A SISTER'S HOPE

Targeting tumor-induced immune suppression: a promising strategy in the battle against breast cancer.

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Background

Immune-regulated pathways influence both breast cancer development and the outcome of conventional therapies like chemotherapy. Killer T cells can specifically recognize and eliminate breast cancer cells and are primed by powerful immune stimulatory antigen-presenting cells, i.e. Dendritic Cells (DC). Unfortunately, breast tumor-derived factors inhibit activation and maturation of DC resulting in defective antigen presentation and consequently defects in T cell responses. These breast tumor derived factors also frustrate normal differentiation of DC and instead promote the development of regulatory DC and myeloid derived suppressor cells (MDSC) that expand regulatory T cells (Tregs), all of which interfere with killer T cell functionality and contribute to tumor progression and spread. This disturbed balance between immune activation and tolerance is thus responsible for the overall inability of the immune system to generate an effective anti-tumor response.

In breast cancer, the first-line primary tumor-draining lymph node, the so-called sentinel lymph node (SLN), represents the first site of tumor-specific T cell activation but also the site where tumor-induced immune suppression most directly interferes with immune activation with reported negative effects on prognosis. In this project funded by A Sister's Hope we target this immune suppression through *in vitro* immune potentiation of the SLN. Breast SLN-derived viable single-cell suspensions offer a unique opportunity to study the tumor-conditioned lymph node microenvironment as well as modulate it *ex vivo.*

Summary of the project

Phase 1 of the study

In order to gain a better understanding of how different immune effector cell subsets are affected by breast cancer we compared the activation state of DC and T cell subsets of breast cancer SLN with healthy axillary lymph nodes (HLN). This project is part of a previously established research collaboration with the Antoni van Leeuwenhoek Hospital (Prof.dr. Emiel Rutgers, NKI-AvL) and was continued in our current A Sister's Hope project. HLN were obtained from consented BRCA1/2 patients undergoing a prophylactic mastectomy without previous medical history of cancer or autoimmune-disease. DC and T cell activation state was determined by extensive multi-color flowcytometric analyses.

Results from preliminary analyses of 14 HLN and 25 breast cancer SLN (re-analyzed from our existing SLN database) reveal that there is indeed immune suppression in breast cancer SLN, evidenced by higher immune suppressive, regulatory T cell (Treg) frequencies found in breast cancer SLN as compared to HLN, which significantly increase upon metastatic involvement of the SLN. Interestingly,

effects of this immune suppression varied between different DC subsets: we previously identified different subsets in lymph nodes, consisting of CD1a(+) skin-derived migratory subsets and CD1a(-) lymph node-resident subsets (see Fig.1). Lymph node resident conventional DC (cDC), characterized as CD1a(neg)CD11c(hi)CD14(neg) showed significantly lower expression of activation/maturation markers in breast cancer SLN as compared to HLN. Furthermore, we observed a further decrease (although not significant) in maturation state in the small subgroup of metastasis positive breast cancer SLN. Most profound suppression was observed in the plasmacytoid DC (pDC) subset, with significantly lower expression levels of maturation markers in breast cancer SLN. For CD1a positive migratory cDC subsets (most likely migrated to the SLN from skin: i.e. dermis derived DC [dDC] and Langerhans cells [LC]) no evidence of immune suppression in breast cancer SLN was found.

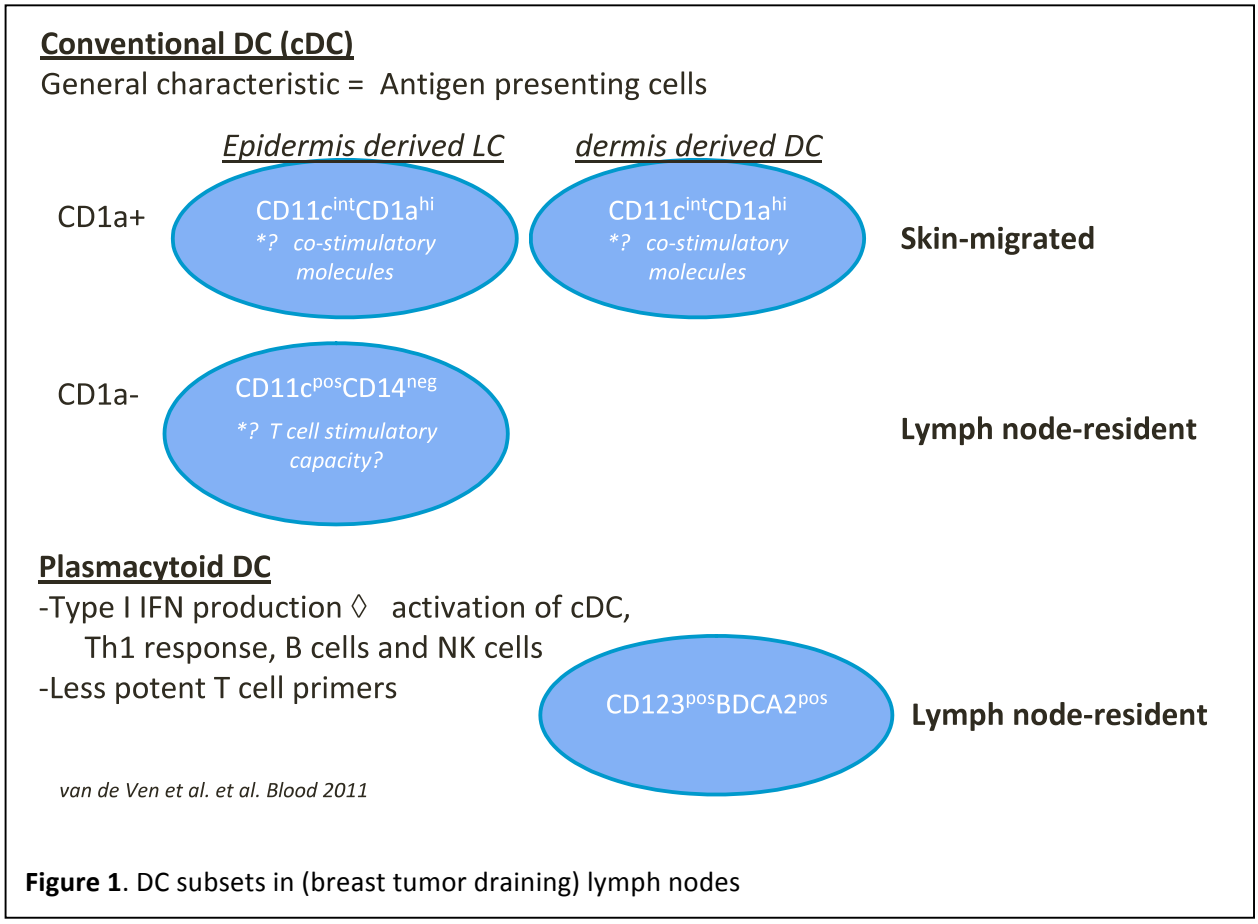

Figure 1. DC subsets in (breast tumor draining) lymph nodes

These results suggest that breast cancer induced immune suppression is primarily mediated by hampered maturation and activation of lymph node resident cDC and in particular pDC. Recent work from our research group has shown that under steady state conditions LN resident cDC, as compared to the more mature CD1a positive cDC subsets, in fact possess superior T cell stimulatory capacities (van de ven etal. Blood 2012). Moreover, BDCA-3 expression was found to be significantly higher in lymph node resident cDC. BDCA-3 is implicated in DC mediated cross-presentation of tumor derived antigens to CD8 cytotoxic T cells. pDC can rapidly release type-I interferons which boost cytotoxic T and NK cells which in turn provide protection against tumor outgrowth and metastasis. Moreover, type-I interferons further activate BDCA3+ cDC and enhance T- cell priming and cross presentation. T cell priming and cross presentation are both vital to the elicitation of an effective anti-tumor immune response. As suppressive conditions in breast cancer SLN appear to preferentially affect these LN-

resident cDC and pDC subsets, the anti-tumor response is therefore likely to be diminished which warrants further research to target and activate these DC subsets in particular.

Phase 2 of the study

In two separate phase 1-2 trials in melanoma patients we have previously established the immune potentiating effect of the immune stimulatory compound CpG, which resembles bacterial DNA and can bind human pDC by a specialized receptor known as Toll-like receptor 9 (TLR9). CpG increased activation of DC subsets (in particular pDC) in SLN and tipped the local immune balance in favor of killer T cell mediated anti-tumor immunity. Tumor-derived suppressive factors bind various receptors on myeloid cells but intracellular molecular signals, activated down-stream from these receptors converge in shared pathways. These down-stream signaling pathways, i.e. STAT3 and p38-MAPK pathways, have recently emerged as master switches of tumor induced immune suppression. Recent work by our group showed that combined interference in the STAT-3 and p38 MAPK signaling pathways almost completely prevented inhibition of human DC differentiation by breast cancer cells and effected superior DC function and T cell activation.

In the second phase of our A Sister's Hope project we set out to study the *ex vivo* effects on DC subsets of (combinations of) targeted agents interfering in down-stream signaling pathways mediating tumor-induced immune suppression (STAT3 and/or p38-MAPK inhibitors) and of immune stimulatory cocktails or CpG in breast cancer SLN suspensions.

SLN were obtained from clinically node negative breast cancer patients undergoing either breast conserving surgery or mastectomy in combination with an SLN procedure in the VU medical center (collaborators Dr Petrousjka van den Tol and Dr Lisette te Velde). After removal of the SLN, the SLN was bisected crosswise and under close supervision of the pathologist cells were scraped from both lymph node surfaces. SLN cell samples were cultured under the following conditions (C): medium only (C1), CpG (C2), CpG + STAT-3 inhibitor (C3), GM-CSF + IL4 (C4) and GM-CSF + IL4 + STAT3 inhibitor (STAT3-i) + p38 MAPK inhibitor (p38-i) (C5) (see Figure 2). Prior to, and subsequent to culture, the activation state of DC and T cell subsets was determined by extensive multi-color flowcytometric analyses.

After 7 days of culture, immune stimulatory effects were most prominently observed in LN resident cDC and pDC, both for cultures containing CpG and CpG in combination with STAT3-i. In LN-resident cDC maturation marker expression was slightly higher for CpG + STAT3i as compared to CpG alone. In dDC and LC subsets maturation marker expression was significantly increased for all culture conditions compared to the negative control (C1), but no obvious differences were found between the different culture conditions (C2 – C5).

pDC subset analyses revealed significant increases in maturation after culture with CpG and CpG in combination with STAT3-i. Moreover, a clear additive effect of STAT3-i was observed.

Superior immune stimulatory effects of CpG in combination with STAT3-i on both cDC and pDC is most likely a result of a combined mechanism. On the one hand STAT3 signaling in DC induced by tumor derived factors directly prevents differentiation into mature DC and blocking STAT3 signaling will revert this process, thus facilitating DC maturation. On the other hand STAT3 is also crucial for the release and signaling of the anti-inflammatory factor IL-10. Increased IL-10 production is a commonly observed but undesirable effect of CpG administration which can cause suppressive Treg

induction. STAT3 inhibition in combination with CpG can thus block CpG induced IL-10 production, resulting in superior immune stimulatory effects of CpG.

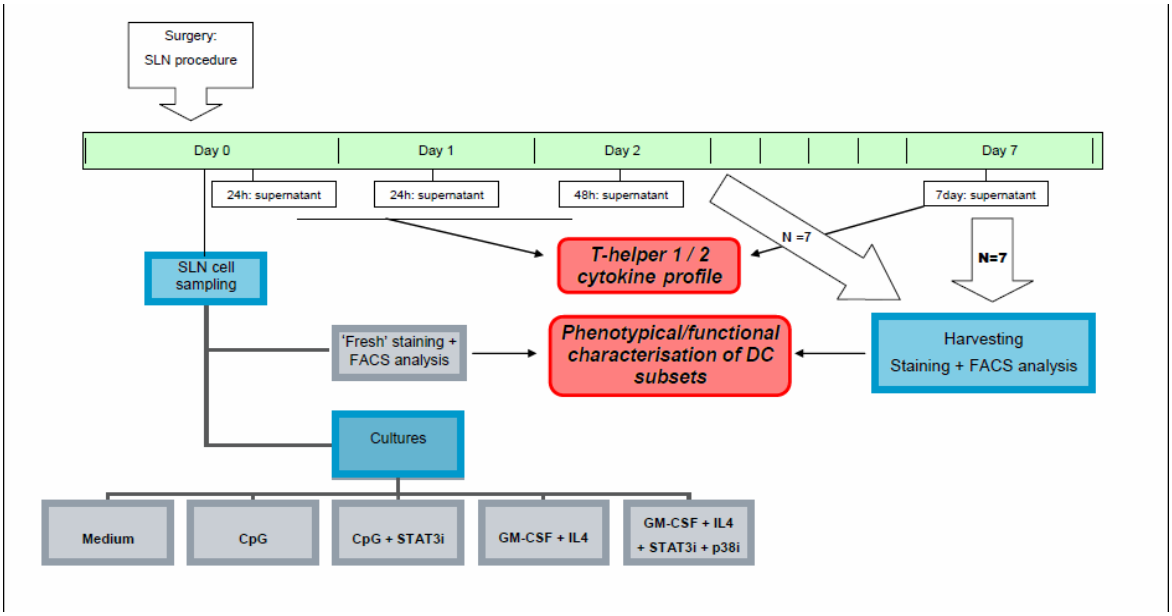

Figure 2: A scheme depicting SLN cultures and analyses performed to assess DC subset activation.

Phase 3: ongoing research

Based on the results of phase 2 we have selected CpG and CpG in combination with STAT3-i as the most promising agents in targeting breast cancer induced immune suppression in SLN. To test if these agents will also result in superior anti-tumor T cell functionality we have now started T cell cultures/expansions with subsequent functional assessment of tumor antigen recognizing T cells. We expect to be able to test at least 10 patients until the end of the project, i.e. by July 2014.

Since 2006 our research group has gathered extensive flowcytometric data on breast cancer SLN. Combined with the SLN included in this A Sister's Hope project, we have now built a database of 70 breast cancer SLN. This will enable us to correlate phenotypic characteristics of the different DC and T cell subsets to clinical parameters such as tumor stage, nodal status and survival. This should provide further information on the relationship between SLN immune status and breast cancer development and metastasis and provide a rationale for immune potentiation of the SLN as part of neoadjuvant therapy regimens.

On al final note, we are currently isolating DC subsets from HLN, and tumor-positive and –negative SLN to perform planned genome-wide transcriptional analyses in order to identify further therapeutic targets to overcome tumor-related immune suppression (collaboration Dr Rieneke van de Ven, VUmc). Overall, and with the support of **A Sister's Hope**, these studies will lead us to the design of novel immune-potentiating therapeutic approaches aimed at strengthening protective immunity in breast tumor-draining SLN to thus prevent tumor spread and metastasis.

Budget

Spent so far (per february 2014):

Salary costs (0.5 FTE Lab tech, 1.0 FTE MD research associate):	€58,834
Lab costs (material):	€15,000

NB: Project will run until 1 July 2014.