Mice deficient for CD137 ligand are predisposed to develop
germinal center-derived B cell lymphoma

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Abbreviations: AID, activation induced cytidine deaminase; BCR, B cell antigen receptor; BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; CSR, class switch recombination; FDC, follicular dendritic cell; FL, follicular B cell lymphoma; DLBCL, diffuse large B cell lymphoma; EAF, ethanol-acetic acid-formol; GC, germinal center; H&E, hematoxylin and eosin; Ig, immunoglobulin, L, ligand; mAb, monoclonal antibody; MACS, magnetic labelled bead cell separation; MFG-E8, milk fat globule-EGF factor 8; NP-CG, 4-hydroxy-3-nitrophenylacetyl-conjugated chicken γ-globulin; PBS, phosphate-buffered saline; PCR, polymerase chain; PNA, peanut agglutinin; SHM, somatic hypermutation; TNF, tumor necrosis factor; WT, wild-type.
Abstract

In the germinal center (GC), B cells proliferate dramatically and diversify their immunoglobulin (Ig) genes, which increases the risk of malignant transformation. The GC B cell reaction relies on crosstalk with follicular dendritic cells (FDCs), to which the costimulatory receptor CD137 on FDCs and its ligand on GC B cells potentially contribute. We report that mice deficient for CD137 ligand (L) are predisposed to develop B cell lymphoma, with an incidence of about 60% at 12 months of age. Lymphoma membrane markers were characteristic of GC B cells. Longitudinal histological analysis identified the GC as site of oncogenic transformation and classified 85% of the malignancies found in about 200 mice as GC-derived B cell lymphoma. To delineate the mechanism underlying lymphomagenesis, gene expression profiles of wild-type and CD137L-deficient GC B cells were compared. CD137L deficiency was associated with enhanced expression of a limited gene set that included Bcl-10 and the GC response regulators Bcl-6, Spi-B, Elf-1, Bach2 and AID. Among these are proto-oncogenes that mediate GC B cell lymphoma development in human. We conclude that CD137L ordinarily regulates the GC B cell response and thereby acts as a tumor suppressor.
Introduction

During the GC reaction, B cells undergo antigen-driven clonal expansion, as well as somatic hypermutation (SHM) and class-switch recombination (CSR) of immunoglobulin (Ig) genes. As a result, memory B cells and plasma cells are formed that provide defense against infectious agents. However, the GC response comes with a risk, since SHM and CSR generate DNA breaks, which can lead to chromosomal translocations. These translocations may place proto-oncogenes under control of the active Ig locus and consequently deregulate their expression.

About 95% of newly diagnosed human lymphomas are of B cell origin, the rest are T cell malignancies. Since B cells and not T cells undergo antigen receptor gene diversification by SHM and CSR, these statistics clearly indicate the added risk of these GC-specific processes for malignant transformation. GC-derived lymphomas are the most common type of B cell non-Hodgkin lymphoma in human and comprise a heterogeneous group of malignancies. A well-characterized GC-B cell derived malignancy in human is follicular lymphoma (FL). This is defined as a nodular lymphoma with a follicular growth pattern. The lymphoma cells morphologically and phenotypically resemble GC B cells. FL is associated with a t(14;18) translocation that fuses bcl-2 and IgH genes and leads to overexpression of the anti-apoptotic protein Bcl-2. GC B cells normally downregulate Bcl-2 and are highly apoptosis-prone, which is required to select B cell clones with a functional and high affinity membrane Ig (B cell antigen receptor, BCR). Apoptosis-resistance is therefore an evident mechanism to promote the malignant transformation of these cells.

FL usually runs an indolent course, but can progress to an aggressive and rapidly fatal disease in the form of diffuse large B cell lymphoma (DLBCL). It has been hypothesized that FL pathogenesis not only relies on intrinsic characteristics of the B cell at risk, such as the acquisition of secondary oncogenic mutations, but also on interactions with the micro-environment. Whether the disease follows an aggressive or indolent course may be determined by deregulation of the crosstalk between transformed B cells and FDC and T cells in the GC. This hypothesis is derived from genome-wide expression profiling. In the datasets from independent groups that performed such analysis, gene signatures pointing to differential involvement of activated T cells, macrophages and FDC were part of the prognosis profile. Also in DLBCL, gene expression profiling has allowed classification on basis of both tumor cell intrinsic and environmental information.
GC consists of a framework of FDCs and activated B- and T lymphocytes that are mutually dependent. Development of the FDC network requires B cells, while FDCs and T cells support the GC B cell response.11,12 FDCs retain antigen-antibody complexes on their surface, which interact with membrane Ig on the B cell and thereby provide the primary signal for B cell survival, expansion and selection.11 Additionally, adhesion receptors, cytokines and various Tumor Necrosis Factor (TNF)/TNF receptor family members play a key role in the GC reaction: Interaction between CD40 ligand on activated T cells and CD40 on GC B cells is critical for GC B cell survival and differentiation. TNF, Lymphotoxin α, Lymphotoxin β, BAFF and their respective receptors also play a reciprocal role in stimulating both FDC network formation and the B cell response. Finally, CD95 on GC B cells in interaction with CD95L on FDC mediates GC B cell survival as well as death.4,12,13

In this study, we report a previously unsuspected role of TNF family member CD137L in the suppression of GC B cell lymphomagenesis in the mouse. The CD137 (4-1BB, TNFRSF9) receptor/ligand system is best known for its role in T cell costimulation. CD137 is acquired by both human and mouse T cells upon their activation and supports survival and memory formation of primed CD8+ T cells.14,15 CD137 is also found on myeloid cells, including macrophages and DC16,17, as well as on FDC18,19, which is most relevant for the current study. CD137L is expressed on activated myeloid cells and activated B cells. Receptor and ligand engage in bidirectional communication.20,21 Although no obvious defects in B cell responses were observed in receptor- and ligand knock out mice14,22, triggering of CD137L promoted B cell proliferation and antibody production in vitro18,20, while triggering of CD137 inhibited T cell-dependent humoral immune responses, either by effects on helper T cells or FDC.23,24 These data suggest that interactions between CD137 on FDC and its ligand on B cells may regulate the GC B cell response.

Apart from CD137, CD27 (TNFRSF7) has been implicated in the GC B cell response. In both mouse and human, B cells acquire CD27 during the GC reaction and its ligand CD70 is expressed on occasional T and B cells in the GC.25,26 In human, B cells retain CD27 after the GC reaction and hence CD27 is widely used as a marker of memory B cells and GC-derived B cell malignancies.25 In the mouse, CD27/CD70 interactions promote B cell expansion in the GC, but do not affect SHM, CSR, or ultimate Ig production.26 Their role in the GC B cell response may be more pronounced in human, because of the more abundant expression of CD27 on GC B cells.25,26

We present here that mice deficient for CD137L are strongly predisposed to develop GC-derived B cell malignancies. The majority of these was categorized as FL according to the "Bethesda
proposals for classification of lymphoid neoplasms in mice' 27, but they were histologically distinct from human FL. CD27-deficiency did not alter the risk of lymphoma development. Based on comparative genome-wide expression profiling of normal GC B cells from wild-type (WT) and CD137L-deficient mice, we have delineated a contribution of CD137L to the GC reaction and thereby identified this molecule as a tumor suppressor.
Methods

Mice
Mice were bred in the animal facility of The Netherlands Cancer Institute under specific pathogen-free conditions and animal experiments were approved by the Experimental Animal Committee of the Netherlands Cancer Institute and conformed to national guidelines. C57BL/6 (WT), CD27-/-, CD137L-/- and CD27;CD137L-/- double deficient mice were generated and phenotyped by polymerase chain reaction (PCR) as described.22,28,29 The mice had been backcrossed for 8-10 generations to a C57BL/6 background.

Pathology
Mice of ages ranging from 6-16 months were killed at specific time points as indicated in Table II and Figure 2. Conventional histopathology was performed on hematoxylin and eosin (H&E)-stained sections prepared from lymph nodes (including Peyer’s Patches), spleen, thymus, bone marrow, lung, liver and kidney that were fixed in ethanol-acetic acid-formol saline fixative (40 : 5 : 10 : 45 v/v) (EAF) and embedded in paraffin. Sections were examined blindly for indications of lymphoma according to Bethesda proposals.27 The sections were reviewed with a Zeiss Axioskop2 Plus microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with Plan-Apochroma (5x/0.16; 10x/0.45; 20x/0.60; and 40x/0.95) and Plan-Neofluar (2.5x/0.075) objectives. Images were captured with a Zeiss AxioCam HRc digital camera and processed with AxioVision 4 software (both from Carl Zeiss Vision, München, Germany).

(Immuno)histochemistry
EAF fixed, paraffin-embedded tissue of spleen and lymph nodes was sectioned and deparaffinized. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ in methanol. Immunohistochemistry was performed for CD3 (clone SP7, Neomarkers), B220 (clone RA3-6B2, BD) and milk fat globule-EGF factor 8 (MFG-E8) (clone 18A2-G10, MBL Medical Biological Laboratories Co., Naka-ku Nakoya, Japan). Antigen retrieval was obtained for B220, CD3 and MFG-E8 by incubation in 0.1 M citrate buffer pH 6 for 30 min at 95°C, followed by cooling to room temperature for 1 h. Sections were blocked in phosphate-buffered saline (PBS) with 4% bovine serum albumin (BSA), 5% normal serum and incubated with primary and secondary antibodies in PBS, 1% BSA, 1.25%
normal serum. Biotinylated goat-derived secondary antibodies were detected by the avidin-biotin-
horseradish peroxidase complex method (ABC; Dako systems), using 3,3-diaminobenzidine-
tetrahydrochloride (DAB, Sigma) as a substrate. For staining with peanut agglutinin (PNA), sections
were incubated with biotin-conjugated PNA (Vector Labs), which was detected by the avidin-biotin
complex (ABC) method. Sections were counterstained with hematoxylin.

**Clonality assay and analysis of somatic hypermutation (SHM)**

Genomic DNA was isolated from WT spleen and from tumor material derived from aged
(CD27;CD137L−/−) mice with late stage disease by proteinase K treatment and ethanol precipitation.
The rearranged JH genes were amplified by PCR, using a set of forward primers to detect most VH
gene families 31 and a single reverse primer CTCCACCAGACCTCTCTAGACA that binds within the
JH4 intronic region. PCR products were resolved by agarose gel electrophoresis and visualized by
ethidium bromide staining. PCR products were purified from gel using the Qiagen Gel Extraction Kit
and sequenced directly on a 3730 DNA analyzer (Applied Biosystems, Foster City CA). JH intronic
nucleotide sequences were compared to sequences from the NCBI database.

**Flow cytometry**

Spleens or tumor cell suspensions were forced through a nylon mesh in IMDM with 8% FCS.
Erythrocytes were lysed in 0.14 M NH4Cl, 0.017 M Tris-HCl, pH 7.2. Cells were incubated with Fc
Block (2.4G2, BD PharMingen, San Diego, CA), washed in staining buffer (PBS, 0.5% BSA, 0.01%
sodium azide), stained with fluorochrome-conjugated antibodies as indicated and analysed using a
FACSCalibur (BD, Franklin Lakes NJ) in conjunction with FloJo software (Tree Star, Ashland OR).
Monoclonal antibodies used were anti-CD45R/B220 mAb RA3-6B2, anti-CD19 mAb 1D3, anti-CD27
mAb LG.3A10, anti-CD70 mAb FR70, anti-CD86 mAb B7-2, anti-GL7 mAb GL7 (from BD
Biosciences, eBioscience or purified from available hybridomas). Goat anti-mouse IgM was from
Southern Biotech.

**Confocal laser scanning microscopy (CLSM)**

Small pieces of spleens used for the microarray experiments were embedded in Tissue-Tek OCT
compound (Sakura), frozen in dry-ice/ethanol and stored at -80°C. Frozen 5-µm cryostat sections
were air-dried overnight, fixed in acetone for 10 min and dried for 1 h. Sections were rehydrated in
PBS and pre-incubated for 1 h at room temperature with 2.4G2 mAb to block Fc receptors for Ig. CD137L was detected by purified mAb TKS-1 (BD Biosciences), followed by Alexa 568-conjugated anti-rat Ig. Sections were incubated overnight at 4°C in a humid chamber with allophycocyanin-conjugated B220 mAb (RA3-6B2, BD) and biotinylated anti-FDC mAb (FDC-M2, eBioscience), followed by FITC-conjugated streptavidin. All antibody incubations were done in PBS, 0.5% BSA, 0.02% sodium azide. Slides were mounted in Vectashield (Vector labs) and analyzed with a Leica TCS NT confocal laser-scanning microscope (Leica microsystems, Germany).

**GC B cell isolation, RNA isolation and amplification**

WT and CD137L−/− mice of 8-12 weeks old were immunized intra-peritoneally with 50 µg chicken γ-globulin conjugated to 4-hydroxy-3-nitrophenylacetyl (NP-CG) in alum or infected intranasally with 25 hemagglutinin units of influenza virus strain A/NT/60/68 (Department of Virology, Erasmus MC Rotterdam, The Netherlands) in 50 µl HBSS. Nine days after immunization or infection, B cells were enriched from pooled spleens and lymph nodes of 4 mice per test group by means of magnetic labelled bead cell separation (MACS) using anti-mouse BD Imag™ CD45R/B220-particles DM, according to the manufacturer’s protocol (BD). The B220+ MACS-sorted populations were stained with anti-GL7-FITC and anti-CD19-PE to sort CD19+GL7+ GC and CD19+GL7− non-GC B lymphocytes by flow cytometry in the presence of propidium iodide on a FACSAria (BD). Sorted GC B cells and non-GC B cells derived from 4 individual mice per test group were pooled and RNA was isolated using the RNeasy kit (Qiagen). Isolated total RNA was subsequently DNase-treated by using the Qiagen RNase-free DNase kit and dissolved in RNase-free H2O. cDNA was generated by Superscript II reverse transcriptase using total RNA as template and an oligo(dT) primer containing a T7 polymerase recognition site. Amplified RNA was generated by in vitro transcription using a T7 RNA polymerase (Megascript T7 kit, Ambion).

**Gene expression profiling**

Microarrays spotted with the Operon v3 oligonucleotide library, covering 20312 different mouse genes according to the ENSEMBL database were obtained from the central microarray facility of The Netherlands Cancer Institute. Amplified RNA (aRNA) was labelled using Cy5- and Cy3-ULS (ULS aRNA Fluorescent labeling kit, Kreatech, Amsterdam, The Netherlands) and fragmented into stretches of 60-200 bases (RNA fragmentation reagents, Ambion), before adding the probes to the
microarray slides. Microarrays were scanned on an Agilent microarray scanner (Agilent Technologies, Palo Alto, CA) and data extraction was done using Imagene 6.0 (BioDiscovery Inc., Los Angeles, CA). One experiment was performed by immunization with NP-CG and one experiment was performed by infection with influenza, each with 4 mice per test group. For derivation of RNA samples, see above. For each microarray analysis, a dye reversal was performed, thus reducing systemic errors due to oligonucleotide-specific dye preferences. Genes found to be differentially expressed in both experiments, i.e. independent of the immunization strategy and with a statistical cut off (P<0.01) were classified as being differentially expressed between samples from wild-type and CD137L−/− mice. Array data have been deposited in ArrayExpress (EBI) under accession number E-NCMF-29.

Statistics
For statistical analysis, we used LogXact 7 software (Cytel inc., Cambridge MA, 2005). P-values were calculated on the basis of the exact logistic locally-weighed polynomial regression.
Results

**CD137L-deficient mice are predisposed to develop B cell lymphoma**

Previously, it was shown that CD137L−/− and CD27;CD137L−/− double deficient mice develop normally and have normal T- and B cell numbers and subset compositions.22,28,29 However, at old age, mice of both genotypes became ill at an unusual frequency and were diagnosed with severe neoplasms in spleen and lymph nodes. Flow cytometric analysis revealed that these were of B cell origin - as hallmarked by expression of CD19 - and in most cases expressed the GC B cell marker GL7 32 (Figure 1A). The GL7 positive cases also stained strongly with the lectin PNA (PNAhigh, see below), which is an additional hallmark of GC B cells 33. Expression of CD27, CD86, CD70 (data not shown) further supported the notion that the GL7+/PNAhigh tumors were derived from GC B cells.25,26 Full blown tumors that consisted primarily of B cells were diagnosed as clonal, using a PCR for Ig JH gene segment usage (Figure 1B). In a normal polyclonal B cell population from WT spleen, PCR products identified multiple rearranged JH gene segments. In contrast, unique PCR products were derived from tumors, denoting a single rearranged IgH locus making use of the JH1, JH2, JH3 or JH4 gene segment (Figure 1B). Nucleotide sequencing of the VDJ junctions confirmed clonality (data not shown). Within the set of five full blown B cell lymphomas depicted, four were PNAhigh (Table I). In three PNAhigh cases, nucleotide sequencing of the IgH locus indicated the occurrence SHM (Table I). SHM frequency was high compared to what is generally reported for normal GC B cells (see e.g. ref. 31). The occurrence of SHM further underlined the GC B cell origin of the GL7+/PNAhigh lymphomas.

To determine the moment of tumor onset, histological examination was performed on mice of different age groups ranging from 6-16 months. Mice were sacrificed independent of presentation of disease symptoms. In total, 89 CD137L−/− mice and 112 CD27;CD137L−/− mice were examined, while 30 WT mice were analyzed for comparison. Spleen, thymus, bone marrow, lung, liver, kidney and various lymph nodes, including Peyer’s Patches were processed for sectioning. Sections were examined blindly and lesions were classified according to defined criteria.27 Lymphoma incidence was plotted as a function of age for mice of each genotype (Figure 2A). Because the number of mice in each age group varied, lymphoma incidence showed a rather high variability. To correct for this, we also calculated the smoothed estimates (Figure 2B), as based on locally weighed polynomial regression, specified in the Methods section. This probability model predicts that CD137L-deficiency increases the risk to develop follicular lymphoma from 5-10% at the age of 7 months to about 60% at
the age of 12 months and 75-90% at the age of 16 months. By using the exact variant of logistic regression, P-values were calculated to determine statistical significance of each data set. Both CD137L<sup>−/−</sup> and CD27;CD137L<sup>−/−</sup> mice had a significant risk to develop lymphoma (P<0.0001) compared to WT mice, but there was no significant difference in the risk between the two knockout mouse strains (P=0.11). We conclude that CD137L-deficiency in mice leads to a significant predisposition to develop B cell lymphoma, while additional CD27-deficiency does not alter this risk.

**Lymphoma classification and incidence**

Lesions were classified on basis of histology according to the “Bethesda proposals for classification of lymphoid neoplasms in mice”. The great majority of the B-cell neoplasia found in our cohort were thus classified as follicular B-cell lymphoma (FL, Supplementary figure 1A; Figure 3A,B). It should be mentioned, however, that mouse FL cannot be considered as the counterpart of human FL due to notable differences in histopathological features. According to the same classification, cases of Small B cell lymphoma (SBCL, Supplementary figure 1B), Diffuse large B cell lymphoma (DLBCL, Supplementary figure 1C) and anaplastic plasmacytoma (PCT-A, Supplementary figure 1D) occurred at low frequencies.

The results are shown numerically in Table II. WT mice showed no malignancies before the age of 13 months. In 3 WT mice in the age group of 13-16 months, pre-neoplastic lesions were found, while 2 mice presented with FL (14% tumor incidence). This lymphoma incidence (see also Figure 2) in aged WT mice of the C57BL/6 strain is consistent with reported data on lymphoma incidence in aged B6;129 mice. In CD137L<sup>−/−</sup> and CD27;CD137L<sup>−/−</sup> mice, pre-neoplastic disease was frequent in all age groups. About 70% of 13-16 month-old mice and about 40% of 10-12 month-old mice had histologically overt lymphoma. Also in the youngest age group of 6-9 months, lymphoma incidence was evident in both strains (7-17%). The great majority of lymphoma cases concerned early- and late stage FL, while the other lymphoma types occurred in low frequency (Table II).

**Histology reveals tumor progression and defines the GC as site of tumor origin**

Histological examination of CD137L<sup>−/−</sup> and CD27;CD137L<sup>−/−</sup> mice revealed a broad spectrum of apparently progressive stages in the development of B-cell neoplasia in spleen, lymph nodes and Peyer’s patches and to a lesser extent in thymus and bone marrow. The earliest morphological changes were related to the B cell follicles that showed expansion of GCs with thin or absent mantle...
zones. In the aberrant follicles, increased frequencies of mitotic and apoptotic cells were observed. These were defined as pre-neoplastic lesions (Figure 4B, II). Progressively confluent nodules were formed by merging of multiple enlarged GCs that were surrounded by a very thin or no mantle zone, in which centrocyte-like and centroblast-like cells were the major components. This presentation was defined as early stage FL (Figure 3A). Mitotic and apoptotic rates in these lesions were high. Disease progression was apparent from the simultaneous presence of pre-neoplastic and early lesions in the same spleen (Figure 3A). For comparison, B cell follicles in spleens of a non-immunized mouse (Figure 3C) and a virus-infected mouse (Figure 3D) are shown.

Further development of the lesions was apparent from their diffuse growth throughout the entire target organ, often with the involvement of liver and kidney. In spleen, the late stage FL lesions remained more or less nodular (Figure 3B), whereas in lymph nodes and Peyer’s patches more diffuse patterns were seen (results not shown). The neoplastic cell populations were centrocyte-like and centroblast-like, but transformed blasts resembling immunoblasts and plasmablasts or other large cells with pleomorphism were often present. Also in these lesions, mitotic and apoptotic rates were relatively high (Figure 3B; Supplementary figure 1A).

To examine the GC origin of the lymphomas, we performed immunohistochemistry. Serial spleen and lymph node sections of mice diagnosed with early- and late stage disease were stained to identify B cells and T cells. This revealed aberrant B cell follicles in the early-stage disease and large nodular B cell masses in the late stage disease, with diminished T cell zones (Figure 4A). Staining with PNA was performed to specifically detect GC B cells.33 GC B cells in spleen of a healthy aged WT mouse served as a control (Figure 4B, I). In the mice diagnosed with pre-malignant lesions, early- and late stage disease, the histologically aberrant follicles appeared to consist largely of GC B cells (Figure 4B, II, III, IV).

Immunohistochemistry with an antibody to MFG-E8 was performed to examine whether the aberrant follicles maintained an FDC network. MFG-E8 is identical to the FDC marker FDC-M1 and was previously shown to specifically detect the FDC network in B cell follicles.35 In the spleens of non-immunized, healthy aged WT mice, antibody to MFG-E8 specifically denoted tight FDC networks in B cell follicles (Figure 5, I). It stained the cell body and dendritic processes of the FDCs that were clustered in the GC, adjacent to the mantle zone (Figure 5, II). FDC networks became expanded but remained intact in GCs of follicles that were greatly enlarged after infection of 8-12 week old WT mice with influenza virus (Figure 5, III, IV). In the spleens of mice diagnosed with early stage FL, FDCs did
not form a restricted network as in normal follicles, but were more widely dispersed throughout the aberrant follicles (Figure 5, V, VI). In late stage FL, FDCs appeared more sparse and were diffusely distributed throughout the lesions (Supplementary figure 2). Together, these data define the GC as origin of the malignant transformation and strongly suggest a gradual progression from pre-neoplastic lesions into early- and then late-stage GC-derived B cell lymphoma.

**CD137L controls the GC B cell response**

Apparently, CD137L deficiency predisposes mice for malignant transformation of GC B cells. This suggests that CD137L ordinarily reduces the risk of malignant transformation during the GC B cell response. A potential role for CD137L during the GC reaction is supported by its presence on GC B cells: Using three color immunofluorescence analysis of spleen sections from WT and CD137L−/− mice by CLSM, we could specifically detect CD137L expression by those WT B cells that were in close proximity of FDC (Figure 6).

After immunization with NP-CG in alum, we examined GC formation in WT and CD137L−/− mice histologically. No differences were found between these two genotypes in terms of numbers or sizes of the GCs, or the cellular composition of the GCs, including centrocytic and centroblastic cells, FDCs, mitotic and apoptotic cells (Supplementary figure 3).

To evaluate the role of CD137L during the GC B cell response at the molecular level, we performed a comparative genome-wide mRNA expression profiling of WT versus CD137L−/− GC B cells. WT and CD137L−/− mice were immunized with NP-CG in alum (Figure 7; GC1) or were infected intranasally with influenza virus (Figure 7; GC2). Spleen and lymph nodes of 4 mice per genotype were harvested at the peak of the response and were pooled to sort GC B cells by flow cytometry on basis of co-expression of CD19 and GL7. Genes differentially expressed in both data sets, i.e. independent of the immunization strategy and with a statistical cut off (P<0.01) are listed in Figure 7. All genes listed are specific for GC B cells, since a concurrent micro-array analysis revealed that they were not differentially expressed in CD19+GL7− non-GC B cells from WT and CD137L−/− mice that were sorted alongside with the GC B cell fraction (Figure 7;non-GC).

The limited number of significant hits thus selected as being overexpressed in CD137L−/− GC B cells included the transcription factors Stat-1, Spi-B, Elf-1, Bcl-6, CIITA and Bach2, the Activation Induced cytidine Deaminase (AID), the NFκB regulator Bcl-10, as well as the double strand DNA repair protein Rad21. Interestingly, the majority of these molecules has been implicated in key
aspects of the GC B cell response. Moreover, Bcl-6 and AID are known to drive GC B cell lymphomagenesis.\textsuperscript{36,37} Genes encoding RAG1-activating gene and the DNA mismatch repair protein MLH1 had a lower expression in CD137L\textsuperscript{+} GC B cells than in WT GC B cells (Figure 7). These data indicate that CD137L modulates the GC B cell response by regulating the expression of genes involved in GC specific processes, including Ig gene diversification. Together, our data strongly support a scenario in which CD137L acts in this way as a suppressor of FL development.
Discussion

We report here that CD137L-deficiency predisposes mice to develop GC-derived B cell lymphoma. Extensive histological analysis of mice in different stages of tumor development allowed us to unambiguously define GC B cells as the target of malignant transformation in the great majority of cases. By unbiased examination of histological sections, 64 out of 76 malignancies found in the large cohort of mice were classified as GC-derived B cell lymphoma. This was underlined by staining with antibody to the FDC marker MFG-E8 that defined aberrant GC B cell distribution within an extended and disordered FDC network in early- and late stage lesions.

Flow cytometric analysis of a limited number of samples indicated the presence of CD27 and its ligand CD70 on these malignant cells (data not shown). From findings in human B cell malignancies, it has been postulated that CD27/CD70 interactions may support survival and/or expansion of transformed B cells. However, we found that CD27 deficiency did not significantly alter lymphoma incidence in CD137L-/- mice, indicating that CD27/CD70 interactions were not vital to lymphoma development in this setting.

Although the GC-derived B cell lymphoma that arises in CD137L-/- mice was classified as FL according to the Bethesda proposals, it has an obviously different histological appearance than human FL. In particular, whereas human FL is indolent, in mouse FL mitotic and apoptotic cells are frequently observed. It is, however, of interest that the expansion of (pre-)malignant B cells in CD137L-/- mice takes place in progressively more disturbed FDC networks. This indicates a follicular growth pattern as observed for human FL. Human FL is associated with t(14;18), leading to Bcl-2 overexpression. In mice, deliberate bcl-2 overexpression under control of the IgM gene enhancer (Eμ) gives rise to follicular hyperplasia, but not lymphoma (reviewed in 4). In conjunction with overexpression of the c-myc gene, bcl-2 transgenesis is potently transforming, but gives rise to pre-B cell lymphomas. Also in other contexts, bcl-2 deregulation or deregulation of other genes instrumental in human B lymphoma development does not reliably mimick human FL in mice. Possibly, the key genes that cooperate with Bcl-2 in human FL development remain to be tested in transgenic mouse models. Alternatively, the GC micro-environment or overall physiology of the mouse is not suitable to generate a model for human FL.

In the present study, we have focused on the mechanism that may underlie the malignant transformation of GC B cells in CD137L-/- mice. For this purpose, we have first documented
expression of CD137L in the B cell follicle. CLSM after immunostaining revealed that a selection of follicular B cells express CD137L, namely those that were in close contact with FDC. Specificity of the staining was underlined by its absence in samples from CD137L−/− mice and by evident membrane localization of CD137L (Figure 6). The FDC themselves did not express CD137L, nor did we find any other non-B cells in the follicle that expressed it. CD137 expression on FDC could not be documented, since appropriate antibodies to detect mouse CD137 in cryosections are lacking. Therefore, we rely on data from the human system in the supposition that CD137L on GC B cells communicates with CD137 on FDC during the GC reaction. The gene array experiments have provided proof that CD137L plays a specific role during the GC B cell response. Given the restricted expression pattern of CD137L in the B cell follicle, we favor the interpretation that CD137L on GC B cells give signals to regulate expression of the genes we have outlined in Figure 7, possibly in response to binding CD137 on FDC. In this scenario, CD137L would act directly as a tumor suppressor in GC B cells.

We have found no differences between 8-12 week old WT and CD137L−/− mice in the histological appearance of GCs, their overall sizes and their frequencies of occurrence at day 9 after immunization with NP-CG. Although we cannot exclude differences in GC maintenance for lack of kinetic analysis, we know that WT and CD137L−/− mice produced fully comparable levels of Ig of all isotypes in response to primary and secondary influenza virus infection. Even mice lacking both CD137L and CD27 had normal serum Ig of all isotypes in this setting.22 However, several data argue for a role of CD137L in regulating the B cell response. In vitro triggering of CD137L with recombinant CD137 costimulated anti-IgM-induced proliferation of both human and mouse B cells.18,20 Agonistic antibody to CD137 suppressed T cell-dependent B cell responses in mice, which was attributed to induction of T cell anergy23, but may have involved T cell-dependent attrition of FDC networks.24 Our study underlines that CD137L plays a role in regulating the GC B cell response. This role may be compensated for by other factors with regards to eventual Ig production, but we find that it plays a crucial role in suppression of GC B cell lymphomagenesis. The genes that were found to be downregulated – either directly or indirectly - by CD137L in GC B cells provide a direct clue as to how such tumor suppression may occur.

The presence of CD137L contributed to downregulation of AID, which is a key regulator of CSR as well as SHM. AID deaminates cytidines in DNA which causes DNA strand breaks and activates error-prone DNA repair pathways. This leads to the introduction of somatic mutations in VDJ
recombined Ig genes, but the SHM machinery may also target potential oncogenes. Accordingly, AID deficiency prevented GC-derived B cell lymphomagenesis in Bcl-6 transgenic mice. CD137L also contributed to downregulation of the transcriptional repressor Bcl-6, which is essential for GC formation and affinity maturation and implicated in human GC B cell lymphomagenesis. Bcl-6 negatively regulates p53 expression, thereby allowing GC B cells to sustain the physiological genotoxic stress associated with high proliferation rate and DNA breaks induced by SHM and CSR. Another major function of Bcl-6 is to inhibit the differentiation of GC B cells into Ig-producing plasma cells. This is mediated by transcriptional repression of the Blimp1 (Prdm1) gene.

CD137L also contributed to downregulation of the transcription factors Bach2 and Spi-B. Like Bcl-6, these factors attenuate Blimp-1 expression and thereby prevent plasma cell differentiation, thus sustaining the GC B cell response. Bach2 is required for CSR and SHM and implicated in human B cell lymphomagenesis. Elf-1, another member of the gene set that is negatively controlled by CD137L is, like Spi-B, a member of the Ets family of transcription factors. It is regulated by the BCR and CD40 and controls IgH enhancer elements and potentially CSR. All these findings indicate that CD137L under normal circumstances promotes exit of GC B cells from the GC towards the plasma cell developmental stage and thereby reduces the risk of malignant transformation in the GC. Downregulation of Bcl-10, a CARD domain protein activates NFκB in response to BCR signaling also fits in a scenario in which CD137L prevents malignant transformation, in this case by interfering with an essential survival pathway of GC B cells. A number of other gene products identified here as potential CD137L targets, such as the DNA repair proteins Rad21 and MHL1, as well as the Stat-1 transcription factor can also be placed in the context of the known literature on the GC reaction, but this becomes more speculative than for the other targets.

Based on our findings, we propose that CD137L controls the GC B cell response, by signaling – directly or indirectly - to the GC B cell to downregulate genes that sustain GC B cell survival and the GC specific processes SHM and CSR. Thereby, CD137L promotes exit of the B cells from a hazardous developmental stage and suppresses the risk of transformation.
Acknowledgements

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Authorship

Contributions: Y.X. made the initial observation. S.M., Y.X., J.-Y.S., V.P. and P.H.K. designed and performed experiments and analyzed results; H.J. and J.B. designed experiments and analyzed results. S.M. and J.B. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


<table>
<thead>
<tr>
<th>Lymphoma</th>
<th>CD19</th>
<th>PNA&lt;sup&gt;high&lt;/sup&gt;</th>
<th>IgM</th>
<th>JH gene usage</th>
<th>SHM number</th>
<th>SHM percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>JH3</td>
<td>23/691</td>
<td>3.3%</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>JH1</td>
<td>18/346</td>
<td>5.2%</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>JH4</td>
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<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>JH2</td>
<td>0/502</td>
<td>0.0%</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>JH2</td>
<td>42/502</td>
<td>8.4%</td>
</tr>
</tbody>
</table>

Lymphomas are identical to those analyzed for JH gene segment usage and clonality in Figure 2. Diagnosis for CD19, PNA and IgM was performed by flow cytometry. Occurrence of SHM in the Ig locus is depicted as number of mutated nucleotides per number of nucleotides sequenced and additionally as percentage calculated from these numbers.
### Table II. Lymphoma classification and incidence

<table>
<thead>
<tr>
<th>CD137L⁺</th>
<th>CD27;CD137L⁻/⁻</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in months (total n)</td>
<td>6-9 (n=28)</td>
<td>6-9 (n=6)</td>
</tr>
<tr>
<td></td>
<td>10-12 (n=30)</td>
<td>10-12 (n=9)</td>
</tr>
<tr>
<td></td>
<td>13-16 (n=31)</td>
<td>13-16 (n=18)</td>
</tr>
<tr>
<td>Pre-neoplastic</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>early stage (FL)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>late stage (FL)</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>DLBCL</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SBCL</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCT-A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lymphoma incidence (2/28)</td>
<td>7%</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>(12/30)</td>
<td>46%</td>
</tr>
<tr>
<td></td>
<td>(22/31)</td>
<td>72%</td>
</tr>
</tbody>
</table>

B cell malignancies observed in CD137L⁺, CD27;CD137L⁻/⁻ and wild-type (WT) mice, per age group. Pre-neoplastic lesions, early- and late stage GC-derived B cell lymphoma were defined as outlined in the Results section. Classification as follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL), small B cell lymphoma (SBCL) and anaplastic plasmacytoma (PCT-A) was done according to Bethesda proposals.27
Legends

Figure 1. Tumors arising in CD137L−/− mice are B cell lymphomas of clonal origin. (A) Flow cytometric analysis of tumor cells, isolated from spleen and stained for CD19 and GL7 with mAbs directly conjugated to PE or FITC, respectively. Mean fluorescence intensity (MFI) is represented for each event analyzed. Forward and side scatter gating was set to focus on tumor cells. (B) Diagnosis of tumor clonality by means of a PCR assessing the relative location of rearranged JH1, JH2, JH3, and JH4 segments from V(D)J junctions. Splenic B cells from a wild-type (WT) mouse were used as control for a polyclonal B cell population. Tumors were in advanced stages and isolated from spleens and liver (case 4) of aged (13-16 months) (CD27−/−)CD137L−/− mice. Further characteristics of these tumors are depicted in Table I.

Figure 2. CD137L−/− and CD27−/CD137L−/− mice are predisposed to develop B cell lymphoma. WT, CD137L−/− and CD27−/CD137L−/− mice of various ages (6 to 16 months) were sacrificed and examined blindly for indications of lymphoma according to Bethesda proposals.27 (A) For each genotype frequencies of lymphoma incidence were determined per age in months. The risk to develop lymphoma was determined, based on locally-weighed polynomial regression. (B) Trend lines for the smoothed estimates were calculated to determine the lymphoma incidence probability for each genotype. Diagnosis of lymphomas is presented in Table II.

Figure 3. B cell follicles and their neoplastic transformation. H&E stained spleen sections from (A) a 16 month old CD137L−/− mouse diagnosed with early-stage FL, showing merging of enlarged GCs; (B) an 11 month old CD27−/CD137L−/− mouse diagnosed with late-stage FL, showing nodular appearance of tumor mass. (C) a 12 month old WT mouse. The GC (G, dotted circle) is small, periarteriolar lymphoid sheath region (*) and mantle zone (MZ) are prominently present; (D) a 10 week old WT mouse at day 8 after influenza infection. The GC is enlarged and the periarteriolar lymphoid sheath region is less pronounced. Original magnifications: A-D 2.5x, inserts 40x.

Figure 4. Lymphomas in (CD27−)CD137L−/− mice are of GC B cell origin. (A) Immunohistochemistry of spleen sections of mice diagnosed with early-stage and late-stage FL, using antibody to B220 to detect B cells and antibody to CD3 to detect T cells (serial sections). (B)
Histochemistry with PNA was performed to define GC B cells. Shown are spleen sections of mice diagnosed with different stages of FL development: I) healthy aged WT control, II) pre-neoplastic lesions, III) early-stage FL, IV) late-stage FL. Original magnification for (A) and (B): 5x. Specific staining is shown in brown and hematoxylin counterstaining in blue.

**Figure 5. Transformed GC B cells in (CD27;CD137L<sup>-/-</sup> mice expand within a FDC network.**
Immunohistochemical detection of FDCs with antibody to the marker MFG-E8 in representative spleen sections of a 12 month old WT mouse (I, II), a 10 week old mouse at day 8 after infection with influenza virus (III, IV), and a representative mouse (n=5) diagnosed with early-stage FL (V, VI). Original magnification: 5x for I, III and V; 40x for II, IV and VI. Specific staining is shown in brown and hematoxylin counterstaining in blue.

**Figure 6. CD137L is expressed on B cells that are in close contact with FDC.** CLSM analysis of CD137L expression on B cells and/or FDCs in GC of immunized mice. Cryostat sections of spleens from (A) WT and (B) CD137L<sup>-/-</sup> mice used for the microarray experiments were stained with mAb to detect CD137L (red), FDC (FDC-M2<sup>50</sup>; green) and B cells (B220; blue). In the WT sample, B cells that are adjacent to FDC express CD137L, as indicated by the pink color. In the CD137L<sup>-/-</sup> sample, this signal is virtually absent, indicating specificity of CD137L detection. Original magnification: 40x.

**Figure 7. CD137L regulates gene expression in GC B cells.** CD19<sup>+</sup,GL7<sup>+</sup> GC B cells were sorted by flow cytometry from spleen and lymph nodes of WT and CD137L<sup>-/-</sup> mice, nine days after immunization with NP-CG (left panel, ‘GC 1’) or infection with influenza virus (middle panel ‘GC 2’). GC B cells of 4 mice per genotype were pooled for each experiment. Messenger RNA was isolated and used for comparative expression analysis by genome-wide micro-array. The heat map depicts transcript levels according to the indicated log ratio between expression in WT versus CD137<sup>-/-</sup> GC B cells (see color scale). Green denotes lower expression and red denotes higher expression in WT GC B as compared to CD137<sup>-/-</sup> GC B cells. The list of genes was selected for statistically significant differential expression (P<0.01) and presence in both experiments. CD19<sup>+</sup,GL7<sup>-</sup> non-GC B cells (right panel, ‘nonGC’) were sorted simultaneously and analysed to confirm GC-specificity of the listed genes.
Figure 1

A

Spleen

GL7-FITC

CD19-PE

Liver

9.8 53.5
9.8 53.5

33.7 3.0
4.2 1.9

3.6 90.3

B

Tumor

JH1
JH2
JH3
JH4

1 2 3 4 5 WT
Figure 2

A

B

Age (months)

Lymphoma incidence probability

CD27;CD137L-/-

CD137L-/-

WT
Figure 3
Figure 6
Figure 7

-3.0  0.0  3.0  \(2\) log ratio

GC1  GC2  nonGC

Leukocyte common antigen (Ptprc)
Lim-bud and heart (Lbh)
Antigen peptide transporter 1 (Tap1)
Moesin (Msn)
Actin-binding LIM protein 1 (Ablim1)
Deoxyctydine kinase (Dck)
Splicing factor, arginine/serine-rich 1 (Asf)
14-3-3 protein zeta/delta (Ywhaz)
Polypyrimidine tract-binding protein 1 (Ptbp1)
Transcription factor Spi-B (SpiB)
Splicing factor, arginine/serine-rich 10 (Sfrs10)
Splicing factor, arginine/serine-rich 10 (Sfrs10)
Mps one binder kinase activator-like 1B (Mobk1lb)
Double-strand-break repair protein rad21 (Rad21)
Myotrophin (Wtpn)
B-cell lymphoma 6 protein (Bcl6)
ADP-ribosylation factor 1 (Arf1)
Glycolipid transfer protein (Gltp)
Phosphoinositol-3-kinase adaptor protein 1 (Pik3ap1)
Zinc finger protein 758 (Zfp758),
ETS-related transcription factor Elf-1 (Elf1)
Signal recognition particle 54 kDa protein (Srp54)
H-2 class I histocompatibility antigen, Q10 alpha chain (H2-Q10)
H-2 class I histocompatibility antigen, K-B alpha chain (H2-K1)
Dipeptidyl-peptidase 1 (Dpp1)
Signal transducer and activator of transcription 1 (Stat1)
B-cell leukemia/lymphoma 10 (Bcl10)
MHC class II transactivator (Ciita)
AT rich interactive domain 1A (Arid1a)
Transcription regulator protein BACH2 (Bach2)
Activation-induced cytidine deaminase (Aicda)
Probable cation-transporting ATPase 13A2 (Atp13a2)
RAG1-activating protein 1 (Raglap1)
DNA mismatch repair protein Mlh1 (Mlh1)
Transmembrane protein 87A (Tmem87a)
Mice deficient for CD137 ligand are predisposed to develop germinal center-derived B cell lymphoma

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