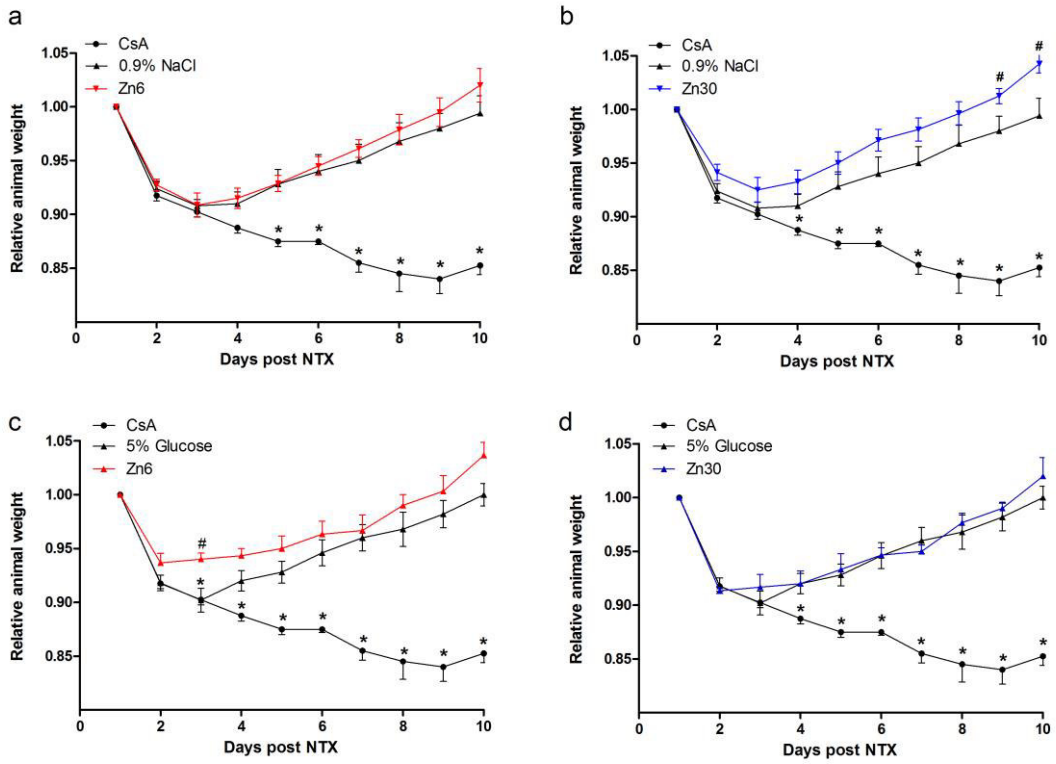


**Fig. 23. Weight-course of differently treated animal groups.**
Rats were treated daily with 15 mg/kg CsA (n=5), 0.9% NaCl (n=5), 6 µg/kg zinc dissolved in NaCl (Zn6) (n=8), 30 µg/kg zinc dissolved in NaCl (Zn30) (n=8), 6 µg/kg zinc dissolved in glucose (Zn6 G) (n=3), or 30 µg/kg zinc dissolved in glucose (Zn30 G) (n=3) respectively. The animals' weight was determined daily. (**a**, **b**) The weight-course of placebo and Zn6 or Zn30 treated animals is displayed as well as (**c**, **d**) the weight course of placebo and Zn6 G or Zn30G treated animals. (n=3). * indicates a significance of at least $p<0.05$ (student's t-test) comparing CsA and the remaining treatments. # indicates a significance of at least $p<0.05$ (student's t-test) comparing the zinc and NaCl treated group and zinc and glucose treated group respectively. Results are shown as mean values + SEM.

Thus, zinc supplementation improves the animals' well-being displayed by elevated body weight.

Since zinc homeostasis is essential for proper immune function and zinc deficiency is associated with an increased percentage of transplant rejections [164, 165], we measured the intracellular free zinc concentration in lymphocytes (figure 24a) and splenocytes (figure 24b) as well as the total zinc amount in the serum (figure 24c) of placebo and zinc treated animals at day 10. An adequate organ and cellular function is amongst others dependent on cellular viability itself, surrounding nutrient milieu, pH, and concentration of essential trace elements. Thus, the zinc amount of the autologous kidney (figure 24d, f) and the allogeneic transplant was determined respectively (figure 24e, g).

Subcutaneous zinc administration of low or high dose (Zn6 G and Zn30 G) provoked a significant increase of the intracellular free zinc level in lymphocytes independent of either low (Zn6 G) or high dose (Zn30 G) was administered (figure 24a). This effect was only detectable when zinc dissolved in 5% glucose was administered. In splenocytes, no change of the intracellular free zinc level was detectable (figure 24b). Measurement of the total amount of zinc in the serum pointed to a slight decrease due to NaCl treatment (figure 24c). In contrast to that, 5% glucose and the treatment with low dose glucose-dissolved zinc significantly increased the serum zinc level compared to NaCl. Comparable to the determined amount of zinc in splenocytes, the zinc amount in the autologous kidneys did not change; neither by placebo nor zinc treatment (figure 24d). Interestingly, the total zinc amount of the allogeneic transplant significantly increased due to high zinc administration (Zn30 G). All residual treatments remained ineffective. The comparison of the treatment with NaCl-dissolved zinc and glucose-dissolved zinc uncovered a significantly elevated zinc amount in the allogeneic transplant due to glucose-dissolved zinc treatment (figure 24g).

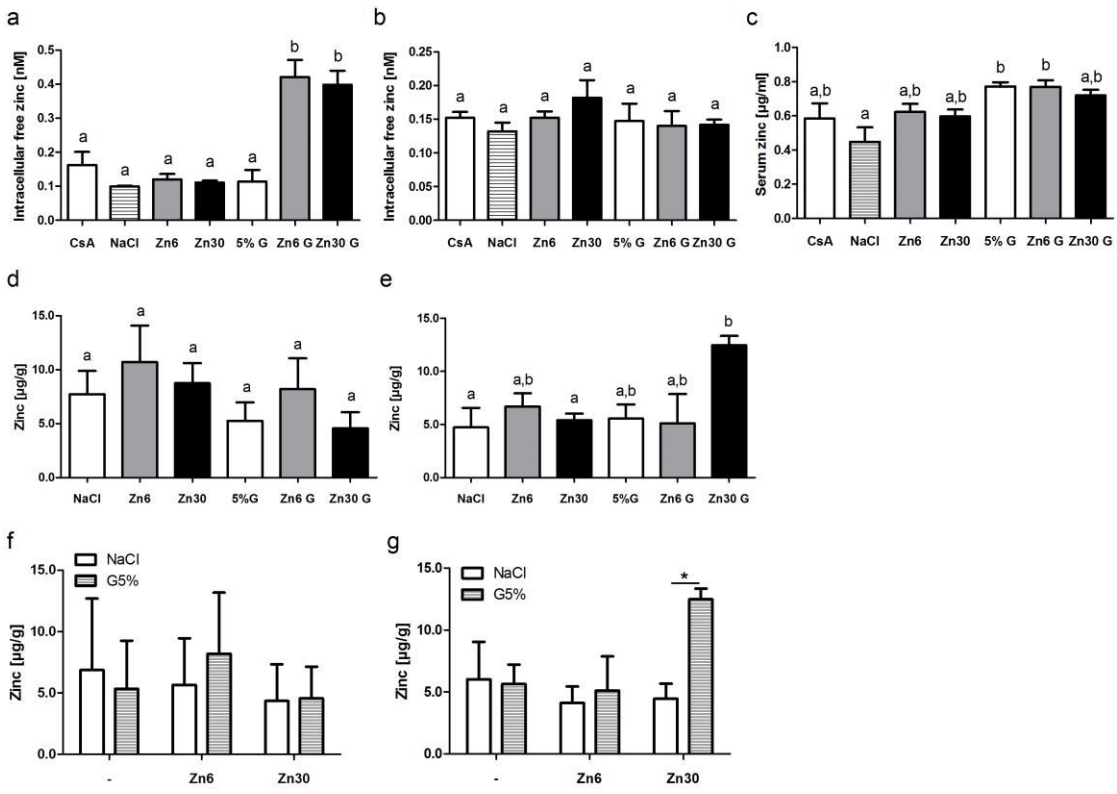


**Fig. 24. Measurement of the intracellular and intra-kidney zinc amount.**
The intracellular zinc concentration was measured in (**a**) lymphocytes and (**b**) splenocytes by Fluozin3-AM using FACS analysis. (**c**) The total zinc amount of the serum, the zinc amount of the (**d**) autologous kidney and (**e**) the allogeneic transplant was determined by AAS. The total zinc amount of the kidney is displayed for the 0.9% NaCl-dissolved zinc treatment (white bars) and the 5% glucose-dissolved zinc treatment (light-grey bars) in (**f**) the autologous kidney and (**g**) the allogeneic transplant. Results are shown as mean values + SEM. Data of (**a**, **b**) were analyzed by One-way ANOVA and Tukey post hoc test, (**d**, **e**, **f**, **g**) were analyzed by student's t-test. Significantly different means do not share the same letters. * indicates a significance of $p<0.05$, ** of $p<0.01$ (student's t-test).

In summary, these results indicate that subcutaneous zinc administration increases the intracellular zinc level and intra-graft zinc level when zinc is dissolved in 5% glucose. In contrast to that, NaCl-dissolved zinc did not alter the intracellular, intra-graft and serum zinc level. Moreover, simple NaCl treatment slightly reduces the serum zinc level. Application of high zinc beneficially affects the well-being of the animals as indicated by elevated body weight. Therefore, the following experiments were performed by using Zn30 dissolved in 5% glucose.

### 4.19.3 Subcutaneous Zinc Administration Beneficially Affects Treg Cell Induction in CsA Treatment

Calcineurin inhibitors, like CsA, are well established and widely-used immunosuppressive agents in transplantation medicine. However, CsA is associated with antagonistic effects on tolerance induction by inhibiting conventional T cells but also Treg cells [71, 72]. Nevertheless, studies suggest a dose-dependent CsA-impact on Treg cells. The administration of high dose CsA (15 mg/kg) in known to induce adverse effects on Treg cells frequency and function, whereas low doses CsA (1.5 mg/kg) is associated with pro-tolerogenic effects, enabling graft tolerance [73, 74]. Zinc treatment is also known to induce tolerance *ex vivo* and *in vivo* [83, 156]. Moreover, zinc administration beneficially affected the animals' welfare in this study by increasing body weight and increasing the intracellular zinc level.

This is why we investigated whether simultaneous zinc administration and CsA treatment induces tolerance. Therefore, high dose CsA treatment (15 mg/kg) and low dose CsA treatment (1.5 mg/kg) were compared to simultaneous CsA and Zn30 administration. The percentage of Treg cells (figure 25a) and Th17 cells (figure 25b) in whole blood and in the spleen (figure 25 c-e) are displayed. The comparison of simple CsA high treatment and CsA high zinc treatment revealed no significant change in $CD4^+Foxp3^+$ Treg cells until day 30. On the contrary, the comparison of simple CsA low treatment and CsA low zinc treatment indicated already a significant upregulation of Treg cells at day 6 (figure 25a). In accordance to that, $CD4^+ROR\gamma t^+$ Th17 cells were significantly reduced due to simultaneous CsA low zinc treatment in contrast to simple CsA low treatment (figure 25b). Again, CsA high zinc treatment was not effective to dampen Th17 cells since only a sporadic diminution of Th17 cells was found compared to simple CsA high treatment. Investigations of resident Treg cells and Th17 cells in the spleen uncovered merely an increase due to CsA low treatment respectively. This effect was more prominent regarding to Th17 cell population (figure 25 c, d). By determination of the Treg/Th17 ratio in the spleen of the CsA low and CsA low zinc treated animals, we uncovered a significant increase in the CsA low zinc co-treatment.

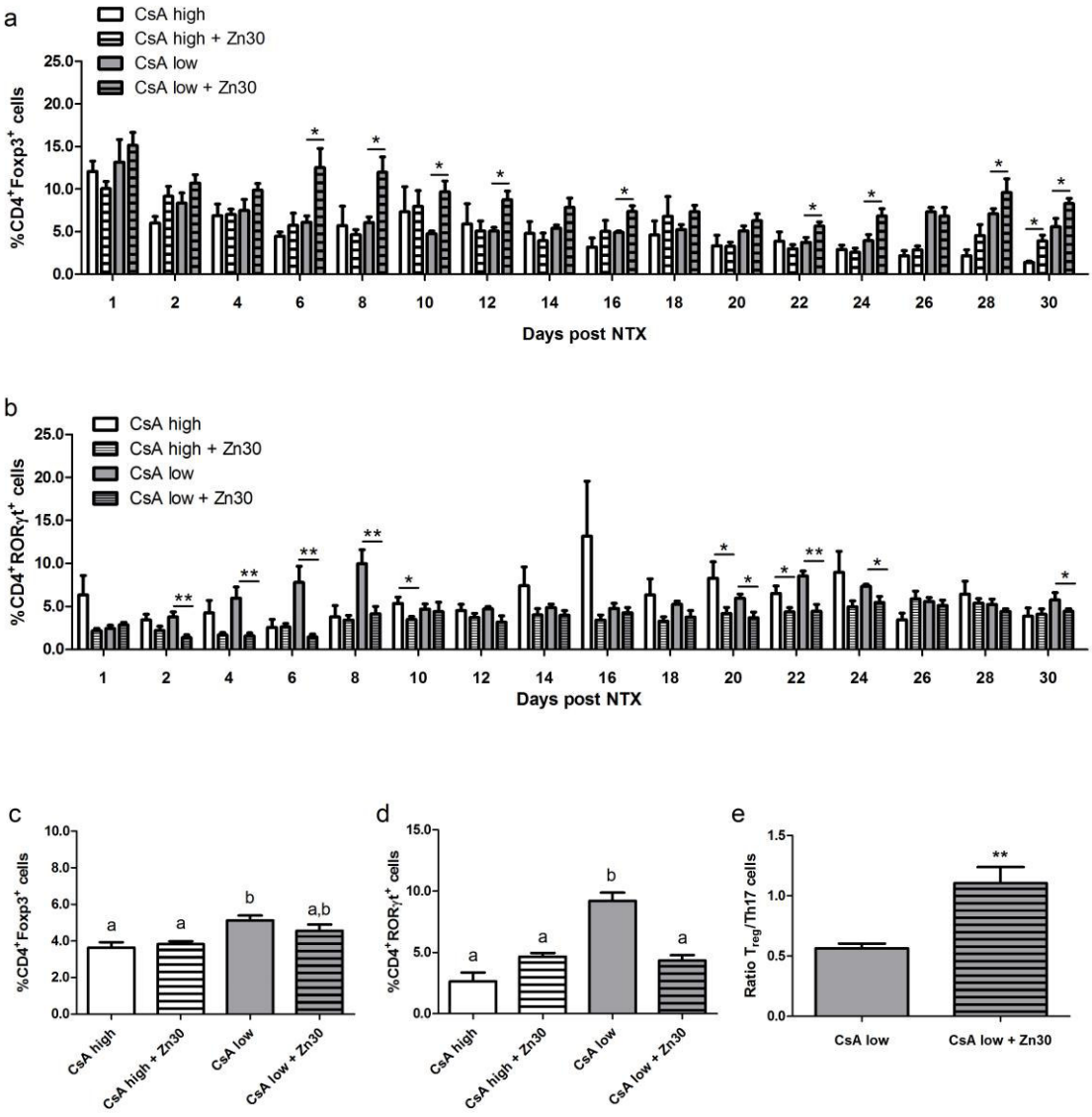


**Fig. 25. Zinc administration beneficially influences immunological tolerance *in vivo*.**
Rats were treated daily with CsA high (15 mg/kg) (s.c.), CsA low (1.5 mg/kg) (s.c.) (n=5) and combined CsA and Zn30 (s.c.) treatment (n=8) respectively. (**a**) Peripheral $CD4^+Foxp3^+$ Treg cells and (**b**) $CD4^+ROR\gamma t^+$ Th17 cells as well as (**c**) of $CD4^+Foxp3^+$ cells and (**d**) $CD4^+ROR\gamma t^+$ cells in the spleen were measured by FACS analysis. (**e**) The ratio of Treg/Th17 cells in the spleen is calculated. Results are shown as mean values + SEM. Data of (**a**, **b**, **e**) were analyzed by student's t-test, (**c**, **d**) by One-way ANOVA and Tukey post hoc test. Significantly different means do not share the same letters. * indicates a significance of $p<0.05$, ** of $p<0.01$ (student's t-test).

Thus, these results indicate that additional zinc administration in low dose immunosuppression provokes an immunomodulation that beneficially influences Treg cell induction and dampens Th17 cells, whereas high CsA treatment negatively influences the immune response by decreasing the percentage of effector T cells and Treg cells.

Furthermore, the animals' body weight as well as intracellular zinc level was investigated (figure 26). In line with the former results, additional zinc administration beneficially

increases animals' welfare indicated by elevated body weight (figure 26 a, b). Moreover, the serum zinc level was significantly increased by zinc administration (figure 26 c). Determination of the total zinc amount in the autologous kidney reveled no difference by any therapy (figure 26 d), whereas a significant increase was detectable in the allogeneic graft (figure 26 e).

Together, these results point to beneficial immunomodulation towards tolerance induction in transplantation, when immunosuppressive agents are used in a lower concentration and are administered in combination with daily zinc treatment.

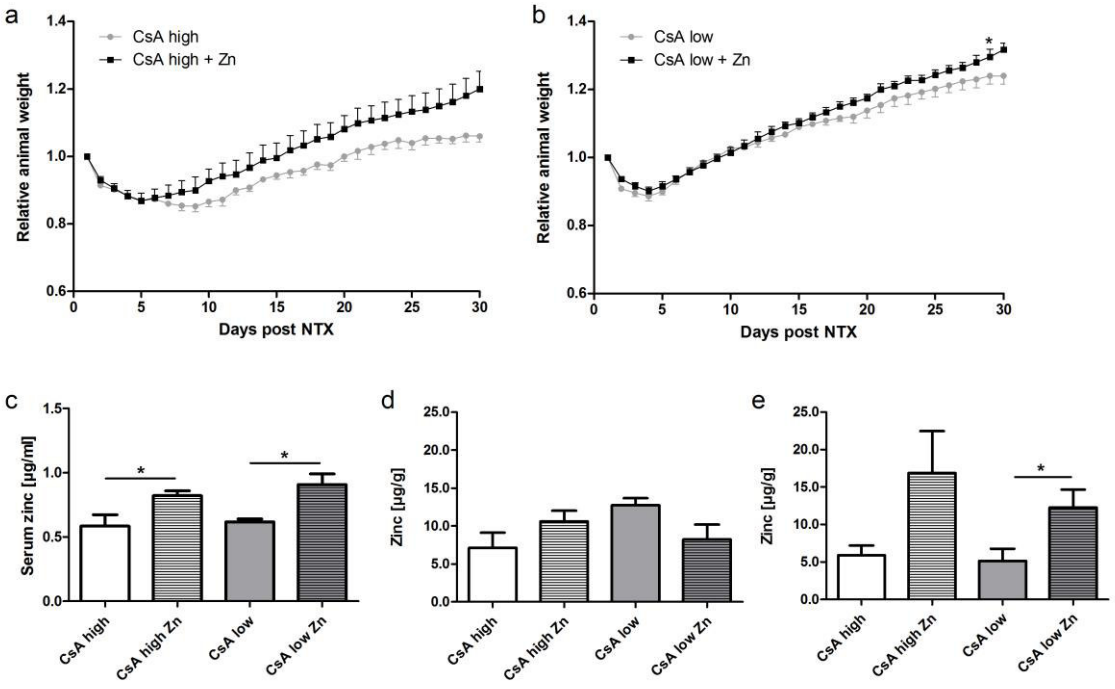


**Fig. 26. Zinc administration in CsA treatment increases animal welfare and the zinc level**
Rats were treated daily with CsA high (15 mg/kg) (s.c.), CsA low (1.5 mg/kg) (s.c.) (n=5) and combined CsA and Zn30 (s.c.) treatment (n=8) respectively. (**a**) The weight course of CsA high treated animals and (**b**) CsA low treated animals is displayed. Measurements of (**c**) the total serum zinc amount and (**d**, **e**) the total zinc amount in (**d**) the autologous kidney and (**e**) the allogeneic transplant were done. Results are shown as mean values + SEM * indicates a significance of $p<0.05$ (student's t-test).

### 4.19.4 Oral Administration of Zinc is Effective for Treg Cell Induction in CsA Treatment

For a clinical more relevant setting, oral zinc application needs to be investigated since the majority of drugs are orally administered. Thus, the therapeutic potential of zinc can be better determined. Therefore, the animals were daily supplemented with Zn30 (oral) and CsA low (s.c.). The percentage and relative amount of Treg cells in whole blood

(figure 27a, b) and in the spleen (figure 27e) as well as the percentage of Th17 cells in whole blood (figure 27c) and in the spleen (Figure27f) was investigated.

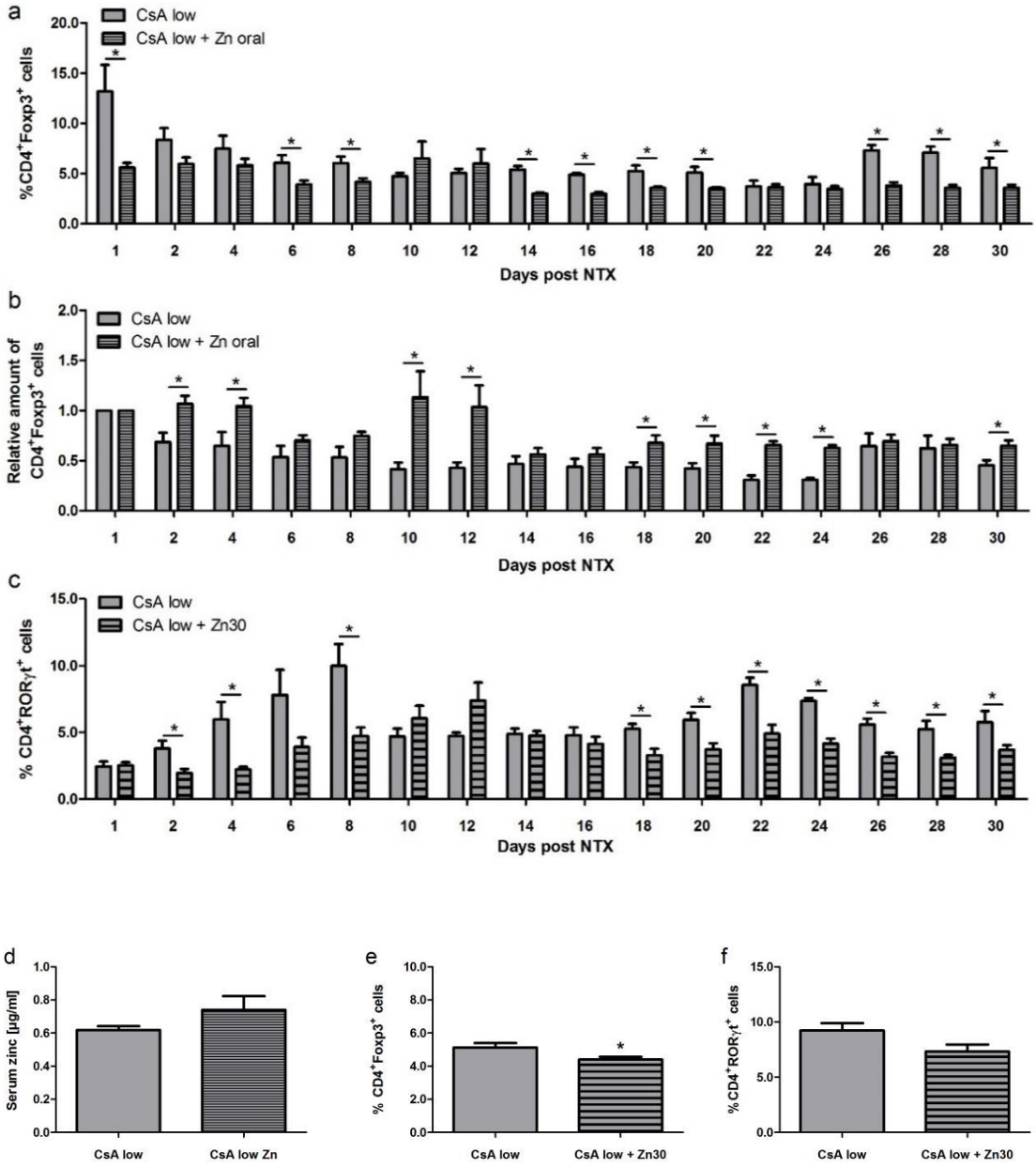


**Fig. 27. Oral zinc administration beneficially influences immunological tolerance *in vivo*.** Rats were treated daily with CsA low (1.5 mg/kg) (s.c.) (n=5) and combined CsA (s.c) and Zn30 (oral) (n=8) respectively. (**a, b**) Peripheral $CD4^+Foxp3^+$ cells and (**c**) $CD4^+ROR\gamma t^+$ cells as well as (**e**) $CD4^+Foxp3^+$ cells in the spleen and (**f**) $CD4^+ROR\gamma t^+$ cells in the spleen were measured by FACS analysis. (**d**) The total zinc amount in the serum was determined by AAS. Results are shown as mean values + SEM. Significantly different means do not share the same letters. * indicates a significance of $p<0.05$ (Student's t-test).

Combined CsA low and zinc treatment provoked a significantly diminished occurrence of $CD4^+Foxp3^+$ Treg cells in whole blood. This effect might be zinc independent since the percentage of Treg cells in the CsA low zinc group at day 1 was already significantly lower compared to the CsA low group. Thus, data were normalized to the control at day 1 (figure 27b). The relative expression of Treg cells in the blood of CsA low zinc treated animals is significantly elevated compared to CsA low single treatment. Consistent with that, the percentage of peripheral Th17 cells is significantly reduced due to zinc treatment. This effect was only found in peripheral blood Th17 cells and not in the spleen (figure 27f). Investigations of Th17 cells in the spleen showed a slight but not significant reduction by CsA low single treatment compared to the untreated control. Instead, a similar, but significant reduction of Treg cells in the spleen was uncovered (figure 27e). The measurement of the total zinc amount in the serum revealed only a slight but not significant increase due to zinc treatment compared to the control (figure 27d).
Thus, orally administered zinc seem to be not as effective as subcutaneously administered zinc, since the oral application did not raise the serum zinc level of the animals. Nevertheless, it also dampens the pro-inflammatory immunoreaction and favors tolerance induction as indicated by a decreased percentage of Th17 cells and simultaneously increased percentage of Treg cells in the blood.

The results of this thesis indicate that zinc administration is highly effective in tolerance induction *ex vivo* and *in vivo* as demonstrated in MLC experiments and kidney transplantations. On the molecular level, zinc administration has not only a beneficial impact on the mRNA expression and protein stabilization of the master transcription factor of Treg cells, Foxp3, but also on the expression of particular transcription factors like IRF-1 and KLF-10 directly affecting Foxp3 expression. Moreover, TGF-β signaling essential in Treg cell differentiation, is supported by zinc administration leading to higher Treg cell induction. Interestingly, TGF-β treatment induces a zinc signal, which indicates a positive feedback mechanism of TGF-β and zinc. Additionally, Treg cells can be classified by an elevated intracellular free zinc level that can not be found in activated T cells. Thus, zinc administration in physiological doses seem to be a promising approach to affect tolerance induction beneficially by altering cellular signaling and the zinc status of the cells itself. Hence, zinc administration should be considered in future treatment of adverse immune reactions in transplantation medicine to improve successful graft acceptance.

# V Discussion

The aim of this thesis was to investigate the influence of the pro-antioxidant zinc on adverse immune reactions. In this context, the capacity of zinc supplementation to modulate the allogeneic immune response in graft versus host disease (GVHD) *in vitro* and in kidney-transplantation *in vivo* was examined. To gain insight into underlying molecular mechanisms responsible for the zinc-related amelioration of those adverse immune reactions, cellular differentiation, transcription factor expression, and signaling pathways were explored.

This study is based on prior investigations showing an immunomodulating effect of zinc in the allogeneic mixed lymphocyte culture (MLC) and in experimental autoimmune encephalomyelitis (EAE), an *in vivo* mouse model for multiple sclerosis (MS) [82, 156]. In line with that, previous studies uncovered that zinc administration in pharmacological doses of 50 µM suppressed interferon (IFN)-γ production in MLC [152, 166].

The secreted level of the Th1-derived cytokine IFN-γ can be used to assess the severity of the alloreaction [151], since MLC and GVHD are mainly characterized by Th1 effector cell responses. In MLC, mainly T cells become activated due to the expression of divergent human leukocyte antigen (HLA) haplotypes of the two donors leading to lymphocyte proliferation, formation of T cell blasts, and production of pro-inflammatory cytokines. Hence, a zinc-mediated reduction of the IFN-γ secretion in MLC reflects diminished T cell responses and the amelioration of the allogeneic immune response.

This study verifies former findings by showing a significant induction of Treg cells in MLC supplemented with zinc compared to untreated controls (figure 1) leading to an amelioration of the alloreaction. Previous studies indicated a zinc-mediated amelioration already after 3 days of MLC incubation by displaying a reduced IFN-γ secretion [167]. This effect was most prominent at day 8, displaying significantly elevated Treg cell induction for the first time. In this study, day 5 was chosen for experimental setups, since a significant upregulation of Treg cells was observed already for the first time and stayed stable until day 8. Additionally, IFN-γ secretion was significantly reduced in zinc-supplemented samples pointing to a dampened immunoreaction due to zinc supply. Besides Treg cells, the differentiation and function of effector T cell populations as Th1, Th2, and Th17 are well known to be altered in immunoreactions as

well and are often associated with allergies, autoimmune diseases, and transplant rejection [8, 168, 169]. Hence, these subpopulations have to be considered to be regulated by zinc supplementation in adverse immunoreactions as well. Indeed, zinc supplementation is known to influence the function and differentiation of T cell populations, as for instance, a shift of Th1 cells towards the Th2 population [127, 170] but interestingly zinc supplementation in MLC specifically affected Foxp3 expression and thus Treg cells (figure 4). The expression of the lineage specific transcription factors T-bet (for Th1 cells), GATA-3 (for Th2 cells), and RORC2 (for Th17 cells) remained unaffected and were comparable to control samples.

Interestingly, former investigations uncovered a moderate increase in the IL-10 secretion, pointing to a stronger Th2 response, while the IFN-γ secretion and therefore the Th1 response was decreased simultaneously. In accordance, the IFN-γ/IL-10 ratio showed a significant shift of IFN-γ (Th1) towards IL-10 (Th2) cytokines [82], which is reported to be an important parameter in transplantation to prognosticate acceptance or rejection and is thus of high clinical relevance [171]. Hence, those results might suggest a zinc-mediated manipulation of the Th2 response. However, no assessment regarding cellular differentiation can be drawn simply by measuring of cytokine secretion. In fact, the reactivity of Th1 and Th2 cells can be altered whereas the differentiation of those cell populations persists unaffected. Moreover, the anti-inflammatory Th2-related cytokine IL-10 is additionally secreted by Treg cells themselves to dampen immunological reactions [172]. Therefore, the altered IL-10 production during zinc supplementation observed in previous studies is most likely due to increased Treg cell differentiation resulting in amelioration of the adverse reaction. This presumption is underlined by elevated expression of Treg cell related surface and intracellular marker (figure 3, 6), as well as by a reduced IFN-γ secretion and reduced cell proliferation (figure 8).

Treg cell differentiation and function is highly dependent on transcription factor activation or repression. Various signaling pathways are involved in those processes and a multitude of signaling molecules interact with each other leading to an altered signal transduction, triggering a particular immune response. The transcription factor Krüppel-like factor (KLF)-10 is well known to be essential in Treg cell function, whereas the transcription factor interferon regulatory factor (IRF)-1 is described to have a negative impact on Treg cell differentiation. Both transcription factors directly regulate

Foxp3 expression and thus influence Treg cell development [48, 49]. IRF-1 is ubiquitously expressed at low basal levels in most types of resting cells including T cells [173]. In the case of stimulation by cytokines like IFN-γ, IRF-1 expression is upregulated. In this study, the analysis of IRF-1 expression in non-activated resting T cells (PBMC) revealed a significant increase upon zinc supplementation compared to untreated controls. This is in line with previous investigations showing a significant increase of IFN-γ due to zinc supplementation *in vitro* [174] and *in vivo* [154]. Interestingly, in activated T cells (MLC), the IRF-1 mRNA and protein expression was significantly lowered due to zinc supplementation. Hence, the IRF-1 expression is oppositionally regulated in resting and activated T cells. In line with that, Foxp3 expression was significantly increased in zinc-supplemented activated T cells in MLC whereas the IFN-γ secretion was dampened significantly pointing to an amelioration of the alloreaction in MLC.

In compliance, another study elicited that IRF-1 deficiency results in a selective and marked increase in highly differentiated and activated Foxp3 expressing Treg cells *in vivo* [48]. Since IRF-1 plays a direct role in the generation and expansion of Treg cells by specifically repressing Foxp3 activity, we showed for the first time that IRF-1 mRNA and protein expression is dampened by zinc supplementation in MLC.

On the other hand, the KLF-10 mRNA expression remained unaffected in resting T cells in PBMC compared to controls, whereas it was significantly upregulated in zinc-treated MLC compared to untreated controls. Consistently, a correlation of KLF-10 expression and IFN-γ secretion reveal elevated KLF-10 expression by simultaneously reduced IFN-γ secretion. This is in line with another study, mentioning KLF-10 to be indispensable for appropriate Treg cell function, because animals carrying a disruption in KLF-10 no longer show Foxp3 activation [49]. In accordance, KLF-10-deficient Treg cells display impaired cell differentiation, altered cytokine profiles with enhanced Th1, Th2, and Th17 cytokine expression. Furthermore, a reduced capacity for suppression by wild-type co-cultured T effector cells, as well as accelerated atherosclerosis in immunodeficient atherosclerotic mice was exhibited [49].

Thus, our study uncovered two novel molecular targets, IRF-1 and KLF-10, modulated by zinc administration in activated T cells in MLC resulting in diminished IFN-γ cytokine production and consequently in the amelioration of the Th1-driven alloreaction by Treg cell induction (figure 28). Hence, zinc can be taken into account as a desirable additive to modulate Treg cell differentiation *in vitro*.

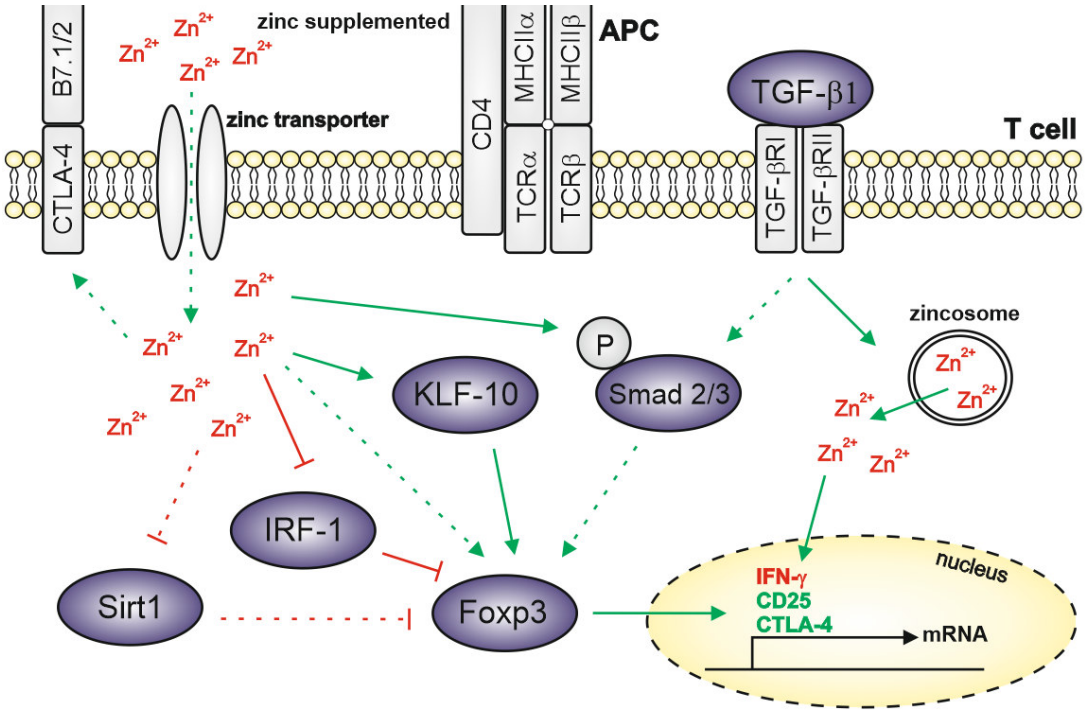


**Fig 28. Molecular targets to induce Treg cells.**
Treg cell development is critically dependent on the expression of various proteins interacting with Foxp3 and on the cytokine/trace element milieu. Treg cells can be induced (green arrows) by TGF-β1 signaling via Smad 2/3 as well as by zinc stimulation. Zinc induces KLF-10, Smad 2/3 phosphorylation and Foxp3 expression, but represses (red arrows) IRF-1 and Sirt1, leading to elevated expression of Foxp3. Foxp3 as well as zinc dampen the IFN-γ production but elevate the expression of CD25 and CTLA-4. Mechanisms responsible for Treg cell induction uncovered in this study are indicated by solid arrows, former findings are indicated by dashed arrows (APC, antigen presenting cell).

Nowadays, studies uncovered antigen-specific Treg cells to be much more potent suppressors of the development of autoimmune diseases than unrestricted Treg cells [35, 36]. Thus, clinical approaches try to generate antigen-specific Treg cells for suppression and prevention of organ-specific and systemic autoimmune disease [35, 37, 175]. However, the use of methods for the isolation of good quality Treg cells adequate for cellular therapy are still under investigation [176].

In our approach, we uncovered an induction of $CD4^+CD25^+CTLA\text{-}4^+Foxp3^+$ antigen-specific Treg cells due to zinc treatment. Zinc administration led to an ameliorated IFN-γ production and dampened proliferation compared to the untreated control. This was also observed after re-activation of the antigen-specific T cells for a second time with the same antigen used during priming. However, re-activation with a foreign antigen showed no difference between zinc supplemented and un-supplemented T cells. In agreement with that, former studies elicited a zinc-induced reduction of IFN-γ production in allogeneic immune reactions without affecting the cellular response to neo-

antigens as shown by tetanus toxoid-triggered immune responses [166]. Thus, the allogeneic immune response is ameliorated specific by zinc treatment, whereas the immune response to neo-antigens remains unaffected.

By analyzing the antigen-specific Treg cell induction, an upregulation of Foxp3 and KLF-10 in first (priming) MLC and second (re-stimulation) MLC was uncovered but not during the expansion phase. Indeed, Foxp3 is well established to delineate Treg cells from other T cell subsets. However, Treg cell-specific markers can also be unspecifically down- or upregulated temporarily under certain conditions. Downregulation was found when Treg cells were transferred to a lymphopenic environment [177], whereas multiple studies mentioned Foxp3 upregulation during cell activation and proliferation in mice and humans [178, 179]. Hence, a strong cell activation during expansion by IL-2 and phytohaemagglutinin (PHA) might contribute to an intermediate maturation stage in which newly generated Treg cells present a versatile behavior before establishing the specified Treg cell character [180]. Most studies agree that under normal conditions Treg cells are very stable and long-lived [181]. Nevertheless, especially induced Treg cells become unstable and loose Foxp3 expression in case of inflammatory or pathogenic conditions [180]. In this study, no stable upregulation of Treg cell-specific markers during the expansion of antigen-specific T cells was measured, confirming the presumption of the aforementioned investigations.

In clinical therapy, the success of a therapy critically depends on the time point, when a therapy is started. Often, it is of main interest whether an already triggered adverse immune reaction can still be modulated by additives and medication, since most adverse immunoreactions are already triggered before diagnosis and therapy. In this regard, the influence of zinc supplementation on antigen-specific T cells that have already been primed by a foreign antigen was explored. Zinc supplementation occurring just before re-activation of expanded T cells still diminished cell proliferation and pro-inflammatory cytokine production compared to untreated controls. Nevertheless, the reaction to a distinct antigen remains unchanged upon zinc supplementation (figure 10). Thus, the antigenic potency of the host remains maintained. Hence, a T cell-mediated alloreaction can probably still be dampened at a late stage after the trigger event occurred. These findings are highly important, since most studies merely uncover beneficial effects of zinc supplementation if administered previously or simultaneously with disease-induction in EAE in mice [156, 182, 183] or allogeneic cardiac transplantation or intraportal islet transplantation in rats [164, 165, 184].

Since Treg cells gain more and more importance in combating diseases in clinical approaches, a multitude of studies investigated possible methods to induce and expand Treg cells *in vitro*. Most of them elicited TGF-β1 to be essential in conversion of Th cells into Treg cells [16, 37, 53]. TGF-β1 itself is known to trigger both pro- and anti-inflammatory immune responses, and, depending on the present cytokine milieu, Treg cells or Th17 cells can be induced [32, 153]. Thus, a strictly regulated cytokine milieu is highly important in TGF-β1-mediated T cell differentiation. Studies indicate that TGF-β1 is crucial in establishing immunological tolerance by inhibiting T cell proliferation as well as cytokine production by Foxp3-dependent and independent mechanisms. Regarding this, a T cell receptor (TCR)/TGF-β1-related induction of Treg cells in mice [28] and man [19] has been shown. TGF-β1 activates the Smad-signaling pathway and Smad-binding elements were found in the conserved non-coding DNA sequence (CNS) 1 region of the Foxp3 promotor [55, 56]. Therefore, an essential role of the Smad signaling pathway in Foxp3 induction and pro-inflammatory cytokine suppression can be assumed. In accordance to that, in this study a TGF-β1-mediated elevation of the Foxp3 expression in MLC experiments was uncovered (figure 14). Additionally, a significant upregulation of Foxp3 due to zinc pre-treatment was displayed. This is in line with our results (figure 3), and with former investigations showing Treg cell induction in MLC [82]. Furthermore, combined zinc pre-treatment and TGF-β1 stimulation led to a significant synergistic elevation of Foxp3 expression (figure 14). In agreement, Smad 2/3 phosphorylation was elevated in MLC and PBMC (figure 13, 14).

Because Smad-binding elements were found in the CNS 1 region of the Foxp3 promoter [55], and zinc promotes Foxp3 stability by preventing proteasomal degradation caused by the histone deacetylase Sirt1 [82], the synergistic effect of the combined treatment could result from both of the triggered mechanism.

This study uncovered for the first time a beneficial effect of a combined zinc pre-treatment and TGF-β1 stimulation on Treg cell induction in MLC and PBMC, due to increased Smad 2/3 phosphorylation as well as Foxp3 expression.

Besides the canonical TGF-β1-triggered Smad signaling pathway, TGF-β1 moreover activates other signaling pathways and molecules including the extracellular regulated kinase (ERK) [185, 186], c-Jun N-terminal kinase (JNK) [187], phosphatidylinositol-3-kinase (PI3K)/Akt [188], and p38 MAP kinase [189, 190]. Those may cooperate with the Smad pathway to regulate cellular function. The p38 MAP kinase was recently

shown, to play an important role in Treg cell differentiation and thus tolerance mediation [57]. Consistent with that, zinc supplementation was shown to induce p38 phosphorylation [136, 191]. Hence, Treg cell induction may be a result of various molecular mechanisms interacting with each other.

In this approach, cell type specific effects of zinc supplementation on Smad signaling activation were revealed (figure 18). Combined zinc and TGF-β1 treatment induced Smad 2/3 phosphorylation in all experimental setups, whereas Smad 1/5/8 phosphorylation was seen only in hepatocytes, monocytes, and PBMC. In T cells, B cells, and MLC no effect was detectable. This is in line with other findings, showing that cellular TGF-β responses are highly cell type-specific, and that the DNA-binding affinities of Smad molecules are relatively weak. Hence, the final transcriptional outcome depends on interactions between a multitude of transcription factors, co-activators, and co-repressors [192]. Furthermore, the ratio of the expressed TGF-β receptor type II (TGF-βRII) and TGF-β receptor type I (TGF-βRI) as hetero-oligomers is essential. Thus, the downstream specificity and signaling of the activated TGF-βRII/TGF-βRI complex is different [193, 194]. High TGF-βRII expression levels in dermal cells are related to a selective activation of Erk 1/2. In contrast, high levels of TGF-βRI in epidermal cells favor the activation of the Smad signaling pathway and Erk 1/2 is not activated [195]. Other studies uncovered a direct link between TGF-β1-mediated Erk 1/2 activation and the stability of Smad 2/3 phosphorylation, resulting in an elevated duration of Smad target gene transcription [186]. Similar feedback mechanism could be conserved in T cells, but other signaling pathways e.g. via other mitogen-activated protein kinases (MAPK) could also be involved. Besides TGF-β1, also zinc is known to differently regulate target genes depending on cell species and experimental setup. For instance, we and others observed a zinc-dependent p38 activation in mice and man [136, 191], whereas Song et al. reported a p38 downregulation but Erk upregulation in pigs [196]. These species-specific observations could also explain the different results obtained in our study in T cell and hepatocyte experiments.

Next to TGF-β1, the anti-inflammatory cytokine IL-10 is also necessary for Treg cell mediated immune suppression [158]. In MLC experiments, a significantly dampened IFN-γ secretion due to IL-10 stimulation was detected (figure 15) pointing to the importance of IL-10 for Treg cell mediated immune suppression. In contrast to former studies, we discovered that IL-10 is not required for Foxp3 maintenance. This might

be due to different experimental setups and different species used for the investigations, since Murai et al. analyzed the IL-10 impact on Foxp3 expression in mice [159]. Thus, IL-10 in fact is an important factor for Treg cell mediated immune suppression in man but is not required for a stable Foxp3 expression as it was shown in rodents. In addition to that, a specific differentiation towards the Treg cell subtype Tr1 could be considered, since the zinc-mediated Foxp3 induction is abolished due to IL-10 stimulation. IL-10 treatment is known to trigger Tr1 cell induction, which in response produce high amounts of IL-10 and TGF-β1. However, in contrast to conventional Treg cells, they do not constitutively express Foxp3 [197]. Hence, Foxp3 expression and IL-10 cytokine production seem to be independently regulated in Treg cells in MLC.
Nevertheless, IL-10, TGF-β1 as well as zinc supplementation led to an amelioration of IFN-γ secretion in MLC (figure 13-15). This is in line with other studies, showing a blunted Th1 cytokine production due to anti-CD3/TGF-β stimulation, and an unresponsiveness to TCR re-stimulation [20]. Additionally, former findings reveal a suppressed IFN-γ production in MLC due to zinc supplementation [82, 152]. Especially pro-inflammatory cytokines, as IFN-γ, are sensitive parameters for possible graft rejection in GVHD and their levels in serum correlate with disease severity. In this regard, zinc treatment as well as TGF-β1 and IL-10 stimulation ameliorated this allogeneic reaction in this study. In accordance to that, former investigations showed a beneficial effect of zinc supplementation in autoimmune diseases like allergy, multiple sclerosis, and transplantation [164, 182, 183]. Hence, allogeneic immune reactions can be modulated by zinc supplementation showing a capacity to downregulate adverse immune reactions due to stabilization of induced Treg cells.
Besides altering transcription factor expression, zinc signals are known to influence cellular function in a direct manner. In particular, T cell function, maturation, and differentiation are strongly zinc dependent processes [136, 144, 145, 198]. A shift of the intracellular zinc concentrations is known to affect signal transduction, e.g. MAPK, Toll-like receptor, or TCR signaling pathways and thus cellular responses can be altered [101, 191]. Therefore, the intracellular free zinc level in $CD4^+$ T cells was investigated after TGF-β1 and IL-10 stimulation (figure 16).
A significant increase in free intracellular and lysosomal zinc in $CD4^+$ T cells upon TGF-β1 stimulation was detected. In contrast to that, no effect upon IL-10 stimulation was seen. Hence, we could show for the first time that TGF-β1 stimulation triggers an intracellular zinc signal, which might be responsible for the altered signaling pathway

activity and protein expression. Thus, an additional possible reason why selectively TGF-β1 but not IL-10 stimulation affects Foxp3 expression in PBMC and MLC beneficially has been revealed.

By further investigations of the intracellular free zinc level in Treg cells, this study demonstrates for the first time that $CD4^+CD25^{hi}$ expressing Treg cells can be distinguished from $CD4^+CD25^{low}$ expressing activated T cells by a significantly elevated intracellular free zinc content (figure 11). Thus, Treg cell development seems to directly depend on the intracellular free zinc level. Additionally, the intracellular free zinc level during T cell priming is highly decisive for T cell differentiation since zinc supplementation favors Treg cell differentiation whereas zinc deficiency favors Th17 cell differentiation (figure 12). This is in line with studies showing an altered T cell differentiation during zinc deficiency, which is often observed in the elderly population or vegetarians [110, 111]. Here, zinc deficiency leads to a shift in T cell subpopulations favoring $CD8^+$ memory T cells as well as to a reduction of the overall T helper cell activity [115]. The Th1/Th2 polarization is altered resulting in a favored Th2 differentiation [170, 199]. Moreover, a non-specific pre-activation, indicated by an increased percentage of $CD25^+$ T cells was shown to lead to an impaired immune function and response to vaccination [200]. Thus, zinc homeostasis is highly essential to guarantee proper T cell differentiation to avoid autoimmune diseases.

The understanding of adverse immune reactions is gaining more and more importance especially regarding transplantation medicine. This is on the one hand due to the lack of suitable amounts of organ grafts and on the other hand due to harmful side effects of the administered immunosuppressive agents provoking early graft failure [63]. Thus, the understanding of tolerance mechanisms in solid organ transplantation is essential to enable long-term graft survival and acceptance. So far, long-term survival of transplanted grafts depends on continuous exposure to combinations of immunosuppressive drugs to prevent a harmful immunoreaction against the graft. Simultaneously, those drugs prevent tolerance induction and subsequently create a dependence on continuous immunosuppression. However, this therapy is necessary but also associated with off-target effects and an increased risk for infections and cancer [63, 201]. In comparison to standard immunosuppressive drugs, Treg cells are considered as smart therapeutic agent due to their antigen-specificity and their capacity to induce other T cells to acquire regulatory functions *in vitro*. Hence, tolerance induction instead of chronic immunosuppression might be enabled by using Treg cells in therapy.

Since zinc is known to induce tolerance by enhancing Treg cell numbers and dampening Th17 cell numbers in EAE and renal, cardiac, and intraportal islet transplantation [156, 164, 165, 202], zinc supplementation was applied in kidney transplantation in rodents to investigate tolerance induction *in vivo* (figure 22). Simple subcutaneous (s.c.) zinc application (NaCl dissolved) did neither induce Treg cells nor dampen Th17 cells. Interestingly Th17 cells were induced by simple s.c. application of 0.9% physiological NaCl solution. Similar results were observed in mice and humans in *in vivo* and *in vitro* studies by administration of high salt diets and high NaCl concentrations (40 mM) respectively [203, 204]. In line with that, an increase of the intracellular free zinc content was only detected in lymphocytes when zinc dissolved in glucose was administered but not when zinc was dissolved in NaCl (figure 24). Thus, a direct negative interaction of zinc and NaCl can be assumed leading to a preference of the pro-inflammatory immune response due to NaCl administration.

In transplantation medicine, pro-inflammatory graft-related immune reactions are tried to impeded. This is achieved by administration of immunosuppressive drugs, like Cyclosporine A (CsA), which are selected based on their ability to prevent immune activation in general. Since T cells are the central players in graft rejection, the most common immunosuppressive drugs target T cell activation and clonal expansion [71, 201]. Due to the well-known side effects of immunosuppressive drugs, it is beneficial to lower the total intake to a minimum while simultaneously ensuring suppression of the anti-graft immunoreaction. Moreover, a lower intake of immunosuppressive drugs is promising likely to establish an immunological tolerance reaction, since the immune reaction is not abolished completely [205]. Furthermore, zinc supplementation might positively support the tolerance immune reaction by inducing Treg cells. Investigations reveal only a sporadic zinc impact on Treg cell induction in 15 mg/kg CsA (CsA high) treatment (figure 25). This indicates that the CsA high dose treatment dampens the whole immunoreaction efficiently and even additional zinc treatment cannot induce a pro-tolerogenic effect. In contrast to that, a stable Treg cell induction and Th17 cell diminution in the 1.5 mg/kg CsA (CsA low) zinc treated group compared to the CsA low-only group was uncovered (figure 25), verifying the former hypothesis. Additionally, the ratio of Treg/Th17 cells was significantly elevated in the spleen (figure 25). In accordance with that, studies already noted a dose-dependent CsA-impact on the differentiation of Treg cells. Herein, an adverse effect of high dose CsA is mentioned regarding frequency and function of Treg cells, but a pro-tolerogenic effect

leading to graft tolerance by applying lower doses was described [73]. Furthermore, other studies uncovered beneficial zinc effects in allogeneic transplantation models. Here, a cardiac transplantation model was used, showing a reduced allograft rejection upon zinc supplementation [164, 184]. Additionally, the function of intraportal islet grafts were maintained by zinc supplementation [165]. Here, possible reasons discussed to be responsible were zinc-mediated inhibition of apoptosis by influencing caspase-3 activity, or scavenging of free radicals. Reduced apoptosis of graft-tissue cells would thus contribute to allograft survival. However, this might be a secondary effect related to the attenuating impact of zinc on the immune reaction. In those studies, a direct effect of zinc supplementation on Treg cells, or a direct effect of caspase-3 inhibition on the Treg cell population was not investigated. In contrast to that, we uncovered a direct effect of zinc supplementation on Treg cell induction, measured by reduced IFN-γ production *in vitro* and elevated Treg cell numbers *in vitro* and *in vivo*. Nevertheless, zinc beneficially affects the former transplantation models, supporting our results of diminished IFN-γ production and elevated Treg cell numbers due to zinc supplementation in antigen-specific T cells *in vitro* and in kidney transplantation *in vivo*.

Moreover, zinc administration in CsA high and CsA low treated groups beneficially influences the animals' welfare as indicated by elevated body weight (figure 26). This is in accordance to our observed results in zinc-only treatment (figure 23) and to former studies showing a similar zinc-related effect on the animals' body weight [156].

For a clinically more relevant setting, oral zinc application was investigated since the majority of drugs are orally administered. Thus, the therapeutic potential of zinc can be determined more precisely. Compared to s.c. administered zinc, oral administration was also sufficient to enhance Treg cell numbers and dampen Th17 cell numbers in peripheral blood in rats (figure 27). This is consistent with preliminary studies, demonstrating a similar zinc-related effect in EAE in mice [206]. However, the intracellular free zinc and total serum zinc amount remained unchanged compared to the vehicle treated group. This might be due to the composition of the diet, because phytates and lignins in the chow are well-known zinc-chelating phosphates leading to a lower zinc absorption [109]. Hence, oral administered zinc in concentrations of 30 µg/kg seem to be not as effective as s.c. administered zinc in modulating the intracellular free zinc and total serum zinc amounts in the allogeneic kidney-transplantation model (figure 27). However, the immune response was still beneficially manipulated to favor

tolerance induction by elevating Treg cell numbers and dampening Th17 cell numbers in peripheral blood in zinc supplemented rats (figure 27).

In summary, zinc treatment in physiological doses favors the Treg cell induction *in vitro* and *in vivo*, which leads to a dampened adverse immune reaction and provokes tolerance. Therefore, zinc could be considered as a useful additive to beneficially influence the immune reaction during transplantation *in vivo*. This is of great importance, since the discovery of Treg cells offers a new paradigm for transplantation research because intra-graft Treg cell frequency seems to correlate with clinical graft acceptance, survival, and function [69, 70]. In a mouse model of transplantation, Treg cell therapy not only prevents rejection, but also allows the induction of new Treg cells with broader specificities [64]. Therefore, Treg cell therapy can potentially turn the graft tissue from a target of immune attack into a tolerogenic organ, which enables its long-term survival.

Collectively, this thesis shows that zinc administration in physiological doses induces tolerance in the allogeneic Th1-driven transplant model in GVHD *in vitro* and in kidney transplantations *in vivo*. On the molecular level, zinc modulates the expression of the transcription factors Foxp3, IRF-1, and KLF-10 as well as the activity of the Smad 2/3 signaling molecule of the TGF-β1-triggered Smad signaling pathway.

However, the overall mechanisms involved in zinc function seem to be more complex. For instance, also Smad-independent pathways, like MAPK signaling are involved in Treg cell differentiation. Moreover, alterations of transcription factor stability by acetylation or ubiquitination, as well as epigenetic modifications, like histone acetylation and methylation or DNA methylation, are involved in Treg cell induction and stabilization, which requires further investigations [57, 106, 207, 208].

Nevertheless, zinc represents a low cost additive for Treg cell upregulation to induce immunological tolerance in adverse immune reactions and should therefore be considered as therapeutic agent for T cell-driven immunological disorders. Hence, a modulation of the T cell response is an attractive approach for the induction and maintenance of allograft-specific tolerance, making long-term immunosuppression unnecessary.

# VI Summary

Proper zinc homeostasis is well known to be essential for adequate immune function as displayed in immune disorders seen upon zinc deficiency. Former studies already postulated beneficial effects of zinc administration in physiological doses concerning autoimmune disease like multiple sclerosis, allergies or graft versus host disease. Thus, an immunomodulatory effect of zinc was anticipated in this study, offering the opportunity to elaborate zinc application as a useful tool in transplantation medicine. The results support earlier postulates of a beneficial effect of zinc supplementation in adverse immune reactions. In addition, here it was shown for the first time that on the molecular level the inhibition of the transcription factor IRF-1 and the induction of KLF-10 is directly involved in zinc-mediated Foxp3 stability. Moreover, the TGF-β1-mediated Foxp3 induction is intensified by zinc administration. An increased stability of Foxp3 subsequently results in enhanced Treg cell levels, which are capable of inducing tolerance in adverse immune reactions and consequential diseases. Moreover, antigen-specific Treg cells can be induced by zinc supplementation, still showing immunomodulatory capacity when induced in late disease progression. This was revealed by dampened pro-inflammatory cytokine secretion and proliferation in MLC *in vitro*. Comparable results were also uncovered in an *in vivo* kidney transplantation model. Cyclosporine A-mediated immunosuppression was beneficially influenced by zinc administration in physiological doses. Zinc administration inducing Treg cells and dampened Th17 cells resulting in an ameliorated adverse immune reaction. In line with that, the animals' welfare was positively influenced represented by elevated body weight.

Collectively, this study illustrates that zinc modulates the adverse immune responses in transplantation. This is due to upregulation of Treg cells and dampening of the pro-inflammatory immune reaction. Understanding the complex immune modulation by zinc may establish zinc therapy as a new therapeutic tool in transplantation medicine. This is of high relevance, since zinc therapy shows no toxic side effects or off target effects compared to immunosuppressive strategies. Moreover, the combined zinc and immunosuppressive therapy seem to be greatly beneficial, since its ability to induce immune tolerance without completely abrogating antigenic immune defense. This might guarantee exceeded long-term graft survival and hence is a topic of broad interest, which warrants further investigation.

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## VIII List of Figures

# IX List of Abbreviations

| | |
|---|---|
| AAS | atomic absorption spectrophotometry |
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| APC | antigen presenting cell |
| APS | ammonium persulfate |
| BSA | bovine serum albumin |
| CD | cluster of differentiation |
| CPM | counts per minute |
| CsA | cyclosporine A |
| CTL | cytotoxic T cell |
| CTLA-4 | cytotoxic T lymphocyte antigen 4 |
| CNS | central nervous system |
| DAMP | and damage-associated molecular patterns |
| DNA | deoxyribonucleic acid |
| EAE | experimental autoimmune encephalomyelitis |
| ELISA | enzyme-linked immunosorbent assay |
| ERK | extracellular signal-related kinase |
| FACS | fluorescence activated cell sorting |
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |
| FSC | forward side scatter |
| Foxp3 | forkhead box P3 |
| GITR | glucocorticoid-induced tumor necrosis factor receptor family related gene |
| GVHD | graft-versus-host disease |
| HSCT | hematopoietic stem cell transplantation |
| HLA | human leukocyte antigen |
| HRP | horseradish peroxidase |
| IFN | Interferon |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IRF | interferon regulating factor |
| KLF | krüppel-like factor |
| MAP | mitogen-activated protein |
| MHC | major histocompatibility complex |
| MLC | mixed lymphocyte culture |
| MS | multiple sclerosis |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NET | neutrophil extracellular traps |
| PAGE | polyacrylamide gel electrophoresis |
| PAMPs | pathogen-associated molecular patterns |
| PBGD | porphobilinogen deaminase |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PE | Phycoerythrin |
| PI | propidium iodide |
| PI3K | phosphatidylinositol-4,5-bisphosphate 3-kinase |

| | |
|---|---|
| PMN | polymorphonuclear cells |
| RmT | room temperature |
| RORC2 | retinoic acid receptor-related orphan receptor C2 |
| RORγt | retinoic acid receptor-related-related orphan receptor gamma |
| ROS | reactive oxygen species |
| SDS | sodium dodecyl sulfate |
| SEM | standard error of mean |
| SSC | side scatter |
| STAT6 | signal transducer and activator of transcription |
| TCR | T cell receptor |
| Teff | effector T cells |
| TEMED | N,N,N’,N’-tetramethylethylenediamine |
| TGF | transforming growth factor |
| Th | T helper cell |
| TPEN | N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine |
| Treg | regulatory T cells |
| Tris | tis-(hydroxymethyl)-aminomethane |
| Zip | Zrt- and Irt-like proteins |
| ZnT | zinc transporter |

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### Publications of this thesis

**Maywald M.**, Meurer S., Weiskirchen R., Rink L. Zinc supplementation augments TGF-β1 dependent regulatory T cell induction. Mol Nutr Food Res. 2016 Oct 29., DOI:10.1002/mnfr. 201600493.

**Maywald M.**, Rink L. Zinc supplementation induces $CD4^+CD25^+Foxp3^+$ antigen-specific regulatory T cells and suppresses IFN-γ production by upregulation of Foxp3 and KLF-10 and downregulation of IRF-1; Eur J Nutr., DOI: 10.1007/s00394-016-1228-7

**Maywald M.**, Kunter, U., Ostendorf. T., Pluemaekers, B., Rink L. Zinc administration beneficially affects kidney transplantation in rats by induction of regulatory T cells and diminishing of pro-inflammatory Th 17 cells; in progress

**Publications related to this thesis**

Rosenkranz E., **Maywald M**., Hilgers R.-D., Brieger A., Clarner T., Kipp M., Pluemaekers B., Meyer S., Schwerdtle T. and Rink L. Induction of regulatory T cells in Th1-/Th17-driven experimental autoimmune encephalomyelitis by zinc administration; J. Nutr. Biochem., DOI 10.1016/j.jnutbio.2015.11.010

Rosenkranz E., Metz C.H.D., **Maywald M**., Hilgers R.-D., Wessels I., Senff T., Haase H., Jäger M., Ott M., Aspinall R., Plümäkers B., Rink L. Zinc supplementation induces regulatory T cells by inhibition of Sirt-1 deacetylase in mixed lymphocyte cultures; Mol. Nutr. Food Res., DOI 10.1002/mnfr.201500524

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Gruber K., **Maywald M**., Rosenkranz E., Haase H., Plumakers B., Rink L. Zinc deficiency adversely influences interleukin-4 and interleukin-6 signaling. J Biol Regul Homeost Agents. 2013 Jul-Sep;27(3):661-71.

## Zusammenfassung

Eine adäquate Zinkhomöostase ist für die Funktion des menschlichen Immunsystems unabdingbar. Unter Zinkmangel wird dieses besonders deutlich, da dieser häufig mit einer Fehlfunktion des Immunsystems assoziiert ist. Durch eine Zinksupplementation hingegen können sowohl zinkmangelbedingte Fehlfunktionen des Immunsystems aufgehoben werden, als auch Autoimmunerkrankungen wie Multiple Sklerose oder Allergien positiv beeinflusst werden. Deshalb wurde in dieser Studie ein immunmodulierender Effekt von Zink in allogenen Immunreaktionen, wie bei Transplantationen erwartet.

In dieser Arbeit konnte erstmalig gezeigt werden, dass eine physiologische Zinksupplementation in der gemischten Lymphozytenkultur *in vitro* auf molekularer Ebene die Expression des Transkriptionsfaktors IRF-1 hemmt bzw. von KLF-10 steigert. IRF-1 gilt als Negativregulator der regulatorischen T-Zellen (Treg), wohingegen KLF-10 eine wichtige Rolle in der Foxp3-vermittelten Treg Induktion spielt.

Des Weiteren konnte eine Steigerung der transformierenden Wachstumsfaktor-β-induzierten Foxp3 Expression festgestellt werden, die letztendlich einen Anstieg der Treg Population bedingt. Außerdem konnten durch eine Zinksupplementation Antigen-spezifische Treg induziert werden, sogar, wenn eine Zinksupplementation erst in einer späten Phase einer allogenen Immunreaktion vorgenommen wurde. Untermauert wurde dieses durch die verminderte Produktion des pro-inflammatorischen Zytokins Interferon-γ und einer verminderten Proliferation unter Zinksupplementation.

Vergleichbare Zinkeffekte wurden in einem Nierentransplantationsmodell in der Ratte *in vivo* beobachtet. Hier konnte unter Zinkgabe eine vorteilhafte Beeinflussung der Cyclosporin A-vermittelten Immunsuppression verzeichnet werden. Trotz der Immunsuppression konnten mittels Zink Treg induziert und pro-inflammatorische Th17 Zellen vermindert werden, was letztendlich zu einer Abschwächung der allogenen Immunreaktion führt. Im Einklang dazu war unter Zinkgabe ein gesteigertes Wohlergehen der Tiere festzustellen, welches durch ein erhöhtes Körpergewicht repräsentiert wurde.

Somit sollte die Zinksupplementation als nützliches Additiv in einer Standardimmunsuppression bei der Behandlung von Transplantationspatienten in Betracht gezogen werden, da hierbei keine toxischen Nebeneffekte bekannt sind und die pro-tolerogene Immunantwort unterstütz wird, sodass eine verlängerte Lebenszeit des Transplantats erwartet werden kann, ohne die protektive Immunreaktion gegen schädliche Pathogene zu beeinträchtigen.