Antigen receptor triggering and apotopic pathways in neoplastic B cells
Mackus, W.J.M.

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The work described in this thesis focuses on the functional consequences of antigen receptor triggering (BCR) of B cells. In addition, the pathways by which programmed cell death is induced and how this might be disregulated in neoplastic B cells, and consequently might lead to B cell malignancy, were studied. Chapter 1 introduces the current understanding of BCR-mediated signaling and apoptosis in B cells and provides an outline for the studies presented. The first part of the thesis (chapter 2 & 3) describes the biochemical analyses of the events upstream and downstream of mitochondrial depolarization mediated by receptor-derived signals such as CD95, BCR and CD40 using the Ramos cell line as an in vitro model for mature B cells. A further understanding of BCR triggering and apoptosis regulation in neoplastic B cells was investigated in cells isolated from B-CLL patients as described in the latter part (chapter 4, 5 & 6).

BCR triggering can result either in induction of cell cycle arrest or apoptosis of B cells. The commitment to apoptosis following cell cycle arrest after BCR triggering was investigated (chapter 2). Triggering of the BCR of Ramos cells with a mAb resulted in both cell cycle arrest and apoptosis. Similarly, incubation of Ramos cells with sodium butyrate (n-But), a direct inducer of cell cycle arrest, was complemented with apoptosis. Still, costimulation of B cells with T cell-derived signals could uncouple apoptosis from growth arrest. Whereas ligation of CD40 or addition of TNF-α enhanced BCR-mediated or sodium butyrate-induced growth arrest, release of cytC from the mitochondria was prevented and apoptosis inhibited. Thus, a BCR-triggered growth arrest is not obligatory followed by apoptosis, providing the B cell an extended timeframe to integrate signals for further B cell selection.

Several investigations regarding the induction of BCR-mediated apoptosis indicate involvement of the mitochondrial apoptosis pathway mediating caspase 3 activation independent of the CD95-mediated apoptosis pathway. Differences in caspase 3 processing suggested the involvement of a specific zVAD-insensitive initiator protease in BCR-mediated apoptosis. Utilizing Ramos cells a comparative analysis of CD95- versus BCR-mediated apoptosis cells shows (chapter 3) that although both apoptosis pathways require the involvement of Bax activation and cytC release they differ in timing and caspase-dependence of mitochondrial membrane depolarization. This might point to engagement of distinct upstream pro-apoptotic mediators in both pathways. Furthermore, initial activation of caspase 3 processing can be mediated by caspase 9, even in the presence of zVAD. No evidence for involvement of a BCR-specific protease is obtained.

During the course of our studies it became apparent that two subgroups of B-CLL patients with a different clinical outcome could be identified based on the percentage of mutations present in the IgVH genes of the BCR. In addition, these two subgroups differentially expressed ZAP-70 mRNA which was suggested to be involved in signaling downstream of BCR triggering. It was investigated how ZAP-70 protein expression correlated with IgVH mutation status and whether the differences in functional outcome of BCR triggering could account for the distinct biological behaviour of the B-CLL subgroups (chapter 4). Expression of ZAP-70
protein in B-CLL cells correlated with the unmutated IgV<sub>H</sub> status as detected by Western blot and confirmed by FACS-analysis. As to spontaneous apoptosis or cytostatic drug-induced apoptosis no differences were observed between IgV<sub>H</sub> unmutated or mutated B-CLL patients. However, in patients expressing unmutated IgV<sub>H</sub> genes BCR ligation reduced the percentage of leukemic cells undergoing spontaneous apoptosis and diminished the sensitivity of these cells to cytostatic drugs. These findings indicate that in unmutated IgV<sub>H</sub> B-CLL BCR signaling interferes with the induction of apoptosis and suggests that chemotherapy might be less effective due to continuous in vivo BCR signaling, explaining the unfavourable clinical outcome in this group of patients.

Insight at the molecular level of apoptosis regulation of B cells was obtained by a comprehensive expression survey of both anti- and pro-apoptosis regulators (RT-MLPA analysis) in normal and malignant B cells (chapter 5). Shifts in the apoptosis gene expression profile of normal B cells alternating from an anti-apoptotic to a pro-apoptotic status and back again were observed during the transition of a naive to a germinal center to a memory B cell stage. Comparison of normal and leukemic B cells identified aberrant expression of anti- but moreover pro-apoptosis regulators which lead to a block in both the CD95-mediated and mitochondrial apoptosis pathways explaining the prolonged cell survival of the leukemic cells. Paradoxically, the apoptogenic BH3-only family members Bmf and Noxa were found to be strongly upregulated. Though their levels were not sufficient for direct induction of apoptosis. Culture of leukemic cells in vitro but moreover in combination with cytostatic drugs induced profound upregulation of the p53-responsive gene Puma which was accompanied with apoptosis. Although no differences in the basic apoptosis gene expression profile comparing IgV<sub>H</sub> unmutated and mutated patients could be detected, cells of the latter subgroup showed more profound Puma upregulation during cytostatic drug-induced apoptosis. These results suggested that the difference in clinical outcome of the two B-CLL subgroups might originate from a different capacity to induce the p53-mediated apoptosis pathway upon triggering of the BCR.

Next to the clonal expansion of B cells B-CLL patients show increased absolute numbers of CD8<sup>+</sup> T cells. It has been suggested that these T cells initiate cytotoxic immune responses against the leukemic B cells in an effort to eliminate the tumor. Since concrete evidence for this hypothesis is missing, the T cell compartment of B-CLL patients and the ability of these cells to mount specific responses was analysed (chapter 6). Immunophenotypical analyses showed significantly increased absolute and relative numbers of CD8<sup>+</sup> cytotoxic effector cells in B-CLL patients when compared to healthy age-matched controls. Antigen-specificity of these CD8<sup>+</sup> cytotoxic effector cells was specifically directed against cytomegalovirus as detected by using tetrameric CMV-peptide complexes. Since the expansion of T cells was not observed in those patients who had not been infected with CMV it rather relates to chronic viral infection than to tumor-specificity indicating that the T cell expansion in B-CLL is not directed against the leukemic cells.

The implications of these studies regarding the funtional consequences of BCR triggering and the identifications of aberrations in the apoptosis pathways of
neoplastic B cells are discussed in chapter 7. The findings imply that the differential clinical outcome of the two IgVH subgroups of B-CLL patients might be related to a different capacity to signal via the BCR and/or to activate the p53-mediated apoptosis pathway. Expression of ZAP-70 appears to be involved and the association of ZAP-70 signaling with B-CLL should be further investigated. The findings have increased the understanding of apoptosis regulation in neoplastic B cells and a model for apoptosis regulation in B-CLL is proposed (chapter 7, Fig. 1). At last, suggestions for future strategies for the development of novel anti-cancer therapeutics are presented.