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Tetraspanin CD37 protects against the development of B cell lymphoma

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Worldwide, B cell non–Hodgkin lymphoma is the most common hematological malignancy and represents a substantial clinical problem. The molecular events that lead to B cell lymphoma are only partially defined. Here, we have provided evidence that deficiency of tetraspanin superfamily member CD37, which is important for B cell function, induces the development of B cell lymphoma. Mice lacking CD37 developed germinal center–derived B cell lymphoma in lymph nodes and spleens with a higher incidence than Bcl2 transgenic mice. We discovered that CD37 interacts with suppressor of cytokine signaling 3 (SOCS3); therefore, absence of CD37 drives tumor development through constitutive activation of the IL-6 signaling pathway. Moreover, animals deficient for both Cd37 and Il6 were fully protected against lymphoma development, confirming the involvement of the IL-6 pathway in driving tumorigenesis. Loss of CD37 on neoplastic cells in patients with diffuse large B cell lymphoma (DLBCL) directly correlated with activation of the IL-6 signaling pathway and with worse progression-free and overall survival. Together, this study identifies CD37 as a tumor suppressor that directly protects against B cell lymphomagenesis and provides a strong rationale for blocking the IL-6 pathway in patients with CD37− B cell malignancies as a possible therapeutic intervention.

Introduction
The majority of B cell lymphomas originate from germinal center–derived (GC-derived) B cells, which is the result of genetic defects during VDJ recombination, somatic hypermutation and class-switching recombination (1). The best-known chromosomal aberration in follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) is translocation of t(14;18), resulting in constitutive expression of BCL-2 and defective apoptosis (2), which is correlated with worse survival in patients with DLBCL (3). However, t(14;18) can also be detected in B cells of healthy individuals, suggesting that the translocation by itself is insufficient and other genetic alterations are required to induce B cell lymphoma (4, 5). Detailed genomic analyses revealed the complexity of different pathways that are recurrently altered in lymphomas, including B cell receptor, Toll-like receptor, Notch, and NF-κB signaling pathways (6, 7). The challenge is to identify the driver mutations of these altered pathways in order to unravel how these genetic aberrations contribute to B cell lymphomagenesis.

IL-6, originally identified as a B cell–differentiating factor, contributes to the growth of many types of cancer, including hematological tumors (8, 9). IL-6 exerts its biological function via a receptor complex composed of the IL-6 receptor α chain (IL-6Rα) and the common signaling receptor gp130 (10) that together activate 3 pathways: the JAK/STAT3 (11), PI3K/AKT (12), and Ras/MAPK pathways (13). Activation of the IL-6 signaling pathway is negatively regulated by suppressor of cytokine signaling 3 (SOCS3), which is transcribed upon DNA binding of STAT3 homodimers (14). Cytosolic SOCS3 translocates to the plasma membrane, in which its SH2 domain binds gp130 to prevent binding and phosphorylation of STAT3 proteins (15, 16). Simultaneously, SOCS3 binds JAK1/2 with its kinase-inhibitory region, which targets these proteins for ubiquitination (17). In B cell lymphoma, proteins of the JAK/STAT3 signaling pathway are frequently overexpressed, contributing to cancer development and progression (18, 19). In addition, autocrine IL-6 production in DLBCL provides proliferative and antiapoptotic signals, and IL-6 levels in serum correlate with the prognosis of the disease (20). To date, the underlying mechanism responsible for the constitutive activation of the IL-6 pathway in cancer is largely unknown.

Tetraspanins belong to the superfamily of transmembrane 4 proteins that form multimolecular complexes with other tetraspanin proteins, integrins, growth factors, and signaling molecules (21–24). Targeting of CD37 is currently under investigation in clinical trials for patients with B cell malignancies, but the molecular pathways have not been fully resolved (25, 26). CD37 is
months of age, \( Cd37^{+/–} \) mice spontaneously developed large neoplasms in mLNs, spleens, and livers in contrast to age-matched \( Cd37^{+/+} \) WT littermates (referred to herein as WT mice; Figure 1, A and B). Extensive analyses of mice at different ages revealed that over 50% of \( Cd37^{–/–} \) mice developed tumors starting at 12 months. In contrast, only approximately 15% of WT mice developed tumors at the age of 22 months (Figure 1B and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI81041DS1), which is in line with the reported tumor occurrence in aged inbred C57BL/6 mice (33, 34).

\( Cd37^{+/–} \) heterozygous mice were healthy and did not develop lymphomas until 20 months of age with the same incidence as WT mice (data not shown). The neoplasms in \( Cd37^{–/–} \) mice were characterized by nodular growth of small and large pleiomorphic B cells, highly expressed on mature B cells and is required for optimal GC function and long-lived antibody production (27, 28). Mice deficient for \( Cd37^{–} \) (\( Cd37^{–/–} \) mice) have impaired humoral and cellular immune responses (29–31). This paper provides the first evidence to our knowledge that CD37 is a novel tumor suppressor that acts to suppress IL-6–driven B cell transformation in vivo.

Results

\( Cd37^{–} \) deficiency predisposes mice to develop spontaneous B cell lymphoma. CD37 is highly expressed on mature B cells and plays a fundamental role in B cell function and humoral immunity (27, 28). Although \( Cd37^{–/–} \) mice develop normally, with unaltered numbers of lymphoid and myeloid cells in lymphoid organs (32), we observed that \( Cd37^{–/–} \) mice became diseased during aging. By 15 months of age, \( Cd37^{–/–} \) mice spontaneously developed large neoplasms in mesenteric lymph nodes (mLNs), spleens, and livers in contrast to age-matched \( Cd37^{+/+} \) WT littermates (referred to herein as WT mice; Figure 1A and B). Extensive analyses of mice at different ages revealed that over 50% of \( Cd37^{–/–} \) mice developed tumors starting at 12 months. In contrast, only approximately 15% of WT mice developed tumors at the age of 22 months (Figure 1B and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI81041DS1), which is in line with the reported tumor occurrence in aged inbred C57BL/6 mice (33, 34). \( Cd37^{–/–} \) heterozygous mice were healthy and did not develop lymphomas until 20 months of age with the same incidence as WT mice (data not shown). The neoplasms in \( Cd37^{–/–} \) mice were characterized by nodular growth of small and large pleiomorphic B cells,
notype of the mature lymphoma cells by flow cytometry. Cd37–/– lymphomas were all negative for IgD, IgM, and IgG, whereas IgA was the most abundant immunoglobulin expressed by the neoplastic cells (Figure 2C and Supplemental Figure 1B). This was in contrast to the few lymphomas that were found in approximately 15% of aged WT mice, which were of the IgG or IgM phenotype (Supplemental Figure 3). Taken together, these data demonstrate that the Cd37–/– neoplasms consist of mature isotype-switched IgA+ B cells that originate from the GC.

Neoplastic Cd37–/– cells are of monoclonal origin and induce disease upon transfer into young mice. To investigate the clonality status of the neoplastic Cd37–/– cells, molecular genetic analysis of the B cell receptor was adapted using fresh lymphoma tissues from Cd37–/– mice and mLN tissue samples from aged WT mice as a control. In normal mLN tissue from WT mice, VJ gene rearrangements of the immunoglobulin heavy chain (IGH) locus demonstrated a Gaussian-like distribution of peaks, which was the result of the presence of some mitotic figures, histocytes, multinucleated giant cells, and focal necrosis (Figure 1C). Immunohistochemical analyses demonstrated that the lymphomas were strongly positive for both B cell markers, B220 and CD19 (Figure 1D). CD3+ T cells and CD68+ macrophages were apparent (Figure 1D), whereas Gr1+ myeloid cells and CD138+ plasma cells were scarce (Supplemental Figure 1A). Quantification of different immune cell subsets in Cd37–/– lymphomas and WT mLN control tissues by flow cytometry confirmed these results (Supplemental Figure 2). These data demonstrate that Cd37 deficiency promotes spontaneous development of B cell lymphoma in vivo.

Neoplastic cells are IgA+ GC-derived tumor cells. Expression of costimulatory molecules (CD86, MHC-II, PNA, and CD43) revealed that the lymphoma cells phenotypically resembled mature GC B cells (Figure 2A). This was confirmed by high expression of the GC markers PNA and GL7 in lymphomas of aged Cd37–/– mice (Figure 2B). Next, we characterized the immunoglobulin phenotype of the mature lymphoma cells by flow cytometry. Cd37–/– lymphomas were all negative for IgD, IgM, and IgG, whereas IgA was the most abundant immunoglobulin expressed by the neoplastic cells (Figure 2C and Supplemental Figure 1B). This was in contrast to the few lymphomas that were found in approximately 15% of aged WT mice, which were of the IgG or IgM phenotype (Supplemental Figure 3). Taken together, these data demonstrate that the Cd37–/– neoplasms consist of mature isotype-switched IgA+ B cells that originate from the GC.
mature isotype-switched IgA + B cells of GC origin and showed a phenotype similar to that of the original transferred tumor (Supplemental Figure 5). Next, neoplastic cells from aged Cd37−/− mice were transferred subcutaneously or intravenously into young WT and immunocompromised mice that lack the lymphocyte compartment, including CD19+B220+ B cells. Whereas tumor cells did not grow in WT mice (data not shown), evident lymphoma growth was observed in the immunocompromised mice, similar to that in young Cd37−/− mice after subcutaneous injection (Figure 3C). Similarly, Cd37−/− neoplastic CD19+B220+ B cells were identified in mLNs (Figure 3D) and spleens (Figure 3E) of immunocompromised mice after intravenous adoptive transfer. Thus, the neoplastic cells derived from aged Cd37−/− mice are of monoclonal origin and can induce disease and exhibit metastatic capacity upon adoptive transfer.

of the polyclonal nature of B cells. In contrast, Cd37−/− B cell lymphomas showed a single dominant peak, indicating a clear clonal IGH rearrangement in all framework regions (referred to herein as FR1, FR2, FR3), indicative of B cell lymphoma (Figure 3A and Supplemental Figure 4). Next, adoptive transfer experiments were performed to explore whether freshly isolated B cell lymphoma cells could be transferred into young (6- to 8-week-old) Cd37−/− mice. Transfer of fresh neoplastic cells from aged Cd37−/− mice rapidly induced tumor growth in healthy recipient Cd37−/− mice irrespective of their age (Figure 3B). The lymphoma cells showed clear metastatic capacity, as several tumor deposits were found in secondary lymphoid organs, including spleen, inguinal, brachial, and axillary lymph nodes. Predominant expression of CD19 and B220 as well as high expression of GL7, CD86, MHC-II, CD43, PNA, and IgA confirmed that all neoplasms resembled mature isotype-switched IgA+ B cells of GC origin and showed a phenotype similar to that of the original transferred tumor (Supplemental Figure 5). Next, neoplastic cells from aged Cd37−/− mice were transferred subcutaneously or intravenously into young WT and immunocompromised mice that lack the lymphocyte compartment, including CD19+B220+ B cells. Whereas tumor cells did not grow in WT mice (data not shown), evident lymphoma growth was observed in the immunocompromised mice, similar to that in young Cd37−/− mice after subcutaneous injection (Figure 3C). Similarly, Cd37−/− neoplastic CD19+B220+ B cells were identified in mLNs (Figure 3D) and spleens (Figure 3E) of immunocompromised mice after intravenous adoptive transfer. Thus, the neoplastic cells derived from aged Cd37−/− mice are of monoclonal origin and can induce disease and exhibit metastatic capacity upon adoptive transfer.
Constitutive activation of the IL-6 signaling pathway in Cd37−/− lymphomas. To gain insight in the mechanism underlying the spontaneous lymphoma development in Cd37−/− mice, we performed quantitative PCR using a Mouse Oncogenes & Tumor Suppressor Genes PCR Array on mLNs from tumor-bearing Cd37−/− mice and age-matched WT littermates. Among the 84 cancer-associated genes, 6 genes were upregulated and 7 genes were downregulated by approximately 2-fold in neoplastic tissue compared with control mLN tissue (see also Supplemental Table 2). Red dots represent genes that were upregulated, and green dots represent genes that were downregulated. (B) Immunohistochemistry analysis of p-AKT and p-STAT3 in mLNs from 18-month-old WT and Cd37−/− mice. Scale bar: 50 μm. Experiments were performed 3 times with 4 mice per group. (C and D) Protein levels of p-STAT3 (76–78 kDa) in mLN and spleen cells from 18-month-old WT and Cd37−/− mice, as detected by Western blot (n ≥ 4 mice per genotype). p-STAT3 protein levels were normalized to total STAT3 levels. Data represent mean ± SEM. *P < 0.05, Mann-Whitney nonparametric test.
Loss of CD37 correlates with upregulated IL-6 signaling and poor survival in patients with DLBCL. To validate the role of CD37 in
To investigate CD37 expression in lymphomas, the authors collected biopsies from 47 patients with de novo GC B cell–like DLBCL (GCB-DLBCL) and 50 patients with activated B cell–like DLBCL (ABC-DLBCL), all of whom were treated uniformly with standard R-CHOP chemotherapy. Gene expression profiling determined the cell-of-origin classification for GCB-DLBCL. Approximately 32% of GCB-DLBCL tumors and 46% of ABC-DLBCL tumors expressed ample CD37 protein at the cell membrane.

**Figure 6.** CD37 controls the IL-6 pathway via SOCS3, and lymphoma development is dependent on IL-6 in vivo. (A) Comparable surface expression of IL-6Rα and gp130 in the plasma membrane of B220+IgA+ B cells from 18-month-old WT and Cd37–/– mice analyzed by flow cytometry (specific antibody staining [black] versus isotype control antibody [gray]). MFI, mean fluorescence intensity. (B) Protein levels of SOCS3 in mLN cells from 18-month-old WT and Cd37–/– mice, as detected by Western blot (noncontiguous lanes from the same gel). SOCS3 protein levels were normalized to actin levels (n = 5 mice per genotype). Data represent mean ± SEM. (C) HEK293 cells were cotransfected (TF) with CD37-GFP (or GFP alone) and SOCS3, followed by immunoprecipitation with anti-GFP or isotype control IgG. Western blots were probed with anti-SOCS3 (26 kDa) and anti-GFP (CD37-GFP, 60–75 kDa, due to heavy glycosylation, ref. 66; noncontiguous lanes from the same gel). (D) Colocalization of CD37 (red) and SOCS3 (green) or control antibody was studied on human JY B cells without (–) or with (+) IL-6 stimulation by confocal microscopy. Colocalization is shown in yellow (white arrows). Manders coefficient was determined with n ≥ 17 cells from 3 independent experiments (each dot represents a single cell). Data represent mean ± SEM. *P < 0.05, Mann-Whitney nonparametric test. (E) Colocalization of CD37 (red) and IL-6Rα (green) or control antibody was studied on human JY B cells by confocal microscopy. Colocalization (yellow) was independent of IL-6 stimulation. Data are shown for B cells without IL-6 stimulation. (F) p-STAT3 (76–78 kDa) levels in NALM-6 cells, which were mock transfected or transfected with CD37-GFP, with or without IL-6 stimulation for 60 minutes. Actin (42 kDa) was used as loading control (noncontiguous lanes from the same gel). (G) Cd37–/–xil6–/– mice are protected against development of B cell lymphoma (n = 26) (see Supplemental Table 1 for details). Original magnification: ×630 (D and E); ×1,260 (insets, D and E).

In conclusion, this study demonstrates the critical role of CD37 in regulating the IL-6 pathway via SOCS3, and highlights the importance of IL-6 in lymphoma development. The findings suggest potential therapeutic targets for the treatment of DLBCL.
months for patients with CD37– tumors, and this median OS was not reached for patients with CD37+ tumors. These findings were confirmed in patients with ABC-DLBCL. Patients with ABC-DLBCL with CD37– tumors had a worse OS (hazard ratio, 2.87; 95% CI, 1.13–7.29; \( P = 0.03 \)) and PFS (hazard ratio, 3.71; 95% CI, 1.55–8.89; \( P = 0.003 \)) compared with patients with CD37+ tumors (Figure 8, C and D). In summary, loss of CD37 in tumors of both patients with GCB-DLBCL and patients with ABC-DLBCL is significantly correlated with activation of the IL-6 signaling pathway and decreased survival.

Discussion

IL-6 has emerged as a critical tumor-promoting cytokine in different types of human cancer, including B cell malignancies (8, 38). Although IL-6 and its downstream effectors are widely studied as therapeutic targets for patients with B cell malignancies, very little is known about the underlying mechanisms that induce constitutive IL-6 signaling in these tumor cells. Here, we demonstrate that deficiency of CD37 leads to spontaneous development of GC-
IL-6 family members in Cd37–/– mice, including IL-11, which was recently reported to play a major role in inflammation-induced tumors due to constitutive STAT3 activation (44). Importantly, we uncovered the underlying molecular mechanism of lymphoma formation in Cd37–/– mice by investigating SOCS3, the major negative regulator of the IL-6 signaling pathway (45). SOCS3 is a tumor suppressor, as demonstrated in conditional Socs3-deficient mice (Socs3 deletion in gastrointestinal epithelial cells) that develop gastric cancer (46). In human B cell malignancies, hypermethylation of SOCS3 leading to hyperactivation of STAT3 has been correlated with worse patient outcome (47, 48). In contrast, increased SOCS3 expression has been reported in melanoma (49) and breast cancer (50). Furthermore, increased SOCS3 mRNA levels in peripheral blood of patients with non-Hodgkin lymphoma have been found to correlate with worse disease outcome (51). We found elevated levels of SOCS3 in Cd37–/– tumors compared with those in WT control tissues, indicating that negative feedback regulation by SOCS3 is defective in the absence of CD37. Indeed, we observed that CD37 directly interacts with SOCS3. Based on these observations, we propose the following model for the role of CD37 in the IL-6 signaling pathway (Supplemental Figure 8).

Upon activation of the IL-6R complex, AKT and STAT3 are phosphorylated, leading to production of SOCS3 and subsequent inhibition of the IL-6 signaling cascade by SOCS3 binding to gp130. CD37 facilitates this inhibition by binding SOCS3 and stabilizing the IL-6R complex in the plasma membrane. In Cd37–/– B cells, SOCS3 cannot bind efficiently to the IL-6R complex, resulting in defective feedback inhibition. Consequently, SOCS3 will accumulate in the cytoplasm due to continuous activation of the IL-6 signaling pathway through autocrine IL-6 production by the tumor cells. In conclusion, the IL-6 derived B cell lymphoma in an IL-6–dependent manner. CD37 is a regulator of SOCS3 and thereby interferes with activation of the IL-6 signaling pathway in B cells.

Gene expression profiling of tumor suppressors and oncogenes in Cd37–/– lymphomas revealed upregulation of Akt1, Stat3, and Jun, which play a central role in the IL-6 signaling pathway and have been implicated in cancer cell survival (18, 19). The constitutive activation of AKT in Cd37–/– lymphomas corresponds to previous work showing that CD37 regulates AKT signaling, leading to B cell survival (27, 39). Importantly, in 68% of human GCB-DLBCL tumors and in 54% of human ABC-DLBCL tumors, CD37 expression was lost, which correlated with increased IL-6 levels and elevated levels of AKT1 mRNA and p-STAT3, validating the findings in Cd37–/– mice.

Bcl2 transgenic mice develop lymphoma at low frequencies (15%–20%) (40) compared with the 50% incidence observed in the Cd37–/– mouse strain at the same age and background. We did not detect enhanced Bcl2 expression in Cd37–/– lymphomas (Supplemental Table 2), indicating that the tumor suppressor activity of CD37 is independent of BCL-2. B cells from young Cd37–/– mice are prone to differentiate toward IgA-producing plasma cells, due to the high IL-6 levels that circulate in vivo (28). Although constitutive expression of the BCL-2 protein has been reported in many human FL and DLBCL tumors (41), we now provide a CD37-dependent mechanism for B cell lymphomagenesis. Our findings are in line with those in studies showing that Il6 transgenic mice are prone to develop FL, plasmacytoma, and DLBCL (42) and IL-6 signaling in B cell malignancies can be independent of BCL-2 (43). The Cd37–/– lymphoma model may serve as a new model for human B cell lymphomas in which the IL-6 signaling pathway is constitutively activated. It will be interesting to investigate other IL-6 family members in Cd37–/– mice, including IL-31, which was recently reported to play a major role in inflammation-induced tumors due to constitutive STAT3 activation (44).

Importantly, we uncovered the underlying molecular mechanism of lymphoma formation in Cd37–/– mice by investigating SOCS3, the major negative regulator of the IL-6 signaling pathway (45). SOCS3 is a tumor suppressor, as demonstrated in conditional Socs3-deficient mice (Socs3 deletion in gastrointestinal epithelial cells) that develop gastric cancer (46). In human B cell malignancies, hypermethylation of SOCS3 leading to hyperactivation of STAT3 has been correlated with worse patient outcome (47, 48). In contrast, increased SOCS3 expression has been reported in melanoma (49) and breast cancer (50). Furthermore, increased SOCS3 mRNA levels in peripheral blood of patients with non-Hodgkin lymphoma have been found to correlate with worse disease outcome (51). We found elevated levels of SOCS3 in Cd37–/– tumors compared with those in WT control tissues, indicating that negative feedback regulation by SOCS3 is defective in the absence of CD37. Indeed, we observed that CD37 directly interacts with SOCS3. Based on these observations, we propose the following model for the role of CD37 in the IL-6 signaling pathway (Supplemental Figure 8). Upon activation of the IL-6R complex, AKT and STAT3 are phosphorylated, leading to production of SOCS3 and subsequent inhibition of the IL-6 signaling cascade by SOCS3 binding to gp130. CD37 facilitates this inhibition by binding SOCS3 and stabilizing the IL-6R complex in the plasma membrane. In Cd37–/– B cells, SOCS3 cannot bind efficiently to the IL-6R complex, resulting in defective feedback inhibition. Consequently, SOCS3 will accumulate in the cytoplasm due to continuous activation of the IL-6 signaling pathway through autocrine IL-6 production by the tumor cells. In conclusion, the IL-6

Figure 8. Loss of CD37 in lymphomas of patients with DLBCL correlates with worse survival. (A and B) Loss of CD37 correlates with significantly poor (A) OS ($P = 0.02$) and (B) PFS ($P = 0.05$) in 47 patients with GCB-DLBCL. (C and D) Loss of CD37 correlates with significantly poor (C) OS ($P = 0.03$) and (D) PFS ($P = 0.003$) in 50 patients with ABC-DLBCL. Log-rank (Mantel-Cox) test was used for statistical analysis.
signaling pathway is constitutively activated, which is caused by defective regulation by SOCS3 due to CD37 deficiency, resulting in malignant B cell transformation and tumor cell survival.

The association of tetraspanins with tumor progression has been established, although the underlying mechanisms have not been fully resolved (23, 24, 52). Little is known about tetraspanin involvement in human B cell lymphomagenesis. CD81 protein is expressed at high levels in normal GC B cells and in DLBCL (53), whereas downregulation of CD9 has been related to lymphoma progression (54). Our data now demonstrate that loss of CD37 in DLBCL is directly correlated with worse OS and PFS, which shows that CD37 may serve as a novel prognostic marker for patients with DLBCL. Sequencing of the CD37 gene in human B cell lymphomas did not reveal obvious mutations (data not shown). Thus, we postulate that CD37 deficiency in human DLBCL is most likely caused by involvement of microRNAs or epigenetic changes, which have been reported for different tumor suppressor genes as an alternative mechanism of gene silencing in cancer (55, 56).

To the best of our knowledge this study is the first to demonstrate that tetraspanin deficiency itself can lead to tumor development. In most human B cell malignancies, including chronic lymphocytic leukemia and different types of non-Hodgkin lymphoma, CD37 is highly expressed by tumor cells (57). Since CD37 can directly couple to B cell survival, CD37 targeting is currently under investigation in clinical trials in patients with B cell malignancies (25, 58, 59). Our study now demonstrates that expression of CD37 is completely lost in 68% of GCB-DLBCL tumors and in 54% of ABC-DLBCL tumors, which has important consequences for selecting patients for CD37-based immunotherapies. Furthermore, we anticipate that patients with CD37 lymphomas may particularly benefit from therapies inhibiting the IL-6 pathway, including monoclonal antibodies that target IL-6 and/or IL-6R (8, 19) and JAK1/2 inhibitors that can reduce p-STAT3 and IL-6 levels in patients with myeloproliferative disorders (60).

Taken together, this study demonstrates that tetraspanin CD37 protects against development of GC-derived B cell lymphoma. We found that CD37 interacts with SOCS3 and thereby induces feedback inhibition of the IL-6 signaling pathways via STAT3 and AKT, providing direct mechanistic insight into the regulation of the IL-6 signaling pathway in B cell biology and lymphomagenesis in vivo. This study is of major clinical relevance in human B cell lymphoma, since CD37 expression in patients with DLBCL is directly coupled to B cell survival, CD37 targeting is currently under investigation in clinical trials and provides a strong rationale for blocking the IL-6 pathway in patients with CD37 B cell malignancies as therapeutic intervention.

Methods

Mice. Cd37−/− mice were generated by homologous recombination (32) and backcrossed to the C57BL/6J background (29). Cd37−/−xIl6−/− mice were generated by crossing Cd37−/− mice with Il6−/− mice (61). WT littermates were matched for age and gender. BALB/cAnNrc–Foxn1nu (nu) mice were purchased from Janvier Labs, and NOD/SCID/IL-2Rγnull (NSG) mice were originally purchased from The Jackson Laboratory. Cd37−/−, Cd37−/−xIl6−/−, and NSG mice were bred in the Radboud University Medical Center Central Animal Laboratory. All mice were housed in top-filter cages and fed a standard diet with freely available water and food.

Patients. Biopsies of 47 patients with de novo GCB-DLBCL and 50 patients with ABC-DLBCL were obtained and stained for CD37 using immunohistochemistry. Patients were diagnosed between 2001 and 2008, lymphomas were classified according to the World Health Organization 2008 classification criteria, and the pathology was reviewed and validated. The median age was 61 years, and all patients underwent standard R-CHOP chemotherapy. Thirty-five patients had died by the last follow-up. The median follow-up interval for the 62 censored patients was 59.6 months (range from 12.2 to 186.7 months). Some of the patients with GCB-DLBCL and ABC-DLBCL had serum available, which was used to evaluate IL-6 levels.

Histology and immunohistochemistry. Collected tissues were fixed in phosphate-buffered 4% paraformaldehyde (PFA) and embedded in paraffin wax. Serial 5-μm tissue sections were stained with H&E prior to microscopical analysis. Two independent pathologists (M.J. Gijbels and K.M. Hebeda) classified the lymphomas as described previously (34). Immunohistochemical stainings were performed on OCT-embedded frozen tissue sections. Tissue slides were dried at room temperature for 30 minutes, fixed in 2% PFA for 10 minutes, and treated with 3% H2O2 in methanol for 15 minutes at room temperature. After blocking Fcγ receptors with normal serum in which the secondary antibody was produced, tissue sections were stained against IL-6 (MP5-32C11, BD Pharmingen), B220 (RA3-6B2, BD Pharmingen), CD19 (ID3, eBioScience), CD3 (Cd3-12, Serotec), F4/80 (CI:A3-1, Serotec), CD68 (FA-11, Serotec), Gr1 (RB6-8C5, BD Pharmingen), PNA-biotin (Sigma-Aldrich), GL7 (GL-7, eBioScience), CD138 (281-2, Biolegend), IgA (C10-1, BD Pharmingen), phospho-STAT3 (Tyr95, D3A7, Cell Signaling Technology), and phospho-AKT (Ser473, D9E, Cell Signaling Technology) for 1 hour at room temperature. Subsequently, tissue slides were subjected to incubation with appropriate biotinylated secondary antibodies for 45 minutes at room temperature, followed by avidin-biotin complex detection (Vectastain, Vector), according to the manufacturer’s instructions. Complexes were visualized using Fast Red Substrate, counterstained with hematoxylin, and mounted with Kaiser’s Glycerin Gelatin (Merck). Immunohistochemistry staining on human lymphoma tissues of patients with DLBCL was performed on formalin-fixed and paraffin-embedded tissue microarrays following the manufacturer’s instructions using monoclonal antibody specific for CD37 (clone 2B8, ThermoFisher Scientific) and p-STAT3 (62), followed by hematoxylin counterstaining. Assessment of CD37 and p-STAT3 protein expression was performed by two pathologists (K.J. Jabbar and K.H. Young) individually blinded from the clinical outcomes. The interobserver agreement was 98% for the samples, and disagreements were resolved by joint review on a multihead microscope.

Immunofluorescent staining of immunoglobulins. Cryostat 4-μm sections were dried at room temperature for 30 minutes and fixed in acetone for 10 minutes at -20°C. Subsequently, tissue sections were treated with 5% normal horse serum for 30 minutes at room temperature. After blocking, tissue sections were stained with AffiniPureFab(α)2 fragment goat anti-mouse IgG (2 μg/ml, Jackson Immunoresearch Laboratories), goat anti-mouse IgM (5 μg/ml, Vector Laboratories), or rat anti-mouse IgA (C10-1, 2 μg/ml, BD Pharmingen) for 45 minutes at room temperature, followed by incubation with appropriate secondary antibodies (Alexa Fluor 488, Life Technologies) for 45 minutes at room
temperature. Sections were fixed in 1% PFA, enclosed in Mowiol (EMD Biosciences), and imaged by fluorescent microscopy (Leica DM).

Flow cytometry and antibodies. Single-cell suspensions were stained for 30 minutes at 4°C in PBS, 0.1% BSA, and 2% normal goat serum, with the following primary mouse antibodies: anti-CD19-APC (ID3, eBioscience), anti-CD3-APC (145-2C11, BD Pharmingen), anti-B220-FITC (RA3-6B2, Biolegend), anti-IGa-FITC (ma-6E1, eBioscience), IgD-FITC (11-26c.2a, BD Pharmingen), anti-CD86-PE (GL1, BD Pharmingen), anti-MHC-II-PE (M5/114.15.2, eBioscience), anti-CD40-PE (3/23, BD Pharmingen), anti-CD43-PE (S7, BD Pharmingen), anti-B220-Alexa Fluor 647 (RA3-6B2, BD Pharmingen), biotinylated anti-IgM (Vector Laboratories), anti-IgA (C10-1, BD Pharmingen), PNA (Sigma-Aldrich), anti-CD138 (281-2, BD Pharmingen), anti-CD37 (WR17, made in-house), anti-IL-6Rα/CD126 (D7715A7, Biolegend), or anti-gp130 (M-20, Santa Cruz Biotechnology). This was followed by incubation with streptavidin conjugated to PE or PerCP or appropriate secondary antibodies (PerCP, BD Pharmingen, or anti-rabbit IgG-Alexa Fluor 488, Life Technologies). Intracellular stainings for α(1,4,5,3)-glucuronidase, anti-CD138 (281-2, BD Pharmingen), anti-CD37 (WR17, made in-house), anti-IL-6Rα/CD126 (D7715A7, Biolegend), or anti-gp130 (M-20, Santa Cruz Biotechnology) was performed as described previously (27). Stained cells were analyzed using a FACScalibur (Becton Dickinson) and FlowJo software version 9.6 (TreeStar Inc.).

Assessment of immunoglobulin heavy chain rearrangements. IGH rearrangements were assessed using the standardized multiplex PCR protocols (35 PCR cycles) developed by the BIOMED-2 concerted action BMH4-CT98-5936 (63). The IGH complete (VDJ) rearrangements were assessed in the 3 framework regions in the IGH variable genes. Multiplex PCR products were monitored by GeneScan analysis on an ABI 3730 platform (Life Technologies) and processed by the Genemapper software (version 4.0, ABI prism). Interpretation of the clonality findings was performed according to the EuroClonality guidelines (64). This test to perform clonality assessment on human samples is validated for use in murine samples.

Adoptive transfer experiments. Tumors from Cd37−/− mice were incubated with collagenase and DNase in order to obtain single-cell suspensions. Freshly isolated tumor cells (5 × 10^6) were injected subcutaneously into the flanks or intravenously into the tail veins of gender-matched 6- to 8-week-old Cd37−/−, WT, or immunocompromised recipients. Tumor growth was measured over time. In case of subcutaneous injection, human endpoint was reached at tumor size >2 cm^3.

RNA isolation and quantitative PCR analysis. For PCR-based gene profiling, the following protocol was used. Total RNA was prepared from Cd37−/− tumor or WT control mLNs using the MagNA Lyser Instrument (Roche) and the RNeasy Kit (Qiagen). Total RNA was reverse transcribed to cDNA using the RT2 First-Strand Kit (Qiagen) following the manufacturer’s instructions. Array plates of Mouse Oncogenes & Tumor Suppressor Genes PCR Array (SA Bioscience) were run according to the manufacturer’s instructions using the ABI/Prism 7000 Sequence Detection System (Applied Biosystems). The ΔCt value was calculated by determining the difference between the Ct value of the gene of interest and the average Ct value of 5 different housekeeping genes (Gush, Hprt1, Hsp90ab1, Gapdh, and Actb). The ΔΔCt value was calculated by subtracting the WT ΔCt value from Cd37−/− ΔCt value. The fold change in the expression of each gene between WT and Cd37−/− mice was calculated as 2−ΔΔCt. For quantitative PCR analysis of individual genes in Cd37−/− tumor cells and WT control cells, RNA was isolated using the Quick-RNA Miniprep Kit (Zymo Research). RNA quantity and purity were determined on a NanoDrop spectrophotometer. RNA was treated with DNase 1 (amplification grade; Invitrogen) and reverse transcription to cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels of the genes of interest were determined with a CFX96 Sequence Detection System (Bio-Rad) with SYBR Green (Roche) as the fluorophore and gene-specific oligonucleotide primers. The primers used are as follows: Casp8 (forward 5′-CACTTTCCCTAGACT-GCAACCG, reverse 5′-TCCACTCTGTCATTCTTCT), Ctnnb1 (forward 5′-CATCTTAAACCCCTTGGCTCGG, reverse 5′-AGTAC-GATTGTCCAGCGAC). Reaction mixtures and program conditions were used as recommended by the manufacturer (Bio-Rad). Quantitative PCR data were analyzed with the CFX Manager software (Bio-Rad) and checked for correct amplification and dissociation of the products. Ct values of the genes of interest were normalized to the Ct value of the housekeeping gene Pbgd.

Determination of IL-6 levels. IL-6 levels in serum and supernatant were measured using standard ELISA procedures. Briefly, NUNC Maxisorp 96-well plates (eBioscience) were coated with capture anti-mouse IL-6 antibody (MP5-20F3, 2 μg/ml, BD Pharmingen) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Wells were blocked with PBS containing 1% BSA and 1% FCS for 1 hour at room temperature, washed, and incubated with 50 μl of sample and standard (2-fold serial dilutions starting from 10,000 pg/ml) (eBioscience). After 2-hour incubation at room temperature, wells were incubated with biotinylated anti-mouse IL-6 (MP5-32C11, 1 μg/ml, BD Pharmingen) for 1 hour at room temperature, followed by incubation with HRP-conjugated streptavidin (1:5,000) for 30 minutes at room temperature. Complexes were visualized using TMB substrate (Sigma-Aldrich), and reaction was stopped by adding 0.8 M H2SO4. Absorbance was measured at 450 nm using an ELISA plate reader (iMark, Bio-Rad). IL-6 levels in sera of patients with DLBCL were measured using the Pelipair reagent set for human IL-6 (M9316, Sanquin) according to the manufacturer’s instructions.

Cell culture, survival assays, and transfection. Freshly isolated Cd37−/− tumor cells were cultured in RPMI 1640 (Invitrogen) containing 10% FCS, 1% ultraglutamate, 1% antibiotic-antimycotic, 1 mM sodium pyruvate, and 30 μM β-mercaptoethanol in the presence or absence of 10 μg/ml anti-mouse IL-6 (MP5-20F3, BD Pharmingen) or appropriate isotype control. For survival assays, viability of the neoplastic cells was assessed by trypan blue exclusion. The human B cell lymphoblastoid JY cell line, human pre-B cell line NALM-6, and HEK293 cells (all from ATCC) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 1% antibiotic-antimycotic, and 1% stable glutamine. Human CD37 construct (39) was cloned into psGFP2-N1 vector, placing the psGFP2 moiety on the C-terminus of the protein. NALM-6 cells were transiently transfected with human CD37-GFP using the SF Cell Line 4D-Nucleofector X Kit and 4D Nucleofector system (Lonza).

IL-6 stimulation. Human NALM-6 pre-B cells (10 × 10^6) or JY B cells (2 × 10^6) were stimulated with 15 ng/ml recombinant IL-6 (CellGro) for 60 or 75 minutes, respectively, at 37°C and 5% CO2.

Determination of SOCS3 and p-STAT3 expression. Freshly isolated WT control and Cd37−/− tumor mLNs (10 × 10^6) were lysed in 150 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100, 100 nM NaVO4, 500 mM NaF, 100 mM PMSF, and Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche) and separated by reducing SDS-polyacrylamide gel electrophoresis. Western blots were incubated with goat
anti-SOCS3 (M-20, 1;1,000, Santa Cruz Biotechnology) or rabbit anti-
pSTAT3 (Tyr^705, D3A7, 1;2,000, Cell Signaling Technology) and rabbit anti-
actin (20-33, 1;1,000, Sigma-Aldrich) or rabbit anti-STAT3 (79D7, 1;2,000, Cell Signaling Technology) as loading control, followed by secondary IRDye anti-goat or anti-rabbit antibodies (LI-COR) and Odyssey infrared detection and Odyssey analysis software.

**Coimmunoprecipitation experiments.** Human CD37-EGFP (pEGFP-
Ni) or empty pEGFP and SOCS3 (SIN-pgk) were transiently cotrans-
fected into HEK293 cells using Metafectene (Biontex). After 16 hours, cells were lysed in lysis buffer (0.5% NP40, 10 mM TrisHCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 pH 10.0, 1 mM NaF, and 1x Protease Inhibitor Cocktail [Pharmingen]). Lysates were precleared with isotype control antibodies bound to protein G-Sepharose beads, followed by incubation with mouse anti-GFP (Roche), mouse anti-CD37 (WR17, made in-house; ref. 65), or mouse IgG (BioLegend) isotype control antibodies. After incubating for 2 hours at 4°C, beads were washed 3 times in lysis buffer and SDS sample buffer containing 5% β-mer-
captoethanol was added. Samples were separated by SDS-PAGE, and Western blots were incubated with rabbit anti-GFP (made in-house) or rabbit anti-SOCS3 (Cell Signaling Technology, 2923) in TBS supple-
mented with 0.3% BSA and 0.1% skim milk powder at 4°C overnight and stained with secondary IRDye anti-rabbit antibodies (Invitrogen), followed by scanning membranes on the Odyssey Scanner (Westburg).

**Copatching experiments.** Human JY B cells were blocked with 3% BSA, 10 mM glycine, and 1% filtered human serum in PBS and stained with primary antibodies against human CD37 (WR17, made in-house; ref. 65), IL-6Ra/CD126 (M5, BD), or isotype controls for 30 minutes at 4°C. Secondary Alexa Fluor 488 or 647 antibodies (Invitrogen) were incubated for 30 minutes at 4°C, followed by 1 hour at 12°C. Cells were fixed with 2% PFA for 15 minutes at 4°C, followed by 15 minutes at room temperature, and mounted on poly-l-lysine–coated glass slides at room temperature. For intracellular SOCS3 staining, the cell mem-
brane was permeabilized with 0.5% saponin, 1% BSA, 10 mM glycine, and 1% filtered human serum in PBS, and cells were stained with anti-
SOC3 (M-20, Santa Cruz Biotechnology) or isotype control for 45 minutes at room temperature, followed by secondary Alexa Fluor 488 or 647 antibodies (Invitrogen) for 30 minutes at room temperature. Glass slides were sealed with Mowiol (EMD Biosciences) on covers-
slips and imaged with an Olympus FV1000 Confocal Laser Scanning Microscope. Images were analyzed using Fiji software and Manders coefficients were determined using the JACoP plug-in.

**Human IL6 and AKTI mRNA expression.** Gene expression analysis was performed on lymphoma tissues from patients with GCB-DLBCL (n = 47), and mRNA expression of IL6 and AKTI was retrieved from the gene expression profiling data obtained from the Affymetrix GeneChip Human Genome HG-U133 Plus 2.0. The log expression values of the probe sets IL6 (205207_at) and AKTI (207163_s_at) were used for analysis.

**Statistics.** Statistical differences in viability of Cd37−/− tumor cells and between CD37+ and CD37− groups were determined using unpaired Student’s t test (1 tailed) or nonparametric Mann-Whitney test (1 tailed, in case of non-Gaussian distribution). Statistical differences in IL-6 production in murine sera were determined using Kruskal-Wallis 1-way ANOVA test. OS was calculated from the time of diagnosis to death from any cause or last follow-up. PFS was calculated from the time of diagnosis to disease progression, relapse, or death from any cause. Patients who remained alive or progression free were censored at last follow-up. Survival analysis was performed using the Kaplan-Meier method with GraphPad Prism 5 (GraphPad Software), and differences were compared using the log-rank (Mantel-Cox) test. All differences were considered to be statistically significant at P ≤ 0.05.

**Study approval.** All murine studies complied with European legis-
lation (directive 2010/63/EU of the European Commission) and were approved by local authorities (CCD, The Hague, the Netherlands) for the care and use of animals with related codes of practice. Studies on human DLBCL tumor tissue were conducted in accordance with Decla-
ration of Helsinki and were approved as being of minimal to no risk or as exempt by the Institutional Review Boards at The University of Texas MD Anderson Cancer Center.

**Author contributions**

ABVS designed the study. CMDW, SV, and ABVS wrote the manu-
script. CMDW, SV, AVDS, and JSIV performed mouse experi-
ments. MVDB, MJG, KMH, PJTAG, JHV, and ABVS character-
bized the B cell lymphoma. CMDW, AVDS, and ABVS performed tissue stainings and in vitro tumor cell experiments. CMDW, SV, and AVDS did adoptive transfer experiments. CMDW and SV performed gene expression studies. CMDW and SE performed molecular studies of the IL-6 pathway. FAJVDL and MBB provided Il6−/− mice and SOCS3 antibodies. KHY organized the lymphoma patient material. KHY, ZYXM, XW, YX, and KJJ performed the studies on human patients with lymphoma under approval of the IRB. CGF and ABVS supervised the work.

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