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CHAPTER 2

Thymus independent class switch recombination is affected by APRIL.

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ABSTRACT

The tumour necrosis factor (TNF) family member A PRoliferation Inducing Ligand (APRIL) is implicated in various B cell processes, such as class switch recombination, plasma cell differentiation and plasma cell survival. This was suggested from initial studies analysing B cell responses in APRIL deficient and transgenic mice and mice deficient for the TNF receptors of APRIL, transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA). Here, we present additional evidence for the importance of APRIL in thymus independent B cell responses, using APRIL deficient and transgenic mice. APRIL deficient mice show an impaired IgA response towards thymus independent (TI) B cell responses, whereas APRIL transgenic mice show exaggerated TI B cell responses. Moreover, antibody titers to TI antigens were sustained in APRIL transgenic mice for a long time and even increased up to 75 days in the case of IgA against NP-LPS.
INTRODUCTION

APRIL and its receptors, B cell maturation antigen (BCMA) and transmembrane activator and CAML interactor (TACI), play a crucial role in various B cell processes, such as class switch recombination (CSR), plasma cell differentiation and plasma cell survival. APRIL has been shown to promote thymus independent (TI) CSR to IgA via engagement of TACI, in vitro by directly stimulating B cells with recombinant APRIL [1, 2] and in vivo by immunising a strain of APRIL deficient mice with the TI type 1 antigen NP-LPS [3]. Besides being a potent inducer of CSR, TACI activation halts proliferation and promotes plasma cell differentiation of activated B cells in response to the TI type 2 antigen NP-Ficoll in vivo [4], and promotes CD40-driven plasma cell differentiation in vitro [5, 6]. In contrast, the function of BCMA is more enigmatic, as BCMA-deficient mice respond normally to immunisation with NP-Ficoll or the thymus dependent (TD) antigen NP-chicken gamma globulin (NP-CGG) [7]. In a later study, BCMA-deficient mice showed impaired survival of long-lived plasma cells towards the NP-CGG antigen [8], thereby implicating BCMA in sustained plasma cell survival.

In line with a role for APRIL in B cell responses, APRIL transgenic (Tg) mice produce increased antigen-specific IgM in response to TD vaccinia virus and increased IgM and IgG in response to NP-Ficoll [9]. In the present study, these observations are extended by comparing NP-LPS and NP-Ficoll B cell responses in APRIL Tg and a second APRIL deficient mouse strain [10].

MATERIALS AND METHODS

Animals
6-8 weeks old APRIL transgenic (Tg) mice, wildtype (WT) littermates, and APRIL deficient mice (kindly provided by Avi Ashkenazi, Genentech), all on a C57BL/6 background were used in the experiments. The APRIL transgenic mouse line expresses human APRIL under the lck-distal promotor, which directs transgene expression to mature thymocytes and peripheral T lymphocytes. Generation of this APRIL transgenic mouse line has been described previously [9]. The APRIL deficient mouse line used, was described earlier [10]. Mice were bred in the animal facility of the Academic Medical Center. All experiments were approved by the institutional animal ethical committee.

NP-ficoll / NP-LPS immunisations
For analysis of humoral responses, mice were immunised intraperitoneally with 100 μL PBS containing either 250 μg 4-hydroxy-nitrophenacetyl–conjugated Ficoll (NP-Ficoll)
or 100 μg NP-LPS (Biosearch Technologies). Blood was collected at different time points after immunisation from the tail vein using a capillary tube, samples were centrifuged, and serum was obtained and stored at minus 80°C. Antibody titers against 4-hydroxy-nitrophenacetyl (NP) were determined in an ELISA.

**ELISA**
Immunoglobulins were assayed by ELISA. To measure anti-(4-hydroxy-nitrophenacetyl)(NP)-specific antibodies, 96-wells ELISA plates (Greiner, Microlon 600) were coated with NP-BSA (Biosearch Technologies) (5 μg/ml) in sodium carbonate buffer (pH 9.6) O/N at 4ºC. The wells were blocked with 1% BSA for 1 hr at 37ºC and incubated with diluted sera for 2 hrs at room temperature. HRP-conjugated isotype-specific antibodies (all from Southern Biotech, Birmingham, AL) were used as revealing antibodies.

**Statistical analysis**
The Student’s T test was used to determine whether differences between experimental groups were significant. P < 0.05 was considered significant.

**RESULTS AND DISCUSSION**
APRIL-deficient mice display decreased IgA responses mainly towards type 1 TI antigens, suggesting that APRIL regulates these responses [3]. To validate whether this was mirrored in a second strain of APRIL-deficient mice [10], the antibody response towards the typical TI antigens NP-LPS (type 1) and NP-Ficoll (type 2) was analysed. Both antigens elicited a strong IgM response that was already detectable at day 3 and reached a maximum around day 8. The response towards NP-LPS was clearly more rapidly induced. However, in contrast to previous findings, no apparent difference in the IgM response towards NP-LPS between wildtype and APRIL-deficient mice was found. Also the IgM response towards NP-Ficoll was not markedly different between wildtype and APRIL-deficient mice, indicating that the lack of APRIL did not prevent B cell activation.

TI antibody class switching to IgG and especially IgA is mediated, at least in part, by APRIL and/or B cell Activating Factor belonging to the TNF Family (BAFF). Therefore the IgG and IgA response towards NP upon TI antigen immunisations was analysed. Class switching towards IgG occurred relatively early in the NP-LPS response. IgG class switching in the NP-Ficoll response was slower, but more pronounced. Although the NP-Ficoll IgG responses were comparable between wildtype and APRIL-deficient
mice, the IgA response was significantly reduced. Class switch recombination after NP-LPS immunisation was also affected in APRIL-deficient mice, since both IgG and IgA were significantly lower in this strain (Figure 1). It is important to note that the IgA response was relatively low, which is consistent with the fact that this antibody is to a large extent secreted into the intestine [11].

Although these data confirm the role of APRIL in class switching after immunisation with TI antigens, they differ significantly from previous findings. In agreement with previous reports, the IgA α-NP response was significantly diminished after NP-LPS immunisation. However, we found no differences in the IgM response against NP-LPS. Furthermore, we observed decreased IgG responses towards NP-LPS and significantly reduced IgA responses towards NP-Ficoll in this strain of APRIL-deficient mice. Apparently, differences exist between the two available APRIL-deficient mouse strains (summarised in Table 1). We therefore set out to further evaluate the role of APRIL in antibody class switching using APRIL Tg mice. APRIL Tg mice display elevated natural antibody levels and increased IgM and IgG responses towards NP-Ficoll [9]. These findings are now corroborated and extended with IgA measurements. In wildtype mice, α-NP IgA levels were low after NP-Ficoll immunisation. In contrast, the APRIL Tg mice showed very pronounced α-NP IgA responses. Moreover, this response was also sustained for over 50 days.

The response towards the typical type 1 TI antigen NP-LPS was also strongly affected by transgenic APRIL expression. IgM, IgG and IgA responses were all significantly elevated. IgM and IgG titers were around two-fold higher at most time points analysed, whereas the difference in IgA titers was much more pronounced. As shown above, this antibody subtype was very low in serum of WT mice, likely due to secretion into the intestinal lumen, but it was clearly detected in APRIL Tg mice. Also here it is striking to observe that the IgA antibodies against NP remained present for a long time, as high titers were still detected 75 days after immunisation and the IgA response did not appear to decrease, but rather increase even at this time point (Figure 2).

Combined with literature findings, our results indicate that APRIL is important for IgA class switching. Previous findings have indicated that APRIL enhances B cells to switch to IgA in vitro [1, 12]. Moreover, BCMA has been reported to provide APRIL-dependent survival to plasma cells in vivo [8]. This could indicate that the responding B cells are initially enhanced in both their activation and class switching to IgA by APRIL, while later effects are rather dependent on survival. In agreement, we have previously reported that APRIL enhances survival of B1 B cells and results in expansion of this pool of cells [13]. Recent evidence also indicates that B1 B cell derived clones are implicated in sustained antibody production upon NP-Ficoll immunisation [14]. In addition, we detected that APRIL can enhance the NP-LPS-induced migration of activated B1 B cells towards mesenteric lymph nodes (GH, unpublished observations). We therefore conclude that
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**Figure 1.** T-independent B cell responses in wildtype versus APRIL deficient mice

**A.** NP specific IgM, IgG and IgA levels in unimmunised mice and on day 3, 8, 14, 28, 70 and 153 after NP-Ficoll immunisation in serum of WT (grey circles) and APRIL deficient mice (white circles). Black lines are group averages and statistical significance versus WT is indicated with *** for $P < .001$, ** for $P < .01$, and * for $P < .05$.

**B.** NP specific IgM, IgG and IgA levels in unimmunised mice and on day 3, 8, 14, 28, 70 and 153 after NP-LPS immunisation in serum of WT (grey circles) and APRIL deficient mice (white circles). Black lines are group averages and statistical significance versus WT is indicated with *** for $P < .001$, ** for $P < .01$, and * for $P < .05$.
B1 B cells are likely responsible for the observed responses, which fits their localisation in the peritoneal cavity where the NP-LPS and NP-Ficoll immunisations took place. Early effects of APRIL likely involve enhanced B1 B cell migration to lymphoid organs, while later effects are probably due to enhanced survival of IgA-producing cells. APRIL Tg mice have high amounts of APRIL in their circulation, which is independent of T cell activation as it is expressed under the constitutive T cell specific lck-promoter. As APRIL is a secreted molecule, it is detected systemically at high levels in these mice. Importantly, T cells are not involved in the responses tested here and as such only serve to produce APRIL. In more physiological settings this production is likely induced upon pathogen recognition and performed by either intestinal epithelial cells or by the dendritic cells that are activated upon pathogen entry. Both cell types have been reported to produce significant amounts of APRIL, which is sufficient for IgA class switching [1, 12, 15]. In more pathogenic situations, such as B cell lymphomas, we have also detected significant amounts of APRIL in the circulation of patients and this is related to patient prognosis [13, 16]. Others have shown that this is likely due to sur-

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<th>APRIL-deficient (Varfolomeev et al. [10])</th>
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<tr>
<td><strong>Basal serum titers</strong></td>
<td>No differences [10]</td>
<td>Decreased IgA [3]</td>
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<tr>
<td><strong>NP-LPS i.p.</strong></td>
<td>No differences in IgM</td>
<td>Decreased IgM and IgA [3]</td>
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<td>Decreased IgG and IgA</td>
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<tr>
<td><strong>NP-Ficoll i.p.</strong></td>
<td>No differences in IgM and IgG</td>
<td>No differences in IgM, IgG3 and IgA [3]</td>
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<td><strong>S. pneumoniae i.v.</strong></td>
<td>Similar PC-specific plasmablast numbers</td>
<td>Not performed</td>
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<td>[10]</td>
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<td><strong>NP-CGG in alum i.p.</strong></td>
<td>No differences in NP-serum titers [10]</td>
<td>Increased IgG response and increased GC formation [3]</td>
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<td><strong>TNP-KLH in CFA i.p.</strong></td>
<td>No differences in NP-serum titers [10]</td>
<td>Not performed</td>
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<tr>
<td><strong>CGG i.p.</strong></td>
<td>Not performed</td>
<td>Increased IgG</td>
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<tr>
<td><strong>Boost with CGG + CTB i.g. + i.n.</strong></td>
<td>Not performed</td>
<td>Decreased IgA [3]</td>
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* pertussis toxin was co-injected and the response was boosted at day 14.
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Figure 2. T-independent B cell responses in wildtype versus APRIL transgenic mice

A. NP specific IgM, IgG and IgA levels in unimmunised mice and on day 3, 8, 14, 28, 42 and 56 after NP-Ficoll immunisation in serum of APRIL Tg (black circles) and WT mice (grey circles). Black lines are group averages and statistical significance versus WT is indicated with *** for P < .001, ** for P < .01, and * for P < .05.

B. NP specific IgM, IgG and IgA levels in unimmunised mice and on day 7, 13, 29, 38 and 75 after NP-LPS immunisation in serum of APRIL Tg (black circles) and WT mice (grey circles). Black lines are group averages and statistical significance versus WT is indicated with *** for P < .001, ** for P < .01, and * for P < .05.
vival of the malignant B cells [17-21] and as such further support our findings that APRIL is enhancing B cell survival and may be associated with prolonged B cell survival after immunisation. Taken together, we conclude that APRIL is an important player in the antibody response towards pathogens that elicit a TI B cell response and mainly dictates class switching towards IgA.

ACKNOWLEDGEMENTS

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Thymus independent class switch recombination is affected by APRIL.

REFERENCES

15. He, B. et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity 2007; 26: 812-826.