The role of APRIL in immunity and tumorigenicity

Hardenberg, G.

Citation for published version (APA):
CHAPTER 4

The HSPG binding domain of APRIL serves as a platform for ligand multimersation and cross-linking.

Fiona C. Kimberley¹, Liesbeth van Bostelen¹*, Gijs Hardenberg¹, J. Arnoud Marquart², Joost C. Meijers², Michael Hahne³ and Jan Paul Medema¹

¹Laboratory of Experimental Oncology and Radiobiology, Room G2-131, Academic Medical Center (AMC), Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
²Experimental Vascular Medicine, Academic Medical Center, Room G1-114, 1100 DD Amsterdam, The Netherlands
³Institut de Génétique Moléculaire de Montpellier (IGMM), CNRS UMR5535, 1919, route de Mende, 34293 Montpellier cedex 5, France

¹F.C.K. and L.B. contributed equally to this work.

Manuscript in preparation
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

ABSTRACT

APRIL (A Proliferation Inducing Ligand or TALL-2 and TRDL-1) is a member of the tumor necrosis factor (TNF) superfamily and binds two TNF receptors: TACI (Transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen). APRIL has tumorigenic properties, but is also important for the maintenance of humoral immune responses. Recently, APRIL was shown to bind heparan sulfate proteoglycans (HSPGs), but little is known about the functional significance of this interaction. In this study we have generated point mutants of APRIL that lack either HSPG-binding capacity or the ability to bind TACI and BCMA. This revealed that HSPG-binding is crucial for IgA production and B cell proliferation. Importantly, we show that the lack of HSPG interaction can be compensated by artificial cross-linking. This indicates that the HSPG-binding domain in APRIL is not necessary to allow for signalling through HSPG, but instead mediates ligand cross-linking and oligomerisation.
INTRODUCTION

A proliferation inducing ligand (APRIL) is a member of the TNF superfamily and was named according to its ability to drive tumor cell growth [1]. APRIL is expressed as a type II transmembrane protein, but unlike many of the other TNF family ligands it is not expressed at the cell surface but is cleaved intracellularly by a furin convertase, to release the active soluble form [2]. The soluble protein forms a non-covalent homotrimer, with high structural homology to several other TNF family ligands [3]. A membrane bound form of APRIL, generated by an alternative splicing event in which the membrane domain of TWEAK fuses with the extracellular domain of APRIL, is called TWE-PRIL [4, 5]. However, a specific function for this form has not yet been shown and its expression pattern not fully delineated.

APRIL binds two TNF receptors: BCMA (B-cell maturation antigen) and TACI, (Transmembrane activator and calcium modulator and cyclophilin ligand interactor) which it also shares with another TNF ligand, BAFF (B cell activating factor or B-lymphocyte stimulator, BLyS). However, BAFF binds another unique receptor, BAFF receptor (BAFF-R or BR3) and plays a crucial role in B cell homeostasis, particularly as a survival factor during B cell maturation [6-9]. The physiological role of APRIL appears primarily to be the stimulation of proliferation and survival of a number of different cell types. It can signal activation of the classical NF-κB survival pathway via both TACI and BCMA and plays an important role in B cell responses (reviewed in chapter 1).

APRIL signalling appears to drive IgA class-switch recombination and IgA production in B cells exclusively via TACI [10-13], while activation of BCMA by BAFF or APRIL appears to be necessary for the survival of long-lived plasma B cells [14]. In addition, both APRIL and BAFF have been shown to enhance survival of plasmablasts from activated human memory B cells, in a BCMA-dependent manner [15]. APRIL also plays a co-stimulatory role in B-cell antigen presentation to T cells by stimulating the up-regulation of important co-factors such as CD40, MHC-II, B7.1 and B7.2; this effect is attributed to signalling through BCMA [16]. APRIL has also been shown to play a role in T cell co-stimulation, but a direct role in T cell biology is not yet clear. Originally APRIL was shown to affect survival of various T cell populations in vitro, but recent evidence suggests that the role of APRIL in physiological T cell responses in vivo is at best limited (chapter 6).

APRIL is expressed by a subset of immune cells and also in non-immune cells such as epithelial cells and osteoclasts [13, 17, 18]. Significantly, APRIL is over-expressed by several solid tumours of varying origin and lymphoid malignancies, inducing an enhancement of proliferation and survival [19-23].

It was recently shown that APRIL can bind to heparan sulfate proteoglycans (HSPG)
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

[24, 25]. This finding immediately posed a number of questions regarding the contribution of the HSPG interaction to APRIL signalling. A direct signalling role was recently proposed in a study showing that APRIL-driven IgA production via TACI required a separate signal from HSPGs [26]. It was proposed that the HSPG binding domain of APRIL forms a direct link with the HSPGs. However, a recent study used antibody-mediated cross-linking of a soluble form of APRIL to show that APRIL-driven TACI signalling requires multimerisation [27]. HSPGs are abundant in the extracellular matrix and therefore it has been postulated that they serve to naturally cross-link soluble APRIL via the HSPG binding domain [24, 25]. In this study we have sought to better understand the role of the HSPG interaction in the context of normal APRIL signalling.

**MATERIALS AND METHODS**

**Cell Culture**

All cell lines were maintained at 37°C with 5% CO2. Human embryonic kidney cells (293T) were cultured in IMDM (Invitrogen) supplemented with 10% FCS with 2mM Glutamine. Mouse splenocytes and purified B cells were grown in RPMI-1640 (Gibco) supplemented with 8% FCS, 2mM Glutamine and Beta-mercaptoethanol at 50 μM.

**Antibodies and Reagents**

Commercial reagents used were as follows: monoclonal anti-Flag-M2 (Sigma), monoclonal anti-Flag-M2-Bio (Sigma), monoclonal anti-Flag-M2 peroxidase conjugate (Sigma), streptavidin-APC (BD Biosciences), heparin sepharose fast flow-6 (Amersham Biosciences), anti-Flag-M2 sepharose (Sigma), anti-human-TACI-PE (clone 1A1-K21M22) (BD Biosciences), Heparin (hospital pharmacy, LUMC Leiden), anti-MHC Class II (I-Ab, BD Biosciences), mouse IL4 and mouse IL6 (Peprotech). Soluble Flag-tagged APRIL, the various APRIL mutants, BCMA-Fc and TACI-Fc were produced and purified in-house (see protein expression and purification).

**Cloning and Constructs**

The APRIL-HSPG-triple and APRIL-HSPG mutants were generated previously [24]. APRIL-WT-triple was generated using Quick Change site-directed mutagenesis (Stratagene) using the following primers: 5’-tagggtgaggacgcctacagccc-3’; 5’-gagaagccagcaggtcattttacctcc-3’; 5’-atatgcagcggtctctcttttggagatattctcg-3’. The APRIL R230A point mutation was generated using a two-stage PCR using the following primer pairs (Invitrogen): primer 1: 5’-caagtgagagcgctgctc-3’ and primer 2: 5’-ctggcccatggtgccgtctgtt-3’; primer 3: 5’-caagacgtgactgcaacgacagtctc-3’
and primer 4: 5’gctctagatcacagtttcacaaa-3’. Primer 1 and primer 4 were then used in the second reaction with the products from the step 1 and an initial annealing step at 60°C to allow association between the two strands. All mutations were confirmed by sequencing. Plasmids expressing human full-length TACI- and full-length BCMA-with an internal C-terminal Flag-tag were generated previously. BCMA- and TACI- human IgG-Fc were generated previously.

**Protein Expression and Purification**

For purification, soluble Flag-tagged APRIL was transiently expressed in 293T cells using large-scale calcium phosphate transfection. Soluble protein was purified from the resulting supernatant using anti-Flag-M2 sepharose (Sigma), according to the manufacturer’s guidelines. Conditioned medium containing soluble APRIL was produced by similar transient transfection of 293T cells in 6-well plates; following transfection cells were changed into RPMI-1640. Both BCMA- and TACI- human IgG Fc were expressed using large-scale transient transfection of 293T cells and purified by running over a protein-A sepharose column according to manufacturer’s guidelines (Sigma).

**Flow Cytometry**

All flow cytometry was carried out on a FACSCalibur (BD Bioscience) and analysed using FlowJo software (Tree Star). To measure receptor binding, 293T cells were co-transfected with BCMA, or TACI, plus GFP at a ratio of 10:1. Transfected 293T cells were harvested after 48 hours in PBS, washed and stained in PBS/1% BSA with the various forms of soluble APRIL, plus or minus Heparin (4 IU/mL). Bound APRIL was then detected with anti-Flag-M2-bio (Sigma) followed by streptavidin-APC (BD Biosciences), and cells gated on GFP to include only positive transfectants.

**Mouse B cell Isolation, Proliferation and IgA Production**

Splenic mouse B cells were isolated from wild-type mice using magnetic activated cell separation (MACS) columns with CD45R/B220 MACS beads (Miltenyi Biotec). The cells were cultured in 96-well round-bottomed microtiter plates at a density of 2 x 10⁶ /well in a final volume of 200 μL. For all assays conditioned medium containing the various forms of soluble APRIL were normalised for expression levels prior to use. To measure proliferation, cells were treated with anti-IgM (Jackson ImmunoResearch) and soluble APRIL in conditioned medium or as purified protein at a final concentration of 1 μg/mL. Cross-linking anti-Flag monoclonal antibody was added to the well at a final concentration of 1 μg/mL. The cells were incubated at 37°C and after 48 hours pulsed with 0.3 μCi (0.011 MBq) of tritiated thymidine ([6-3H] Thymidine, GE Healthcare) for 18 hours, before harvesting. To measure IgA production, mouse B cells were cultured
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

and treated with APRIL, as above. Following incubation for 6 days, supernatant was collected and assayed for IgA content by ELISA. Briefly, ELISA plates were coated with 2 μg/mL anti-mouse-Ig (Southern Biotech), blocked with PBS/1% BSA and incubated with the collected supernatant. Bound IgA was then detected with HRP labelled anti-mouse-IgA (Southern Biotech). As a control, cells were treated with 10 μg/mL LPS (Invivogen) plus 1 ng/mL of human TGFβ (Sigma-Aldrich).

MHC Class II up-regulation Assay
Splenitic mouse B cells were purified and seeded in 96-well round-bottomed microtiter plates at 3 x 10⁵/well. The cells were stimulated with mouse IL-4 and IL-6 at a concentration of 0.4 ng/mL and treated with the various forms of APRIL at a final concentration of 0.5 μg/mL, plus or minus anti-Flag at a final concentration of 1 μg/mL. The cells were harvested after 48 hours, stained for MHC class II an analysed by flow cytometry.

Immunoblotting and Immunoprecipitations
Conditioned medium containing the various forms of soluble APRIL was quantified by western blotting using anti-Flag-M2-HRP (Sigma) to visualise. The intensity of the resulting bands was compared and the medium normalised for subsequent assays. To compare the binding of the various forms of soluble APRIL to heparin, quantified supernatant was immunoprecipitated by mixing with heparin sepharose beads (Sigma) and incubated at RT for 1 hour. The beads were then washed three times with 0.5M NaCl and three times with PBS, boiled in reducing sample buffer and subjected to western blotting. The bands were visualised using anti-Flag-M2-HRP (Sigma). To compare binding of APRIL to TACI and BCMA, purified receptor Fc-fusion proteins were incubated with protein-A plus the APRIL supernatants, and processed as above.

Surface Plasmon Resonance
SPR measurements were carried out on a BIACore 2000 instrument (BIACore, Uppsala Sweden) using PBS, at 25°C. In order to capture APRIL in the correct orientation for binding, anti-Flag monoclonal antibody (diluted in sodium acetate pH 4.5) was covalently immobilized to a CM-5 sensorchip (BIACore) using the BIACore Amine Coupling kit according to manufacturer’s guidelines. One flow cell was chemically activated and de-activated, but not coated with antibody and used as a reference lane to control for background binding. The various forms of soluble APRIL were captured onto the chip directly via the N-terminal Flag-tag. BCMA- or TACI- Fc were diluted in PBS and then injected over all four flow cells in five consecutive injections (3 minutes) with increasing concentrations ranging from 1-250 nM. The binding curves were analysed using ClampXP Biosensor Data Analysis software, version 3.5. Because of the nature of the
proteins (dimer binding a trimer) a two parallel binding reaction was chosen to model an equation for the binding curves, since this best approximates to the real binding kinetics and gave the better overall fit for the data. The KD was calculated using the kd and ka from the first binding reaction which gave the most significant contribution to the overall binding. Each KD was calculated from one simultaneous fit of curves from three separate experiments. Background and non-specific binding were subtracted using the binding curves generated in the reference flow cell.

**RESULTS**

**APRIL-induced B cell proliferation, but not IgA production, is enhanced by antibody-mediated ligand cross-linking.**

It has been shown previously that APRIL can co-stimulate B cells to drive proliferation and that this co-stimulatory effect is entirely dependent upon antibody-mediated ligand cross-linking [25]. In our experiments, mouse splenic B cells were stimulated via the B cell receptor (BCR) with anti-IgM and co-stimulated with supernatant containing soluble APRIL (aa 88-233), with and without cross-linking antibody (monoclonal anti-Flag). At a range of anti-IgM concentrations there was a co-stimulatory effect of soluble APRIL, which was enhanced, but not dependent, on antibody-mediated cross-linking (Figure 1A). Importantly, APRIL-induced co-stimulation was concentration-dependent (data not shown).

**Figure 1.** APRIL-induced B cell proliferation but not IgA production is enhanced by antibody-mediated ligand cross-linking. (A) Mouse splenic B cells were stimulated with anti-IgM (to activate BCR) at a range of concentrations and co-stimulated with conditioned medium containing soluble APRIL, plus or minus cross-linking anti-Flag monoclonal antibody. Proliferation was measured using tritiated thymidine. All samples were analysed in triplicate. (B) Mouse splenic B cells were treated with conditioned medium (in three-fold dilutions) containing soluble APRIL plus or minus cross-linking anti-Flag monoclonal antibody. Cell supernatant was assayed for IgA content by ELISA. All samples were analysed in triplicate and error bars represent standard deviation (S.D.) of the mean.
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

Since different biological effects require different thresholds for signalling, we examined the requirement for APRIL oligomerisation using a second readout for APRIL activity. APRIL has been shown to mediate antibody class switch recombination and IgA production in B cells exclusively via TACI [10-13]. Therefore, we stimulated mouse splenic B cells, with conditioned supernatant containing soluble APRIL, with and without anti-Flag, then measured soluble IgA in the supernatant on day 6 (Figure 1B). Interestingly, soluble APRIL alone was effective in stimulating IgA production, but there was no increase with the addition of anti-Flag, suggesting that antibody-induced oligomerisation was not a requirement, and did not enhance signalling in this context. Thus, from our data it appears that APRIL signalling for IgA production and proliferation does not require antibody-mediated cross-linking.

The HSPG domain of APRIL is required for effective production of IgA.

In order to test whether the HSPG binding domain of APRIL forms a platform for natural ligand cross-linking, we cloned and expressed several mutant forms of soluble APRIL, lacking either partial or complete HSPG binding, or with functional HSPG binding but lacking the capacity to bind TNF receptors (Figure 2). The exact HSPG binding domain of APRIL was mapped previously using known HSPG consensus sequences, mutagenesis studies and by deletion of the N-terminal domain [24, 25]. Specifically, the HSPG site has been mapped to two regions: (1) A hydrophobic motif at the N-terminus (QKQKKQ) and (2) three basic amino acids on the opposite side of the molecule to the TACI/BCMA binding site: R146, H220 and R189.

![Figure 2. APRIL HSPG-Binding. (A) A schematic to illustrate the point mutations made in the various APRIL constructs and the terminology used throughout the paper. The numbering of amino acids in the APRIL constructs is based on the peptide sequence given by the UniProtKB/Trembl entry Q8NFH7, which represents the sequence of the immature unprocessed form. (B) Structure of the APRIL-BCMA complex (visualised using Cn3D and PDB co-ordinate 1XU2) with the specific mutations highlighted. The position of the N-terminus is highlighted at K113 as only this part of the hydrophobic motif (QKQKKK113Q) is present in the structure. The R230A mutation within the BCMA and TACI binding pocket is also highlighted.](image-url)
We used site-directed multi-mutagenesis to generate several mutant clones: (1) WT-triple - containing three point mutations: R146S, R189S and H220E; (2) APRIL-HSPG - containing three point mutations in the hydrophobic domain in which lysine is replaced by alanine (Q109A110Q111A112A113Q); (3) APRIL-HSPG-triple – in which all six amino acids were mutated, at both these sites. Finally, as a control we generated a form of APRIL capable of binding HSPGs but lacking the ability to bind either TACI or BCMA. Based on the crystal structure of the TACI-D2-APRIL complex, we mutated a key arginine within the proposed receptor binding region, to alanine: R230A [28]. In order to validate these proteins we examined binding capacity using immunoprecipitation (Figure 3A). Conditioned supernatant containing the various forms of soluble APRIL was incubated with protein-A beads plus either TACI- or BCMA- Fc, or directly with heparin sepharose, to examine binding to the receptors and HSPG respectively. Immunoprecipitation with both BCMA- and TACI- Fc indicate that all the mutant forms of APRIL, except APRIL-R230A, retain the ability to bind both BCMA and TACI. The R230A mutant shows complete loss of binding to both receptors. However, APRIL-R230A retains its ability to bind HSPGs, as indicated by effective immunoprecipitation with heparin sepharose. In agreement with previously published data, the APRIL-triple mutant does not lose the ability to bind to HSPGs, while mutations in the hydrophobic N-terminal region (QKQKKQ) lead to complete loss of binding, as observed for the HSPG and HSPG-triple mutants [24]. To test activity of the various proteins, supernatant containing soluble protein was normalised for APRIL expression and used to treat mouse B cells. After 6 days, IgA production was assayed (Figure 3B). The result was a significant decrease in IgA production for both the HSPG and HSPG-triple APRIL mutants. However, the APRIL-HSPG-triple mutant was less active than the APRIL-HSPG mutant, indicating that whilst the hydrophobic N-terminal region (QKQKKQ) is essential for HSPG binding, mutations at both sites are necessary for complete loss of activity. This may be explained by a difference in affinity for HSPGs or ability to bind in the correct orientation, and not easily detected

Figure 3. The HSPG domain of APRIL is required for effective production of IgA, but loss of HSPG-binding capacity does not affect binding to either TACI or BCMA. (A) Western blot following immunoprecipitation of the various forms of APRIL in order to validate HSPG and TNF receptor binding. Conditioned supernatant containing soluble APRIL was incubated with either BCMA- or TACI- Fc fusion protein plus protein-A, or with heparin sepharose beads directly. The samples were then washed and run on an acrylamide gel. Bound APRIL was visualised using anti-Flag-HRP. To confirm normalisation of the APRIL supernatants used in IP, these were blotted directly with anti-Flag (lower panel). (B) Mouse splenic B cells were treated with conditioned medium containing the various soluble forms of soluble APRIL. The supernatant was analysed for IgA content by ELISA following a 6-day incubation. Each sample was analysed in triplicate. Statistical analysis was carried out using the Student’s T-test (* p<0.05, ** p<0.001). (C) Comparative affinities of the receptors for the WT and HSPG-triple mutant form of APRIL as measured using BIAcore. The given affinities are in nM and represent the calculated KD based on a simultaneous fit for data from three separate measurements. (D) 293T cells transiently transfected with TACI were stained with various form of soluble purified APRIL, with and without heparin, and analysed for positive staining by flow cytometry. Key: Solid lines - staining with APRIL, dashed lines – APRIL plus heparin, filled grey – Unstained control, grey dotted line – unstained control plus heparin. All samples were analysed in triplicate and error bars represent S.D. of the mean.
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

A. APRIL

B. 

C. Affinities in nM

<table>
<thead>
<tr>
<th></th>
<th>BCMA-Fc</th>
<th>TACI-Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT APRIL</td>
<td>2.67 +/- 0.03</td>
<td>1.18 +/- 0.02</td>
</tr>
<tr>
<td>APRIL-HSPG-triple</td>
<td>1.37 +/- 0.02</td>
<td>1.6 +/- 0.02</td>
</tr>
</tbody>
</table>

D. APRIL binding
by IP. Finally, the APRIL-R230A mutant, lacking TNF receptor binding capacity, does not show any activity above background levels. This shows that any potential signal for IgA production coming from the HSPGs is redundant without TNF-receptor binding.

**Loss of HSPG binding capacity does not affect binding to BCMA or TACI**

We chose to study WT-APRIL and APRIL-HSPG-triple mutant since this shows complete loss of HSPG-binding, and also used the R230A mutant as a control. Since the APRIL HSPG-triple mutant contains a total of six point mutations, it is possible that these lead to disruptions in overall protein folding. Although the immunoprecipitations show that the mutant retains TNF receptor binding capacity a difference in binding affinity would not necessarily be detected. Therefore, we purified both WT and HSPG-triple APRIL and used BIACore to measure the exact affinity of the two forms for TACI and BCMA. A CM5 BIACore chip was coated with anti-Flag monoclonal. Purified APRIL was then bound to the chip and either TACI- or BCMA- Fc flowed over at increasing concentrations. The exact affinity of the ligand for receptor was calculated using software to model kinetics for a two-step binding reaction (Figure 3C).

Several papers report affinity measurements for binding of APRIL to both BCMA and TACI [29-32]. However, these reported affinities are quite variable due to differences in the techniques and proteins used. Our measured affinities were in the nano-molar range, as previously reported [29-32]. Significantly, the affinity of the HSPG-triple mutant for both TACI and BCMA was comparable to that of WT APRIL, indicating that the point mutations in the HSPG binding domain do not affect overall protein folding. Therefore, decreased activity of the HSPG-triple mutant is not related to loss of TNF receptor binding capacity.

Although BIACore is a reliable method to define affinities, the measurements still may not reflect a true physiological affinity, since the influence of HSPG interactions are not considered. Hence, the physiological binding affinity for the receptors may be different when the interaction is analysed at a cellular level. Therefore, the mutants were further validated for binding capacity by cell staining and flow cytometry (Figure 3D). Both TACI and BCMA are absent on 293T cells, but these cells contain surface HSPGs. Therefore, cells were co-transfected with GFP and full-length BCMA or full-length TACI then stained with the various forms of soluble APRIL and gated on GFP-positive cells. Since heparin can compete for HSPG binding, the cells were also stained in the presence of heparin, to show specific binding to BCMA or TACI.

The binding of WT-APRIL to GFP-only transfected cells indicates HSPG binding; this binding can be completely competed off with heparin. For the TACI- and BCMA- transfected cells the binding can only be partially competed off as a result of the additional
receptor binding. The APRIL-HSPG-triple mutant, as expected, shows no binding to GFP-only transfected cells, but there is distinct binding to the BCMA and TACI transfected cells. The addition of heparin in this case does not influence the level of binding, indicating that this is specific binding to the TNF receptors. The binding of R230A to the GFP-only transfected cells indicates HSPG binding which can be completely removed by the addition of heparin. The R230A mutant shows no additional binding in the transfected cells, which is further proof that it cannot bind either BCMA or TACI. Combined, these observations confirm that the HSPG-triple mutant binds to both TACI and BCMA, but is unable to interact with HSPGs. The R230A APRIL mutant cannot bind to either BCMA or TACI, but retains HSPG binding.

**Antibody-mediated ligand cross-linking of APRIL can compensate for loss of the HSPG binding domain**

In a recent study it was suggested that APRIL driven IgA production by TACI requires an additional signal from HSPGs and that the HSPG domain on APRIL is required to mediate this signal [26]. Therefore, if the APRIL-HSPG domain is mutated, then additional co-stimulatory signals from HSPGs would be lost.

In order to test this we used a purified form of the APRIL-HSPG-triple mutant, since this shows complete loss of HSPG-binding.

Splenic mouse B cells were treated with purified proteins, plus or minus cross-linking antibody, and IgA production measured (Figure 4A). As observed with supernatant containing soluble APRIL (Figure 1A), antibody cross-linking had no additional effect on the signal from purified WT-APRIL. However, antibody cross-linking of the non-HSPG binding mutant could completely compensate for the loss of HSPG binding, and restored the level of activity to that of the WT. The non-TNF receptor binding mutant, APRIL-R230A, showed no activity even with antibody cross-linking. Combined, this data strongly suggests that the HSPG domain of APRIL does not mediate a separate signal, but instead serves simply as a platform for natural ligand cross-linking and oligomerisation.

To extend this, we also tested purified APRIL on mouse splenic B cells in a proliferation assay, plus or minus anti-Flag (Figure 4B). Soluble APRIL alone was sufficient to co-stimulate proliferation, and as previously observed with supernatants (Figure 1A) the addition of anti-Flag cross-linking antibody served to enhance the signal, but was not essential for activity. Cells treated with HSPG-triple mutant alone did not proliferate above background levels, but addition of anti-Flag restored activity.

To validate these findings in a separate read-out, we used APRIL-induced up-regulation of MHC class II on B cells [16]. We treated mouse splenic B cells with both WT and HSPG-triple (non-HSPG binding mutant), and measured MHC class II expression
by flow cytometry (Figure 4C). Cells treated with WT alone up-regulated MHC class II, but this was further enhanced in the presence of cross-linking antibody. Cells treated with the HSPG-triple APRIL mutant showed low (or background) levels of MHC class II expression in the absence of antibody. However, in the presence of cross-linking antibody the level of MHC class II expression was strongly enhanced and comparable to that of the WT. This is further evidence that the HSPG binding domain mediates natural cross-linking, since cross-linking with monoclonal antibody can compensate for loss of HSPG binding and restore signalling capacity.

Figure 4. Antibody-mediated ligand cross-linking of APRIL can compensate for loss of the HSPG binding domain. Mouse splenic B cells were treated with purified forms of APRIL (WT, HSPG-triple and R230A) at a final concentration of 1 μg/mL plus or minus cross-linking anti-Flag monoclonal antibody. LPS and TGFβ served as a positive control. All conditions were set-up in triplicate. (A) Supernatants were assayed for IgA content by ELISA, following 6-day incubation. (B) Cells were assayed for cell proliferation after 48 hours using tritiated thymidine. (C) Cells were incubated with 0.5 μg/mL of soluble APRIL and stained for the expression of MHC class II after 48 hours. All samples were analysed in triplicate and error bars represent S.D.
DISCUSSION

The observation that APRIL can bind HSPGs, in addition to the TNF receptors BCMA and TACI, was an intriguing one. Indeed, recent evidence suggesting that HSPGs can signal in their own right prompted questions regarding the role of this interaction in canonical APRIL signalling [33]. Using well-characterised soluble forms of APRIL we have shown that the HSPG-binding domain serves as a platform to mediate natural ligand cross-linking. We found that WT-APRIL alone was capable of inducing both proliferation and IgA-production, but that the non-HSPG binding form of APRIL was inactive in these assays. However, activity was restored with the addition of cross-linking antibody.

In a recent study it was shown that signalling through TACI requires ligand multimerisation [27]. In the case of BAFF, this was evidence for a physiological function for a 60-mer form of BAFF identified previously by crystallography [34]. In addition, antibody-mediated cross-linking of WT-APRIL was shown to be necessary for APRIL-dependent MHC class II up-regulation via TACI [16]. However, no higher order oligomers have been reported for APRIL, so the question is how multimerisation of APRIL might occur in a physiological setting. Based on our data we propose that multimerisation is achieved naturally by interactions with the HSPG-binding domain, but that for certain stimuli (possibly TACI-dependent) additional ligand cross-linking can serve to enhance the signal.

In a physiological setting it can be imagined that secreted soluble APRIL is captured by HSPGs onto the extracellular matrix, with the effect being two-fold: (1) to retain soluble APRIL at the cell surface ready for signalling, and (2) to cross-link and orientate APRIL for efficient signalling. Figure 5 provides a schematic to explain our hypothesis. Under in vitro conditions, antibody-mediated cross-linking can mimic the natural effect of HSPGs, and hence compensate for the loss of HSPG binding in the APRIL mutant. In the case of WT-APRIL, additional cross-linking may serve to super-cluster the receptors and enhance signalling, depending on the threshold for a given signalling event, though this phenomenon is unlikely to be physiologically relevant.

Of note is the difference we observed between the antibody effects in the two assays. In the proliferation assay, WT-APRIL was sensitive to further enhancement by antibody-mediated cross-linking, whilst the activity of WT-APRIL in IgA production assays was unaffected. The reason for this difference is not entirely clear, but may reflect a difference in the particular TNF-receptor used in each case. Depletion of activated B cells (via anti-CD43) gave no APRIL-response (data not shown), suggesting that we are definitely measuring IgA production, rather than class-switch. IgA production could therefore be a TACI-independent event and simply survival of pre-existing IgA produc-
a. In-vitro antibody cross-linking of APRIL:

Soluble APRIL trimers:

Receptor:

TACI or BCMA

Recruitment of internal adaptor proteins

SIGNAL

b. Natural and enhanced cross-linking of WT-APRIL by cell surface HSPGs:

HSPGs

Antibody

SIGNAL

(Enhanced) SIGNAL

Figure 5. Schematic to illustrate natural cross-linking of APRIL by HSPGs on the extracellular matrix. (A) An illustration of how in vitro cross-linking by monoclonal antibody can mimic that of natural HSPGs on the extra-cellular matrix. In the case of the non-HSPG binding mutant, one can imagine how addition of antibody might mimic the natural HSPG cross-linking effect and therefore compensate for loss of binding. In order to deliver an effective signal, WT-APRIL needs to bind receptors in functional signalling complexes, leading to recruitment of internal adaptor proteins and downstream signalling. Physiologically HSPGs on the surface could help to cluster APRIL and achieve this multimeric assembly at the cell surface. Further antibody cross-linking of WT-APRIL could lead to ‘super-clustering’ and hence enhanced signalling. (B) The cross-linking effect from HSPGs may occur in a direct (cell to cell) or lateral (same cell) fashion. In the case of TACI, signalling may be complicated by separate TACI-HSPG interactions.
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

ing cells, via BCMA.
Hence, the idea of multimerisation put forward by Bossen et al in essence is in agreement with our findings. We can only postulate that the reason for the discrepancy between our observation and theirs, regarding the necessity for artificial cross-linking of APRIL, is due to the fact that they did not use the naturally processed form of APRIL (starting at Q109 instead of A105), which may lack full HSPG binding capacity [27]. However, for IgM secretion and plasmablast survival they reported some activity of WT-APRIL alone, but observed a 6-fold enhancement with the addition of anti-Flag. Importantly, in our hands APRIL-induced co-stimulation was diluted out at lower concentrations (data not shown). This may also explain the discrepancy with previously published data suggesting that APRIL-induced proliferation is dependent upon cross-linking, since they used dilutions of conditioned medium in which the APRIL concentration could be very low [25]. However, a recent paper, using B220 purified splenic B cells, shows that WT-APRIL alone is sufficient to induce proliferation in these cells [35]. This same study examined plasmablast survival in bone marrow and reported pro-survival activity of WT-APRIL (starting at A105) alone, but decreased survival with the use of the non-HSPG binding form (starting at H115); cross-linking was not tested in this context. This is thought to be primarily a BCMA-dependent response [16], but may also be a combination of signalling through both TACI and BCMA.

Unlike previously published data, we did not see any evidence for a separate signal from HSPGs, but instead antibody cross-linking was sufficient to restore activity of mutant APRIL [26]. In this study, cross-linking antibodies against TACI and HSPG were used to stimulate cells in the place of soluble APRIL, and it was shown that both antibodies were required for effective production of IgA. However, it was not formally shown that the TACI antibody used was indeed cross-linking. In addition, TACI has recently been shown to interact with HSPGs in its own right, so it is possible that the HSPG antibody actually served to cross-link TACI via these interactions [36]. Moreover, the authors used heparitinase treated cells which would eliminate not only surface HSPGs for binding APRIL, but also any TACI-HSPG interactions. The contribution of this interaction to APRIL-TACI signalling is not yet known. The use of the non-HSPG binding mutant in our study means that these interactions would remain intact. Thus, our experiments better reflect the direct effect of APRIL-HSPG binding.
The described mechanism for natural oligomerisation of soluble APRIL is no surprise in light of the behavior of other members of the TNF superfamily. It is well-described that many TNF family ligands require cross-linking or oligomerisation in order to deliver an effective signal [37, 38]. The minimum requirement for Fas signalling has been shown to consist of two adjacent Fas-Fas Ligand (FasL) complexes and confocal microscopy has shown that receptor ligation leads to the formation of oligomeric complexes [38, 39].
So, the arrangement of receptors and ligand at the cell surface is crucial for recruitment of signalling molecules to deliver an effective signal. Differences in the requirement for ligand presentation can distinguish between the role of soluble and membrane-bound forms of the same ligand and result with different receptor specificities [40, 41]. The role of HSPGs in APRIL signalling is reminiscent of FGF, where heparin interactions place FGF in the correct orientation for signalling [42]. It can be envisaged that APRIL is both concentrated, and positioned for signalling, on specific cells such as follicular B cells that express high levels of HSPGs.

Finally, it still remains that the HSPG interaction in different cells may have another role altogether, since several cell lines that do not express either TACI or BCMA appear to respond to APRIL in terms of survival and/or proliferation [24, 43]. Again, whether this occurs as a direct signalling event or whether there is another, unidentified receptor for APRIL is not known.

ACKNOWLEDGEMENTS

The work in this study is supported by TI-Pharma, Dutch Cancer Society grants nr 2003-2812 and 2007-375 and Stichting Vanderes. We would like to thank the animal caretakers at the AMC.
The HSPG binding domain of APRIL serves as a platform for ligand multimerisation and cross-linking.

REFERENCES