

4 Discussion

4.1 Dysregulation of Apoptosis in Cancer

One of the hallmarks of cancer is evasion of programmed cell death. There is now a growing number of examples for dysregulation of apoptosis in cancer cells (reviewed in [213, 214]). Defects in apoptotic signalling allow tumour cells to live longer than their normally limited lifespan and render them independent of growth signals, provision with nutrients and oxygen, attachment to an extracellular matrix and cellular shut-down mechanisms after genetic alterations.

Upregulation of the *bcl-2* oncogene due to chromosomal translocation next to the immunoglobulin heavy-chain (IgH) locus in follicular lymphoma illustrated the significance of increased expression of anti-apoptotic proteins in this type of haematologic malignancy and serves as an overall paradigm (reviewed in [213, 215, 216]). Besides overexpression of anti-apoptotic *bcl-2* family proteins, loss of the caspase activator Apaf1 [217], blockade of the granzyme B/perforin pathway through PI-9/SPI-6 [218], upregulation of caspase 8 inhibitor cFLIP [219-221] and defects in death receptor signalling due to receptor downregulation or dysfunction [222] have been documented in malignancies.

There is now mounting evidence that aberrant expression of IAP family members is linked to cancer. Survivin, an IAP with one BIR domain [223], is highly expressed during development, but not in most terminally differentiated adult tissues [224, 225]. It has been shown, however, that survivin is present in transformed cell lines and the majority of the most common cancers including solid malignancies like lung, colon, breast, pancreas and prostate cancer as well as haematologic malignancies [226]. Furthermore, several melanoma and non-melanoma skin cancers have also been reported to be invariably positive for survivin expression [227, 228]. Moreover, in a global gene expression analysis with ca. four million transcripts it was found that survivin was one of the most prominent tumour-associated transcripts [229]. It has been demonstrated that

expression of survivin is correlated to unfavourable prognostic features or worse clinical outcome or in colorectal cancer, breast carcinoma, esophageal cancer, bladder cancer, neuroblastomas, non-small cell lung cancer and diffuse large B-cell lymphomas [230-239, 240 162, 241].

Zhang *et al.* provided evidence that expression of XIAP in melanoma cells protects them from TNF-related apoptosis-inducing ligand (TRAIL) -induced apoptosis [242]. Another group revealed that high-level expression of XIAP blocks caspase-3 and -9 activation in cytosolic extracts of Hodgkin's lymphoma derived B cells [243]. Interestingly, Hodgkin and Reed-Sternberg cells of primary tumour tissue stained moderately or strongly positive with an anti-XIAP mAb in paraffin sections.

The mucosa-associated lymphoid tissue (MALT) lymphoma t(11;18)(q21;q21) translocation effectively replaces the prodomain of the human paracaspase MALT1 with the three BIR domains of c-IAP2 [244]. The resulting fusion protein that does not contain a RING domain was found to markedly activate transcription from an NF- κ B responsive promoter and thereby reveals a potential oncogenic mechanism.

A recent study revealed a possible reason for the overexpression of IAPs in tumours [245]. It was shown that cancer cells can survive with low proteosomal activity in the presence of high expression of Tri-peptidyl-peptidase (TPP II). The lower rate of protein degradation leads to increased IAP levels. Expression of IAP family members gives tumour cells a survival advantage since they inhibit caspase dependent apoptotic pathways (Fig. 7) and hence make cells less vulnerable to broad spectrum of apoptotic stimuli including adverse growth conditions and conventional cancer therapy. Interestingly, the novel IAP that we identified, ML-IAP, is widely expressed in melanomas, a type of cancer that is particularly resistant to chemotherapeutic drugs and ionising radiation. It would be of interest to study the compensatory mechanisms which allowed growth of the antigen-loss variants in patient K030 despite chemotherapy. The patient's cell line M34 that expresses only very low levels of ML-IAP may facilitate this endeavour.

Moreover, other groups and our laboratory have found evidence that the role of ML-IAP is not limited to melanoma. The chromosomal location of ML-IAP, 20q13, has been found to be frequently amplified in several types of neoplasms and our own immunohistochemistry stainings revealed ML-IAP expression in a wide variety of human solid tumours and haematologic malignancies (manuscript in preparation).

4.2 Vaccine Enhances ML-IAP-Specific Humoral Response – The Important Role of Anti-Tumour Antibodies

We revealed that ML-IAP is a target of the patient's humoral response. While ML-IAP-specific antibodies are present prior to enrollment, vaccination augments antibody-mediated immunity as seen in increased anti-ML-IAP antibody titres and isotype switching. Our data based on 92 serum samples from melanoma, lung and prostate cancer patients revealed that in a subset of cases significantly elevated antibody levels against ML-IAP are present. None of the 67 healthy blood donors tested displayed any increased humoral activity. Interestingly, other studies revealed that cancer patients have elevated antibody levels against IAP family member survivin [246, 247]. Furthermore, a very recent finding showed that patients with gastrointestinal tumours display elevated levels of anti-ML-IAP antibodies [248]. Taken together, these results indicate that the incidence of elevated anti-ML-IAP antibody levels is correlated with several types of cancer. Moreover, we identified several other targets of patient K030's humoral response (Table 1) and showed previously that vaccine treatment increased antibodies against ATP6S1, a putative accessory unit of the vacuolar H⁺-ATPase complex [249].

Several studies allowed new insights into the role of antibodies in anti-tumour immunity. Experiments with Fcγ receptor-deficient mice revealed that Fc receptors are necessary for passive and active immunisation to melanoma [250]. O'Reilly *et al.* showed that the humoral response contributes to the rejection of

her2/neu positive tumours in a murine GM-CSF-based cancer vaccine model [137]. Moreover, Forni's lab showed in a murine model that the eradication of established her-2/neu carcinomas required the combination of several humoral and cellular mechanisms [251]. The majority of BALB- μ Ig deficient and BALB-Fc γ RI/III deficient mice failed to reject the tumour post-vaccination despite temporary tumour regression and delayed growth.

Initially, the mechanisms by which antibodies against cytoplasmic TAAs such as ML-IAP aid anti-tumour responses were not clear. It is now well documented that antigen bound by antibodies, so-called immune complexes (ICs), play an important role in DC maturation and crosspriming [252, 253]. ICs are taken up through Fc γ receptors by DCs and a unique pathway leads to the proteasomal degradation of the antigen. Subsequently the endocytically acquired antigen is presented on MHC class I, thereby linking humoral and cellular immunity. Recently the role of ICs in anti-tumour immunity has been shown in several studies. Dhodapkar *et al.* found that coating tumour cells with anti-tumour mAbs lead to enhanced crosspresentation of tumour-derived cellular antigens and generation of tumour-specific killer T cells by DCs [254, 255]. Another group showed that DCs loaded with ovalbumin-based ICs can protect mice against a later challenge with an ovalbumin positive melanoma cell line and also can cure mice with early established tumours [256].

The abovementioned studies clearly support an important role for anti-TAA antibodies in crosspriming and rejection of established tumours. The GM-CSF-based vaccine contributed to the anti-tumour reactivity by eliciting higher anti-ML-IAP antibody titres. The functional significance of the isotype switching to IgG4 that we observed is currently not fully understood and will require further investigation.

4.3 CD4⁺ T Cells are Significantly Contributing to Tumour Rejection and Recognise ML-IAP

Like the humoral response, the cellular immune response was augmented by treatment with autologous irradiated GM-CSF-secreting melanoma cells. While no TILs had been present in resected metastases prior to enrollment, tumour tissue became inflamed, necrotic and heavily infiltrated with CD4⁺ and CD8⁺ T cells. Our findings strongly suggest the presence of CD4⁺ T cells that recognise ML-IAP in the patient. We detected high levels of anti-ML-IAP IgG (whose secretion is CD4⁺ T cell dependent), paraffin-embedded tissue sections showed CD4⁺ TILs in necrotic tumour tissue and we documented the strong proliferative response of a 95% CD4⁺ T cell population in the presence of recombinant ML-IAP protein.

The data obtained indicate an important role for CD4⁺ T cells in the GM-CSF vaccine-elicited anti-tumour response. These observations are in accordance with previous results obtained in murine models. It is well established that CD4⁺ T cells help prime CD8⁺ T cell responses [257]. Recent experiments with a murine fibrosarcoma model showed that direct priming alone is a very inefficient mechanism, while CD4⁺ T cell-dependent crosspriming of CD8⁺ T cells led to rejection of the tumour [258]. Moreover, CD4⁺ T cells secrete cytokines such as IL-2 that are necessary to maintain CD8⁺ T cell functions [259]. Finally, CD4⁺ T cells themselves can cause tumour regression in the absence of CD8⁺ T cells by recruiting effector cells such as macrophages, eosinophiles and other cell populations [135] as well as by secreting cytokines such as IFN- γ [260, 261]. In murine vaccination studies it was demonstrated that not only adoptive CD4⁺ T cell transfer but also injection of a MuLV-derived Th epitope conferred protection against a later challenge with a MHC class II negative, virus-induced tumour cells [262].

The central role of CD4⁺ T cells in anti-tumour immune reactions underscores the significance to employ treatment strategies involving both MHC class I and MHC class II antigens. Vaccines based on whole tumour cells or full length proteins encompass all potentially presentable epitopes. Since many clinical trials have been exclusively based on MHC class I peptides as a source of antigen in the past, the lack of CD4⁺ T cell involvement may be one reason for the lack of a potent anti-tumour response.

4.4 CD8⁺ TILs Can Kill ML-IAP Expressing Cells Through Caspase-Independent Pathways

CD8⁺ cytolytic T cells are important effector cells mediating tumour killing. Post-vaccination we observed CD8⁺ T lymphocytes in necrotic tumour tissue and revealed that a subset of these CD8⁺ T cells was clearly ML-IAP-specific. Moreover, we established not only the presence of CD8⁺ T cells recognising ML-IAP by tetramer assay, we also showed functional activation in the presence of ML-IAP peptides by IFN- γ secretion (ELISPOT assay) and specific lysis of ML-IAP bearing target cells (cytotoxicity assay). Recent findings suggest that it is of importance to validate the presence of antigen-specific T cells with more than one assay system [263]. Discrepancies between ELISPOT IFN- γ secretion and tetramer binding revealed dissociation of effector function from TCR/tetramer complexes half-life.

ML-IAP renders cells less vulnerable to a wide variety of apoptotic stimuli and can inhibit caspase activation. In this context, it is important to note the ability of CTLs to trigger caspase-independent cell death pathways through granzymes. Recent reports revealed that granzyme A can activate the DNase NM23-H1 [264] and target apurinic endonuclease-1 (Ape1), the rate-limiting enzyme of DNA base excision repair [265]. It was also shown that inhibitor of caspase-activated deoxynuclease (ICAD) serves as a substrate for granzyme B

which leads to DNA fragmentation mediated by caspase-activated deoxynuclease (CAD) [266, 267].

Our results reveal that stimulating T cell immunity against ML-IAP bearing tumour cells results in effective antigen-specific killing of target cells *in vitro* and that the occurrence of ML-IAP-specific CD8⁺ T cells is correlated with killing of ML-IAP expressing tumour cells *in vivo*.

4.5 Loss of Tumour-Associated Antigen During Course of Treatment

Immunohistochemistry with an anti-ML-IAP mAb showed that antigen-loss variants emerged post-vaccination, evading the ML-IAP-specific immune responses. The emergence of the ML-IAP negative melanoma cells is correlated with a lack of TILs, inflammation and necrosis. Moreover, at the same time the patient's overall clinical condition deteriorated and subsequently died.

Several reports documented immune escape by loss of antigens in melanoma [268-270]. Yee *et al.* observed the appearance of antigen loss variants in melanoma patients after treatment with *ex vivo* expanded MART-1 and gp100-specific CD8⁺ T cells [271]. While preinfusion tumour immunostaining revealed moderately intense expression of gp100, tyrosinase and MART-1, postinfusion biopsies from relapsing or residual nodules showed selective loss of the targeted antigens.

Our finding that treatment resulted in the selection of antigen-loss variants strongly indicates T cell reactivity against antigen-bearing tumour cells *in vivo*. Progressing disease in patient K030 reveals, however, that the effective immune response against ML-IAP expressing melanoma cells could be evaded by downregulation or loss of expression.

4.6 Implications for Future Vaccine Development

A continuing interest of our group is the development of easily applicable therapeutic vaccines against melanoma.

ML-IAP is expressed in a significant proportion of melanomas and several other types of cancer, while its expression in normal tissues is highly restricted. Importantly, our results showed that it is possible to develop potent humoral and cellular anti-ML-IAP reactivity that is associated with widespread tumour necrosis without triggering autoimmunity. Thus it seems worthwhile to pursue ML-IAP as a component of future vaccines.

The observation of ML-IAP antigen loss variants, however, has essential implications for future vaccine studies. While antigen loss validates the effectiveness of the ML-IAP-directed immune response our results clearly indicate that it will be necessary to target antigens that are vital for tumour cell proliferation besides ML-IAP. A possible target fulfilling this criterion is human telomerase reverse transcriptase (hTERT)[272], which is widely expressed in tumours but also in proliferating normal cells. The p53-interacting protein MDM2 is overexpressed in cancers and also a potential target antigen [273]. Furthermore, it is conceivable to vaccinate against other IAPs, most notably survivin whose expression render the malignant cells less vulnerable to conventional anti-cancer therapy such as chemotherapeutic drugs or irradiation. This approach could be combined with conventional chemo- and radiotherapy. It will be necessary to investigate, however, how ML-IAP will work as part of a larger group of TAAs given the principle of immunodominance (reviewed in [274]). Novel hierarchies emerge among TAAs when multiple dominant epitopes are given in a single inoculum. Likewise, new hierarchies appear when an immunodominant TAA is lost following immune selection but multiple other TAAs remain on the tumour cell.

Our group is currently investigating the use of the MHC class I negative cell line K562, as an irradiated, GM-CSF-secreting so-called bystander line that can be administered in combination with target antigen peptides or protein.

Moreover, it may be of benefit to transfect GM-CSF-secreting K562 cells with TAAs and the appropriate MHC class I molecule(s). These approaches would greatly facilitate vaccine production. Treatment would be possible even if very little or no tumour tissue could be obtained for viral transfection.

Alternatively, it seems promising to inhibit IAP activity by pharmacologically exploiting the binding mechanism of IAP antagonists. As outlined above, IAP regulatory proteins Smac/DIABLO [275] and Omi/HtrA2 interact with ML-IAP [196] through their IAP-binding motif (IBM), a four amino acid sequence lead by alanine. Initial studies revealed that coexpression of Smac or addition of Smac peptides abrogates the ability of ML-IAP (and XIAP) to inhibit cell death and thereby sensitising cells to pro-apoptotic stimuli [195]. Continuing this approach, the laboratory of Fairbrother screened peptide libraries for binding to XIAP and ML-IAP and demonstrated that amino acid substitutions within the binding motifs may allow for increased selectivity to ML-IAP relative to XIAP [276]. Therefore it seems feasible to specifically block the activity of certain IAPs such as ML-IAP with peptides. The strategy to sensitise tumour cells for chemotherapeutic agents and radiation could be a useful tool in conjunction with conventional cancer treatment.

Finally, it is important to point out that our current GM-CSF-based vaccination approaches are carried out in late stage cancer patients with a significant tumour burden who are often severely immunocompromised and have been extensively treated before. After fully addressing concerns regarding safety and efficacy it will be of great interest to move vaccination to an earlier disease stage.

4.7 Investigating the Distribution and Biological Function of ML-IAP Splice Variants

Our finding that a third isoform of ML-IAP exists that lacks the RING domain raises the question about the splice variants' different biologic activities. The absence of the RING domain which is currently believed to play an important role in regulating the expression level of interacting caspases as well as ML-IAP itself, may affect the ability of inhibiting apoptotic signalling *in vivo*. A RING-less ML-IAP could have an increased half-life and therefore be a more potent caspase inhibitor. Several splice variants have been discovered for the IAP family member survivin. One of the isoforms, survivin-2B, which retains a part of intron 2 as a cryptic exon, showed a marked reduction of its anti-apoptotic function [234, 277]. Human clear cell renal cell carcinomas exhibit a stage-dependent relative decrease of this isoform [278]. In this context, it will be of interest to explore the expression levels and biologic function of ML-IAP splice variants in normal and malignant tissues. We are in the process of investigating which ML-IAP epitope is recognised by our monoclonal antibody 3F9 and whether other splice variants can be detected with it. We would like to examine if the third isoform compensates for the downregulation of the two RING-bearing forms in the late stage tumour tissue samples of our patient. Furthermore, we constructed a retroviral vector with a RING-less ML-IAP; functional studies are pending.

4.8 Importance of Identifying the Murine Form of ML-IAP

The identification of the murine form of ML-IAP will allow to conduct pre-clinical vaccination studies targeting this antigen in murine tumour models such as the melanoma line B16. Moreover, it will be possible to study the ML-IAP directed humoral and cellular responses in much greater detail. The kinetics of and recognised epitopes by emerging ML-IAP-specific T cells and antibodies will be of interest. Since relatively little is known about the biologic role of ML-IAP it will

be appealing to explore its function in transgenic and gene deficient mice. One of the questions is whether ML-IAP and XIAP have a compensatory function, given that XIAP and ML-IAP share many biologic activities (inhibition of caspases 9 and 3, DIABLO/Smac acts as an antagonist) but XIAP-deficient mice lack a phenotype [279]. Studies with ML-IAP- and XIAP-double-deficient mice should be able to address this question. Furthermore, due to our finding that ML-IAP is expressed in a variety of haematologic malignancies such as lymphomas we are interested in investigating whether transgenic mice that express ML-IAP under a B cell-specific promoter will develop lymphoproliferative disease.

4.9 Concluding Remarks

Late stage melanoma patients who were enrolled in a phase I trial and received autologous, irradiated GM-CSF-secreting melanoma cells mounted an impressive anti-tumour response that resulted in inflammation, dense lymphocytic infiltrates and significant necrosis of tumour lesions. In our studies we identified ML-IAP, a previously unknown IAP family member, as a tumour rejection antigen. ML-IAP is linked to cancer by its anti-apoptotic function and its high-level expression in a wide variety of solid and haematologic malignancies. We revealed that a GM-CSF-based vaccination strategy elicits potent humoral and cellular immune responses against ML-IAP-expressing tumour targets in a late stage melanoma patient. Since also no autoimmune reactivity was observed, it seems promising to incorporate ML-IAP-directed immune responses in future immunotherapies. Future clinical studies based on this TAA, however, will require additional measures to prevent the tumour from immune escape by losing ML-IAP expression. It also will be of interest to investigate the precise signalling pathways of ML-IAP and its potential role in neoplastic transformation in murine models. The major findings mentioned in this thesis have been recently published in the Proceedings of the National Academy of Sciences [280].