

● Immunobiology of antigen presenting cells

Several different cell types have thus far been identified that efficiently present antigen (1-3). Early work has focused on B-cells and macrophages due to their natural capacity to take up, process and present antigen (4-9). B-cells are now mainly studied for their capacity to generate antibodies e.g. using hybridoma technology or in tumor immunology in the context of antigen discovery (SEREX) (10,11). Research on antigen presentation has meanwhile clearly shifted to dendritic cells (DC).

CD40-Activated B-Cells as Antigenpresenting Cells: From Immunobiology to Immunotherapy

Michael S. von Bergwelt-Baildon^{1,4},
Lee M. Nadler² and Joachim L. Schultze^{1,3}

¹Molecular Tumor Biology and Tumor Immunology, Medical Faculty, University of Cologne, Cologne, Germany; ²Department of Adult Oncology, Dana-Farber Cancer Institute; Department of Medicine, Brigham and Women's Hospital; Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA; ³ATABIS GmbH, Cologne, Germany; ⁴AG Zoophysiology and Verhalten, FB Biologie, Geo- und Umweltwissenschaften, Carl von Ossietzky University, Oldenburg, Germany



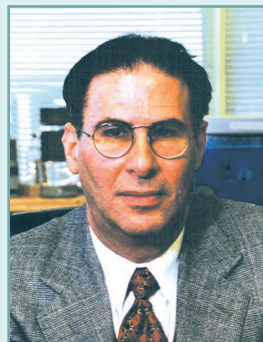
Michael von Bergwelt-Baildon, M.D., Ph.D. studied Medicine and Human Biology at the Universities of Freiburg and Paris. He earned his M.D. degree (Dr. med.) from the University of Freiburg and his Ph.D. degree (Dr. rer. nat.) from the University of Oldenburg. From 1999-2002 he worked as a fellow of the Deutsche Krebshilfe and the Lymphoma Research Foundation of America at the laboratory of Lee M. Nadler at the Dana-Farber Cancer Institute in Boston on CD40-activated B-cells and the characterization of novel "universal" tumor antigens. He is currently working as a research group leader in the laboratory for Molecular Tumor Biology and Tumor Immunology (Joachim L. Schultze/Jürgen Wolf) at the Department for Hematology/Oncology, University of Cologne. His research focus is the characterization of defects of antigen-presenting cells in cancer patients and the pharmacogenomics of tumor immunotherapy.

specific antigen uptake via surface Ig molecules (7). This is followed by an upregulation of costimulatory molecules and presentation of peptides in the context of MHC class II. Upon cognate interaction CD4⁺ T-cells upregulate CD40L and in turn costimulate B-cells via CD40. B-cells are implicated in the priming of CD4⁺ cells against protein but not peptide antigens *in vivo* (3,15). Resting and naïve B-cells appear to tolerize T-helper cells while CD40 activation is linked to improved antigen presentation (16-18).

They represent a complex system that is highly efficient at surveying peripheral tissue, taking up and presenting antigen to induce MHC-class I and II restricted T-cell immunity *in vivo* and *in vitro* (12). Since their initial description by Steinman et al. they have been extensively studied and as "nature's adjuvant" they have become a focus of research attempting to define immunity and develop immunotherapy (12-14).

● B-cells as antigen presenting cells

B-cells are considered to function as "secondary APC" using highly



Lee M. Nadler, M.D. Senior Vice President, Experimental Medicine at Dana-Farber Cancer Institute. Dr. Nadler received his MD from Harvard Medical School in 1973. After residency training at Columbia-Presbyterian and training at the National Cancer Institute in tumor immunology, he completed a medical oncology fellowship at DFCL, where he joined the staff in 1980. He is currently senior vice president of Experimental Medicine at Dana-Farber and Professor of Medicine at the Harvard Medical School. He serves in numerous leadership roles in both Dana-Farber/Partners CancerCare and Dana-Farber/Harvard Cancer Center. Dr. Nadler has been awarded with the Richard and Hinda Rosenthal Foundation Award, American Association of Cancer Research in 1998 for his achievements in translational research and more recently in 2002 with the 7th AACR-Joseph H. Burchenal Clinical Cancer Research Award for his pioneering efforts in the discovery, development, and characterization of monoclonal antibodies for the diagnosis and treatment of human B-cell malignancy.



Joachim L. Schultze, M.D. studied medicine at the University of Tübingen, Germany. He joined the Dana-Farber Cancer Institute as a postdoctoral fellow in 1993 where he joined the faculty in 1996. In 2002 he was appointed Professor for Tumor Immunology at the University of Cologne. He also has been awarded the Sofja-Kovalevskaja Award of the Alexander von Humboldt Foundation as well as other awards including the Translational Research Award of the Leukemia and Lymphoma Society, and the Senior Investigator Award of the Multiple Myeloma Research Foundation. He is author of more than 50 publications and as a scientist in tumor immunology, he was involved in the discovery of telomerase as an almost universal tumor antigen.

● Antigen presentation in tumor immunology

Although anti-tumor immunity has great potential, there is compelling evidence for a specific pattern of deregulation of the immune response in cancer patients: It has been suggested that immune dysfunction is antigen specific at the onset of malignant disease and becomes more generalized with progression. Quantitative as well

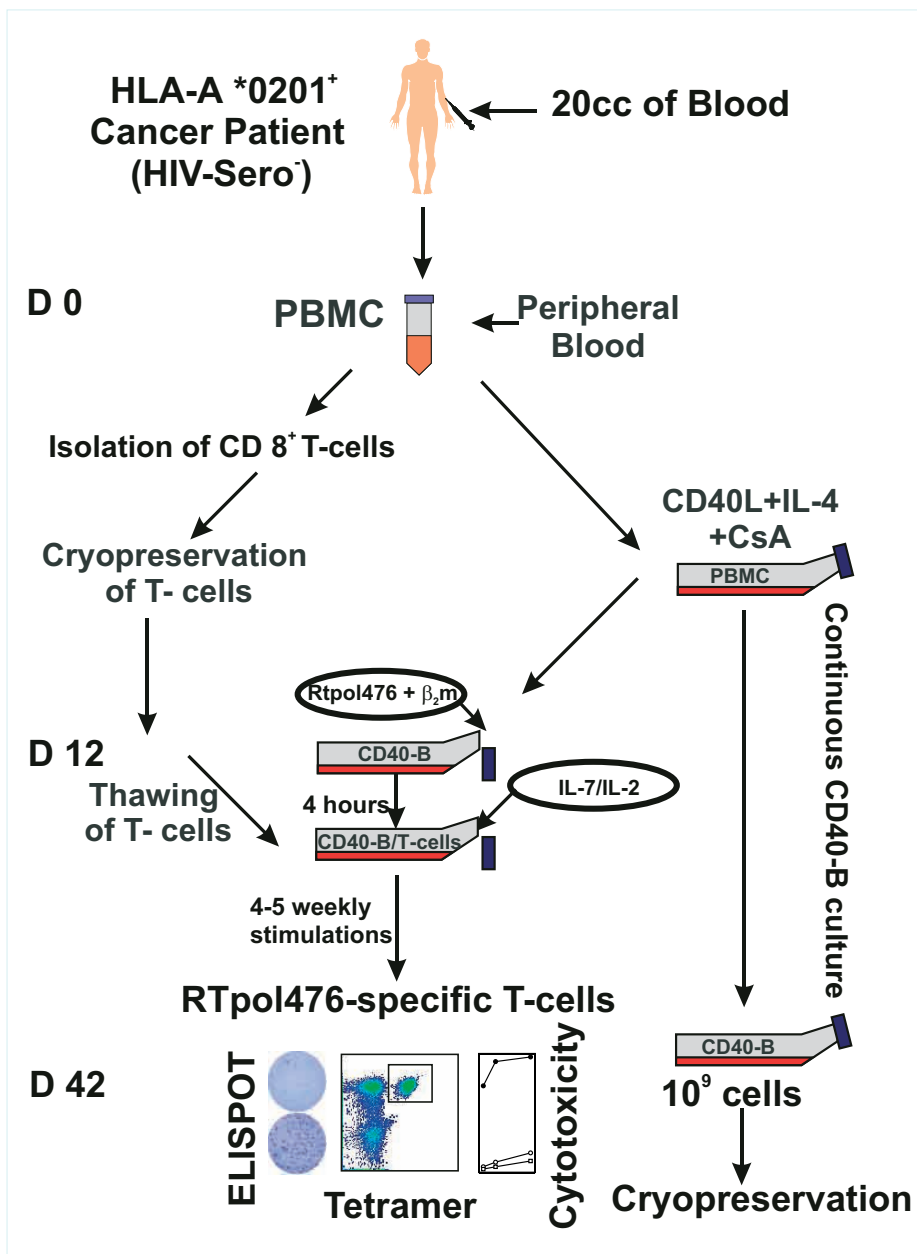


Figure 1: Priming CD8⁺ T-cells in cancer patients using CD40-B. PBMC and CD8⁺ T-cells are isolated from the peripheral blood of HLA-A*0201⁺ donors. CD40-B are expanded from PBMC through continuous culture with IL-4, CD40L and CsA. Starting day 12 of culture CD40-B are loaded weekly with the HLA-A*0201 binding peptide from reverse transcriptase polymerase (HIV) “RTpol 476” and beta-2-microglobulin. Cells are irradiated and cocultured with autologous CD8⁺ T-cells in the presence of IL-2 and IL-7. After 5-6 stimulations antigen-specific T-cells are detected by ELISPOT, tetramer stain or cytotoxicity assay.

as qualitative alterations have been described in cancer patients: Certain tumors such as renal cell carcinoma and multiple myeloma have been demonstrated to be marked by poor infiltration or low number of circulating DC (19-21). In cancer patients tumor-antigen-specific immune responses are impaired by the absence of costimulation and IL-12 secretion as well as the promotion of Th2/Th3 differentiation (22-25). Cytokines, actively secreted by tumors, such as IL-10 or TGF- β have been demonstrated to inhibit T-

cell activation by DC (26,27). IL-10 furthermore promotes DC apoptosis and skews the differentiation of monocytes from DC to macrophages (28,31). B-cells have been implicated in the failure of tumor control by the immune system. Qin and colleagues showed that presence of B-cells in the priming phase results in a reduced “help” for CTL mediated tumor rejection in combination with a non-productive humoral response (32).

Taken together, novel therapies attempting to control

tumor growth should potentially be designed to overcome defects in antigen presentation.

● *CD40-activation in normal and malignant B-cells*

Significant improvement of antigen presentation in the context of normal B-cells as well as B-cell tumors of various histologies was demonstrated by our group and others to be induced by CD40-signaling (15,33-37). CD40, a member of the family of tumor necrosis factor receptors (TNFR), is expressed throughout B-cell development and is implicated in cell-survival and differentiation (38-41). Its physiologic ligand CD40L (CD154) is expressed primarily on CD4⁺ T-cells within hours after ligation of CD3 (42,43). CD40/CD40L signaling stimulates monocytes, B-cells and DC to differentiate, proliferate and increase antigen presentation (34,35,44-47). CD40/CD40L interaction is critically involved in cognate T/B-cell interaction (48-50). Deletion of the CD40-L gene in mice or administration of anti CD154 antibodies therefore results in the abrogation of germinal center formation during immune responses (51,52). CD40 is widely expressed in immune cells, keratinocytes, carcinomas and fibroblasts, while the expression of CD40L is mainly limited to activated CD4⁺ cells, granulocytes, DC and activated B-cells (41,53-55). Signaling through CD40 involves several second messengers (e.g. PTK, PI-3 kinase, C γ 2) and members of the TRAF family and Jak3 ultimately initiates activation of transcription factors like NF- κ B, c-Jun and NFAT (41,56,57). CD40 activation has a plenitude of effects concerning differentiation and proliferation of B-cells depending on the state of differentiation including a “switch” to production of different immunoglobulin isotype classes and, in combination with B-cell receptor signalling, prevention of apoptosis (41).

● *Antigen presenting cells for cancer immunotherapy*

Despite the fact that DC are the most prominent APC and the only APC activating naïve T-cells *in vivo*, DC are still marked by technical difficulties that limit translation to a standard tool in laboratory research, diagnostic or therapy: DC are not homogenous and represent different cell types including

Application	Background	Status
antigen discovery	e.g. "reverse immunology" approach requires powerful platform to identify epitopes recognized by T-cells	active
adoptive T-cell transfer	treatment of infections or tumors by <i>ex-vivo</i> expanded patient T-cells requires autolog. <i>in vitro</i> APC-system that can be used for multiple rounds of stimulations	in preparation
immune assessment	antigen-specific immune intervention requires quality control platform	active
vaccination	cellular adjuvants should be generated at great quantities and low cost	in preparation
MHC-class II restricted anti-tumor immunity	increasing evidence demonstrates importance of CD4 ⁺ T-cells for tumor control needs to be further characterized and translated to immunotherapy	active

Table 1: Applications of the CD40-B system.

tolerogenic cells (12,58). They are rare in the peripheral blood (<1%) and are therefore usually isolated from apheresis products or after *in vivo* expansion (59-62). Furthermore, DC have a limited life span *in vitro* and are difficult to expand. Successful use as a laboratory tool and as cellular adjuvant on the contrary requires large numbers of efficient, standard quality antigen-presenting cells (APC) for repetitive, possibly life-long stimulations and vaccinations (63,64). To circumvent these problems alternative approaches have focussed on so-called "artificial APC". Using well-characterized, easily expandable cells investigators are attempting to "engineer" APC e.g. by introducing genes of interest (65-68). While significant amounts of well-defined APC can thus be generated, they can only be developed to have properties currently known to be of importance for efficient antigen presentation. Professional APC on the contrary certainly express an array of cyto- and chemokines, surface and other molecules that are important for their function and have not yet been identified and therefore will not be considered for aAPC. Furthermore, these approaches frequently rely on allogeneic or xenogeneic cells. This adds an additional risk (e.g. immunogenicity, infection, oncogenicity) for the patient to the aspect of gene-therapy, which is subject to strict regulatory limitations and currently under review (69,70). We therefore hypothesized that it would be advantageous to extend the sources of APC to combine technical features of aAPC and biological properties of professional APC.

● *The CD40-B system*

CD40-activated B-cells can be generated without difficulties from small amounts of peripheral blood mononuclear cells (PBMC). Key culture factors are CD40-signaling, interleukin-4 (IL-4) as a growth factor, and cyclosporin A (CsA) to prevent outgrowth of other cell types particularly T cells. After 12-14 days of culture CD40-B cell cultures are >95% pure B cells and very efficient APC (36,37). PBMC from 20cc of peripheral blood can be expanded to $\sim 2 \times 10^9$ CD40-B cells over a period of 6 weeks. Expansion of CD40-B cells past day 65 is frequently possible without loss of function (36). These cells express significant amounts of adhesion, costimulatory and MHC molecules at densities comparable to DC. CD40-B cells can be antigen-loaded for weekly stimulations of autologous or HLA-matched T-cells inducing primary and secondary Ag-specific MHC class I and MHC class II restricted T cell responses (36,37), (and von Bergwelt-Baildon et al., unpublished results). Furthermore, CD40-B cells have been demonstrated to be readily transducible using retroviral vectors encoding for model recall antigens. APC function was unaltered and antigen-specific T-cells were induced that used several HLA-alleles as restriction elements (71).

To address the requirements of a cellular adjuvant for clinical application quality control strategies have been developed to perform simple monitoring of antigen presentation and antigen loading. Furthermore, culture conditions

have been adapted for clinical use including the introduction of recombinant, trimeric CD40-L without loss of function (37). This new generation of the CD40-B system has been pre-tested *in vitro* and demonstrated to function equally well in cancer patients and healthy donors (37).

● *Current and future applications of the CD40-B cell system*

Ongoing and future experiments intend to validate CD40-B cells as potential cellular adjuvant for active immunotherapy. Apart from vaccines CD40-B cells are currently already in use or under study as APC system for various applications:

Classical T- or B-cell based approaches to identify antigens as targets for anti-tumor immune intervention focus on the analysis of existing anti-tumor responses (72,73). Alternative strategies attempt to identify candidate antigens using an approach termed "reverse immunology" (74-77). For this approach powerful epitope screening systems are essential. Based on previous experiences with CD40-B cell based tumor antigen discovery approaches (75,78) a "pooled T-cell screening system" was established relying solely on the use of CD40-B cells as APC. This system allows simultaneous screening for up to 50 candidate epitopes and has led to the identification of more than 15 potential tumor antigens. (von Bergwelt-Baildon et al., unpublished results).

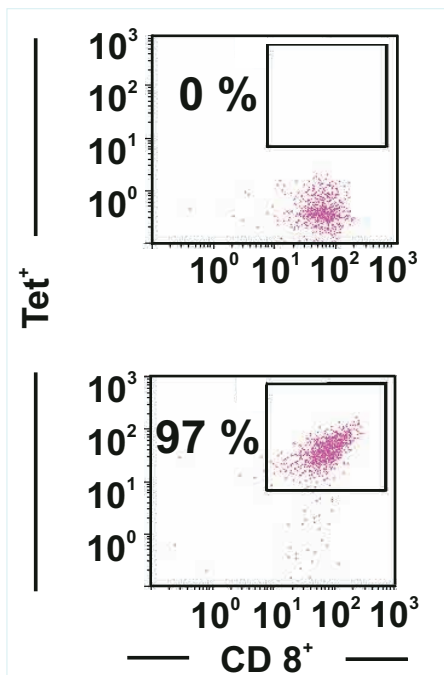


Figure 2: Generation of antigenspecific T-cells using CD40-B cells as APC. Visualization of CD8⁺ T-cells specific for MART-1(EAAGIGILTV) peptide in the context of HLA-A*0201 using tetrameric MHC/peptide complexes. Upper panel: MHC/Tax (control tetramer); lower panel: MHC/MART-1 tetramer. Results are shown after priming with MART-1 HT(ELAGIGILTV) peptide-loaded HLA-A*0201⁺ CD40-B and 7 weekly restimulations.

Adoptive transfer of *in vitro* expanded T-cells has developed as an important tool to treat life-threatening viral infections in the setting of immune-suppression in transplant recipients and as an experimental treatment for malignancies (79,80). These strategies similarly depend on powerful APC systems.

Any kind of immune intervention - either activation or suppression - can only be developed if an exact quality control is possible by means of a powerful immune assessment. One important factor is the quantity of patient samples. Due to the very limited amounts of available patient samples *ex vivo* culture systems that can efficiently expand cells are of extreme importance. The CD40-B system in combination with multiplex immune assessment procedures is a powerful platform to assess immunity and thus improve the design of immunotherapy.

Recent discoveries have demonstrated the crucial role of

MHC-class II restricted responses in anti-tumor immunity (81-83). The characterization of MHC-class II restricted responses therefore has become the focus of an increasing research effort. CD40-B cells could also serve as a tool in the context of MHC class II restricted epitope discovery. Encouraged by initial data indicating that the CD40-B system can be translated to MHC class II, current efforts are focusing on antigen uptake and processing in the context of MHC class II, induction of primary and secondary CD4⁺ T cell responses as well as analysis of T_{H1}/T_{H2} bias, (von Bergwelt-Baildon et al., unpublished results).

CD40-B cells can be generated in great numbers and therefore possibly facilitate 'dose-escalation' of vaccination. The beneficial effect of frequently repeated vaccinations with significant numbers of cells in the tumor-bearing host has been clearly demonstrated by Ochsenbein et al. in a murine model (63). Regression of well-established tumors was only achieved when antigen-loaded APC were applied repetitively over a period of 3 weeks. In contrast to DC, B cells are normally not surveying subcutaneous tissue and *ex vivo* generated CD40-B cells might therefore be incapable of migration into draining lymph nodes. However, in mice B-cells injected subcutaneously are indeed capable of migrating to lymphoid organs (84). Preliminary data in our laboratory suggest thus far that human CD40-B cells might also have the potential to home to secondary lymphoid organs, (von Bergwelt-Baildon et al., unpublished results). Future studies will have to clarify if CD40-B cells have the capacity to interact with T-cells in a tumor bearing host upon vaccination. Furthermore, novel genomic and proteomic approaches will be employed to characterize CD40-B from healthy individuals and cancer patients in order to understand biology and improve therapy.

Ultimately only trials in cancer patients will be able to define if only DC can issue the "licence to kill" (85) or whether CD40-B represent a promising alternative.

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