CD40 activation: potential for specific immunotherapy in B-CLL

M. von Bergwelt-Baildon¹, B. Maecker¹, J. Schultze² & J. G. Gribben¹*

¹Department of Medical Oncology, Disease Center for Hematologic Neoplasia, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA; ²Molecular Tumorbiology and Tumorimmunology, Department for Hematology/Oncology, University of Cologne, Germany

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Despite encouraging scientific and therapeutic advances, chronic lymphocytic leukemia (CLL) principally remains an incurable disease. Allogeneic transplantation represents the only curative approach, but is marked by high mortality. Novel and less toxic treatment modalities are needed. Immunotherapeutic approaches have clearly demonstrated potential effectiveness in CLL and other B-cell malignancies. To successfully direct immunity against CLL, highly immunogenic tumor cells or tumor-antigen-loaded antigen-presenting cells are necessary. The CD40–CD40L interaction has been shown to significantly increase antigen presentation in normal and malignant B-cells. Here we discuss biology and potential therapeutic applications of the CD40-system in CLL.

Key words: antigen presentation, CD40, CD154, CLL, immunotherapy

Introduction

More than 7000 cases of chronic lymphocytic leukemia (CLL) are diagnosed in the USA every year, with an incidence of three in 100,000. While this disease classically occurs in older people with a median age at diagnosis of 70 years, an increasing number of patients are younger. Although younger and older patients have a similar survival, analysis of the relative survival rates showed that the disease has a greater adverse effect on the expected survival probability of the younger population, since the older patients often die of causes unrelated to their CLL. This makes the development of curative, rather than palliative, regimens even more important. Classically palliative treatment approaches have consisted of observation, steroids, alkylating agents and the use of purine analogs as single-agent therapy. Monoclonal antibodies have demonstrated promising results and have fueled the interest in targeted immunotherapy in CLL [1, 2]. More recently, novel treatment approaches have included the use of combination chemotherapy often including the use of monoclonal antibodies, and there is increasing use of approaches such as autologous and allogeneic transplantation that may have curative potential but at the cost of significant toxicity and treatment-related mortality [3].

Molecular and immunological characterization of B-CLL

CLL is characterized by the accumulation of a clonal population of malignant CD5+ tumor cells [4]. Their physiological, non-malignant counterparts, so-called ‘B-1 cells’, are part of the innate immune response and characterized by the secretion of polyspecific immunoglobulin (Ig) [5]. These antibodies display affinity to various structures including auto-antigens, and have been implicated in autoimmune disorders including rheumatoid arthritis and systemic lupus erythematosus [6]. CLL cells often express autoimmune Ig and autoimmune phenomena including idiopathic thrombocytopenic purpura (ITP) (2–4%) and autoimmune hemolytic anemia (AIHA) (10–20%). CLL cells are small cells that are predominantly arrested in the G0/G1 stage of the cell cycle. They are characterized by the co-expression of CD5, CD19 and CD23. Furthermore, B-CLL cells have low expression of a truncated B-cell receptor (CD79a/CD79b) [7]. They express major histocompatibility complex (MHC) class I and II, CD54 (ICAM-1), CD27, CD40 and CD154 (CD40L) in up to one-third of cases [4]. CLL cells, in common with other B-cell malignancies, fail to express or express only weakly adhesion or costimulatory molecules including CD80 and CD86, which are essential for the function of any antigen-presenting cell (APC). Chemokine receptor expression on B-CLL cells including CXCR4, has been implicated in the tendency of B-CLL cells to home to the marrow [8].

CLL cells not only lack costimulatory molecules, they also actively secrete immune inhibitory cytokines including IL-10 and TGF-β, which suppress antigen-specific responses in advanced cancer and granulomatous diseases, such as sarcoids [9, 10]. Furthermore, T-cells in CLL patients are often dysfunctional as demonstrated by poor IL-2 secretion and proliferative responses to mitogens or alloantigens [11].

CD40 in normal B-cells and B-CLL

CD40 is a molecule of the family of tumor necrosis factor receptors (TNFR), which is expressed throughout B-cell development and is implicated in cell survival and differentiation [12, 13]. Its
physiological ligand CD40L (CD154) is a member of the TNF family [14]. CD40/CD40L interaction stimulates B-cells, dendritic cells (DC) and monocytes to proliferate, differentiate, upregulate costimulatory molecules and increase antigen presentation [15–18]. In particular, cognate antigen recognition by the T-cell receptor (TCR) induces an upregulation of CD154 on the CD4+ T-cell, enhanced signaling via CD40 and upregulation of CD80/CD86 on the APC. This in turn amplifies the activation of the T-cells and promotes their differentiation thereby modulating humoral immune responses [19]. Congenital deficiency of CD154 in humans, the X-linked Hyper-IgM Syndrome (HIGM), leads to recurrent bacterial infections due to an inability to mount sufficient antibody class switching [20]. Patients with B-CLL have immune defects similar to those observed in patients with inherited CD154 deficiency. While CLL-cells express variable amounts of functional surface molecules to those observed in patients with inherited CD154 deficiency. Anti-apoptotic effects of CD40 signaling

CD40 ligation induces expression of CD95, a receptor for apoptotic signals, but paradoxically provides a strong NF-κB mediated survival signal to B-CLL cells in vitro [4, 23]. This is interesting since increased apoptosis has been shown for other B-cell malignancies such as multiple myeloma [24]. Correspondingly, CD40 activation of B-CLL cells reduces fludarabine-induced apoptosis in vitro [23]. It remains to be determined if these findings can be reproduced in vivo.

CD40 activation induces APC function in B-CLL cells

In order to investigate how T-cell unresponsiveness could be overcome we and others studied the effect of CD40 signaling in B-cell malignancies: Ranheim was the first to demonstrate that CD40 activation of B-CLL upregulates B7 molecules and that these cells present alloantigen significantly better than unstimulated B-CLL cells [15]. Correspondingly, surface expression of costimulatory molecules can be upregulated and T-cell unresponsiveness to allo- and tumor antigens overcome in several other B-cell malignancies [25–28]. Buhmann et al. showed an increased expression of adhesion molecules and presentation of antigen to autologous T-cells. Interestingly, allogeneic CLL cells induced the expansion of CD8+ cells whereas autologous cells induced Th-like CD4+ cells [29]. We have examined the antigen-presenting capacity of CD40-activated CLL cells and DC pulsed with apoptotic bodies of CLL cells. Both APC types were capable of generating T-cell lines that proliferate specifically in response to unstimulated CLL cells. However, whereas cytotoxic responses against stimulated and unstimulated CLL cells could be repeatedly generated by allogeneic healthy donors, autologous cytotoxicity against CLL cells was rarely detected. T-cells isolated from patients with CLL could recognize and lyse allogeneic-stimulated and unstimulated CLL cells, demonstrating that cytotoxic T-cells from these tumor-bearing patients are functionally intact [30]. Therefore, additional mechanisms, e.g. lack of CD4-mediated help, must be responsible for the inability to induce autologous cytotoxic responses.

Another approach to upregulate accessory and costimulatory molecules on B-CLL cells is adenviral transfection of CLL cells [31]. Initial studies showed technical difficulties, suggesting that transfection of the human molecule might be more difficult than the murine homolog and that activation might be a prerequisite of successful transfection with CD40L (CD154) [32, 33]. Using an optimized adenovirus-free packaging system Wendtner et al. first demonstrated efficient infection of resting CLL cells using recombinant adeno-associated virus (rAAV) vectors encoding CD40 ligand (MOI 100; efficiency 97%). This resulted in upregulation of CD80 in infected and noninfected bystander cells, inducing proliferation of allogeneic T-cells [34]. This effect can be amplified by transduction of leukemic cells with the interleukin-2 gene [35]. Briones et al. demonstrated the potential of this approach in a murine lymphoma model: vaccination using A20 lymphoma cells optimized adenovirus-free packaging system Wendtner et al. first demonstrated efficient infection of resting CLL cells using recombinant adeno-associated virus (rAAV) vectors encoding CD40 ligand (MOI 100; efficiency 97%). This resulted in upregulation of CD80 in infected and noninfected bystander cells, inducing proliferation of allogeneic T-cells [34]. This effect can be amplified by transduction of leukemic cells with the interleukin-2 gene [35]. Briones et al. demonstrated the potential of this approach in a murine lymphoma model: vaccination using A20 lymphoma cells adenovirally transduced with CD40-L successfully induced rejection of a lethal dose of parental cells [36]. Furthermore, recent observations suggest that difficulty in CD40L transfection can be overcome by simple coculture of CLL cells with CD40L-transfected bystander cells [37].

To better quantify the effect of CD40 ligation on B-CLL cells and to optimize the approach for vaccination strategies we dissected the effects of CD40 activation on CLL cells by quantifying the density of adhesion, costimulatory and MHC molecules. B-CLL cells activated via CD40 in the presence of IL-4 and INF-γ upregulate the adhesion molecule CD58, CD80 and MHC-II molecules to levels comparable with immature dendritic cells (iDC), while MHC class-I molecules were induced to levels higher than iDC. Correspondingly, presentation of alloantigen by CD40-CLL cells was comparable to normal CD40-activated B-cells and strongest when activated in the presence of IL-4 and INF-γ (Figure 1). Non-activated B-CLL cells secreted little IL-6 or IL-12. Similar to normal B-cells, IL-12 but not IL-6 secretion could be upregulated when CD40-CLL cells were cultured in the
presence of INF-γ, suggesting that this feedback mechanism is still intact in B-CLL cells [38]. While CD40 activation and INF-γ also induced IL-10 and TGF-β secretion by B-CLL cells, the addition of IL-4 antagonized this effect. Furthermore, CD40-activated B-CLL cells induced T-cells to secrete GM-CSF and INF-γ. This secretion is reduced when B-CLL cells were cocultured with IL-10 and partially restored when INF-γ was present in the B-CLL culture. Thus generation of CD40-CLL cells for vaccination purposes might be optimized by co-activation with IL-4 and/or INF-γ and simultaneous blockade of IL-10 (Schultze et al., unpublished results).

These in vitro data have been supported by in vivo studies showing that co-injection of murine leukemia cells and CD40L-transfected fibroblasts into BALB/CBYJ mice induced a CD4-, CD8- and NK-cell-mediated immune response to pre-existing tumor and prolonged survival [39].

**Table 1. CD40-B cells and CD40-CLL cells as APC for vaccination**

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<th>Mechanism</th>
<th>Advantage</th>
<th>Disadvantage</th>
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<tr>
<td>CD40-B plus antigen</td>
<td>Antigen-loaded professional APC</td>
<td>Tumor antigens need to be known</td>
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<td>Antigen specificity</td>
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<td>Generation in large quantities</td>
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<td>CD40-CLL</td>
<td>Enhancement of immunogenicity of tumor APC</td>
<td>Generation of tumor APC</td>
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<td>Tumor specificity</td>
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<td>Independence of known tumor antigens</td>
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APC, antigen-presenting cells.

Use of normal CD40-activated B-cells for the presentation of tumor-derived antigens

An alternative approach is the use of professional APC loaded with the antigen of interest for vaccination. This strategy is limited by the fact that relevant tumor rejection antigens must be known and loaded onto the cells and are not naturally expressed as when using CD40-CLL cells. On the other hand, professional APC, such as DC, more likely induce efficient immune responses than engineered tumor cells. The effect of CD40 activation on normal B-cells can be used to generate professional, autologous APC to treat CLL (Table 1; Figure 2). We have previously shown that CD40-activated B-cells (CD40-B cells) can be generated efficiently from peripheral blood and significantly express adhesion, MHC and costimulatory molecules and present antigen [40]. We have now further optimized this system and developed methods to generate large amounts of CD40-B under quality controlled, clinical conditions in cancer patients. Furthermore, it is the first cell other than a DC that has been shown to prime naïve T-cells in vitro [18]. These or other APC could be loaded with CLL-specific tumor antigens and used for active or passive immunotherapeutic approaches [41]. The idiotype of CLL is a protein unique to tumor cells and could therefore play a role in the development of highly targeted therapy in CLL. It has been demonstrated that the idiotype can be the target of T-cell-mediated immunity in mice and humans [42–46]. We have previously shown that peptides derived from the framework, the conserved parts of the immunoglobulin, can be used to induce tumor-specific CTL in CLL and other B-cell malignancies. Targeting these epitopes that are shared by a number of CLL patients could be a more broadly applicable though specific approach [47]. Furthermore, these epitopes can be rendered more immunogenic amino acid substitutions increasing MHC binding affinity [48].

In order to increase the therapeutic relevance, the identification of further tumor antigens that are shared by many patients possibly beyond the CLL cohort is warranted. One possible approach is the characterization of anti-tumor antibody responses in CLL patients using the SEREX (serological identification of antigens by recombinant expression cloning) methodology [49]. Furthermore, using novel genomics tools such as differential gene expression, genes overexpressed in B-CLL (and/or other malignancies) can be identified and tested for their immunological potential [50].

Vaccination using B-CLL cells transfected with CD40L

Of the several possible approaches to bring CD40 activation of B-CLL cells to the clinic, the first clinical trial was conducted with adenovirally transfected B-CLL cells expressing the CD40L as a transgene (Ad-CD154) in 11 patients [51]. One time injection of Ad-CD154-transduced B-CLL cells induced an upregulation of immune accessory molecules on bystander tumor cells, high blood levels of IL-12 and INF-γ, and an increase of blood T-cells. In particular, B-CLL-specific T-cells increased in frequency post-vaccination. Clinically, a reduction in leukemia cell counts and lymph node size was demonstrated without signs of autoimmunity [51].

**Future directions**

Treatment approaches using CD40-activated B-CLL cells have shown promising preliminary results, but there are still several issues that need to be addressed: (a) efficient transfection with human CD40L under clinical conditions, (b) role of the transfected versus the non-transfected CLL cells, (c) activation via clinical-grade soluble CD40L or CD40 mAb and (d) the therapeutic value of these approaches.

Treatment at early stages or post-transplantation will more likely be settings in which induction of anti-tumor immunity
could prove beneficial. It remains to be determined, if cellular adjuvants can be substituted by FDA-approved reagents that can be produced on a large scale.

Taken together, there is a clear incentive to further develop CD40-based strategies into novel therapies for CLL. However, several biological questions need to be addressed and in light of increasing regulatory difficulties for cellular therapies and the fast development of alternative approaches, it needs to be carefully evaluated whether these exciting but complex vaccines have the chance to become standard care for CLL.

References

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