**Project 1 AG Prof. Jahrsdörfer:**
**Development of regulatory B cells as cellular immunotherapeutic agent**

**Summary:** The main task of B cells in the immune system is considered their antigen-specific differentiation into antibody-producing plasma cells. Less is known about their potential to act as immunoregulatory cells. Recently, we demonstrated that human B cells secrete the cytotoxic serine protease granzyme B (GzmB) after activation of their B cell receptor and stimulation with interleukin 21 (IL-21). We further showed that these GzmB⁺ B cells exhibit a strong antiproliferative effect on T cells, which is reminiscent of regulatory T cells. The current project is designed to investigate the regulatory effect of these so-called GraB cells in more detail and to develop them as immunoregulatory cell therapeutic agent for inflammatory diseases such as transplant rejection, graft-versus-host disease (GvHD) and various autoimmune diseases.

**Detailed description:** The acute phase cytokine interleukin 21 (IL-21) is regarded as key cytokine for the differentiation of B cells into plasma cells. IL-21 is secreted by fully activated CD4⁺ T helper lymphocytes, among other cells. Complete T cell activation, which results from simultaneous stimulation of the T cell receptor (TCR) and the co-stimulatory receptor CD28, is associated with both IL-21 secretion and strong upregulation of CD40 ligand (CD40L, CD154). In contrast, activation of the TCR in the absence of CD28 co-stimulation results in continued secretion of IL-21 by T helper cells, but no relevant upregulation of CD40L. We demonstrated that such incompletely activated T helper cells, instead of driving B cells into plasma cell differentiation, induce strong expression of the serine protease granzyme B (GzmB) in B cells. Furthermore, we showed that GzmB-expressing B cells are able to induce apoptosis in select target cells such certain tumor cells, although they produce no perforin. This may be explained by perforin-independent ways for GzmB to be taken up into the target cell cytosol such as by heat shock proteins, by viral and bacterial proteins, or by the mannose-6-phosphate receptor. GzmB-secreting B cells may therefore be involved in the early defense of viral and bacterial infections and in tumor immunosurveillance, before antigen-specific T cells resume this task and B cells further differentiate into plasma cells.

Due to the absence of perforin, the above-described cytotoxic function of GzmB-secreting B cells represents only one side of the coin. Mouse models show that IL-21 can induce regulatory B cells. These murine regulatory B cells are phenotypically and functionally characterized by their capacity to produce the immunosuppressive cytokine IL-10, but no GzmB. Consequently, the main effect of such so-called B10 cells is an IL-10-mediated modulation of the cytokine profile of T cells with inhibition of IFN-γ and TNF-α. In contrast, B10 cells do not directly influence the proliferation and viability of effector T cells, which functionally distinguishes them from regulatory T cells. As described above, IL-21 induces large quantities of GzmB in human B cells, but only low amounts of IL-10. This effect is synergistically enhanced in the presence of antigen-specific stimulation of the B cell receptor (BCR). Therefore, we hypothesized that GzmB-expressing human B cells may develop an antigen-specific regulatory function towards effector T cells. In contrast to mice however, this immunoregulatory effect may primarily depend on GzmB-mediated mechanisms, similarly to what is described for regulatory T cells and tolerogenic plasmacytoid dendritic cells (pDCs).
Figure 1. Illustration of the difference between full T cell help compared with incomplete T cell help for B cell differentiation. Stimulation of both the T cell receptor (TCR) by MHC/peptide complexes and CD28 by B7 molecules on antigen-presenting cells result in full activation of CD4 T helper cells (left panel side). Such fully activated T cells secrete IL-21 and express high levels of CD40 ligand (CD40L). This enables them to induce the differentiation of plasma cells from B cells, which receive stimulation of their B cell receptor (BCR) by specific antigens at the same time. In certain situations the TCR of CD4 T cells is stimulated in the absence of co-stimulation via CD28 (right panel side). For example, in the case of an HIV infection the viral protein Nef is able to directly stimulate the TCR. This results in incomplete activation of the T cells, which respond with IL-21 secretion, but no upregulation of CD40L. After interaction with such incompletely activated T helper cells, BCR-stimulated B cells differentiate into GraB cells with regulatory potential.

Indeed, our recent work confirms that GzmB-expressing B cells exhibit a strong antiproliferative effect on effector T cells. To delineate GzmB-expressing B cells from IL-10-secreting B10 cells, we termed this type of human regulatory B cells GraB cells. An important underlying mechanism of the immunosuppressive effect of GraB cells consists of GzmB-mediated degradation of the TCR ζ-chain. Since various studies encourage the hypothesis that tumor-infiltrating B cells may support an immunosuppressive milieu in solid tumors, we started screening paraffin-embedded tissue sections of different tumors for the presence of GraB cells. This screening yielded several tumor entities including carcinomas of the breast, the ovary, the cervix and the colon, which were infiltrated by both GzmB-expressing B cells and IL-21-expressing T cells. These findings show that GraB cells may directly contribute to an immunosuppressive milieu within solid tumors, comparable to what has been described for regulatory T cells in the past.

Meanwhile we were able to prove the in-vivo existence of viable GraB cells. We demonstrated that in untreated HIV patients up to 98% of the B cells circulating in the peripheral blood express GzmB and exhibit GzmB-dependent regulatory potential towards T cells. These GraB cells in HIV patients are also characterized by enhanced expression of CD5, CD43, CD86 and CD147. The fact that they do not produce IL-10 distinguishes them both phenotypically and functionally from regulatory B10 cells. Again, the most obvious feature of their regulatory activity is the direct GzmB-dependent degradation of the TCR ζ-chain of co-cultured effector T cells, resulting in a significantly reduced proliferative capacity.

The reason for the development of GraB cells in HIV patients appears to be the viral protein Nef, which is able to directly stimulate the TCR of CD4 T helper cells, independently of the presence of antigen-presenting cells. As described above, this results in incomplete activation of such T helper cells with strong secretion of IL-21, but no enhanced expression of CD40L. Isolation of such IL-21+CD40L T helper cells from HIV patients and co-culture with...
B cells from healthy subjects show that this constellation indeed triggers the differentiation of interacting B cells into GraB cells (Fig. 1). As expected, the addition of agonistic CD40L multimers to such co-cultures allows enhanced plasma cell differentiation from B cells. As proof-of-principle, we confirmed the existence of GraB cells in a patient with a congenital mutation of the nuclear-factor-κ-B essential modulator (NEMO), which results in disrupted CD40L signaling. The majority of peripheral B cells from this patient were GraB cells and strongly suppressed T cell proliferation and viability. Our data show that GraB cells represent a highly effective regulatory B cell population in humans, which may contribute to the impairment of cellular immunity in HIV patients and certain congenital immune defect syndromes. They may also explain ineffective antibody responses in HIV patients after vaccination. Therefore, the use of agonistic CD40L multimers as adjuvants may possibly enhance vaccination outcomes in this group of patients.

Previously we found that GzmB-expressing CD5+ malignant B cells from patients with chronic lymphocytic leukemia are able to induce apoptosis in each other in a GzmB-dependent manner. Later we discovered a CD5+ GzmB-expressing B cell population with similar properties in patients with autoimmune diseases such as systemic lupus erythematosus (SLE). We therefore assume that CD5+ GraB cells are not only able to regulate the cellular immune response, but also their own survival. Interestingly, CD5+ B cells from cord blood exhibit a significantly higher potential to express GzmB after IL-21 stimulation than CD5- B cells. This suggests that CD5+ GraB cells may also play a role for the successful induction of tolerance during pregnancy. The primary role of the CD5+ GraB cell population may be the regulation of T cells from the maternal circulation in the fetal blood, thereby preventing their activation and expansion by fetal antigens. The lack of co-stimulatory signals from professional APC (which are activated only in the presence of danger signals such as TLR agonists, e.g. in the course of infections) ensures that CD4+ T helper cells express IL-21 only, but not CD40L. Consequently, these T helper cells may differentiate fetal CD5+ B cells into immunoregulatory GraB cells, which subsequently suppress effector T cells of similar antigen specificity. Support for this hypothesis comes from a related area of immunology, namely organ transplantation. Recent data from a French research group suggest that GzmB-expressing CD5+ B cells play an important role for the induction of tolerance towards renal transplants. Of note, the mechanism of this tolerance induction also depends on GzmB, but not IL-10, suggesting these regulatory B cells are identical with the GraB cells described by our own group. It appears promising to further investigate the role of GraB cells in different physiological and pathophysiological situations, which may involve the induction or the loss of immune tolerance. Such situations may include pregnancy and abortion, various autoimmune diseases, acute and chronic GvHD after stem cell transplantation, and graft rejection after solid organ transplantation.

In summary, GraB cells represent a novel type of immunoregulatory B cells, which appear to complement the effect of B10 cells in humans. GraB cells are centrally involved in various pathophysiological processes and can be differentiated ex vivo from both naïve and memory B cells. Further investigation of GraB cells may pave the way for their future therapeutic and diagnostic use. The quantification of GraB cells in patients with tumors or infections for example may allow prognostic predictions about the clinical course of the diseases. One step ahead, ex-vivo generation of GraB cells or their in-vivo induction may represent a novel approach for the therapy of inflammatory diseases such as GvHD or autoimmune diseases (Fig. 2). On the other hand, pharmacological inhibition of GraB cells in terms of an immunological checkpoint blockade may enhance alternative immunotherapeutic approaches for the treatment of tumors and infectious diseases (Fig. 3).
Figure 2. Cartoon illustrating potential collection and ex-vivo induction of human regulatory B cells. Abbreviations: GvHD=graft-versus-host-disease, NEMO=nuclear-factor-κ-B essential modulator.

Figure 3. Potential checkpoints for the inhibition of GraB cells. Both suppression of B cell surface receptors including the B cell receptor (BCR) itself, the IL-21 receptor or the Fcγ receptor as well as inhibition of signaling molecules further downstream may interfere with the immunoregulatory function of GraB cells (1). On the level of CD4+ T helper cells, the stimulation of CD28 or inhibition of CTLA-4 may induce CD40L, which may shift the differentiation of interacting B cells into plasma cells instead of GraB cells (2). A similar effect would result from direct stimulation of CD40 on B cells (3). Another possibility to interfere with GraB cell function may be direct suppression of functional GzmB (4).
References


