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Immune suppression

Zinc in pharmacological doses suppresses allogeneic reaction without affecting the antigenic response

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Summary:

Zinc is an essential trace element for the immune system. Previously it was shown that zinc inhibits alloreactivity. In our present *in vitro* experiments, it is shown that zinc maintains the antigenic potency of the host while blocking the allogeneic response. These results were observed in experiments using tetanus toxoid as a well-established recall antigen and the mixed lymphocyte culture as an *in vitro* model for allogeneic reaction. To prove the *in vivo* relevance, an *ex vivo* experimental setup was established. This involved participants taking zinc orally for 1 week. Here it is shown that *in vivo* zinc application induced the same effect of blocking the mixed lymphocyte culture without influencing tetanus toxoid stimulation. So far, no clinical application studies have been performed, but the observed selective suppression of allogeneic reaction by zinc is the first step towards a new generation of immunosuppressants.

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Introduction

The mixed lymphocyte culture (MLC) is an established clinical method used prior to bone marrow transplantation.[1–3] It detects major histocompatibility class II (MHC-II) differences[4] and minor lymphocyte-stimulating (mls) antigens.[5] Normally, T-cell proliferation is measured in the MLC; however, it has now been shown that cytokines are more sensitive parameters for possible graft rejection.[1,6] Danzer *et al*[7] have shown that the T_H1-cytokine interferon (IFN)-γ plays a crucial role in the MLC as it serves as a sensitive parameter for direct T-cell activation.

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In transplantation medicine the most common way of immunomodulation is by immunosuppression with substances like FK 506 or cyclosporine A (CsA). This leads to immune deficiency in the patient.[8] Severe infections are feared complications and other negative side effects such as nephrotoxicity[8] harm the graft and indicate the limiting factors of current immunosuppressants. In conclusion, an optimal effect of immunomodulation is inhibition of allogeneic reactions and maintenance of the potency to react to antigens without toxic side effects. Currently, no medications are available to achieve this effect.

Zinc is a very important trace element for all organ systems, especially for the immune system.[9,10] The zinc deficient organism shows impaired functions in all kinds of immune cells.[11] *In vivo*, natural killer (NK) cell activity, phagocytosis by macrophages and neutrophils and the generation of the oxidative burst were impaired by decreased zinc levels.[12,13] The cell counts of B and T lymphocytes were reduced during zinc deficiency.[14,15] Antibody production by B cells and the killing activity of cytolytic T cells were also disrupted.[16,17] Zinc supplementation and optimal intake restored impaired immune functions and decreased the incidence of infections *in vivo*.[18] The actual recommended daily intake of zinc is 11 mg/day (Food and Nutrition Board, USA, 2001). The physiological plasma zinc concentration is about 12–16 μM.[11] Zinc in concentrations at seven to eight times the physiological zinc level inhibited IL-1 type I receptor-associated kinase (IRAK), resulting in suppression of interleukin-1 (IL-1)-mediated T-cell activation.[19] Based on these observations, further experiments were performed, and aCampo *et al*[20] found that zinc in concentrations at three to four times the physiological level did not decrease T-cell proliferation *in vitro* nor showed immunosuppressive effects *in vivo*, but suppressed allogeneic reactions in the mixed lymphocyte culture.[20]

In the following study, the question is investigated whether zinc would be able to selectively suppress the immune system in order to find a new way of treating patients in transplantation medicine.

Materials and methods

Preparation of lymphocyte cultures

For the *in vitro* experiments, peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats of young

healthy blood donors. After density centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany), the cells at the interface were collected. These cells were washed twice with phosphate-buffered saline (DPBS; BioWhittaker, Verviers, Belgium) and resuspended in RPMI1640 medium (BioWhittaker) containing 10% heat-inactivated low-endotoxin fetal calf serum (PAA Laboratories, Linz, Austria), 1% L-glutamine (200 mM), 1% penicillin/streptomycin (10 000 U/ml/10 000 μg/ml) (all obtained from Biochrom) at a final concentration of 2×10^6 cells/ml.

For the *ex vivo* experiments, blood was obtained from the participants and cells were isolated as described above.

Zinc preparation

Zinc sulfate (Sigma, Deisenhofen, Germany) was dissolved in sterile water to achieve a zinc stock solution of 80 mM, which was then sterile filtered. A further dilution in unsupplemented serum-free medium (Ultradoma, BioWhittaker) achieved the final concentrations of 500 and 1000 μM. The PBMC were preincubated with these zinc solutions (both populations of the two-way MLC) at a volume of 10% of the final culture volume 15 min before the MLC were performed as described previously by aCampo *et al*.[20]

Culture conditions

For every MLC-experiment, a two-way MLC was used. PBMC from two different donors were mixed in equal amounts (0.5 ml of each) into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). Various combinations of 20 μg/ml tetanus-toxoid (a kind gift from Dr Halex, Behring-Werke, Marburg, Germany), 250 ng/ml toxic-shock-syndrome-toxin-1 (TSST) (Toxin Technology, Sarasota, FL, USA), 50 or 100 μM zinc or 20 μg/ml CsA (Alexis Corporation, Lausen, Switzerland) were added to the controls (one population of PBMC in volumes of 1 ml) and to the MLCs. The cultures were incubated with no change of medium for 5 days at 37°C in a 5% humidified CO_2 atmosphere. The culture supernatants were harvested and frozen at −20°C for storage and thawed only once for cytokine determination.

It is known that the PBMC of some donors show a considerable IFN-γ release after they are treated with zinc.[21] Furthermore, the PBMC of some donors do not show an IFN-γ release after they are treated with tetanus toxoid (TT). Inclusion criteria were created to guarantee identical experimental conditions for the MLC:

- The donors' blood must not react upon zinc supplementation, since possible increased IFN-γ levels in a MLC due to zinc addition do not allow us to discriminate the stimulus of IFN-γ release (zinc prestimulation or allogeneic reaction).
- The donors' blood had to react to TT, a common recall antigen, and TSST, a potent T-cell activator, so that nonresponders could be discriminated from inhibition.

Determination of cytokines

For the quantification of the cytokine release into the supernatant, an enzyme-linked immunosorbent assay (ELISA) technique was performed (Bender Med Systems, Vienna, Austria). IFN-γ was measured in picograms per milliliter at a wavelength of 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Zinc supplementation study

Six healthy male volunteers were recruited to take part in a small approved study to investigate the effects of zinc supplementation on the MLC. All participants gave written consent. Zn-aspartate (Unizink 50, Koehler Pharma, Alsbach, Germany) lozenges were administered, each of which contained 50 mg of zinc DL-aspartate (10 mg of pure zinc).

Over the period of 1 week, volunteers took one lozenge every 2 h during the daytime (80 mg a day). This dose is about seven to eight times the RDA, which is the same dosage used to treat the common cold.[22]

After 7 days, PBMC were isolated from the blood of the six volunteers and were used for preparing two-way MLC as described above. For this, PBMC of each participant were mixed with PBMC of the other participants in every possible combination, each combination representing one separate MLC, resulting in 15 MLC.

Zinc analysis by atomic absorption spectroscopy

Serum was diluted in a 1:10 ratio and the probes were measured by atomic absorption spectroscopy (AAS) (1100 B, Perkin-Elmer, Überling, Germany). The concentrations were given in mg zinc per liter (normal range: 0.69–1.49 mg/l).

Test of time-dependence

PBMC were isolated from blood given by four healthy donors. The PBMC were used to mix two-way MLC as described above. PBMC of each donor were mixed in every possible combination with PBMC of the other three donors. Thus, six MLC were obtained. After 10 days, the experiment was repeated in exactly the same way with the same donors.

Viability tests

Flow cytometry was used to investigate the toxicity of zinc and consequently the viability of the PBMC. Propidium iodide (PI) staining was performed by using a stock solution of 1 mg/ml (Sigma). Cells (1×10^6/ml) were incubated with 10 μl of PI stock solution for 20–30 min to allow intercalation of PI in double-stranded DNA. Finally, PI staining was measured at a wavelength of 620 nm in a flow cytometer (Beckman Coulter, Krefeld, Germany).

Statistical analysis

For the statistical analysis SPSS was used (SPSS GmbH Software, Munich, Germany). Significance was proven either by Student's *t*-test or by Wilcoxon signed-rank test.

Results

Antigen recognition is not influenced by zinc

Previously, aCampo *et al*[20] showed an influence of zinc on the MLC. In this experiment, the question was investigated whether zinc would also inhibit the response to antigenic stimulation by conventional antigens like TT.

After cell isolation, PBMC were incubated in various combinations with zinc in a concentration of 100 μM (zinc 100), TT and CsA (Figure 1). In order to guarantee equal conditions, the supernatants were harvested at day 5, which has been shown to be the maximum time point of IFN-γ release in the MLC.[23] Controls did not show an increased level of IFN-γ. PBMC incubated with 100 μM zinc present a slightly elevated IFN-γ level due to two outliers out of 14 volunteers, which were excluded from further experiments (see inclusion criteria). No donor had to be dismissed because of a missing response on TT. TT treated cells with or without zinc addition show a significantly higher release of IFN-γ. Zinc did not influence the reaction to TT, whereas CsA as an established immunosuppressant did inhibit the reaction to antigenic stimulation. This observation led us to the conclusion that antigen recognition is not influenced by zinc addition.

Alloreactivity in the MLC is inhibited by zinc

In order to investigate whether the proven reactions to antigens and the known inhibition of alloreactivity[20] could coexist in the same setting 12 donors who fulfilled the inclusion criteria were selected and MLC studies were performed. IFN-γ release after zinc addition in concentrations of 50 and 100 μM (MLC 50 and MLC 100) and after CsA (MLC CsA) treatment was significantly reduced as compared to the MLC without supplementation (Figure 2).

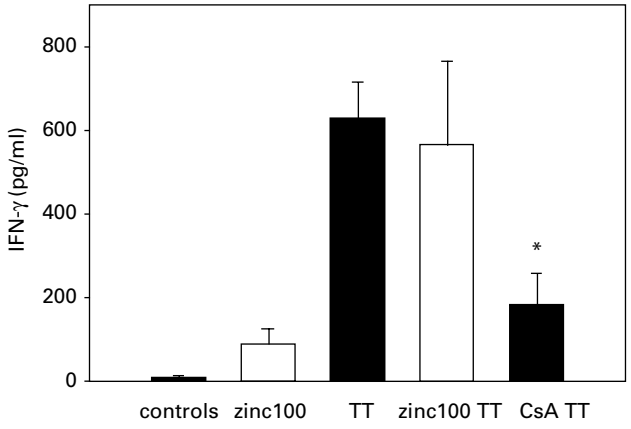

Figure 1 Zinc does not reduce IFN-γ release after antigenic stimulation *in vitro*. Influence of zinc on antigenic stimulation measured by IFN-γ release of PBMC of healthy blood donors. PBMC (controls) show no prestimulation and 100 μM zinc ions (zinc100) do not show stimulative effects except for two outliers. PBMC show a positive response on TT. Preincubation with zinc did not reduce the antigenic capacity of TT (zinc100 TT and TT). Preincubation with 20 μg/ml CsA reduced the IFN-γ release upon stimulation with TT (CsA TT). Mean values and s.e. of $n=14$. IFN-γ release of TT-treated PBMC is significantly decreased by CsA preincubation. $^*P<0.001$ (*t*-test).

Cell viability depends on added amount of zinc

In regard to these *in vitro* results, it is important to know whether the decreased IFN-γ levels after zinc addition were caused by toxic elimination of IFN-γ-producing cells. Flow cytometry over 5 days showed that only zinc addition in a concentration of 100 μM had a toxic effect on cells (Figure 3). However, these dead cells were not responsible for IFN-γ production because the IFN-γ release of PBMC was not influenced by 100 μM zinc during tetanus toxoid treatment (Figure 1), and the IFN-γ release of MLCs treated with 50 and 100 μM zinc are equivalent (Figure 2).

Oral zinc intake prevents allogeneic reaction

These *in vitro* results showed that zinc was able to discriminate between allogeneic and antigen-specific

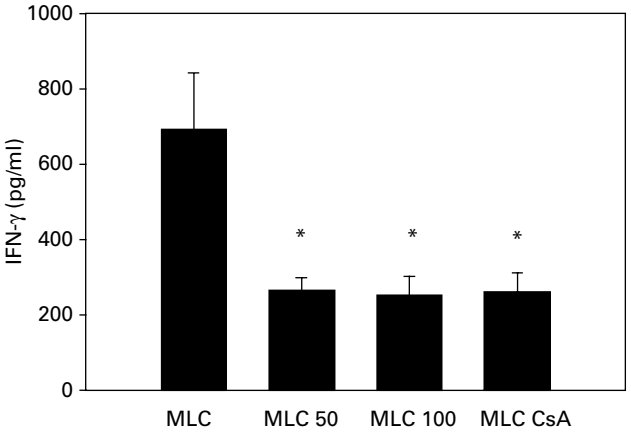

Figure 2 Zinc inhibits the MLC comparable to CsA. Preincubation of PBMC with 50 μM zinc (MLC 50) or 100 μM zinc (MLC 100) or 20 μg/ml CsA (MLC CsA) significantly reduced the IFN-γ release as compared with the untreated MLC (MLC). Mean values and s.e. of $n=16$. IFN-γ production in the MLC after supplementation is significantly decreased to appropriate control. $^*P<0.001$ (*t*-test).

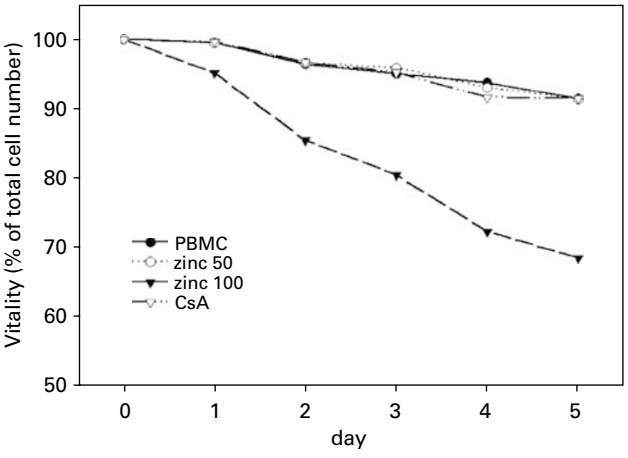

Figure 3 Zinc addition in a low concentration shows no toxicity *in vitro*. Direct toxic side effects of zinc on the viability of PBMC were tested by flow cytometry analysis. Unsupplemented PBMC (●), low zinc supplementation in a concentration of 50 μM (○) and 20 μg/ml CsA supplementation (▽) lead to a comparable cell loss. A high zinc supplementation in a concentration of 100 μM (▲) leads to an increased level of non-vital cells. Mean values and s.e. of $n=3$ (*t*-test).

responses. To come closer to the *in vivo* situation of allogeneic stimulation, an *ex vivo* setup was established to prove the selective capacity of immunomodulation by zinc. In order to transfer these *in vitro* results to an *ex vivo* setting, a group of six healthy male volunteers was recruited. They took zinc orally and the *in vitro* experiments were repeated. Before and after zinc intake, zinc serum levels of the participants were measured. 1 week of zinc intake led to significantly ($P<0.016$, *t*-test) increased serum levels, but they did not exceed the normal physiological values. Full blood profiles were not assessed since it is known that zinc supplementation has no effect on circulating levels of peripheral blood leucocytes and lymphocyte subsets in healthy donors.[24] These *ex vivo* experiments confirmed the *in vitro* results. IFN-γ release of PBMC was compared prior to and after zinc intake. Zinc supplementation had no effect on IFN-γ production by PBMC compared prior to zinc intake (Figure 4). Zinc intake caused no inhibiting effects on TT and TSST stimulation.

Referring to the inclusion criteria, none of the volunteers had to be excluded. Prior to zinc intake a MLC was performed in the same manner as described above for the *in vitro* MLC. The PBMC of each of the six participants was mixed with one another in every possible combination, resulting in 15 MLCs. After zinc intake, this experiment was repeated. In comparison to the MLC prior to zinc intake, decreased release of IFN$-\gamma$ after 1 week of zinc supplementation was observed (Figure 5). Zinc intake reduced the IFN-γ release in the MLC in every donor combination. An increased inhibition of the MLC was observed in cases where higher IFN-γ release occurred prior to zinc intake. The inhibition of the MLC could be interpreted as allogeneic differences[20] not being recognized by the immune cells.[7]

In order to test whether the reduced release of IFN-γ could be attributed to two different points in time when blood was given, the following experiment was set up. PBMC of four male donors were isolated and mixed in every combination, obtaining six MLCs. After 10 days, this experiment was repeated with the same donors. Different points in time were not responsible for the reduced IFN-γ release of the MLC since there is no significant difference comparing the same MLC combinations at different times (Figure 6). However, there is a great difference in the MLC if female donors are included (data not shown). Therefore, the experiments as well as the zinc supplementation were done with male donors only.

Discussion

After organ or bone marrow transplantation, the immune system must be suppressed to prevent the graft being rejected by the host or the host being attacked by the immune cells of the graft. These reactions are known as host-versus-graft disease (HvGD) or graft-versus-host disease (GvHD). To reduce these effects, bone marrow transplant patients must take immunosuppressant medications, for example, CsA, usually at high dosages for 6–9 months. Although organ transplanted patients have to take CsA at a lesser dosage, they have to take it for the rest of their lives. These medications are broadly applied and *in vitro* they show an inhibitory effect on T-cell proliferation in the MLC.[25]

Unfortunately, these medications have severe negative side effects such as superinfection with opportunistic germs. This occurs because these medications suppress both alloreactivity and the ability of the immune system to fight against antigens.[26] None of the common immunosuppressant medications maintain the important capability of immune cells to react to antigen. Over the last few decades,

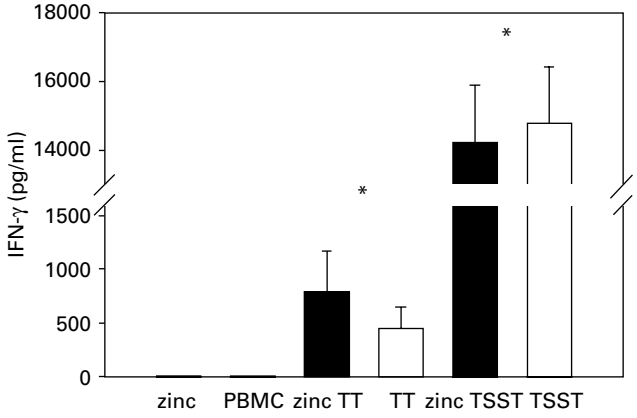

Figure 4 Zinc intake does not influence the reaction to antigen. *Ex vivo* experiments to investigate the influence of zinc intake (10 mg every 2 h, 80 mg/d, one week) on antigenic stimulation were performed. When compared with unstimulated PBMC the zinc stimulated PBMC (zinc) show no additional IFN-γ release. After zinc intake (zinc TT and zinc TSST) antigenic or superantigenic reaction is still present when compared to non-zinc-supplemented stimulation (TT and TSST). Mean values and s.e. of $n=6$. IFN-γ production by stimulants is significant to appropriate control. $^*P<0.001$ (*t*-test).

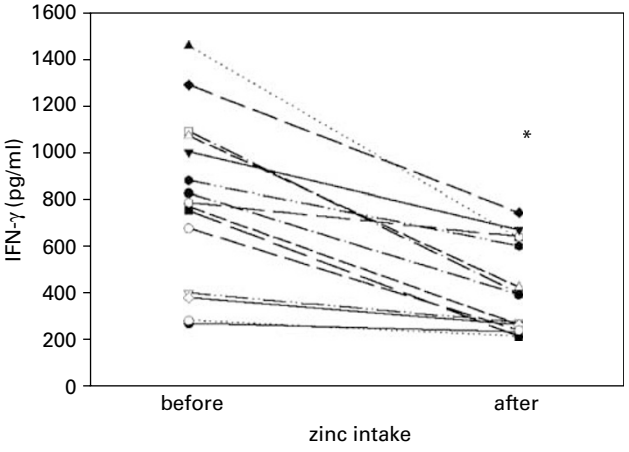

Figure 5 Zinc intake inhibits alloreactivity in every donor combination. Influence of zinc intake (10 mg every 2 h, 80 mg/d) for 1 week on the MLC is shown. IFN-γ release in the MLC after zinc intake shows a reduction in every single donor combination resulting in a significant reduction of the mean value. There was a greater IFN-γ reduction in MLC after zinc intake in samples which had higher IFN-γ levels prior to zinc intake. Values of $n=15$ experiments before and after zinc intake. IFN-γ release in the MLC after zinc intake is significantly reduced to nonsupplemented MLC. $^*P<0.001$ (Wilcoxon-test).

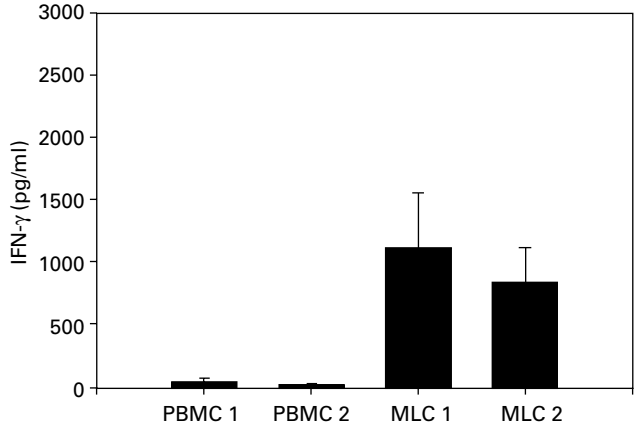

Figure 6 IFN-γ release is not time-dependent. Influence of time on IFN-γ release by MLC is shown. MLC (MLC 1) at the first point in time compared to the MLC (MLC 2) at the second point in time does not show a significant difference in IFN-γ release. Mean values and s.e. of $n=6$. PBMC 1 and PBMC 2 are obtained from the first and the second point in time and are used as controls. Mean values and s.e. of $n=4$.

the inhibition of this crucial immune reaction to antigens had to be tolerated in order to save the graft. An optimal effect of immunomodulation must fulfil three conditions: (i) alloreactivity must be suppressed (see common medications), (ii) reactivity to antigens must remain and iii) no toxic side effects may occur. aCampo *et al*[20] proved inhibition of alloreactivity by zinc using the MLC as an *in vitro* model of transplantation.[20] This important observation suggested that zinc might inhibit the immune cells' reaction to antigen as observed with other immunosuppressants.

However, here in this study it is shown that *in vitro* zinc has no influence on the antigenic reactivity of immune cells. Also, the suppression of responses on allogeneic differences in the MLC by zinc was reproduced as previously shown by aCampo *et al*[20] while maintaining the potency of the host to react to antigens, which had not previously been shown. *In vitro* zinc addition at a concentration of 50 μM is proven to be an optimal nontoxic medication of immunomodulation that retains the potency of immune cells to react to antigen. The *ex vivo* experiments confirmed the *in vitro* results. In the *ex vivo* experiments, an inhibition of alloreactivity was observed while the reaction to antigen and superantigen was not influenced.

During the last few decades, different investigations on zinc ions have been performed, but the exact mechanisms of how zinc influences different types of immune cells is still unknown. Furthermore, it remains unknown how T cells recognize allogeneic MHC molecules leading to strong T-cell activation. Different hypotheses have been proposed which provide evidence for both a peptide-dependent and a peptide-independent alloreactivity.

On the one hand, an alloreactive T-cell clone is able to recognize a peptide, which is presented by a MHC-I molecule with apparent high affinity.[27] On the other hand, a current model suggests that polymorphic determinants on allogeneic MHC molecules, which are recognized by alloreactive T cells, are exclusively contributed by the MHC's α-helices.[28] Thus, there would be a very high density of such determinants on one target cell, and this situation would allow the activation of T cells of much lower affinity than those T cells which are involved in peptide-dependent self-MHC-restricted reactions. Presumably, both hypotheses play a role in alloreaction since it was found that the closer the foreign MHC molecule is related to the T cell's MHC, the higher is the proportion of peptide-specific, alloreactive T cells.[29] However, taking into account that zinc ions can interact with zinc binding sites on the surface of MHC molecules (histidine 81),[30] a low-affinity binding during allogeneic recognition could be disturbed by such an interaction. This could be one explanation why zinc is able to reduce MLC.

Increasing binding affinity implicates stronger intracellular signaling pathways. Cell activation is transmitted via an intracellular cascade of molecule interactions commonly mediated by phosphorylation of amino-acid residues. One enzyme that is influenced by zinc is the IRAK. Zinc inhibits IRAK,[19] and this leads to a suppression of IL-1 mediated T-cell activation. The strength of intracellular signal transduction depends on this degree of binding affinity. The findings indicate that zinc can probably alter the low affinity-induced intracellular pathway caused by allogeneic stimulation. However, it fails to disturb the pathway induced by high-affinity binding caused by the antigenic reaction because intracellular zinc levels cannot reach the threshold to inhibit phosphorylation activity. This hypothesis indicates a new and selective way of immunomodulation by zinc.

Another hypothesis suggests signal transducer and activator of transcription (STAT) proteins are responsible for this phenomenon. STATs were known to be important mediators of signaling by cytokine receptors,[31] and recently, it was published that STAT proteins might also play a role in T cell receptor (TCR) signaling. STAT5 was shown being transiently phosphorylated on a tyrosine residue in response to antigenic TCR stimulation.[32] This stimulation was mediated by the protein tyrosine kinase Lck, and the use of Lck-deficient T-cell line or the use of T cells with a mutant STAT5 protein resulted in a reduced antigen-stimulated proliferation. On the other hand, studies with alloreactive human $CD4^+$ T cells showed tyrosine and serine phosphorylation of STAT3 after TCR stimulation.[33] Thus, these findings suggest that there were different signaling pathways, which were activated due to allogeneic or antigenic TCR stimulation. Zinc might be capable of interfering with STAT3 selectively resulting in a disturbance of the allogeneic reaction, whereas STAT5 remained unaffected by zinc addition and responses to antigens were still possible.

In conclusion, zinc is an essential trace element for the whole organism that fulfils all conditions of optimal immunomodulation. Zinc could become an extraordinarily important substance in transplantation medicine that carries no toxic side effects as it could reduce the rate of graft rejection as well as the danger of further infections. Further clinical studies have to be performed but the first step towards a more effective way of treating patients in transplantation medicine has been taken.

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