Chapter 6

REDUCED B CELL MEMORY FORMATION IN MICE CONSTITUTIVELY EXPRESSING THE CD27 LIGAND, CD70

Kiki Tesselaar¹,², Bianca Heemskerk², Ramon Arens¹,³, Marinus H.J. van Oers³ and René A.W. van Lier¹,²

¹Laboratory for Experimental Immunology, Academic Medical Center (AMC), Amsterdam,
²Department of Immunobiology, CLB, Sanquin Blood Supply Foundation and Laboratory for Experimental and Clinical Immunology, Amsterdam
³Department of Hematology, AMC, Amsterdam,
The Netherlands
SUMMARY

Interaction between the TNF-receptor family member CD27 and its ligand CD70 gives a costimulatory signal for plasma cell formation and IgG secretion in vitro. To test the role of CD27/CD70 interactions on specific antibody formation in vivo we analysed humoral immune responses in transgenic (TG) mice, constitutively expressing CD70 on B cells. CD70TG B cells have no intrinsic defect to proliferate or differentiate into immunoglobulin producing cells after stimulation in vitro. While anti-TNP IgM titers in T-cell-independent type-II (T1-II)(TNP-ficoll) humoral responses were not influenced by the constitutive CD70 expression, IgG anti-TNP titers were elevated. In T-cell-dependent immune (TD) responses to TNP-KLH, CD70TG mice and WT mice mounted similar primary antibody responses. Markedly however, no germinal centres (GC) were formed in CD70TG mice. Consequently, secondary responses were impaired in CD70TG mice, as evidenced by severely reduced anti-TNP IgG1 serum levels and lack of affinity maturation. Together, these data suggest that CD27/CD70 regulate B cell differentiation by promoting signals that favour plasma cell formation rather than germinal centre formation, resulting in reduced B-cell memory formation in CD70TG mice.

INTRODUCTION

T and B cells that are activated during immune responses can differentiate into cells with different phenotype and function. Regulation of this process is not only necessary to effectively eliminate the Ag but will also ensure the formation of immunological memory and the consequent improved immune response after reencounter with the antigen. In T cell dependent immune responses the main decision in B cell differentiation concerns the choice between differentiation along the plasma cell pathway or the memory cell pathway. Different molecular controls like transcription factors, cytokines and TNF-receptor family members have been described to regulate this decision in B cell differentiation

CD27 is a TNF-R family member that regulates T cell expansion and T and B cell differentiation. CD27 expression is found on the majority of T and NK cells and subsets of B cells. In man the CD27 B cell populations are enriched for class-switched Ig expression and are solely responsible for Ig production after in vitro stimulation. Immunohistological analysis shows CD27 expression on marginal zone B cells, isolated B cells within primary follicles and on B cells present in GC's. Together these data imply that CD27 is only expressed on antigen experienced B cells. Indeed, all CD27 B populations have undergone somatic hypermutation and therefore CD27 is a marker for human memory B cells. In mice, a low percentage of splenic B cells express CD27 but expression can be strongly enhanced after stimulation with anti-IgM Ab and anti-CD40 mAb (K. Tesselaar, unpublished data).

CD27 exerts its function after ligation by its ligand CD70. CD70 expression has been characterised in man and mouse and seems to be similar in both species (chapter 3). In vitro analysis has shown CD70 expression on mature DC's and antigen activated, but not naive, T and B cells. Furthermore, antigen-induced CD70 expression on lymphocytes is regulated by cytokines and costimulatory signals. Accordingly, CD70 expression in vivo is very restricted,
even during an ongoing immune response, and seems to limit the interaction period between CD27 and CD70.

In vitro studies have extensively shown that CD27 ligation on human B cells increases the number of Ig producing plasma cells, and augments Ig production in cell cultures. Markedly, the effect of CD27 ligation on B-cell proliferation is limited. In vivo, the absence of CD27/CD70 interaction, either in CD27 knock out (KO) mice or after injection of a blocking anti-CD70 mAb, did not to hamper the production of Ag-specific Ig after influenza infection or induction of experimental autoimmune encephalomyelitis. In apparent contrast to the in vitro studies, it has recently been reported that injection of a presumably stimulatory anti-CD27 mAb reduces Ag specific titers after primary NP-CCG.

Functional consequences of CD27/CD70 interaction in vivo may be obscured by the abundance of molecular interactions regulating immune responses. To create an in vivo situation in which CD27 is present as a dominant co-stimulatory signal we generated transgenic mice, which constitutively expresses the murine CD70 molecule on all B cells. The increased CD27/CD70 interaction in these mice leads, surprisingly without deliberate immunisation, to increased T-cell expansion and effector-cell differentiation, thereby highlighting the function of CD27 on T cells. The increased number of effector T cells in CD70TG mice disturbs B cell development resulting in a progressive reduction of a normally-composed peripheral B cell compartment. To resolve the role of CD27/CD70 interactions on B-cell memory and plasma cell formation in vivo we herein studied humoral immune responses in CD70TG mice.

**MATERIALS AND METHODS**

Mice: C57black/6, CD70TG C57black/6, TCRα− C57black/6 and CD70TGxTCRα− mice were bred in the facilities of the Netherlands Cancer Institute and CLB under specific pathogen free-conditions. Mice used for experiments were between 4 and 12 weeks of age at the start of the experiment. All animal experiments were carried out according to institutional and national guidelines and approved by the Experimental Animal Committees of the respective institutes.

Immunisation: Mice were immunised at the indicated age with 100 μg TNP-KLH, 100 μg TNP-KLH emulsified in alum or TNP-AminoEthylCarboxymethyl-FICOLL (TNP-AECM-FICOLL, Biosearch Technologies, Novato, CA) in PBS (i.p.) at day 0 (primary immunisation) or day 28 (secondary immunisation) after start of the experiment. Sera were collected at the indicated days after immunisation (day 0, 7 or 14, 28 (prior to secondary immunisation) and 35). TNP-KLH was prepared by treatment of KLH (Sigma, MO) with picrylsulfonic acid (Sigma) using standard methods.

Monoclonal antibodies: Anti-mouse CD70 mAb (clone 3B9 (chapter3), was produced at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands. Anti mouse-B220 (RA3.6B2), CD3ε (145-2C11), CD16/CD32 (2.4G2), TER-119 (all ATCC, Rockville, MD)) and CD27 LGA 3A10(3) mAbs were purified from hybridoma
supernatant and FITC conjugated or biotinylated according to standard procedures. Anti-CD40 (HM-40.3), CD3e-allophycocyanin (APC, 145-2C11), CD19-FITC (1D3), and CD138-PE (281-2) were purchased from Pharmsingen (San Diego, CA). Biotinylated Peanut-Agglutinin (PNA) was purchased from Pierce (Rockford, IL). Unlabelled and biotinylated goat anti-mouse (GAM)-IgM, GAM-IgG1, GAM-IgG2a, GAM-IgG2b and GAM-IgG3 (Southern Biotechnology Associates (SBA), Birmingham, AL) were used in ELISA. For B cell stimulation GAM-IgM F(ab')2 was obtained from Jackson ImmunoResearch (West Grove, PA).

Determination of B cell characteristics in vitro: For B-cell purification, CD19+ cells were positively selected from splenocytes using goat anti-CD19 MACS microbeads beads and MACS (MS') separation columns following the manufacturers instructions. Purity of the resulting populations was tested by immunofluorescence with anti-CD19 and anti-CD3 mAbs and exceeded 95%. Purified B cells (10^5/well) were cultured in 96-well microtiter plates at 37°C for the indicated period of time in 200 µl Iscove’s modified Dulbecco’s medium (IMDM, Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS, Euro Biochem, Bierges, Belgium), gentamycin (43 µg/ml), and β-mercaptoethanol (0.0035%). Triplicate cultures were stimulated with the indicated stimuli: LPS (5 µg/ml, Sigma), GAM-IgM F(ab')2 (5 µg/ml), IL-2 (50 units/ml), anti-CD40 mAb (5 µg/ml). For determination of Ig production, culture supernatants were harvested 7 days after start of the culture. Triplicate cultures were pooled and Ig levels were determined by ELISA. Proliferation was measured by [3H]-thymidine incorporation. Sixteen hours prior to harvesting, cells were pulsed with 0.2 µCi/well [3H] thymidine (Amersham, Buckinghamshire, UK). After harvesting, [3H]-thymidine incorporation was measured in a liquid scintillation counter. FACS analysis was performed at the indicated time as described elsewhere.

Enzyme-linked immunosorbent assay: Anti-TNP-specific Ig levels in serum were measured by ELISA. Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 1 µg/ml TNP30-BSA in coating buffer (0.1 M sodiumcarbonate buffer (pH 9.7) for 16 hours at 4°C. This was followed by alternate washing and the subsequent incubation steps, at room temperature: 2% milk in PBS (1 hour). Serum samples diluted in high performance ELISA buffer ((HPE, CLB), 3 h), biotinylated GAM-Ig of the indicated isotype ((0.1 µg/ml in HPE), 1 hour), streptavidin-conjugated horse radish peroxidase ((poly-HRP, CLB diluted 1/10,000), 45 min). After the final wash, substrate was added (0.1 mg/ml TMB (3,3',5,5'tetramethylbenzidin, Merck, Darmstadt, Germany)) and 0.06% hydrogen peroxide in 0.1 M sodium acetate (pH 5.5). The reaction was stopped with 2 M H2SO4 and OD was measured at 450 nm. For determination of difference in antibody affinity, plates were coated with TNP30-BSA or TNP2.5-BSA. For measurement of Ig concentrations plates were coated with 1 µg/ml unlabeled GAM-IgM or a mixture of GAM-IgG1, IgG2a, IgG2b and IgG3. Subsequent steps in the ELISAs were performed as described above. Ig concentrations were calculated from linear standard curves
generated with affinity-purified mouse IgM or IgG (SBA). Relative anti-TNP titers were determined using a serum with high anti-TNP titers as a standard.

Immunohistology: Spleen sections and LN were embedded in O.C.T. compound (Tissue-Tec, Miles Inc. Torrance, CA) and snap-frozen in liquid nitrogen. Acetone fixed tissue sections (5-7 μm) were incubated for 2 hours with appropriately diluted antibodies in PBS/0.5% BSA. Biotinylated antibodies were visualised with Alexa Fluor™ 488- or Alexa Fluor™ 594-conjugated streptavidin (Molecular probes, Eugene, OR). Slides were mounted in Immuno Fluore Mounting Medium (ICN Biomedicals Inc., Aurora, OH) and fluorescent staining was analysed using a Leica DM RA microscope (Leica microsystems, Weilberg, Germany)

RESULTS AND DISCUSSION

Functional characteristics of CD70TG B cells. To assess whether the constitutive CD70 expression on B cells caused any intrinsic defect in cellular functions, purified CD70TG and WT B cells were stimulated with LPS. As shown in figure 1A, B cells from CD70TG mice had a slightly enhanced potency to proliferate in response to LPS (similar data were found for stimulation with anti-CD40 mAb +/- anti-IgM, data not shown). The determination of the percentage of CD138^{bright} cells and the production of IgM and IgG was used to test the ability to differentiate along the plasma cell pathway. After stimulation with LPS substantial numbers of CD138^{bright} cells and large amounts of IgM and IgG were produced in vitro. As is shown figure 1B and C, CD70TG B cells were capable to differentiate into CD138^{bright} and Ig producing cells.

![Figure 1](image.png)

**Figure 1: Characteristics of CD70TG B cells.**
(A) Proliferative capacity and (B) the capacity to differentiate into CD138^{bright} and (C) Ig producing cells of purified B cells derived from WT (white dots and bars) or CD70TG, F1393 (black dots and bars) mice were determined. (A, B) Purified B cells obtained from 4-week-old mice were stimulated with LPS and (A) [3H]thymidine incorporation was measured. (B) Percentage of CD138^{bright} cells was determined by FACS analysis and (C) IgM and IgG concentrations in supernatants after 7 days of culture were measured using ELISA. Concentrations were calculated using a standard concentration curve. Shown is one representative experiment out of 3 experiments
Humoral immune responses in CD70TG mice: T cell independent responses. After having confirmed the intrinsic functional competence of CD70TG B cells in vitro, the effect of persistent CD70 expression was first analysed in T-cell-independent responses. In order to overcome the differences in B cell numbers TI-II responses were analysed after immunisation of CD70TG/TCRa" and TCRα" mice with TNP-ficoll. In CD70TG/TCRα" mice no TCRαβ+ T cells, which in CD70TG mice are primarily responsible for the disturbance in B cell development, are present and B cell development proceeds normally. Serum samples were collected prior to and 6 days after immunisation and analysed for the relative presence of anti-TNP Ig of the different isotypes. Tissue sections were prepared of spleens derived 6 days after immunisation. Immunohistochemistry showed no overt difference in the number of splenic anti-TNP IgM producing cells (data not shown). Concordantly, as is shown in figure 2, both WT and CD70TG mice produced equal amounts of anti-TNP IgM, the main isotype in TI-II responses. Surprisingly, anti-TNP Ig levels of all other isotypes were elevated in CD70TG mice. This effect was most pronounced for IgG3 and IgG2a.

Although ample studies have shown the effect of CD27 crosslinking on plasma cell differentiation, a role for CD27 in isotype switching is unreported. It is unlikely that the effect we observe is caused by direct triggering of CD27 on B cells since others have shown that CD27 upregulation on mouse B cells is dependent on CD40 signals, which are absent in this model. Moreover, we did not find any CD27 expressing B cells in spleen of these immunised mice (data not shown). Further, although the precise mechanisms for class switching in TI-II responses

Figure 2: T-cell-independent type 2 responses in CD70TG/TCRa" mice.
Nine-week-old TCRα" and CD70TG/TCRa" mice were immunised with TNP-Ficoll (100 µg, i.p.). Isotype specific anti-TNP titers were determined by TNP-specific ELISA. Shown are the titers in the median responding TCRα" and CD70TG/TCRa" (F13) mice (n=3-5, each phenotype). Results are expressed as OD₅₀ values. Shown is one representative experiment out of 3 experiments.
are unclear, cytokines, locally produced by T and NK cells which are in close proximity to the TNP-antibody forming cells in vivo\textsuperscript{154}, and have been shown to regulate this process\textsuperscript{155}. CD27 is expressed on NK cells and in vitro crosslinking of CD27 on these cells is sufficient to stimulated NK cell proliferation and IFN-γ production\textsuperscript{53}. In parallel with the effects on T cells in CD70TG mice\textsuperscript{93}, it is possible that CD27 expressed on NK cells is functionally crosslinked in CD70TG mice. It can thus be speculated that in CD70TG mice the Th-II response develops in an IFN-γ enriched environment. Since IFN-γ promotes class switching to IgG3 and IgG2α in Th-II responses\textsuperscript{156}, this could explain the results in the CD70TG mice. Increased anti-Ag specific titers of all class switched isotypes are also found after induction of Th-II responses in the presence of an agonistic anti-CD40 mAb in vivo\textsuperscript{157,158}. Taking in account that CD40 triggering on B cell is a potent inducer of CD70\textsuperscript{38} (chapter 3) this might in fact result in enhanced CD27/CD70 interaction. Although the cellular and molecular basis remains to be defined it appears that CD27/CD70 interactions create a microenvironment that promotes class switching in Th-II responses.

Humoral immune responses in CD70TG mice: T cell dependent responses.
To analyse T cell dependent responses, 4-week-old mice were immunised with the hapten-protein conjugate TNP-KLH without adjuvant, or with the more immunogenic alum precipitated form of the Ag. At the indicated time points after immunisation (day 0, 7, 14, 28 and 35) serum samples were collected and mice were sacrificed (day 14 or 35). Secondary

![Figure 3: T-cell-dependent responses in CD70TG mice.](image)

Primary and secondary antibody responses to hapten-protein conjugates were measured after immunising 4-week-old WT and CD70TG mice (F13\textsuperscript{93}) with TNP\textsuperscript{30}-KLH. Mice were injected at day 0 (primary immunisation) and day 28 (secondary immunisation) after start of the experiment. Anti-TNP IgM and IgG1 titers were determined by TNP-specific ELISA. Shown are the relative titers (arbitrary units) of individual mice as determined by a reference anti-TNP sera. Shown is one representative experiment out of 4 experiments (2 experiments with TNP-KLH and 2 experiments with TNP-KLH in alum).

![Figure 4: Affinity maturation in CD70TG mice.](image)

To determine the presence of high affinity anti-TNP IgG1-antibodies, anti-TNP IgG1 titers of sera from WT or CD70TG mice (F13\textsuperscript{93}) were determined 7 days after secondary immunisation with TNP\textsuperscript{30}-KLH or TNP\textsuperscript{30}-KLH in alum by ELISA after coating with TNP\textsuperscript{30} BSA and TNP\textsuperscript{30} BSA. Shown are the OD\textsubscript{450} values for a given dilution after coating with TNP\textsuperscript{30} BSA and TNP\textsuperscript{30} BSA. Each symbol represents an individual mouse.
Immunisation was performed at day 28 after immunisation. In this protocol the number of splenic B cells in CD70TG declines from number at 9 weeks of TNP-IgM and IgG1 levels were comparable in WT and CD70TG mice at day 7 (data not shown) and day 14 after immunisation (figure 3). These results show that despite the reduced B cell numbers, CD70TG mice were capable of mounting normal primary Ab responses. Similar results were obtained after immunisation with TNP-KLH in alum.

Secondary responses in CD70TG mice were severely disturbed. After either type of immunisation anti-TNP IgG1 titers were approximately 50 fold lower in CD70TG mice than in WT mice. Activation and differentiation of memory cells into antibody forming cells (AFC) is the main pathway leading to increased Ag-specific Ig levels cells during a secondary response\(^{159,160}\).

The induction of anti-TNP IgM titers in CD70TG mice after secondary immunisation showed that the reduced anti-TNP IgG1 in CD70TG mice were not due to a general lack of B cells, but reflect a specific effect on the number and/or function of memory B cells in CD70TG mice.

Memory B cells are formed in germinal centres and express, due to the process of somatic hypermutation and affinity selection in this microenvironment, Ig with increased affinity for the immunising Ag. Disturbed memory cell formation might be the result of disturbed GC reactions in CD70TG mice. Determination of the affinity of IgG1 for TNP in the CD70TG mice strengthened this suggestion, i.e. in CD70TG mice high affinity anti-TNP IgG1 was absent after secondary immunisation (figure 4). The GC reaction was directly analysed by immunohistochemistry. Two weeks after primary immunisation mice were sacrificed and spleen sections were analysed for the presence of GC. As is shown in figure 5, PNA\(^{+}\)

**Figure 5**: Germinal centre formation in CD70TG mice.
Immunohistochemical analysis of spleen section 14 days after primary immunisation of 4-week-old mice WT (upper panels) or CD70TG (lower panels) with TNP\(^{253}\)-KLH in alum. Spleen sections were stained with the B cell specific anti-B220 mAb. Consecutive spleen sections were stained with PNA for the detection of GC-B cells. See also supplement.
GCs are present within the primary follicles in WT mice after immunisation with TNP-KLH. In CD70TGG mice however, primary follicles are present but no apparent GC can be found. Thus in CD70TGG mice secondary humoral responses are disturbed by the lack of GC reactions in the primary response and the consequent lack of memory B cell formation.

Multiple mechanisms can explain the changes observed during TD responses in CD70TGG mice. During T cell dependent responses antigen-activated B cell migrate into the outer T zone where they interact with antigen activated T cells. Signals from the antigen activated T cells, importantly crosslinking of CD40 by CD40L, help to decide whether the B cells will remain in the extra follicular foci (EFF) and become an antibody forming cell, or will migrate back into the primary follicle and start a GC reaction\textsuperscript{149,161}. Changed T cell helper activity would be an obvious mechanism to explain the phenomena observed in the CD70TG mice. It was shown that in the CD70TG mice differentiation of T cells into IFN-\(\gamma\)-secreting effector T cells is promoted\textsuperscript{93}. IFN-\(\gamma\) directly regulates proliferation and class switching of anti-CD40 mAb stimulated B cells in vitro\textsuperscript{162,163} but has not been implicated to regulate plasma cell differentiation or GC formation. However IFN-\(\gamma\) has been described to down-regulate CD40L expression\textsuperscript{164} and could by this means regulate the B cell differentiation process. Beside the increase in IFN-\(\gamma\) expression the enhanced CD27/CD70 interaction may also have changed the quality or quantity of T cell helper function, resulting in changed B cell differentiation. Initial analysis of T cell recall responses points to a mechanism in this direction since these recall responses are not only lower for CD70TG T cells but addition of CD70TG splenocytes as APCs also lowers recall responses of WT T cells (data not shown).

Data with human cells suggest that CD27 crosslinking on B cells promotes plasma cell differentiation\textsuperscript{29,152}. In analogy, enhanced crosslinking of CD27 by constitutively expressed CD70 would be another obvious explanation for the results in the CD70TG mice. In this model, the enhanced CD70 expression in CD70TG mice would instruct CD27\(^+\) B cells to stay in the EFF. Concordantly, B cells could no longer migrate in to the primary follicle and start a GC reaction. Consequently no memory B cells will be generated.

Taken together the analyses of T\textsuperscript{93} and B cell responses in the TG mice that express CD70 as a dominant costimulatory ligand support the idea that CD27 ligation results in enhanced effector cell formation. Although this response may be beneficial in initial coping with antigen, long-term memory responses are strongly undermined. The pathophysiological effects of chronic effector T-cell formation is dramatically demonstrated by the fact that CD70TG mice succumb from T-cell immunodeficiency (chapter 5). Likewise, we here provide evidence for a B-cell immunodeficiency. The strong effects of constitutive CD70 expression on both T and B cells underlines that effector cell formation needs to be highly regulated to maintain homeostasis in the antigen-experienced lymphocytes pool.