11 Summary

Screening a variety of natural compounds and derivatives had revealed appreciable antileishmanial activity for numerous polyphenols. These drugs inhibited the intracellular survival of *Leishmania major* and *L. donovani* parasites, while being largely ineffective against their extracellular promastigote form. Toxicity for the experimental host, macrophage-like RAW 264.7 cells (MΦ), was minimal. This indirect antileishmanial effect could best be explained by an immunomodulatory activity of the polyphenols inducing antimicrobial processes in MΦ. This hypothesis found confirmation in functional immunoassays showing the release of cytotoxic molecules and proinflammatory cytokines by polyphenol-treated MΦ.

For a better understanding of the immunomodulatory and antiparasitic effect of polyphenols, this work was now expanded to include other *Leishmania* species and polyphenolic compounds. Furthermore, molecular and genetic immunoassays were adapted to supplement the functional data.

The tested compounds included simple phenols, B-type proanthocyanidines, and hydrolyzable tannins as well as galloylated flavonoid glycosides and caffeic acid esters. In general, the appreciable antileishmanial activity and low general toxicity of selected polyphenols could be confirmed. Especially hydrolyzable tannins such as corilagin and geraniin exhibited very low IC_{50}-values with intracellular *Leishmania* parasites. Amongst the caffeic acid esters, monomeric caffeic acid, salvaniolic acid K and salvaniolic acid L were the most active as were those galloylated flavonoids that did not contain additional galloyl groups such as kaempferol and quercetin derivatives. All these drugs also activated MΦ for the release of tumor necrosis factor (TNF)-α and interleukin (IL)-6. Gallic acid, the hydrolyzable tannins, the galloylated flavonoid glycosides, and to a minor extent also some of the caffeic acid esters additionally induced interferon (IFN)-activity which could be attributed, by ELISA technique, to IFN-α. All these tests were performed with seven different pathogenic *Leishmania* species: Old World *L. major*, *L. donovani*. *L. tropica*, *L. aethiopica*, *L. killicki*, and New World *L. amazonensis*, *L. guyanensis*. Basically, the tests with

different *Leishmania* species gave similar results (in individual experiments killing of the South American species required higher drug concentrations). Accordingly, work was continued with *L. major* and *L. donovani* only.

In all experiments on MΦ activation, IFN-γ plus bacterial lipopolysaccharide (LPS) was used as a positive control. In contrast to this highly reliable and robust control, MΦ activation with polyphenols depended on experimental details. Tested polyphenols activated only resting MΦ. Addition of polyphenols to IFN-γ and/ or LPS failed to enhance activation but rather reduced NO-release and intracellular *Leishmania* kill compared to either stimulus alone. This effect was largely independent of the sequence and interval in which these products were added. For example, gallic acid reduced LPS-induced NO and *Leishmania* kill irrespectively of whether it was given up to three hours before, simultaneously, or three hours later. 3-galloyl shikimic acid, 3,5-digalloyl shikimic acid, the proanthocyanidine hexamer, corilagin, catechin, and EGCG revealed similar effects. When the time interval was expanded to 24 h, this inhibitory effect of polyphenols was no longer detectable. These results might reconcile our results with others reporting both activating and inhibiting immunomodulation by polyphenols.

A further finding of special interest for parasitologists is, that phagocytosis and intracellular persistence of *Leishmania* organisms already result in low-level MΦ activation. Moreover, the parasites act as a priming signal that is prerequisite for polyphenols to induce appreciable MΦ activation. For example, polyphenol-treated non-parasitized MΦ released only marginal amounts of TNF-α, IL-6 or IFN, whereas *Leishmania*-parasitized MΦ released these cytokines at high concentrations when stimulated with certain polyphenols.

Employing the reverse-transcriptase polymerase-chain-reaction (RT-PCR) technique, MΦ response to selected polyphenols was also investigated at the level of cytokine gene-expression. First, naive or *L. major*-infected MΦ were treated either with IFN-γ+LPS or with gallic acid. Again, only parasitized MΦ were susceptible to polyphenol activation, which strongly supported the results at protein secretion level described above. Transcription of IL-1 and TNF-α genes was evident within 2 hours of adding gallic acid to *Leishmania*-parasitized MΦ, IL-12, IL-18, IFN-α, IFN-γ, and iNOS within

4-6 h, and IL-10 within 10 h. As IL-10 possesses multiple immunoinhibitory functions, this might indicate an end of the activation phase at the genetic level. Transcripts of IL-12, IL-18, IFN-α, and IFN-γ were no longer detectable 18 h post activation, whereas IL-1, IL-10, TNF-α, and iNOS were. With the exception of IFN-γ, this cytokine gene expression pattern was identical with that induced by IFN-γ+LPS.

Polyphenol-induced cytokine gene expression analysis was then expanded to 3-galloyl shikimic acid, 3,5-digalloyl shikimic acid, proanthocyanidine hexamer, corilagin, catechin, and EGCG and performed 4 h after these drugs had been given to naive, *L. major*- or *L. donovani*-parasitized MΦ. The gene expression profiles induced by these polyphenols resembled that of gallic acid. Compounds such as 3-galloyl shikimic acid and catechin that had shown the least effects at the cytokine protein level, also seemed to induce less gene expression, indicating that both methods yield comparable results. Interleukin-12, which plays a central role in cell-mediated anti-leishmanial immunity, did not seem to be induced by these drugs at all, whereas 3,5-digalloyl shikimic acid showed intermediate, and proanthocyanidine hexamer, corilagin, and EGCG the strongest activating capacities, including IL-1 and IL-12 in their profiles. Corilagin and EGCG also induced IFN-α gene expression. Inducible NO-synthase gene expression was detected in all samples, again with a clear correlation between the relative intensities of gene expression and functional, i.e. antiparasitic efficacy. Induction of IL-12 was also monitored by ELISA at the protein level. While IFN-γ+LPS induced MΦ release of IL-12 irrespective of their infection status, only Leishmania-infected MΦ released IL-12 upon stimulation with tested polyphenols. In line with the gene expression data, IL-12 titers were highest in cultures treated with corilagin, EGCG, and proanthocyanidine hexamer, and lowest in those treated with 3-galloyl shikimic acid.

Taken together, these findings strongly support the hypothesis, that antileishmanial effects of polyphenols result largely, if not exclusively, from activation of antimicrobial mechanisms in the host cell. Direct effects of tested polyphenols on promastigote *Leishmania* parasites were low to below detection level. Nevertheless, some selective effects of polyphenols on the intracellular amastigote form cannot be ruled out. The tested polyphenols have a profound effect on MΦ, e.g. in inducing a broad range of cytokines with regulatory functions in innate as well as adaptive immune

response. Interleukin-12, for example, stimulates natural killer (NK) cells for IFN-γ release, and promotes an antigen-specific T cell response of the Th1-type. Both elements enhance cell-mediated defense against intracellular pathogens. The finding, that tested polyphenols act primarily on infected MΦ and not on naive MΦ is of major importance. Provided the same applies in vivo, this could mean that polyphenols are efficacious exclusively at the sites of infection. A systemic, uncontrolled activation of MΦ always bears the danger of serious immunopathological side effects. Such focusing of beneficial immunomodulation to areas where the parasites are present bears a great advantage of antiparasitic therapy with polyphenols.