Summarizing Discussion and Future Prospects:

Partly adapted from:
Dendritic cell plasticity in tumor-conditioned skin: CD14+ cells at the cross-roads of immune activation and suppression

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Front Immunol. 2013 Nov
25;4:403.
Summary

Tumors abuse myeloid plasticity to re-direct dendritic cell (DC) differentiation from T cell stimulatory subsets to immune suppressive subsets that can interfere with antitumor immunity. Lined by a dense network of easily accessible DC the skin is a preferred site for the delivery of DC-targeted vaccines. Various groups have recently been focusing on functional aspects of DC subsets in the skin and how these may be affected by tumor-derived suppressive factors. IL-6, Prostaglandin-E2 and IL-10 were identified as factors in cultures of primary human tumors responsible for the inhibited development and activation of monocyte-derived DC. IL-10 was found to be uniquely able to convert fully developed DC to immature macrophage-like cells with functional M2 characteristics in a physiologically highly relevant skin explant model in which the phenotypic and functional traits of “crawl-out” DC were studied. Mostly from mouse studies, the JAK2/STAT3 signaling pathway has emerged as a “master switch” of tumor-induced immune suppression. Our lab has additionally identified p38-MAPK as an important signaling element in human DC suppression, and recently validated it as such in ex vivo cultures of single-cell suspensions from melanoma metastases. Through the identification of molecular mechanisms and signaling events that drive myeloid immune suppression in human tumors, more effective DC-targeted cancer vaccines may be designed.

Dendritic cell subsets and their plasticity in human skin: impact on cancer vaccination

Skin is the largest human organ and its direct contact with the outside environment requires tightly regulated surveillance mechanisms to keep potentially harmful intruders at bay. For this purpose, human skin is densely populated with patrolling myeloid cells, such as Langerhans cells (LC) in the epidermal outer layer and various dermal dendritic cell (DDC) subsets and macrophages in the dermal layer 1,2. It has been elegantly shown that different profiles of pattern recognition receptors present on the various myeloid subsets lining the skin makes them exquisitely specific in the recognition, uptake and either direct elimination of pathogenic microbes, or in presentation of pathogen-associated antigens for subsequent activation of the adaptive immune system 3-5. Interaction of a pathogen with pathogen-recognition receptors on DC induces activation of downstream signaling pathways that result in their enhanced ability to process and present pathogenic antigens and in their migration to the draining lymph nodes, accompanied by phenotypic and morphological maturation, and priming of antigen-specific T or B lymphocytes 6. Whereas initially studies concerning DC subsets in human skin mostly involved the most predominant subsets, i.e. CD1a+Langerin+ LC, CD1a+ DDC and CD14+CD1a- DDC 7-9, the characterization of new surface markers and deeper phenotypic and functional analyses now show that further distinctions can be made 10-13. From our own work and that of others, it has become clear that beside epidermal LC and dermal macrophages at least five migratory DDC subsets can be distinguished 13,14, i.e. CD1a+CD14- DDC, CD1a+CD14+ DDC, CD1a-CD14+ DDC, and two double negative subsets. An important issue that as yet remains unresolved is whether all these DC populations represent genuine subsets, or whether they are part of the same DC subset in various states of activation or
differentiation. A growing number of studies now point to the existence of an inter-related population of cutaneous DC and macrophages in flux, trans-differentiating into each other as directed by environmental cues. This has direct consequences for the type of immune responses that will ensue, as different migratory DC sub-populations have now been directly linked to the induction of different types of immunity and have different capacities to cross-present antigens for the activation of cytotoxic CD8 T cells, a process crucial for the induction of anti-tumor immunity. Roughly, CD1a+ mature LC and DDC subsets have been linked to type-1 T cell mediated immunity, whereas CD14+ immature DDC subsets have been linked to the induction of humoral immunity and expansion of regulatory T cells (Treg); see Figure 1 for a schematic overview. Recent evidence suggests that tumors like melanoma abuse the balance between these subsets to effectively escape immune recognition. In order for DC-targeted vaccines delivered through the skin to be effective, tumor-induced immune suppression should be overcome and T cell-stimulatory DC subsets selectively targeted. Here, we discuss mechanisms of tumor-imposed DC suppression in the skin microenvironment and how these may be counteracted in aid of DC-based immunotherapy.

**LC and CD1a+ DDC: T cell activation**

Klechevsky et al. first described a functional dichotomy between human LC and CD14+ DDC with the former preferentially activating CD8+ T cells and the latter B cells. In recent publications primary human LC have been shown to be superior inducers of Th22 cells (including conventional variant αβ-T cells restricted through CD1a) . IL-22 has an important
barrier function in homeostasis and safeguards the integrity of epithelial layers, but is also involved in pathological skin conditions like psoriasis. Furio et al. reported a superior ability of migratory LC over DDC to induce either Th1 or Th2 responses 20. Of note, DDC in this report consisted of CD1a-CD14- double-negative DDC with a potentially lower capacity for T cell activation than CD1a+ DDC. Mathers and co-workers showed that while LC were superior Th17 inducers, human CD14+ DDC had the ability to skew Th cells to either a Th1, Th2, or Th17 profile, depending on their environmental conditioning, number, and activation state 21. To further delineate T cell-stimulatory properties of freshly isolated human LC vs. CD1a+ DDC, we undertook a genome-wide transcriptional profiling analysis which revealed CD1a+ DDC to express a far wider range of adhesion and co-stimulatory molecules, chemokines, and cytokines (and at higher levels), pointing to a putatively superior migratory and T cell stimulatory ability over LC in steady state conditions 22. Using a human cell line model of LC and CD1a+ DDC differentiation, we confirmed these data and showed DDC to be superior activators of cytotoxic CD8+ T cells. Importantly, this was validated in the same study by a comparative assessment of the ex vivo ability of human skin-emigrated LC vs. DDC subsets to prime HLA-A2-matched CD8+ T cells against an epitope derived from the MART-1 melanoma antigen 23. While LC and CD1a+ DDC were equally effective in priming allogeneic Th cells, DDC primed significantly higher rates of MART-1 recognizing CD8+ T cells at a higher functional avidity. Of note, Banchereau et al. have recently linked the superior effector CD8+ T cell priming capacity of LC and CD1a+ DDC to their release of IL-15 into the immunological synapse 10.

CD14+ DDC: T cell tolerization

CD14+ migratory DDC are discernible from dermis-resident CD14+ dermal macrophages through their surface expression of CD1b and CD1c 24. In a comparative analysis with CD14- DDC, CD14+ DDC were shown to be poor inducers of allogeneic T cells and to require high DC:T cell ratios for Th1 induction 25. This relative inability of CD14+ DDC to induce Th1 cells was related to their release of IL-10 and TGFβ1. We and others have found CD14+ DC to carry low levels of co-stimulatory molecules, to display a poor T cell priming capacity, and to be characterized by the expression of CD141/BDCA3 (Thrombomodulin), a marker that has been linked to a human DC subset with cross-priming ability 11,13,26. These CD14+BDCA3+ migratory DDC in a report by Chu et al. were shown to constitutively release IL-10 and to induce T cell hypo-responsiveness and Tregs 11. Moreover, they were able to cross-present self-antigens and inhibit skin inflammation in an in vivo transplantation model. These data point to an important role for this subset in T cell homeostasis. Banchereau et al. have pin-pointed the inability of CD14+ DDC to prime effector CD8+ T cells to their release of IL-10 and TGFβ 10 and the expression of Ig-like transcript 4 (ILT4) and ILT2 27.

This thesis: intermezzo 1

I. Tumor-related DC suppression in the skin environment

Of all known immune suppressive cytokines IL-10 is the only one which has been reported to block DC maturation 28-32. In Chapters 2 and 6 we therefore investigated the effects of IL-10
on DC maturation from human skin and MoDC, respectively. In Chapter 2 we characterized different migratory DC subsets from human skin and studied how their migration and T cell-stimulatory abilities were affected by conditioning of the dermal microenvironment by a panel of cancer-related suppressive cytokines. Our group previously showed the effects of the cancer-associated suppressive cytokine IL-10 on the phenotype and allogeneic T cell priming ability of human skin-emigrated DC. In this study we more extensively profiled various DDC subsets and studied the effects of cancer-related and cytokine-mediated conditioning of the human dermis on the subset composition of the skin-emigrating DC population as well as consequences for the induction and skewing of CD4+ and CD8+ T cell differentiation. Our findings show the unique ability of IL-10 to affect DC migration, leading to shifts in migration of DC subsets which clearly impact the induction of both functional Th and CD8+ T cells. The 48h-migrated DC from skin explants taken from breast tumor-overlying skin, showed similar low frequencies of mature CD1a+ migratory DC and high frequencies of immature CD14+ subsets as observed subsequent to IL-10 conditioning. In a comparative analysis with CD14- DDC, CD14+ DDC were previously shown to be poor inducers of allogeneic T cells and to require high DC:T cell ratios for Th1 induction. In this study we showed that dermal conditioning by IL-10 increased B7-H1/PD-L1 expression levels on all migratory DDC subsets and led to high IL-10 release levels upon CD40L-mediated stimulation. This resulted in Th2 skewing and induction of IL-10 transcription and release upon co-culture with CD4+CD25+ T cells, and an inability to prime CD8+ effector T cells. In keeping with these observations, Banchereau et al. have shown that IL-10 released by CD14+ DDC is mainly responsible for their inability to prime functional CTL. Moreover, CD14+ DDC from human skin have been shown to induce regulatory T cells (Treg). Our data are in line with these findings, demonstrating both superior expansion of CD4+CD25hiFoxP3+ Tregs from allogeneic monocyte-depleted peripheral blood lymphocytes and conversion of CD4+CD25hiFoxP3+ Tregs from CD25+ Th cells by predominant CD14+ DDC migrated from IL-10 conditioned skin. Clearly, this tumor-modulated capacity of migratory skin DC subsets to induce effector T cell responses may negatively impact DC-targeted tumor vaccine approaches. DC-targeted tumor vaccines delivered through the skin should therefore incorporate compounds aimed at overcoming tumor-related DC suppression and ensuring predominant migration of mature LC and DDC subsets equipped to induce anti-tumor effector T cells without collateral Th2 and Treg induction. Autologous tumor cell-based vaccines provide a wide range of relevant tumor antigens and personalized neo-epitopes based on individual tumors’ unique antigenic mutanome signatures. However, tumor-derived factors may hamper in situ maturation of DC and thus interfere with the generation of effective anti-tumor immunity. As the skin is a preferred site for tumor vaccine delivery, in Chapter 3 we investigated the influence of primary colon carcinoma-derived soluble factors on the maturation state of migrating DC in a human skin explant model. Perhaps surprisingly, primary tumor-derived supernatants (TDSN) enhanced the phenotypic maturation state of skin-emigrated DC, resulting in an increased T cell priming ability. In the case of monocyte-derived DC (MoDC) a similar TDSN-induced maturation induction was found to entirely depend on cyclooxygenase (COX)-regulated prostaglandins, in line with
previous observations for MoDC differentiation. In contrast, the increase in skin-emigrated DC maturation was completely prostaglandin-independent, as evidenced by the inability of the COX inhibitor indomethacin to abrogate this TDSN-induced effect. Although TDSN conditioning effected a drop in IL-12p70 release by the skin-emigrated DC and induced a predominant Th17/Th22 transcriptional profile in subsequently stimulated T cells, Th cell subset differentiation, as assessed by intracellular cytokine expression upon polyclonal priming and re-stimulation, was not affected. Comparative analysis of phenotypic DC profiles and transcriptional profiles of primed Th cells suggests that the observed maturational effects in skin-derived DC may have been induced by tumor-derived GM-CSF. This study indicates that soluble factors derived from whole-cell colon tumor vaccines will not negatively impact DC migration and maturation in human skin, but rather induce DC maturation that will facilitate the priming of a poly-functional Th cell response. This bodes well for whole-cell tumor vaccines delivered to patients through intradermal injection.

Tumors abuse DC plasticity to undermine immunity: a central role for CD14+ DC

A large number of studies attest to the remarkable plasticity of the myeloid lineage; tumors abuse this phenotypic plasticity to re-direct myeloid differentiation towards the development of immune suppressive subsets that effectively interfere with anti tumor immunity. Consequently, tumors are often characterized by an infiltrate of immature macrophage-like cells and a lack of infiltrating DC, which is generally a poor prognostic sign. We and others have shown that DC differentiation from monocytes can be blocked by tumor-derived soluble factors (most notably IL-10, IL-6 or PGE2) resulting in the development of CD14+ macrophage-like cells with poor T cell stimulatory abilities (so-called M2-type macrophages) and with T cell suppressive activity (Figure 2). Beside monocytes, fully differentiated DC can be recruited to the tumor microenvironment, where they may lose their characteristic CD1a expression through the suppressive action of IL-10, as shown for melanoma metastases. A growing number of studies indicates the unique ability of tumor-associated IL-10 to convert even fully differentiated DC to CD14+ suppressive macrophage-like cells, IL-10 is generally expressed at high levels in the microenvironment of metastatic melanoma and can either be directly derived from tumor cells or from infiltrating immune cells. Among a panel of tumor-associated suppressive factors, we found IL-10 uniquely able to convert DC to immature macrophage-like cells in two human model systems: 1) a physiologically highly relevant skin explant model in which we studied the phenotypic and functional traits of “crawl-out” myeloid cells and 2) an in vitro model of tumor-conditioned DC maturation in which we functionally assessed CD14+ and CD14+ DC that had developed from monocyte-derived DC (MoDC) during IL-10-exposed maturation. In all above mentioned cases the tumor-induced M2-like cells shared some striking traits: an immature CD14+BDCA3+DC-SIGN+CD16+ phenotype and macrophage-like morphology (Figure 2), a disturbed balance in the release of immunosuppressive IL-10 (high) vs. immunostimulatory IL-12p70 (low), high expression levels of the T cell-inhibitory molecule B7-H1/PDL-1, and lower priming efficiency of allogeneic Th cells and of CD8+ (killer) T cells, the latter specifically recognizing the melanoma antigen MART-1, but binding epitope/MHC
complexes with low avidity. In studies assessing CD1a and CD14 expression on DC from human skin explants, we showed the intracutaneous cytokine balance to be important for the subset composition of migrated DC. Indeed, we have found compelling evidence that LC and CD1a+ DDC can actually trans-differentiate during and after migration from human skin explants to a CD14+ macrophage-like state in an IL-10-dependent fashion. Dermal conditioning by IL-10 or by topical application of irritants resulted in a shift among migrated DC from a mature CD83+CD1a+ state to an immature CD83-CD14+ macrophage-like state, passing through a CD1a+CD14+ intermediate stage. Based on the fact that these CD14+ cells also expressed CD1c they were classified as DC rather than macrophages. Moreover, topical application of irritants to epidermal sheets showed that trans-differentiation from LC to macrophage-like DC depended on the presence of dermal fibroblasts and could be blocked by IL-10 neutralizing antibodies. A similar observation has been described in mice, where the presence of a subcutaneous tumor resulted in a DC-to-macrophage shift, with macrophage-like cells producing immune-suppressive factors such as IL-10, iNOS and Arginase. Importantly, this trans-differentiation among DC that had migrated from human skin was preventable by co-injection of the DC-activating cytokines GM-CSF and/or IL-4 prior to skin explant culture. Consistent with their expression of the M2-macrophage marker CD163, IL-10-converted CD14+ cells induced IL-10 and FoxP3 mRNA expression in allogeneic Th cells as well as a Th2-like cytokine profile and Treg expansion. Consistent with these tolerogenic qualities,
IL-10-induced CD14+ macrophage-like MoDC expressed high levels of immune suppression-related transcripts such as Indoleamine 2,3-dioxygenase (IDO), IL-4Rα, IL-6R, TGFβ1, HIF1α, and VEGFA. Activation of a HIF1α transcriptional signature has been reported in tumor-associated macrophages, even under normoxic conditions. This is in line with the transcriptional and cytokine release profiles we observed for CD14+ IL-10-conditioned DC, which revealed coordinated expression of HIF1α, TGFβ, VEGFA, MMP3, MMP9, IL-8 and TNFα, all of which can contribute to such tumor-promoting processes as endothelial cell migration and proliferation and tumor growth and invasion. In conclusion, tumor-related suppressive factors can divert DC during differentiation and even during and after maturation towards a macrophage-like state with immune-suppressive and pro-angiogenic and pro-tumor invasive properties (Figure 2). Interestingly, in DC migrating from human skin, BDCA3 and DC-SIGN expression levels showed a very significant inverse correlation with CD83 maturation marker expression, indicating the utility of these markers for the identification of immature DC. Indeed, they marked CD14+ skin-emigrated DC as the least mature population with poor co-stimulatory properties. In keeping with these observations, DC that had migrated from skin explants taken from breast cancer mastectomy specimens, predominantly consisted of the CD14+ DC subset with a macrophage-like morphology. Normalized distribution (i.e. more mature and less immature DC subsets) was observed for explants taken from patients that had received neoadjuvant chemotherapy, a clear indication that prevailing migration of the immature CD14+ subset was tumor-related. From our observations we conclude that combined expression of CD14, BDCA3, DC-SIGN, CD16, PD-L1, and CD163 provides a phenotypic profile useful for the identification of M2 macrophage-like subsets with immune-suppressive and tumor-promoting characteristics that arise during tumor-conditioned differentiation or maturation of human DC. We and others have found evidence of phenotypically similar subsets in breast, colon, head and neck, renal cell, glioma, and melanoma tumors. Indeed, in single-cell suspensions derived from a panel of six metastatic melanoma tumors, we observed by multicolour flow cytometry analysis, that CD14+ cells, co-expressing both DC-SIGN and BDCA3 and detectable in a range of 1-38%, significantly outnumbered CD1a+ DC, which were virtually absent (ranging from 0.05 to 0.1%). BDCA3 expression has recently been reported on skin-derived CD14+ DC that induced inflammation-attenuating Tregs. Combined with its association with cross-presenting DC subsets, this is highly suggestive of cross-tolerizing ability for BDCA3+ DC. As yet, the functional significance of BDCA3/CD141 in either cross-presentation or immune suppression remains largely unclear, but some clues are emerging. Its Lectin-like domain can down-regulate NF-κB and mitogen-activated protein kinase (MAPK) pathways and might thus interfere with DC maturation and drive IL-10 release and Th2 skewing. In keeping with this notion, BDCA3+ blood DC promote Th2 skewing and in vitro generated or skin-derived CD14+BDCA3+ DC release elevated levels of IL-10. In addition, DC-SIGN can negatively impact DC activation resulting in prolonged and increased IL-10 transcription. Both DC-SIGN and BDCA3 may thus contribute to the immune-suppressive activity of tumor-modulated CD14+ cells. Recently, the role of non-coding RNAs or microRNAs (miRNAs) in myeloid cell plasticity and functionality has also been studied. In mice, tumor-associated miRNAs were
found to modulate the survival and longevity of DC, miR-223 was described to negatively regulate and miR-150 to positively regulate the cross-presenting abilities of LC, the TGFβ-associated miR-27a was reported to inhibit DC-mediated differentiation of Th1 and Th17 cells, and in an allergy setting miR-23b was shown to induce tolerogenic DC through inhibition of the Notch1/NF-κB pathway. In man, this field of research remains largely unexplored, though miR-155 was shown to regulate the M1/M2 macrophage balance by targeting the IL13-Receptor α1, thereby reducing M2 polarization.

This thesis: intermezzo 2

II. Targeting DC subsets in the skin and skin-draining lymph nodes

Direct targeting of antigens to stimulatory DC subsets in vivo is an attractive and intensively studied vaccination approach. Unfortunately, so far very few studies have been carried out in human model systems that are representative of complex 3-D tissues. While DDC may easily be targeted through intradermal (i.d.) injection, LC targeting usually involves transcutaneous approaches. Romani and colleagues showed that i.d. injected antibodies directed at the C-type Lectin DEC-205 were able to pass the basal membrane and specifically bind LC in the epidermis, suggesting that even dermal delivery of large proteins can result in targeting of epidermal LC. Upon dermal delivery, depending on their size, vaccine vehicles may thus either be taken up by LC in the epidermis, DC in the dermis, or in fact drain to lymph nodes where they may be taken up by lymph node-resident DC. To increase DC-targeted vaccine efficacy, there is therefore an urgent need for phenotypic and functional characterization of human Dendritic Cell (DC) subsets residing in lymphoid tissues as well as in skin.

Finding the optimal vector for in vivo targeting of DC is obviously important for DC-targeted vaccination strategies. In Chapter 5 we therefore tested a panel of subgroup C/B chimeric and fiber-modified adenoviruses (Ad) for their relative capacity to transduce human DC. We made use of in vitro generated Langerhans Cells (LC) as well as of ex vivo human skin and melanoma-draining lymph node (LN) derived DC. Of the tested viruses the C/B-chimeric Ad5/3 virus most efficiently transduced in vitro generated LC. It was previously reported that Ad5/3 transduced human monocyte-derived DC by binding to the B7 molecules CD80 and CD86. We similarly showed high-efficiency intradermal transduction of mature skin DC to be mediated through binding to CD80/CD86 and not to interfere with subsequent T cell priming. We conclude that Ad5/3, in combination with DC-activating adjuvants, represents a promising therapeutic tool for the in vivo transduction of mature DC, and may be less likely to induce unwanted side effects such as immune tolerance through the infection of non-professional antigen-presenting cells. We previously showed CD40 targeting of Ad5 vectors to enhance selective transduction of human LC and CD1a+ DDC in situ, while simultaneously ensuring their stable maturation and T cell stimulatory capacity after migration from full-thickness skin explants. Similarly, intradermally delivered CD46-binding Ad35 viruses transduced CD1a+ LC and DDC with high efficiency, allowing for specific CD8+ T cell activation after migration from human skin explants. A panel of retargeted Ad vectors with favorable high-efficiency in situ DC-transducing and/or activating abilities will provide an attractive platform for effective in vivo
**DC-targeted prime-boost vaccination schedules allowing for the generation of powerful anti-tumor immunity.**

**Therapeutic activation and targeting of DC in the skin and its lymph catchment area**

Beyond the local suppressive environment at the site of the tumor, the immune suppressive effects of the tumor stretch to draining lymph nodes where anti-tumor T cell responses should be primed. Sentinel lymph nodes (SLN) are the first-line tumor-draining lymph nodes and as such bear the brunt of tumor-induced immune suppression 59. We have identified and characterized four conventional DC subsets in melanoma SLN, two of which were positively identified as skin-derived CD1a+ LC and DDC, and the remaining two (CD1a-CD14+ and CD1a+ CD14+) as LN-resident subsets with varying levels of BDCA3 and DC-SIGN expression 60. Deeper invasion of the primary melanoma in SLN tumor negative patients was related to a reduced activation state of skin-derived DC subsets in the SLN 61,62. Also, lower frequencies of the skin-derived subsets were found in tumor positive SLN as well as a reduced activation state of LN-resident DC subsets (our own unpublished data). These findings indicate a local suppressive effect of the primary tumor on the activation state of skin-derived DC which then migrate to the SLN and lymph node metastasis-related suppression of SLN-resident DC subsets, and are in keeping with tumor-induced conditioning of the microenvironment (skin or SLN, respectively). Moreover, they suggest that primary melanoma-mediated suppression of activation and migration of skin DC enables local metastasis.

In two Phase II clinical trials we have demonstrated that localized intradermal administration of DC-stimulatory agents such as GM-CSF and CpG oligodeoxynucleotides (ODN), i.e. TLR9 ligands, led to increased activation of DC subsets in SLN of melanoma patients and tipped the local cytokine balance in favor of cytotoxic T cell mediated antitumor immunity 63-66. Although in man CpG ODN don’t directly bind to conventional DC, we nevertheless observed maturation induction of conventional DC subsets, most likely through CpG-induced cytokine release by plasmacytoid DC 66. In our human skin explant model we have similarly tested the effects of intradermal delivery of a panel of TLR-ligands on migratory DC and found a unique ability of the respective TLR2 and -3 agonists peptidoglycan (PGN) and polyribosinic-polyribocytidylicacid (Poly I:C) to enhance the T cell–priming ability of skin-emigrated DC, which, in the case of PGN, was accompanied by Th1 polarization 67. Surprisingly only small effects of the tested TLR-ligands on phenotypic DC activation were observed. This may have been due to induced IL-10 release, which might have been counter-acted by simultaneous signalling modulation 68,69. Indeed, evidence for the therapeutic efficacy of combined STAT3 inhibition and CpG ODN was previously provided by Kortylewski and colleagues, showing superior immune stimulatory effects of CpG by eliminating collateral STAT3-mediated suppressive effects 70,71. This observation indicates the potential clinical efficacy of immune therapeutic approaches that mediate immune potentiation on the one hand (e.g. through stimulatory cytokines and TLR-L) and eliminate immune suppression on the other (e.g. through signaling interference). Both may be required to achieve effective tumor vaccination, particularly when the vaccine in question is targeted to DC under tumor-related immune suppressive conditions.
This thesis: intermezzo 3

III. Targeted signaling interference to induce resistance to tumor-related suppression in DC

Velten et al. previously described the development of a population of monocyte-like cells during the in vitro maturation of IL-10-exposed monocyte-derived DC (MoDC), which de novo re-expressed CD14 on the cell surface. Interestingly, these CD14+ cells also expressed BDCA3 (CD141/thrombomodulin), which was absent from monocytes and iDC. BDCA3 has since been linked to DC subsets with particularly powerful cross-priming abilities. In Chapter 6, we investigated and compared the T cell-activating abilities of two DC populations arising upon cytokine-induced MoDC maturation in the presence of IL-10, i.e. CD14+ and CD14- matured DC (mDC). CD14+BDCA3+ DC constitute a physiologically relevant subset that trans-differentiate from human skin-derived CD1a+ DC and Langerhans Cells (LC) during IL-10-conditioned migration and that is present in human breast and melanoma tumors and predominates DC migration from breast tumor-overlying skin (see Chapter 2). Compared to normally matured mDC, both CD14+ and CD14- IL-10-mDC secreted reduced levels of IL-6 and IL-12p70, a feature shared with M2 macrophages. IL-10-induced CD14+ mDC were phenotypically characterized by a low maturation state and high levels of BDCA3 and DC-SIGN and as such closely resembled CD14+ cells infiltrating melanoma metastases. Compared to normally matured DC, CD14+ DC were found to express high surface levels of B7-H1, to more preferentially induce Th2 cells, to have a lower allogeneic Th cell and tumor antigen-specific CD8+ T cell priming capacity, and to induce proliferative T cell anergy. In contrast to their CD14+ counterparts, CD14- monocyte-derived DC retained allogeneic Th priming capacity but induced a functionally anergic state, completely abolishing the release of effector cytokines. This might be related to particularly low CD40 expression by the CD14- mDC. The relatively poor priming efficiency displayed by both CD14+ and CD14- IL-10-mDC of MART-126–35L-specific CD8+ T cells with low avidity indicates that both subsets may be deficient in terms of supporting effective anti-tumor immunity. This is further supported by the increased expression by both subsets of transcripts like IDO, STAT3, TGFβ, VEGFA, IL-4Ra, and IL-6R, all of which have been related to immune-suppressive myeloid subsets. Transcriptional and cytokine release profiling further indicated a more profound angiogenic and pro-invasive signature of the CD14+ DC than normally matured DC or CD14- DC matured in the presence of IL-10. Importantly, STAT3 mRNA interference prevented development of the IL-10-induced CD14+ phenotype, ensuring normal DC maturation, and providing a potential means of therapeutic intervention. As demonstrated in Chapters 2 and 6, the efficacy of DC-targeted tumor vaccines is hampered by high levels of tumor-derived IL-10 which interferes with optimal DC development and maturation. Interference in intracellular signaling cascades downstream from the IL-10 receptor might improve DC development and activation and thus enhance vaccination efficacy. In the studies described in Chapter 7 we therefore performed exploratory functional screens on arrays consisting of >1000 human kinase peptide substrates to map the major pathways involved in DC development and its inhibition by IL-10. The resulting alterations in the kinome profile
pointed to glycogen-synthase kinase-3β (GSK3β) as a pivotal kinase in both DC development and suppression. GSK3β inhibition blocked human DC differentiation both from CD34+ precursors in human MUTZ-3 cell line model and from monocytes, which was reflected by maintained expression of CD14, accompanied by decreased levels of IL-12p70 secretion and a reduced capacity for T cell priming. More importantly, adenoviral transduction of MoDC with a constitutively active form of GSK3β induced resistance to the suppressive effects of IL-10, i.e. prevented trans-differentiation of maturing DC to a CD14+ macrophage-like state. Enforcing GSK3β activity might thus provide a means to bolster the efficacy of DC-targeted vaccines. Indeed, based on these findings and those from Chapters 5 and 6, we propose that DC-targeted Ad vectors carrying genes encoding tumor antigens as well as shRNA targeting STAT3 and/or constitutively active GSK3β, will provide powerful tumor vaccines that can selectively pulse skin-resident or -derived DC with relevant tumor antigens while simultaneously endowing them with resistance to tumor-induced immune suppression.

**Signal transduction pathways acting as master switches of tumor-induced DC suppression: targets for therapeutic intervention**

Tumor-derived suppressive factors bind various receptors on myeloid cells but down-stream signals may converge in shared pathways. Mostly from mouse studies, the JAK2/STAT3 signaling pathway has emerged as a “master switch” of tumor-induced immune suppression 73. We have additionally identified p38-MAPK as an important signaling pathway in human DC suppression, and validated it as such in in vitro DC cultures and in ex vivo cultures of single-cell suspensions from melanoma metastases 38. From a panel of tumor-associated suppressive factors (including PGE2), we found only IL-6 and IL-10 to induce STAT3 phosphorylation during human MoDC development. As we had previously identified prostaglandins as the main culprit of suppressed DC differentiation by supernatants from single-cell suspensions of metastatic melanoma tumors 33 it was not surprising that STAT3 inhibition alone could not prevent this suppression; for this, combined JAK2/STAT3 and p38-MAPK inhibition was required. Importantly, combined interference in the STAT3 and p38 pathways completely prevented inhibition of DC differentiation by all tested tumor supernatants (n=18, derived from both primary tumors and tumor cell lines, together encompassing eight different histological origins) and led to superior DC functionality, evidenced by increased allogeneic T cell reactivity with elevated IL-12p70/IL-10 ratios and Th1 skewing 38. Most importantly, combined STAT3 and p38 inhibition supported a shift from CD14+ monocyte-like cells to CD1a+ DC in metastatic melanoma single-cell suspensions, indicating a potential for improved DC differentiation in the tumor microenvironment 38. Of note, siRNA-mediated knockdown of STAT3 only, did effectively prevent the generation of CD14+ cells during IL-10-modulated MoDC maturation induction 17. As this was also achieved by transduction of the DC with constitutively active GSK3β this is consistent with cross-talk between these pathways (Oosterhoff et al, manuscript submitted). GSK3β controls the Wnt/β-catenin signaling pathway, which is involved in cell-cell communication, embryonic development and cancer, and has also been implicated in DC differentiation 74. The involvement of the Wnt pathway in DC differentiation was also indicated
by kinomic profiling analyses of DC precursors carried out by us, revealing down-regulation by a differentiation-inducing cytokine cocktail of Casein Kinase 2 (CK2) activity, based on phosphorylation of a substrate derived from T cell Transcription Factor-4 (Tcf); Tcf together with Lymphoid enhancer binding factor (Lef) forms an activational transcription factor complex acting downstream from Wnt and β-catenin. Of note, also p38-MAPK signaling is known to lead to GSK3β inactivation and activation of the canonical Wnt/β-catenin signaling pathway, although the exact signaling intermediates are as yet unknown. p38/ERK1/2 signaling can also induce COX2 activity and release of PGE2, which in turn can induce GSK3β phosphorylation and Wnt signaling.

Altogether, these data point to different tumor-associated factors (i.e. IL-10, IL-6, PGE2) exerting their suppressive effects at various stages of myeloid DC development through converging and communicating signaling elements encompassing the JAK2/STAT3, p38-MAPK and GSK3β/Wnt pathways (Figure 2). To specifically address tumor-induced myeloid suppression it is important to further dissect these signaling pathways and possible cross-talk between them in tumor-associated myeloid subsets in order to identify specifically acting and clinically relevant therapeutic targets. The advent of small-molecule kinase inhibitors and RNAi-based therapeutics now enables targeting not only of tumors, but also of their stroma, and should facilitate re-programming of tumor-associated myeloid cells, as well as tumor-modulated DC subsets in the skin, in support of anti-tumor immunity.

In conclusion, tumor related suppressive factors can divert DC during differentiation and maturation towards an M2 macrophage-like state with T cell-suppressive and pro-angiogenic and pro-tumor invasive properties. JAK2/STAT3, GSK3β/Wnt and/or p38-MAPK signaling interference, combined with local immune potentiation, may counterbalance tumor-imposed suppression of DC subsets, minimizing the induction and trans-differentiation of CD14+ M2-like DC with T cell suppressive characteristics, and thus set the stage for effective tumor vaccination through DC-targeted approaches.

**Future prospects**

As immunotherapy is gaining ground in clinical oncology, so is the realisation that the immune suppressive hold of tumors on their microenvironment needs to be broken to fully realize the potential of a new generation of treatment options. Not just immunotherapy, but also angiostatic therapy and even conventional chemotherapy, can be undermined by resistance mechanisms originating from the tumor stroma. From mouse models the overriding influence of tumor-infiltrating myeloid cells in this respect is becoming very clear, yet, for humans the *in vivo* phenotype-function relationships of these subsets remain largely obscure. Specific markers to identify suppressive populations are lacking entirely. A case in point was provided by a recent publication from the group of Dr. Stephen Rosenberg which called into question the T cell-suppressive qualities of melanoma-infiltrating CD14+HLA-DRdim cells, generally accepted in the field as MDSC. This demonstrates that there is an urgent need for operational *in vitro* subset definitions to be validated in clinical samples. Despite a plethora of mouse studies, this
issue has not yet been comprehensively addressed in a human setting. Research based on physiologically relevant human models is urgently needed for a better understanding of tumor induced immune suppression. This should start with myeloid subset profiling by transcriptional and proteomics analyses. Human myeloid cell suspensions from cancer patients and healthy volunteers need to be characterized to more accurately define separate myeloid subsets and to assess their activation state. After that major subsets can be sorted and subjected to genome-wide transcriptional and proteomic profiling to gain insight into their specialized functions and to identify novel markers for subset classification. Functionality of the sorted subsets in terms of T cell activation/suppression or promotion of endothelial cell growth/invasion, should then be tested in vitro with various assays. To assess active signaling pathways in tumor-conditioned DC differentiation an unbiased mass spectrometry-based approach should be employed. With this approach actual protein phosphorylation profiles can be determined to identify novel and specific targets to address in immunotherapeutic strategies. These analyses are expected to reveal druggable down-stream targets in the STAT3, p38 MAPK, and GSK3β/Wnt pathways -as well as new targets in as yet unidentified pathways, that confer resistance to tumor-induced suppression of DC development.

A disturbed balance in myeloid subset development and functioning leads to a profound state of immune suppression, both regionally, in and around tumors, and systemically in more advanced disease stages. Clinical experience with tumor vaccines has made it clear that cancer immunotherapy can only be successful if the immune system is operating at optimal efficiency. Gabrilovich and Nagaraj have suggested that, for cancer immunotherapy to be effective, it will have to be applied together with therapies aiming to eliminate tumor-related immune suppression 79. Myeloid cells, and M2-macrophages and MDSC in particular, are emerging as an obvious therapeutic target in this regard 82. From the studies comprising this thesis, we propose a dual approach to generate and facilitate an effective anti-tumor T cell response:

1) DC-targeted vaccination through the skin: by intradermal delivery of for instance an Ad5/3 or a CD40-targeted Ad vector encoding a tumor rejection antigen, combined with i.d. GM-CSF, selective transduction of mature human LC and CD1a+ DDC is achieved in situ, while simultaneously ensuring their stable maturation and T cell stimulatory capacity after migration from full-thickness skin explants 7,83-85. Based on findings from this thesis this approach may be further strengthened by incorporating STAT3 shRNA and/or constitutively active GSK3β encoding sequences in the DC-targeted Ad vector, which will endow the transduced DC in the dermal microenvironment with resistance to the suppressive effects of tumor-related IL-10 and thus ensure their proper maturation, migration and subsequent T cell priming.

2) Signaling interference in the tumor microenvironment to ensure a T cell-stimulatory myeloid compartment: Both at the level of the tumor and of its immune infiltrate signaling pathways are being identified that perpetrate and mediate immune suppressive effects, respectively. An ever increasing number of clinically available kinase inhibitors now make it possible to combine vaccination with systemic administration of these inhibitors to tip the myeloid balance in the microenvironment of tumors from suppressive MDSC and M2-macrophages to activated
DC in support of T cell-mediated immunity. Based on our findings a combination of small-molecule inhibitors targeting the JAK2/STAT3, p38-MAPK, and GSK3β/Wnt pathways would be attractive candidates for this purpose. Unfortunately, STAT3, p38-MAPK and GSK3β all control important developmental and metabolic pathways, which may preclude their direct clinical targeting. Future research should therefore aim to identify more specifically acting downstream signaling targets or to achieve targeted delivery of signaling interference e.g. through kinase inhibitor encapsulating nanoparticles or shRNA encoding viral vehicles. Interestingly, conditioning of the tumor microenvironment by certain cytostatic drugs might actually achieve the same objective. In a recent publication paclitaxel was shown in a melanoma model to decrease MDSC numbers and their suppressive activity in the tumor microenvironment through inhibition of p38-MAPK signaling.

Beside powerful DC-targeted vaccine approaches and elimination of immune suppressive signaling networks in the tumor microenvironment, a third level of therapeutic immune control is provided by immune checkpoint inhibitors with proven clinical efficacy like anti-CTLA-4 and anti-PD-1. With the clinical application of these combination therapies within reach, we may be on the verge of an exciting new era for cancer immunotherapy.
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Summarizing discussion

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