Discussion

A concomitant interaction of the innate immune system and the adaptive immune system is the cause for all the diverse immune responses. Innate arm relies on the interactions of PAMP (pathogen associated molecular pattern) and PRR (pattern recognition receptor), whereas adaptive arm majorly relies on the interactions of MHC I, MHC II, BCR (B cell receptor) and TCR (T cell receptor). The key players of innate immune system are non-clonal recognition receptors. These include the C-type lectin Receptors (CLR), Toll-like receptors (TLRs), the retinoic acid-inducible gene I-like receptors or RIG-I-like receptor (RLRs), the nucleotide oligomerization domain-like receptors or NOD-like receptor (NLRs) and cytosolic DNA sensors (110). However, the key receptors of adaptive immune system are clonal in nature. They are present on B cells and T cells that recognize antigens or the processed peptides, in an explicit manner. DCs are one of the connecting links between the innate and the adaptive immune responses. This unique attribute of DCs has made them a suitable candidate for vaccination strategies in immunotherapy protocols.

DC-based vaccination is an attractive immunotherapeutic strategy, because of its ability to induce tumor-specific T-cell responses, which offers the desired anti-tumor effects with minimal toxicity. Allogenic DCs generated from healthy donors (HLA matching or partially matching) have been used in clinical trials as DC vaccines. These dendritic cells were able to initiate a CTL response and induce regression of the tumor (2). Allogeneic DCs can also be generated from CD34\(^+\) cells derived from umbilical cord blood. Our earlier studies had shown that UCB can serve as an alternative source for generating a homogenous DC population with high cell numbers (35-36, 111). However, since UCB are naïve cells, there was a concern whether the DCs generated from them would elicit a CTL response as potent as that generated by peripheral blood-derived DCs, and whether they could serve as good candidates for vaccines. To gain a better insight into this, we performed a systematic comparative study of standard PBL monocyte-derived DCs and UCB-derived DCs, with emphasis on CTL characterization and, in vitro and in vivo CTL assays. We also made an effort to enhance the functionality of DCs by exogenous addition of arachidonic acid in the culture media at the differentiation step.
In the first study, DCs generated from the two sources were found to be similar in morphology and phenotype. Their functional attributes like antigen uptake capacities, the ability to migrate towards a chemokine gradient of CCL-19 and MLR activity (i.e. potent immunostimulatory capacity) were equivalent. The CTLs generated were examined for activation markers (CD69 & CD25), Granzyme A and B and enumeration of MUC1 peptide STAPPVHNV-specific CTLs by Streptamer staining. The cytokines secreted in the co-culture system were more favorable for a Th1 response. Our data indicate that CTLs from UCB-DCs are not only equivalent but superior to CTLs from PBL-DCs in some respects. Cytotoxic T lymphocytes are important constituents of an adaptive immune system. We tested the level of the known HLA-A*0201 restricted MCF-7 associated antigen, MUC1- STAPPVHNV peptide, by the streptamer assay and found that the pool of CTLs had a high percentage of MUC1-specific CTLs. Thus, a pure antigen-specific CD8\(^+\) T cell population could be generated, which could eventually be sorted out to give a single antigen-specific response in CTL assays. Our data is in agreement with those of Irina Fernandez et al. (112) who described an in vitro system for the generation of functional, antigen-specific T cells from human stem cells, which could eventually provide a readily available cell source for adoptive transfer immunotherapies and also enable better understanding of human T cell development.

Defense against virally-infected and malignant cells depends on the action of cytotoxic T lymphocytes and natural killer cells. Although these use several mechanisms to eliminate target cells, the principal event is secretion of granule components, comprising proapoptotic serine proteases (Granzymes) and the pore-forming protein toxin (Perforin). Target cells were synergistically killed by the pores formed by the perforin and various unscheduled apoptotic pathways initiated by the granzymes (113). Target cell recognition by cytotoxic T lymphocytes induces the secretion of cytotoxic granular content towards the target and the induction of death (114). There are five classes of granzymes in human, which are expressed from three different gene clusters. Granzyme A and granzyme B are the most abundant granzymes. Among all the granzymes, B is most generically examined. It cleaves after aspartic acid residues similar to the caspases and induces death in target cell by activating the key executioner caspase-3. Bid can be directly cleaved by granzyme B, which subsequently activates the intrinsic pathway of apoptosis. Granzyme B acts as a
tryptase, which induces cell death independent of caspase cascade. The immune system has to be at par and vie with the plethora of infectious organism and mutations leading to self-altered cells or tumors, which have complicated multiple strategies to circumvent apoptosis and destruction by immune responses (115). We detected high levels of granzyme A and B in UCB-DC-CTLs, further highlighting their potential killing ability. The in vitro CTL activity was evaluated by their efficiency to kill MCF-7 (target cells), and we observed that CTLs from both sources exhibited equivalent killing efficiency. We also witnessed the evidence of cytotoxic activity specific to tumor antigens in the in vitro CTL assay, and not against nonspecific targets like H1229 and SH-SY5Y. These data clearly suggested that UCB-DCs/CTLs are not only as potent as PBL-DCs/CTLs, but they showed enhanced features as well. Chang et al. (116) have also compared PBL-DCs with UCB-DCs using in vitro assays and they report similar findings. They have shown that cord blood stem cells-derived DCs had quicker and greater ERK and Akt phosphorylation, and weaker p38 phosphorylation, than peripheral blood monocyte-derived DCs, when stimulated with LPS. Upon activation of these signalling cascades the maturation of DCs is accomplished by changes in morphology, phenotype and functional properties. Signaling pathways play a decisive role in the differentiation, survival, expression of costimulatory molecules and antigen presentation capacity of DCs. In our study we have laid emphasis on characterization of CTLs obtained via pulsed DCs, from HLA-A*0201 positive UCB/PBL samples and also focused on in vivo CTL assays using xenograft of MCF-7-luc-F5 in female NOD/SCID mice.

To the best of our knowledge, we provide here for the first time, direct evidence in xenograft model of solid tumor, an adoptive T cell therapy by using ex vivo expanded CTLs having the antitumor activity. These CTLs were generated from HLA-A*0201 positive UCB and PBL samples. We found that UCB/CTLs and PBL/CTLs exhibited immunotherapeutic effects against MCF-7-luc-F5 solid tumors in female NOD/SCID mice. It is evident from the IVIS data that there is a significant remission in tumors, ten days after CTL infusion. The H&E and immunofluorescence staining of tumors sections revealed substantial homing and infiltration of CD8+ T cells. It needs to be determined whether frequent booster doses of CTLs over longer period could achieve complete regression. It is also important to generate DCs by our method from other sources like
CD34+ from mobilized peripheral blood or enriched apheresis samples and test their killing efficacy, using different cell lines for xenograft experimental models. In conclusion, UCB-DCs or the CTLs derived from them could be used effectively in allogeneic antitumor vaccines for immunotherapy, as alternative to peripheral blood or bone marrow derived DCs/CTLs.

Cord blood has many advantages over other sources like ready availability, low risk of severe GVHD and presence of stem cells with high proliferative potential. Umbilical cord blood stem cells have longer telomeres and thus have a longer survival. Monocytes from PBL are terminally differentiated cells, and therefore, cannot proliferate. The major disadvantage of umbilical cord blood is the low number of stem cells but this is taken care of by the expansion step in our method. Cord blood banks across the globe can be utilized for this purpose. When a stored sample is revived for transplantation, a small aliquot can be removed to generate DCs, which could then be transplanted into the patient, where they would migrate to lymph nodes and could elicit a strong T cell response against any residual tumor cells. Colin de Haar et al. have already started such pre-clinical trials in the therapy of pediatric AML using UCB-DCs (37). Hutten et al. describe the first pre-clinical evidence for the suitability of UCB-DC either for the induction or for the reactivation of minor histocompatibility antigen (MiHA) HA-1-specific cytotoxic T cells. Their findings are of clinical significance in transplanted patients suffering from hematological malignancies (38).

Knowledge arising from results of many clinical trials, have shown concrete evidence that tumor/tissue associated antigen pulsed DCs are capable of eliciting an effective anti-tumor T-cell responses. Since production of clinical grade DCs/CTLs, using good manufacturing practices (GMP) production facility for vaccination protocols is a time consuming and cost intensive process, DCs/CTLs once generated can be frozen in small aliquots and can be kept ready to use. This would significantly expedite the subsequent vaccinations (117). Likelihood of batch to batch variation from cultures increases as well, which could be minimized if large number of cells are generated in one go and then cryopreserved. Beatrice S-Thurner et al. showed that the administration of antigen primed mature monocyte-derived cryopreserved DCs induced Th1 response against MHCII restricted
tumor peptides in patients with metastatic melanoma (118). Smita K. Nair *et al.* administered cryopreserved DCs primed with the carcinoembryonic antigen peptide-1 (CAP-1) to HLA-A2-positive patients with advanced carcinoembryonic antigen expressing malignancies (119). Similarly in our study we could cryopreserve pulsed DCs for long term i.e. three years period without much decrease in viability and phenotype.

In the second study, we again demonstrated for the first time that the addition of AA at the differentiation step has beneficial effects, thus further improving the quality of DCs generated from umbilical cord blood. The maturation status plays a decisive role in antigen presentation, costimulation and ultimately adjudicates whether the outcome will be immunogenic or tolerogenic. DCs used in cancer immunotherapy should have strong immunogenic response. DCs exhibit their anticancer effect by capturing the tumor antigens and their subsequent presentation (5-8). Many DC-based clinical trials for cancer treatment have shown its safety and feasibility (8). The clinical efficacy of this therapy still needs to be improvised. Advances in biology lead to frequent improvements in the vaccine production protocols and therapeutics (4, 120-124). Recent reports also illustrate that there is emerging evidence that PGE2 plays crucial roles in reciprocal crosstalk between dendritic cells and natural killer cell biology. Several NK cell functions (lysis, migration, proliferation, cytokine production) are influenced by PGE2, accentuating the role of PGE2 on DC–NK cell crosstalk and its subsequent impact on immune regulations in normal and immunopathological processes (125). Some of the previous reports show that *in vitro* generated DCs are less efficient in migration and other functional activities (126-128) due to use of the cytokine IL-4 in the protocols (39). IL-4 is known to adversely affect the AA metabolism. So we hypothesized that exogenous addition of AA in the culture medium may improve the functional activities of DCs.

The AA⁺ DCs showed higher antigen uptake compared to AA⁻ DCs underscoring the beneficial effect of AA addition to the cultures. Another important feature of DCs is their capacity to stimulate the proliferation of T lymphocytes in an allogeneic MLR and to generate effector CTLs. The inflammatory nature of many cancers creates an immunosuppressive environment that leads to suppression of DC-instructed effector CD4⁺ and CD8⁺ T cell responses (126). The enhanced MLR in the culture system with AA may
DCs generated for immunotherapy purpose should have cytokine profile which supports the Th1 type of response. In other words, secretion of low levels of IL-10 and high levels of IL-12 is a desirable character for the DCs to be used in the vaccination regimen. In our culture system AA addition resulted in significantly improved IL12/IL10 ratio thus further improving their antitumor ability. Pawel Kalinski et al. (133) have shown that though PGE2 is reported as a suppressive inflammatory factor, it also contributes to the initiation of primary immune responses by facilitating the cytokine-induced final maturation and the increase in immunostimululatory capacity of DCs, confirming the role of PGE2 as a Th2-promoting factor, acting at the APC level. Enzymes cyclooxygenases (COX-1 and COX-2) convert the AA released by cPLA2 to PG endoperoxide H2, which is the precursor of series 2 prostanoids such as PGD2 and PGE2. Unlike COX-1, COX-2 is an inducible
enzyme involved in the sustained production of prostanoids by many cell types (134). Notably, COX-2 activity is necessary for strong Ab response following vaccination; especially when vaccines are poorly immunogenic or the target population is poorly responsive to immunization (135). Thus the enhanced expression of COX-2 mRNA as seen in AA⁺ DCs may be favorable for their use, as an anticancer agent. As per our expectation, AA⁺ DCs exhibited a better \textit{in vitro} chemotaxis, T cell stimulation, CTL activity, Th1 favorable cytokine profile, antigen uptake and \textit{in vivo} migration. However one wonders which of the following metabolites of AA, from the downstream pathway like LTB4, cysteinyl leukotrienes, 12-15-hetes, PGE2, and PGD2 are actually responsible for the beneficial effect. In future we propose to address these issues of delineating the pathway by appropriate use of pharmacological inhibitors.

\textit{In vitro} manipulation of cellular vaccines is crucial for their successful use in clinics and thus our methodology, though it shows an incremental increase in the output, may add a new dimension in improvising the production of a potent immunotherapeutic agent. UCB-derived DCs and CTLs show great potential, as their functionality is at par to the standard source and can serve as an alternative allogenic source for DC generation in near future. Substantial progress made in the field of human immunology has opened new vistas for the genesis of novel vaccine strategies. Protocols will have to be tailored according to the prognosis of individual’s mutations. We have traversed a long way since the first clinical trial with autologous pulsed DCs to stimulate antitumor immunity in the host (136). We are still in the quest to find the answer to the main problem i.e. what all is required to evoke a therapeutic immunity against cancer, which emerges by evading the defense system of the host. Thus our findings will be helpful in the better contriving of DC based vaccines for personalized cancer immunotherapy with enhanced functionality.