Signal regulatory protein alpha in phagocyte function

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Chapter 8
Summary and general discussion

Phagocytes are the cornerstones of the immune system. They destroy invading pathogens and initiate adaptive responses via antigen presentation. Moreover, phagocytes have important roles in normal homeostasis, which includes amongst other things i) the removal of apoptotic cells and aged erythrocytes and platelets during development and adult life, ii) the resorption of bone during bone formation, maintenance and repair, which is mediated by highly specialized osteoclasts, and iii) the clearance of tumor cells. The basic hypothesis underlying the research described within this thesis was that the activity of phagocytes is not only regulated by receptors that detect stimuli for the triggering of the various phagocyte effector functions, like e.g. the Toll-like receptors that induce cytokine production, but that there are endogenous pathways that inhibit these functions as well. This is a well-established theme for natural killer cells, the activity of which is controlled by killer inhibitory receptors. Killer inhibitory receptors (KIR) recognize broadly expressed MHC class I molecules and the binding of these 'self' molecules by KIRs triggers signaling via the recruited tyrosine phosphatases SHP-1 and SHP-2. This mechanism prevents the killing of healthy MHC class I-expressing cells[1]. We anticipated that similar mechanisms may act to control other immune cells, including phagocytes, and that these would act to limit immune mediated damage to the host. Indeed, a broad range of inhibitory receptors have been identified, differing in their expression pattern, ligand recognition and/or precise signaling properties, and some of these are also expressed on phagocytes[2]. In this thesis we have focused on the role of SIRPα, which appears to be one of the most prominent inhibitory receptors on phagocytes in terms of expression and function. This thesis describes a number of studies aimed to understand the role of SIRPα in phagocytes, as well as the malignant counterparts of their progenitors i.e. acute myeloid leukemia.

The SIRP multigene family and evolution

SIRPα is the best described family member of the SIRP family. The SIRP family is a diverse multigene family of immune receptors, which includes inhibitory SIRPα, activating SIRPβ, non-signaling SIRPγ and soluble SIRPδ members. An overview of the composition of the different SIRP family members present in mammals and birds and their known immunological properties has been provided in chapter 2. It is clear from this that the SIRPs represent a typical example of a 'paired receptor' family, which includes both members with an inhibitory as well as activating signaling potential. Interestingly, there appears to be a single relatively well-conserved prototypic inhibitory SIRPα receptor in each species. Furthermore, there are variable numbers of activating SIRPβ-like molecules present within the different phylogenetic groups of species, which apparently arose by multiple duplication events from a prototypic SIRPα. This strong and relatively recent diversification of SIRPs could well be the result of a species-specific natural selection mechanism, such as infection (also see below). Another aspect of particular interest is that the members of the multigene SIRP family show close relationships to the antigen receptors, in particular the TcR, because they possess a joined VJ segment (i.e. encoded within the same exon) combined with C1-

type Ig-like domains found only in proteins associated with antigen recognition such as TcR, Ig itself, MHC antigens and β2-microglobulin[3;4]. Furthermore, in analogy to TcR and KIR, which interact with MHC, SIRPα interacts with the broadly expressed 'self' molecule CD47[5]. It has recently been shown by site-directed mutagenesis and a high-resolution X-ray crystal structure of co-crystals of the SIRPα N-terminal V-type Ig domain and the CD47 extracellular domain that the interaction between SIRPα and CD47 relies to a great extent on an extended DE loop in SIRPα[6;7]. In this respect SIRPα also resembles the TcR, which uses the DE loop to recognize MHC peptide. Based on the similarities we have previously proposed a direct evolutionary relationship between antigen receptors, TcR and BcR, on one hand, and SIRPs on the other[4]. It seems likely that the rearranging antigen receptors arose from a primordial non-rearranging Ig-superfamily by the insertion of a RAG-containing transposon some 500 million years ago, somewhere before the appearance of jawed vertebrates[8] and that this further evolved into the rearranging adaptive immune system which, as we know now, exists in most vertebrates. It seems reasonable to assume that a primordial SIRP-like molecule was in fact the target for this initial transposition event, which therefore provided the basic molecular foundation for antigen and 'self' recognition in adaptive immunity. As a consequence, any information on the current function of SIRP family members is of potential relevance for understanding the evolution of antigen receptors.

CD47-SIRPα interactions control the phagocyte NADPH oxidase

Phagocytes play a central role in the host defense against invading microorganisms. One of the most important anti-microbial activities of phagocytes is the respiratory burst, which is mediated by the NADPH oxidase enzyme complex, that is assembled at the phagosomal and plasma membrane of the phagocyte upon ingestion of microbes, such as bacteria and fungi[9]. This enzyme converts oxygen into superoxide, using NADPH as electron donor[10]. Superoxide forms the basic compound from which other, generally more aggressive, reactive oxygen species (ROS) are generated[9]. High concentrations of ROS are toxic for the invading microorganisms, but may also cause damage to the host. Therefore controlling of the respiratory burst is crucial. Over the years a large number of microbial stimuli have been described to activate the respiratory burst, but little or nothing is known about the mechanisms that control the production of ROS. In chapter 3 we have shown, by employing human phagocytic cells and phagocytes from SIRPα-mutant mice, that SIRPα acts to limit activity of the phagocyte NADPH oxidase burst. This regulation of the NADPH oxidase by SIRPα depends on interactions with CD47 and on intracellular signaling via SHP-1 and/or SHP-2. Moreover, these signals act by selectively suppressing expression of $gp91^{phox}$, the key catalytic component of the phagocyte NADPH oxidase complex. Somewhat surprisingly the outgrowth of *Salmonella* bacteria was enhanced in SIRPα-mutant mice, and we believe, although this was not proven, that this could in fact be the direct consequence of the enhanced level of 'collateral' respiratory damage, that can be expected to occur in these mutants. If so, the respiratory burst capacity of phagocytes *in vivo* appears to be optimized by a fine-tuned expression of its components, in

particular gp91^{phox}, which allows maximal respiratory killing of microbes, while limiting respiratory damage to the host. Both a defective expression of NADPH oxidase components, which occurs in the primary immunodeficiency chronic granulomatous disease (CGD), as well as exaggerated expression, as present in e.g. the SIRPα-mutant mice, leads to an increased outgrowth of bacterial infection[11].

SIRPα functions as an inhibitor of osteoclast bone resorption

In addition to their immunological functions, phagocytes, and in particular macrophages, also play a central role in homeostasis. In bone, a specialized macrophage cell type, the osteoclast, is essential for bone resorption, which is critical during bone growth and repair as well as during normal bone turnover. Disturbance of the formation and function of the osteoclast leads to bone disease, such as osteoporosis and therefore bone formation by osteoblasts and bone resorption by osteoclasts have to be strictly coordinated in the body. Interestingly, in *motheaten* mice, with a deficiency or loss-of-function mutation in SHP-1, it has been shown that SHP-1 is involved in regulation of osteoclast formation and activity[12;13]. However, the putative inhibitory receptors that mediate SHP-1 recruitment and activation in osteoclast have not been identified. In chapter 4 we have shown that SIRPα is expressed and associated with SHP-1 in osteoclast. In spite of a normal osteoclastic differentiation, the bone resorption capacity of osteoclasts from SIRPα-mutant mice was significantly enhanced. Consequently, SIRPα-mutant mice have a reduced cortical bone mass. This identifies SIRPα as a selective non-redundant inhibitor of osteoclast function. Moreover, our results also provide insight into the mechanism of regulation of osteoclast bone resorption by SIRPα. In particular, osteoclasts from SIRPα-mutant mice show an enhanced formation of actin rings. For proper osteoclast bone resorption the formation of actin rings is required, which form an intrinsic part of an adhesive structure, the so-called sealing zone. Through this zone an isolated acidic microenvironment is created between osteoclast and bone, in which bone matrix is degraded by proteases[14;15].

Actin ring formation involves a complex of $\alpha_v\beta_3$ integrins, Syk, c-Src and ITAM bearing proteins, such as DAP12 and the FcR γ-chain. Upon ligand binding $\alpha_v\beta_3$ triggers autophosphorylation and activation of the tyrosine kinase c-Src. Activated c-Src phosphorylates the tyrosines in the ITAM bearing proteins, DAP12 or FcRγ, which in turn triggers the association and activation of Syk. Activated Syk phosphorylates a variety of proteins which mediate organization of the actin cytoskeleton[16]. We propose that SIRPα, and perhaps other myeloid inhibitory receptors as well, act to counterbalance the organization of actin induced by ITAM mediated signals (this is illustrated in figure 7 of chapter 4). It will be interesting to establish at which level(s) the proposed SIRPα-SHP-1 signal integrates with the $\alpha_v\beta_3$ integrin/c-Src/ITAM bearing proteins/Syk/actin pathway.

CD47-SIRPα interactions form a barrier for antibody-mediated elimination of tumor cells

Previous studies had provided evidence that SIRPα negatively regulates the phagocytosis of host cells upon interaction with the broadly expressed 'self' molecule CD47 on those host cells[17-19]. CD47-SIRPα interactions have for instance been shown to prevent the clearance of red blood cells and platelets by macrophages *in vivo*[20;21]. CD47-SIRPα interactions also exert a negative influence when the clearance of host cells is induced or promoted by antibodies directed against such cells, as apparent in antibody-mediated hemolytic anemia or immune idiopathic thrombocytopenic purpura (ITP), suggesting that FcR signaling, and as a consequence effector function, can be negatively regulated by SIRPα[22]. This has suggested that perhaps the antibody-dependent clearance of tumor cells by macrophages could also be negatively regulated by CD47 on the tumor cells. In chapter 5 we have investigated the involvement of the CD47-SIRPα interaction in the antibody-dependent elimination of tumor cells. Indeed, interference with the molecular interaction between CD47 and SIRPα with suitable antagonistic antibodies significantly enhances Ab-mediated phagocytosis and destruction of tumor cells *in vitro*. In addition the SIRPα-mutant mice showed reduced numbers of metastasis after treatment with therapeutic anti-tumor antibody in comparison to the SIRPα wild type mice. Taken together, this study shows a new intrinsic mechanism that relies on CD47-SIRPα interactions and likewise acts to limit the destruction of host cells by phagocytes. Importantly, this mechanism may hamper the therapeutic effects of anti-tumor antibodies and therefore the killing of the tumor cells during cancer. We anticipate that an appropriate CD47-SIRPα antagonist would be able to support antibody therapy against human cancer and this should definitely be further explored.

Can SIRPα be targeted by pathogens?

In view of the growing body of evidence for a role of CD47-SIRPα interactions in controlling phagocyte effector functions it would not be surprising if this pathway was targeted by pathogens for the purpose of evading immunity. Of interest, indirect evidence is emerging that this may indeed be the case. For instance, essentially all poxviruses encode a homologue of CD47, termed vCD47. Of interest, recent evidence demonstrates that the vCD47 protein of the myxoma poxvirus is expressed on the surface of infected cells and is required for the production of a lethal infection in its host. Moreover, vCD47 contributes to the suppression of macrophage activation[23]. Although this study did not provide direct evidence for a interaction of SIRPα and vCD47 it would be of interest to investigate this option further. Clearly, a potential targeting of SIRPα would expect to be an efficient mechanism for immune evasion by poxvirus.

Another observation of interest in this context is that at least ten distinct human SIRPα polymorphic variants have been indentified[18]. Of interest, variation does occur in particular in the CD47-binding V-type Ig-like domain of SIRPα. On the other hand, most variation does not appear to be present in the regions that actually mediate interaction with CD47, as has now been firmly established by site-directed mutagenesis and high

resolution X-ray crystallography[6;7;24]. One potential driving force for the diversification in these regions is to escape pathogen binding to SIRPα that might otherwise lead to a suppression of phagocyte functions. It will be of interest to investigate whether for instance the poxviral vCD47 can only bind to certain SIRPα allotypes. In contrast, the activating SIRP molecules might not be important for homeostatic regulation, but may rather play a role in pathogen binding. Consistent with this the human SIRPβ1 activating receptor does not bind human endogenous CD47[25]. Clearly, in order to understand the potential significance of the various SIRP family members and their polymorphic variation, it will be important to explore their interaction with endogenous and poxviral CD47 in more detail.

SIRPα is a pro-apoptotic receptor in AML that is downregulated in particular AML subtypes

SIRPα is not only expressed on mature phagocytes but also at low levels on hematopoietic stem cells (HSC)[26-28]. Also acute myeloid leukemic (AML) cells express SIRPα. In analogy to the development of lymphocytes, which is critically regulated by antigen receptor (TcR or BcR) activity, it seemed possible that also normal and/or leukemic myeloid development is regulated by SIRPα. The role of SIRPα in AML was investigated in particular in chapters 6 and 7. In chapter 6 we have demonstrated a strong correlation between SIRPα expression and AML subtype. In particular, there was a relatively low expression of SIRPα in immature and granulocytic subtypes (M0-M3), as compared to normal HSC, and a relatively high expression in the mature monocytic (M4, M5) subtypes. These data are consistent with the idea that SIRPα constitutes a myeloid differentiation marker and suggest that SIRPα downregulation may be a common feature of the more immature and granulocytic AML types. In t(8;21) AML the low expression level of SIRPα appears to involve epigenetic gene silencing, as shown by the use of inhibitors of DNA-methylation and histone deacetylation. Furthermore, we provide evidence that SIRPα can provide growth inhibitory signals in AML, at least in the ones with a t(8;21) or t(15;17) translocation. We have found that the reconstitution and ligation of SIRPα in t(8;21) AML Kasumi-1 or t(15;17) APL NB4 cells promotes their apoptosis. Finally, the SIRPα-derived pro-apoptotic effect was shown to synergize with established chemotherapeutics.

SIRPα as a potential target for therapy in cancer, autoimmunity and osteoporosis

Previous chapters have demonstrated that SIRPα is involved in a variety of myeloid cell functions, including both homeostatic as well as host-defence related ones. However, manipulation of SIRPα may perhaps be exploited in a variety of diseases. The inhibitory effect of SIRPα on the bone resorption provides a rational basis for the design of therapies for osteoporosis and other bone remodeling diseases. For instance, the appropriate agonistic antibodies are anticipated to have a beneficial effect on bone formation by promoting inhibitory signaling in osteoclasts. Furthermore, agonistic antibodies against human SIRPα that are able to promote apoptosis in AML cells could be of value in the treatment of AML, likewise in combination with conventional agents

such as chemotherapeutics and/or all-*trans*-retinoic acid (ATRA). Of relevance, the development of drug resistance is the limiting factor in the therapy of AML[29]. The complete remission (CR) rate reaches 85-90% with standard induction chemotherapy for newly diagnosed pediatric patients. However, about 30-50% of the children that achieve a CR, relapse from minimal residual disease cells that apparently survived chemotherapy[30]. Moreover, the routinely used chemotherapeutic drugs have severe side effects. SIRPα triggering may enhance both the efficacy and/or allow a reduction in dose of the chemotherapeutics, which could potentially limit the severe side effects of chemotherapy.

Concluding remarks

Taken together, the work described in this thesis has provided novel information about the functions of SIRPα in myeloid cells and into the mechanisms by which the specialized immunological and homeostatic functions of phagocytes are controlled. Importantly, our findings strongly support the idea that the regulation of immune cells by 'self' signals is not only limited to lymphocytes and NK cells, but also includes phagocytes and may therefore be a general property of immune cells. A major challenge for the future will be to identify which other functions in phagocytes are subject to regulation by CD47-SIRPα interactions. Finally, it will be important to explore the therapeutic potential of SIRPα targeting.

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