Based in part on
Targeting the acute myeloid leukemic stem cell compartment by enhancing tumor cell-based vaccines.
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GENERAL DISCUSSION AND
FUTURE PERSPECTIVES
With an increasing number of clinical successes in targeting both solid and leukemic tumors by tumor infiltrating lymphocytes (TILs) or T cells engineered to express chimeric antigen receptors (CARs), our increased knowledge on DC biology, and the recent remarkable breakthroughs with checkpoint blockade, the field of tumor immunotherapy is clearly advancing. With these advances and the realization that personalized treatment holds the key to the future for anti-cancer therapy, the use of patient-derived whole tumor cells as a source of tumor or leukemia associated antigens (TAA or LAA) warrants more intensive clinical exploitation. Acute myeloid leukemia (AML) is particularly attractive in this regard, as tumor cells are often readily accessible in the blood. However, loading DC with e.g. apoptotic tumor cells can interfere with DC function due to their generally immunosuppressive nature. It was demonstrated in mice that microvesicles released during apoptosis, i.e. blebs, can induce DC maturation, whereas the larger apoptotic cell remnants (ACR) did not. We therefore set out to explore the use of apoptotic blebs (0.2 μm to 0.6 μm in diameter) as a source of TAA for DC loading, as compared to ACR (2 to 10 μm). Since the isolation of blebs from apoptotic cells requires a specific isolation protocol involving stringent centrifugation steps, it is safe to assume that most studies conducted in the past were using ACR and discarded apoptotic blebs.

**Apoptotic fractions as cell-associated antigen sources: ACR vs blebs**

We have demonstrated in chapter 2 that blebs are more readily ingested by monocyte-derived dendritic cells (MoDC) as compared to ACR. Next to a larger percentage of MoDC that had taken up the antigen, bleb-loaded MoDC also up-regulated CCR7, gained in chemotactic reactivity towards the lymphoid homing chemokine CCL19, and possibly retained a higher level of motility after ingesting blebs as a consequence of their smaller size as compared to ACR. Next to an increase in migration, bleb-loaded MoDC also induced TH1 skewing in an MLR and a higher antigen-specific CD8+ T cell outgrowth with higher antigen-specific functional avidity, as compared to ACR-loaded MoDC. It remains elusive what the exact underlying mechanism is that drives the observed increase in homing and priming ability of bleb-loaded MoDC. It might be explained by an increase in activation state of these DC, but this was not reflected by a detectable increase in expression levels of co-stimulatory molecules, other activation markers, or T cell driving cytokines. In any case, our data do show that blebs are a more potent source of antigen as compared to ACR, in terms of DC functionality and subsequent anti-tumor T cell activation. It would be of interest to next analyze the vaccination and anti-tumor efficacy of bleb versus ACR loaded DC in an animal model.
MoDC types

Although we have shown that MoDC cultured in the presence of GM-CSF and IL-4 take up blebs very efficiently and are potent primers of CD8$^+$ T cells, it has been suggested that monocytes differentiated in the presence of GM-CSF and IFN$\alpha$ have an increased capacity to cross-present antigen. Comparative studies between MoDC that were differentiated in the presence of these different cytokine cocktails were previously conducted, however, none have so far used the commonly accepted differentiation protocols, i.e. a 5-day protocol for GM-CSF/IL-4 MoDC (IL-4 MoDC), and a 3-day differentiation protocol for GM-CSF/IFN$\alpha$ (IFN$\alpha$ MoDC), and tested different antigen sources. As IFN$\alpha$ MoDC were described to be more potent in cross-presentation, in chapter 3 we evaluated in vitro whether IL-4 or IFN$\alpha$ MoDC were preferred vehicles for bleb-based vaccine delivery. We observed little differences based on phenotype and cytokine production between the two MoDC types, and what little differences there were, completely disappeared following maturation of the DC subsets. Clear differences were observed when we analyzed the phagocytic capacity of both subsets, as IL-4 MoDC were more potent in the uptake of both soluble antigen and apoptotic blebs, which could not be explained by a higher expression of receptors possibly involved in antigen uptake (CD36, Lox-1, CR3 and CR4) or cross-presentation (Clec9a). In an MLR, IL-4 MoDC were also more potent in inducing CD4$^+$ T cell proliferation, which could not be ascribed solely to the increased uptake of blebs. The most profound differences were observed in the ability to cross-present long peptides or prime antigen specific CD8$^+$ T cells using blebs as a source of TAA. In agreement with earlier reports that IFN$\alpha$ MoDC were more potent in cross-presentation, our data revealed that IFN$\alpha$ MoDC indeed were more potent in the cross-presentation of soluble antigen. When peptides were exogenously loaded on the HLA class I molecules, both MoDC types were capable of efficient CD8$^+$ T cell priming. However, our priming analysis clearly showed that IL-4 MoDC were superior in cross-priming CD8$^+$ T cells against bleb-derived antigen as compared to IFN$\alpha$ MoDC. This may be due to their superior ability to take up blebs. These differences in endocytic capacity have consequences for applying either MoDC type in DC-based vaccination strategies; IL-4 MoDC would be preferential for use in combination with TAA derived from whole-cell tumor preparations, whereas long-peptide based DC vaccination efficacy may be enhanced by using IFN$\alpha$ MoDC.

Cell line model

Although there is increasing interest in the use of IFN$\alpha$-driven MoDC for tumor immunotherapeutic purposes, mainly due to their perceived superior ability for CTL cross-priming, currently no cell line model has been described for studying IFN$\alpha$-driven
DC differentiation. We have previously described that the human MUTZ-3 AML cell line can be differentiated into MUTZ-DC using a rapid 3-day culture protocol, driven by GM-CSF, IL-4, TNFα and low-dose mitoxantrone\textsuperscript{11}. These MUTZ-DC are now commonly used as a model for IL-4 driven DC differentiation (or TGFβ-driven Langerhans Cell (LC) differentiation), but might also be useful for studying IFNα DC development. We therefore analyzed the use of the MUTZ-3 cell line in chapter 4, as a progenitor for inducing IFNα MUTZ-DC differentiation. Our data indeed show that IFNα-induced MUTZ-3 DC display all the classic DC characteristics, such as e.g. marker expression profile (e.g. CD40, CD83, CCR7) and have a characteristic DC-like morphology. Moreover, matured IFNα MUTZ-DC migrated in response to CCL19, were capable of taking up both soluble and cell-derived antigens, and to cross-prime CD8\(^{+}\) T cells. The MUTZ3 cell line thus offers a useful, standardized and sustainable model to further dissect in detail IFNα induced DC differentiation.

Targeting cutaneous DC subsets: intradermal delivery of apoptotic blebs or ACR

As an alternative strategy to tumor vaccination with \textit{ex vivo} generated and antigen-loaded DC, intradermal administration of TAA and the subsequent in situ loading of skin-resident DC is an attractive strategy, since \textit{ex vivo} loading of DC and subsequent administration of the vaccine to the patient is both laborious and costly. Moreover, whereas only a small percentage of injected DC reach the vaccination site draining lymph nodes, \textit{in vivo} administration and DC targeting of antigens relies on the physiological mechanisms in place to ensure immune activation by tissue-resident DC subsets (reviewed in \textsuperscript{12}). In chapter 5 we therefore determined the capacity of skin-resident DC to take up i.d. delivered leukemia-derived ACR or blebs, and to subsequently cross-present associated antigens. To this end we made use of a near-physiological 3D human skin explant model in which we injected leukemic cell line-derived ACR or blebs i.d. Although apoptotic cells are known to modulate DC function\textsuperscript{1–6}, we did not observe clear differences in the number of DC migrating from the explants, nor in their phenotype or functionality, by the i.d. delivery of either apoptotic cell fraction prior to explant culture. Based on \textit{in vivo} T cell responses in multiple clinical trials\textsuperscript{13–19}, it is clear that cell-derived antigen administered in the skin must be taken up by residential antigen presenting cells, however, direct evidence thereof and which human DC cell subsets exert this function is lacking. The data obtained from our study shows that all skin-resident subsets have the ability to ingest injected ACR and blebs. Importantly, co-administering IL-4 and GM-CSF, which serves as a maturation cocktail for skin DC, did not clearly affect the uptake of ACR, whereas the uptake of blebs by CD1a\(^{+}\)CD14\(^{-}\), CD1a\(^{+}\)CD14\(^{+}\) and CD1a CD14\(^{+}\) dermal DC
(DDC) was significantly enhanced. Our data further show that blebs are ingested more efficiently than ACR by skin DC, in line with the data obtained for MoDC. Interestingly, and supportive of the dogma that macrophage-like cells have an increased ability to ingest exogenous antigen, we showed that CD14+ DDC, previously reported to share many phenotypic, morphological and functional characteristics with macrophages20,21 indeed were most potent in ingesting apoptotic material, which was especially evident with ACR. Of significance is our observation that CD1a+CD14- DDC take up blebs with such efficiency that they constitute almost 15% of all the cells (mainly DC) that migrate from the skin explants. It was shown previously that the CD1a+CD14- DDC subset is the most potent in terms of T cell activation12,21,22. Combined with our finding that administration of blebs in the skin leads to a clear enhancement of the cross-presentation of an associated tumor antigen by skin-emigrating DC (using MART-1 as a model antigen), this argues in favor of the use of tumor/leukemia-derived apoptotic blebs as a skin DC-targeted vaccine.

**LAA-specific CD8+ T cell priming in AML patients**

Optimization of DC-based vaccines, LAA sources and routes of administration of the vaccine are essential. However, it is obviously important to also take the patient’s T cell compartment into account, since it is known that the immune system is (temporarily) impaired following chemotherapy23. Whether CD8+ T cell can be primed for LAA following chemotherapy remained unclear and we therefore set out to determine the ability to prime LAA-specific CD8+ T cells from healthy donors, and AML patients in short-term (<6 months) and long-term (>1.5 years) complete remission (CR), as described in chapter 6. Wilms’ tumor 1 (WT1)-specific and preferentially expressed antigen of melanoma (PRAME)-specific CD8+ T cells could be primed in respectively 40% and 75% healthy donors (HD). In contrast, we were unable to prime WT-1 or PRAME-specific CD8+ T cells in patients with short-term CR, which was likely the result of chemotherapy induced leukopenia or an overall tolerogenic state. Despite being unable to prime PRAME-specific CD8+ T cells in both short- and long-term CR patients, we were able to prime WT-1-specific CD8+ T cells in 1 AML patient with long-term CR. In addition, we did show that a PRAME-specific CD8+ T cell clone primed in HD, recognized and lysed both target cell lines and primary AML cells in an antigen-specific and HLA-I-restricted manner. Taken together, our data suggest that T cell priming in AML patients that underwent chemotherapy is severely impaired, especially in patients that underwent recent chemotherapy (<6 months). However, engineered T cell therapies, e.g. T cell receptor transfer or chimeric antigen receptor modification, hold promise in AML patients and should be further investigated. Importantly, patients that have received a hematopoietic stem cell transplantation could still be eligible for DC-vaccination strategies, especially in combination with
donor-lymphocyte infusions (DLI). In addition, the T cell compartment of patients in early stages of AML or with pre-malignant disease (Myeloid Dysplastic Syndrome [MDS]) might be less affected and these patients might thus be more amenable to vaccination-based therapies.

**Towards an optimized apoptotic bleb-based tumor vaccine**

In this dissertation we have analyzed the use of apoptotic microvesicles (blebs) as a source of TAA for loading DC, as well as determined the most potent human DC subtypes capable of taking up and processing blebs for subsequent T cell activation: we have made head-to-head comparisons between IL-4 and IFNα MoDC, between IL-4 and IFNα MUTZ-DC and between the different skin-resident DC subsets. In all DC subsets and model systems that we tested, blebs were more efficiently ingested, and DC loaded with blebs induced superior CD8⁺ T cell responses in terms of priming efficiency and CD8⁺ T cell avidity, as compared to the ACR that were more commonly used in previous studies. Our data thus suggest that blebs are a potent source of TAA in the context of a whole tumor-based DC vaccination strategy.

A downside of using blebs is their limited availability in patients with (metastasized) solid tumors, as a large number of tumor cells would be required to obtain sufficient numbers of blebs for vaccination purposes. To circumvent this issue, a “bleb bank” could be generated as an alternative for patient-derived blebs, by isolating blebs from different cell lines and/or primary cells of the same cancer type that the patient is treated for, and administering this allogeneic bleb mixture (allo-bleb) to the patient. By carefully selecting different cell lines, numerous TAA may be included in the vaccine preparation, which, importantly, would not be HLA-restricted. Beside containing TAA, (allo-)blebs contain the outer membrane of the apoptotic cell they are derived from, and allo-blebs can therefore provide local immune stimulation and adjuvanticity due to allo-recognition of deviating HLA:peptide complexes, which is likely beneficial for the subsequent recruitment of immune cells and induction of an efficient immune response. Allo-blebs would moreover be a well-characterized and standardized product, which can be produced and handled under cGMP conditions very easily, as microvesicles are resistant to freezing induced damage, and, in case of direct intradermal administration, can be injected in a low volume as a single injection. However, a big advantage of the use of autologous tumor-derived blebs remains the content of patient-specific TAA.

Several improvements can be envisioned to an (allo-)bleb vaccine, e.g. 1. guiding their uptake, 2. increasing their immunogenicity, and 3. altering their antigen content. 1. Blebs have been shown to have a reduced number of sialic acid residues on their surface, which acts as an eat-me signal. Altering the glycosylation of blebs could enhance their
uptake in general, or by specific DC subsets. 2. Increasing the immunogenicity of blebs can enhance subsequent antigen presentation and T cell activation. Previously, it was described that especially anthracyclines can induce immunogenic apoptosis, by the translocation of endoplasmic reticulum (ER)-resident proteins, e.g. calreticulin, to the cell surface. We have generated blebs using the anthracycline drug mitoxantrone, which indeed increased co-stimulatory molecule expression and inflammatory cytokine production by MoDC (unpublished data). However, subsequent T cell responses where completely abolished, likely through the leakage of mitoxantrone from MoDC that had ingested blebs. These data do suggest that blebs can be generated which are more immunogenic, but the anthracycline concentration used for the induction of apoptosis needs to be addressed. 3. Recently, it has been described that the defective ribosomal product (DRiPs) content of autophagosomal preparations is efficiently cross-presented.

It will be interesting to determine whether proteasome inhibition would lead to translocation of DRiPs into blebs and whether this would increase their immunogenicity. It remains to be determined what the minimally required bleb dose would be in order for an autologous bleb-based vaccine to be efficacious. As few DC are capable of activating a multitude of T cells, in theory, only a handful of DC need to reach the secondary lymphoid organs (SLO), in order to orchestrate an anti-tumor immune response. Therefore, a relatively small number of blebs would likely suffice to induce an efficient immune response. Likely to be of greater importance is the nature of the co-administered adjuvants, as they induce DC maturation, enhance DC migration and survival, and can enhance antigen presentation, as well as shape the subsequent immune responses towards effective antitumor immunity (through TH1 skewing). Moreover, knowledge from the field of leukocyte trafficking has taught us that DC transfer information on tissue localization to T cells, and that tissue-resident immune cells are imprinted with a tissue-specific signature. These data argue in favor of the delivery of blebs, or DC loaded with blebs, in the proximity of the tumor and its draining lymph nodes, to ensure proper imprinting of the ensuing T cell response.

A prerequisite for the efficacy of bleb-based vaccines is a properly functioning immune system. Since we showed that T cell priming is impaired in AML patients in short-term CR, and to a lesser extent in long-term CR, active immunization is likely to be severely impaired in these patients. It would be of interest to determine whether LAA-specific (memory) CD8+ T cells can be primed/expanded from the bone marrow compartment, since the bone marrow is a reservoir for memory T cells and it has been described that especially memory T cells can survive chemotherapy in the bone marrow. Moreover, it should be explored whether additional checkpoint blockade can enhance T cell activation/priming by bleb-loaded DC, especially in AML patients. Although using checkpoint blockade as a monotherapy might be insufficient in AML patients, due to the
low mutation rate of AML\textsuperscript{35}, it will be of interest to determine whether T cell priming by bleb-loaded DC can be achieved in AML patients in long-term (as well as short-term) CR in combination with checkpoint blockade (CTLA-4 or PD-1). Nevertheless, from our own clinical experience (and that of others) the use of (allo-)blebs (as well as other vaccines depending on active immunization) in AML patients will remain difficult\textsuperscript{36}. In contrast, patients that have received hematopoietic stem cell transplantation are more likely to respond to active immunization, especially in combination with DLI. In this setting, it is of importance to gain more insight in the effect of immunosuppressive drugs on \textit{in vitro} T cell priming or activation by bleb-loaded DC. Preferentially, vaccination should be attempted when patients are off immunosuppressive regimens. In addition, direct intradermal administration of (allo-)blebs would be preferential compared to \textit{ex vivo} loaded DC, as this radically reduces the costs for treatment and we have shown blebs to be ingested very efficiently by skin-resident DC. Moreover, co-administration of IL-4 and GM-SCF should enhance (allo-)bleb uptake by stimulatory CD1a\textsuperscript{+}CD14\textsuperscript{-} dermal DC and facilitate the recruitment of other immune cells. An additional benefit of direct administration of (allo-)blebs is that clinical translation should be relatively easy, since the vaccine can be irradiated to the extreme, in order to prevent any viable cells to remain in the preparation and its production would not involve the logistically challenging and costly \textit{ex vivo} generation of (autologous) DC. A small-scale phase I clinical trial should provide more insight in the safety of the (allo-)bleb vaccine itself. When proven safe, it will be of great interest to start an add-on phase I study to introduce additional checkpoint blockade in order to increase the chance of inducing a potent immune response. A small scale clinical study has already demonstrated that using CTLA-4 blockade (Ipilimumab) following HSCT does not increase graft-versus-host-disease\textsuperscript{37}, and should therefore be tested in combination with active vaccination.

\textbf{Future perspectives: towards an integrated immunotherapy for AML}

Before any immunotherapy (bleb-based or otherwise) which aims at targeting AML blasts or, more particularly, the LIC, can be successful, it is likely that we will need to address the immune suppressive microenvironment in the bone marrow. AML-LIC are suggested to localize in the stem cell niches of the bone marrow, since bi-directional interactions of LIC with bone marrow stroma can induce quiescence of the LIC resulting in chemotherapy resistance\textsuperscript{38-40}, and can hamper immune responses as well. In order to break LIC quiescence, promoting the progression of LIC proliferation by targeting the F-box protein Fbxw7, a protein essentially involved in the c-Myc dependent regulation of cell proliferation, could increase LIC susceptibility to immune targeting\textsuperscript{41}. In order for LIC to take refuge in stem cell niches, it is essential to interact with the bone marrow stroma...
Summary, general discussion and future perspectives

for adhesion and survival. The CXCR4/stromal cell-derived factor-1 (SDF-1/CXCL12) axis has been described to be essentially involved in homing of HSC and LIC to the bone marrow. Disrupting the binding of CXCR4, expressed on LIC, by antagonistic antibodies, could potentiate immunotherapy aimed at targeting LIC, by making them more accessible to immune effector cells and keeping them away from the tumor-conditioned immune suppressive bone marrow microenvironment. Moreover, interaction of the very late antigen (VLA)-4 integrin on leukemic cells and fibronectin expressed on stromal cells, has been described to induce resistance to drug-induced apoptosis. Importantly, disruption of VLA-4 binding by blocking antibodies (combined with chemotherapy) induced a 100% survival rate in a murine AML model. No clinical trials aiming at disruption of integrin binding in AML as a complementary strategy have been conducted thus far. Blocking integrins using Cilengitide (αVβ5 and αVβ3) in glioblastoma patients has demonstrated to significantly improve the median survival, compared to historical controls and a comparable control study. These data demonstrate the importance of modulating the interaction(s) of LIC with bone marrow stroma, and is likely to be essential in enhancing the efficacy of LIC-targeted immunotherapy.

Although a substantial amount of research has demonstrated the existence of the LIC within the CD34+/CD38 - compartment, it will be essential to gain more insight into the LIC phenotype in order to increase the number of potential targets. Due to the selective expression of proteins in LIC, it could be instrumental to isolate this subpopulation from patients in order to generate a vaccine. It might well be that microvesicle-based vaccines (like blebs) become the giants of whole tumor cell vaccination, as these protein sources are more efficiently cross-presented and contain an array of LAA. It will be of interest to determine whether bleb-based vaccines derived from either AML blast cells or LIC have differential effects on OS. However, using solely a whole cell preparation will not suffice, due to the ability of cancer cells to de-differentiate and the presence of increased regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC). In that context, anti-TNFα antibodies could enhance therapy, since this cytokine is essentially involved in both the arrest of differentiation of MDSC and tumor de-differentiation. Additionally, inhibiting Tregs using cyclophosphamide can enhance vaccination efficacy. Targeting the tumor microenvironment is increasingly acknowledged as an essential step for eradicating the tumor, as its survival relies on interactions with its microenvironment. Lenalidomide (also applied in AML) has been shown to alter the myeloid microenvironment in CLL, and could shape the bone marrow environment in such a way that LIC become more susceptible to immune targeting. The selective expression of TIM-3 on LIC, and its involvement in engraftment, could argue for using TIM-3-targeting antibodies during vaccination. An additional benefit being that TIM-3 expressing antigen-specific T cells are described to be dysfunctional, and blocking
CHAPTER 7

TIM-3-TIM-3L interactions improves T cell function. Additional targeting of immune checkpoint molecules (e.g. CTLA-4, PD-1) is highly efficacious and has been shown to be safely applicable in the combination with an allogeneic whole cell GVAX vaccine. Whether such a strategy can be additionally complemented with antibodies that boost T cell responses (such as OX40), remains to be determined, but it will be necessary to release all regulatory brakes that have been pushed by the cancer, in order for its efficient eradication (figure 1). We are on the verge of unleashing the full potential of the immune system on AML by the use of enhanced whole-cell vaccines, and it is to be expected that

Figure 1: Enhancing the targeting of Leukemia Initiating Cells (LIC) by an integrated bleb-based immunotherapy approach. Eradicating AML requires the targeting of the LIC. Using the LIC as a whole (apoptotic) tumor cell source for the preparation of a bleb-based vaccine is likely to enhance the efficacy of AML-based vaccines. After intradermal injection, the bleb vaccine is taken up by APC and the antigen is presented to T cells recognizing their cognate antigen in the lymph nodes. The activation of LIC-specific T cells can be boosted by providing an agonistic antibody directed against OX40. T cell functions can be down-modulated by the release of suppressive factors by AML and the presence of suppressor cells (Tregs & MDSC). Inhibiting Tregs (using cyclophosphamide) and MDSC (possibly using anti-TNFα antibodies) can enhance leukemia-specific T cell function. Moreover, antagonistic antibodies directed against CTLA-4, PD-1 and TIM-3 can enhance T cell function. An additional beneficial effect of targeting the latter is its expression on LIC, which can potentially further enhance the efficacy of this proposed integrated immunotherapy.
we will see clinical trials initiated in the near future, pushing back the boundaries of overall survival faster than we have seen in the last two decades.

**Concluding Remarks**

In summary, in this dissertation we have studied the possible utility of apoptotic blebs as a source of TAA/LAA in anti-tumor/leukemia vaccination, and studied which DC type is best suited to be targeted for the delivery of blebs. Although the use of blebs as a whole tumor-based vaccine has clear beneficial effects, many challenges remain to prepare the most optimal DC vaccine in terms of adjuvants, and technical and physiological homing characteristics of the administered vaccine. In the past few years, interest has increased dramatically to study the role of microvesicles (blebs, exosomes, DRibbles, etc.) in cell communication and their use as vehicles of TAA for anti-cancer therapies. These microvesicles could well become the new giants in immunotherapy.

**REFERENCES**


Summary, general discussion and future perspectives


