Year : 2012

CHARACTERISATION OF HETEROMERS AND OLIGOMERS IN THE TNF FAMILY OF LIGANDS AND RECEPTORS

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Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

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Lausanne 2012

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur	Prof. Christian **Widmann**
Directeur de thèse	Monsieur	Dr Pascal **Schneider**
Experts	Madame	Prof. Fabienne **Tacchini-Cottier**
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le Conseil de Faculté autorise l'impression de la thèse de

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intitulée

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Lausanne, le 6 décembre 2012

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Christian Widmann

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Acknowledgements

Summarising and documenting four years of physical and mental efforts in a manuscript of 100 pages demands the mention of all the contributors.

To begin with first and foremost, I thank my thesis director and supervisor Dr. Pascal Schneider for having me as a part of his group and giving me an opportunity to perform my PhD studies in his laboratory. I thank him profusely for all his supervision, guidance and support that helped me in many ways in my learning process, developing a certain amount of critical thinking and in the completion of my studies.

I thank all the people scientifically associated with my projects. Laure Willen from the group of Pascal Schneider for contributing to a great extent in various cloning and purification of recombinant proteins. Mai Perroud for her help in screening of heteromers. Luc Lebon, a summer student for contributing to the confirmatory immunoprecipitation experiments. Dr. Edith Hummler and Mrs. Anne-Marie Mérillat from TAF, UNIL for their kind help with screening of ES cells and Southern blot for the knock-in mice project. Olivier Donzé from Adipogen for his help with purification columns and endotoxin tests. Many thanks to people in the animal facility, namely Helena Maria for her advice and help with mice manipulations.

I thank the past and present members in the group of Pascal Schneider namely Christine Kowalczyk, Vanessa Pierroz, Nathalie Dunkel. Cristian Roberto Smulski. Thanks to Sonia Schupbach for her help with French translation of my thesis abstract.

It was a pleasure and honor to have shared the same space with (late) Prof. Jurg Tschopp in our lab meetings and all past group activities. I thank all the past and present members of his group for their scientific advice and the overall collaborative atmosphere in the group. Many thanks to Manuele Rebsamen and Aubry Tardivel from the Tschopp group for their overall support and willingness to help with various scientific matters in the laboratory ever since I joined as a PhD student.

A special thanks to Prof. Hans Acha Orbea for all his help and advice during my PhD studies.

The completion of my thesis deserves a thank you to all those who made my studies and stay in Lausanne memorable. This includes people from both inside and outside our Department of Biochemistry.

Many many thanks to Eric Yu not only for all the scientific discussions, helping me as much as possible for 'discussion' of this manuscript but also for his cordial and friendly self and the great times spent in and outside the department.

Thanks to all the members of the group of Margot Thome and Fabio Martinon for the wonderful atmosphere on fourth floor of CIIL. Thanks to special friends and colleagues from the group of Sanjiv Luther for all their help with reagents and methods and also the good times outside work.

Thanks to the department administrative staff namely Madame Jayet, Simine Aslan and Françoise Flejszman. Thanks to Philipe Margot for his help at the Magasin.

Thanks to all my friends and family members in Lausanne and abroad for their good wishes.

Last and most importantly I thank my parents profusely and immensely for their tremendous support, wishes and blessings.

Summary

TNF family ligands and receptors fulfill a number of functions, mainly in the immune system. For example, the ligands BAFF and APRIL control growth and survival of mature B cells at various stages of differentiation. TNF family ligands usually form homotrimers, but heteromers have also been described for lymphotoxin α1β2 and for BAFF and APRIL. Interestingly, twenty BAFF homotrimers can assemble into virus-like particles coined BAFF 60-mer, which are superior to BAFF 3-mer regarding their ability to signal in primary B cells.

A screen was performed in 293T cells, by co-transfecting differently tagged ligands, to identify six novel heteromers. The specificity of these novel heteromers, however, did not correspond to that of orphan receptors in the TNFR family.

Little is known about heteromers of BAFF and APRIL, in particular their receptor-binding specificity and their ability to signal. A method to produce and purify heteromers of defined stoechiometry was developed, and the resulting reagents were used to demonstrate that $BAFF_2APRIL$, like BAFF, binds to all BAFF receptors - namely BAFFR, TACI and BCMA -, while $APRIL_2BAFF$ and APRIL only binds to TACI and BCMA. Heteromers could signal via their cognate receptors, sometimes as potently and sometimes less potently than homomers, depending on the receptors. A promising system to measure the activity of single-chain homo- and heteromers *in vivo* was set up: it measures mature B cell rescue upon administration of single-chain ligands into BAFF-ko mice.

To tackle the question of the physiological importance of BAFF 60-mer, a point mutation that prevents assembly of mouse BAFF into 60-mer while retaining its ability to form trimers was identified. This mutation (E247K) was introduced by homologous recombination into mouse embryonic stem cells that are now being used to generate knock-in mice.

Results obtained in this work will help to better understand the role of various BAFF and APRIL forms that are elevated in a several autoimmune diseases.

Résumé

Les ligands et récepteurs de la famille du TNF joue un rôle prédominant dans le système immunitaire. Par exemple, les ligands BAFF et APRIL contrôlent la croissance et la survie des cellules B matures à différents stades de différenciation. Ces ligands existent souvent sous forme d'homotrimères (3-mer), bien que des héteromères aient été décrits pour la lymphotoxine α1β2 et pour BAFF et APRIL. Dans le cas de BAFF, vingt trimères peuvent, telle une particule virale, s'assembler en 60-mer qui surpasse le 3-mer pour signaler dans des cellules B primaires.

Un crible effectué dans des cellules 293T, par co-transfection de ligands différemment marqués, a permis d'identifier six nouveaux heteromères dont la spécificité n'a, hélas, pas correspondu à celle d'un récepteur orphelin de la famille du TNFR.

Les connaissances sur la spécificité de liaison aux récepteurs et la capacité à signaler des heteromères de BAFF et d'APRIL sont fragmentaires. Une méthode pour produire et purifier des heteromères "simple chaîne" de stoechiométrie déterminée a été mise au point, et les réactifs ainsi obtenus utilisés pour démontrer que $BAFF_2APRIL$, comme BAFF, lie tous les récepteurs de BAFF - c'est-à-dire BAFFR, TACI et BCMA -, alors qu'$APRIL_2BAFF$ et APRIL ne lient que TACI et BCMA. Les héteromères peuvent transmettre des signaux, parfois aussi bien et parfois plus faiblement que les homomères, selon les récepteurs. Un système prometteur pour mesurer l'activité des ligands simple chaîne *in vivo* a été mis au point. Il mesure la réapparition de cellules B matures dans des souris déficientes pour BAFF après administration des ligands.

Pour s'attaquer à la question de l'importance physiologique du 60-mer de BAFF, une mutation empêchant l'assemblage en 60-mer sans affecter la capacité à former des trimères a été identifiée. Cette mutation (E247K) a été introduite par recombinaison homologue dans des cellules souches embryonnaires de souris qui sont utilisées pour obtenir des souris déficientes en BAFF 60-mer.

Les résultats de ces travaux contribueront à mieux cerner le rôle des différentes formes de BAFF et d'APRIL produites en excès dans plusieurs maladies auto-immunes.

1. Introduction.

1.1.1. Immune system and immunology.

The immune system is a network of cells, tissues, and organs that work together to defend the body against attacks by "foreign" invaders. These are primarily microbes—tiny organisms such as bacteria, parasites and fungi, or viruses that can cause infections. The human body provides an ideal environment for many microbes. It is the task of the immune system to maintain the homeostasis of an uninfected body by preventing their entry or destroying them.

Study of the immune system and its components is the subject of immunology. The concept of immunity and thereby immunology came from the observation that individuals who survived and recovered from certain infectious diseases were thereafter protected from the disease. Observations dating as far back as a plague in Athens, to attempts of inducing immunity by the Chinese and Turks in the 12th century and even later English physician Edward Jenner's technique of inoculating fluid from cowpox pustule as a form of vaccination, have developed and increased the importance of studies in immunology [1].

The immune system has two broadly classified compartments – the innate immune system which is mostly referred to as non-specific immune responses and the adaptive or acquired immune system considered to form specific immune responses.

1.1.2. Innate immunity.

Innate immunity comprises of four main defensive barriers: anatomic, physiologic, endocytic and phagocytic, and inflammatory. Physiological barriers include temperature, pH, oxygen tension and

other soluble factors. For example, chickens display innate immunity to anthrax because of their high body temperature which inhibits its growth. The low pH in stomach does not allow survival of some organisms in the stomach. A hydrolytic enzyme like lysozyme can cleave peptidoglycans in mucous secretions [1].

Endocytic and phagocytic barriers function by engulfing macromolecules and internalizing them. Endocytosis can either be receptor-mediated or by pinocytosis carried out by non-specific membrane invagination. Specialized cells like blood monocytes, neutrophils and tissue macrophages participate in phagocytosis while endocytosis is carried out by virtually all cells.

Inflammatory response are characterised by increased blood flow, capillary permeability and influx of phagocytic cells. It is a response involving several complex interactions leading to a cascade of events mediated by chemical mediators.

The recognition of pathogens or danger signals are accomplished by cells of the innate immune system that express pattern recognition receptors (PRRs) These include the membrane bound Toll-like receptors (TLRs), the retinoic acid-inducible gene I-like receptors (RLRs), the nucleotide oligomerization domain-like receptors (NLRs, also called NACHT, LRR and PYD domain proteins) and cytosolic DNA sensors [2]. Secreted PRRs include complement receptors, collectins, pentaxrin proteins. The PRR can sense two types of signals. One is Pathogen Associated Molecular Patterns (PAMPs). PAMPs include bacterial lipopolysaccharide "endotoxin" (LPS→TLR4), bacterial flagellin, lipoteichoic acid, lipoproteins and peptidoglycan (→TLR1,-2,-6), mannose residues, N-formylmethionine, fungal glucans, endogenous heat shock proteins, extracellular matrix molecules, and nucleic acid variants associated with viruses (vRNA→TLR3, unmethylated cytosin-guanosin dinucleotide (CpG islands)→TLR9, dsRNA) and bacteria (bacterial DNA, unmethylated cytosin-guanosin dinucleotide (CpG)→TLR9). Effector cells of innate and adaptive immunity employ nonclassical pathways to secrete alarmins when they are activated by PAMPs or other alarmins. Endogenous alarmins and exogenous PAMPs therefore elicit similar responses, and can be considered subgroups of a larger set, the damage-associated molecular patterns (DAMPs)[3] .

1.1.3. Adaptive immunity.

Acquired or adaptive immunity involves specifically recognizing and selectively eliminating foreign microorganisms and molecules. Unlike innate immunity, acquired immunity displays specificity, diversity, memory, self and non-self recognition. But acquired immunity is not independent of innate immunity. Phagocytic cells are closely involved in activation of specific immune response. The cells of the adaptive immune system comprise of lymphocytes like B and T cells and antigen presenting cells like dendritic cells and also macrophages. Two broadly classified branches of acquired immune system involves humoral and cell mediated immunity. Humoral immunity originated from the concept of conferring immunity to a non-immune individual and this involved interaction of B cells with antigens and their subsequent proliferation into antibody secreting plasma cells. The resulting complex of an antibody bound to an antigen can be eliminated by fusing to receptors on phagocytic cells or can activate the complement system leading to the lysis of foreign organism [1].

T cells that proliferate and differentiate into effector T cells in response to antigen are responsible for cell-mediated immunity, which can be carried out by immune T cells. T helper cells as well as Cytotoxic T Lymphocytes serve as effector cells in cell mediated immune reactions [1].

The most well-known receptors on B and T cells among many are the BCR and TCR respectively. These molecules are instrumental in antigen recognition mediating further signaling events. The B cell receptor consists of two heavy and two light chains composed of a variable region responsible for antigen recognition and diversity and a constant region responsible for the effector function. The diversity is created by gene re-arrangement in the form of somatic hypermutation: the variable region is encoded by V, D and J segments. Both light and heavy chain contribute to antigen-binding site generating a repertoire of as high as 10^{13} for the BCR. The constant region undergoes class

switching to generate antibodies of differing isotypes, which accomplish varying functions [1].

1.1.4. Organs and Cells of the Immune System.

On the basis of function immune organs can be categorized as primary (central) and secondary (peripheral) lymphoid organs.

Also known as bursa of Fabricus in birds the primary site of B cell maturation is bone marrow. Most of the cells of the immune system originate in the bone marrow (Fig 1), where many of them also mature. They then migrate to guard the peripheral tissues, circulating in the blood and in a specialized system of vessels called the lymphatic system. The other primary lymphoid organ is thymus, a flat, bilobed organ situated above the heart. Each of the lobules are organized into two compartments: cortex which is packed with thymocytes and the medulla sparsely populated with thymocytes. Immature lymphocytes (T cells) generated during hematopoiesis in the bone marrow mature and become committed to a particular antigenic specificity in the thymus [1, 4].

The secondary lymphoid organs include lymph nodes and spleen**.** Lymph nodes are encapsulated bean shaped structures containing a reticular network packed with lymphocytes, macrophages and dendritic cells. They are found clustered at junctions of the lymphatics and serve as the first organized structure to encounter most antigens [4].

Spleen is a large, ovoid secondary lymphoid organ situated high in the left abdominal cavity. Spleen primarily functions to filter blood and trapping blood borne antigens and thus responds to systemic infections. The spleen also consists of two compartments namely the red and the white pulp. The red pulp consists of a network of sinusoids occupied by macrophages and numerous erythrocytes and is the site of destruction and removal of old and defective red blood cells. The white pulp surrounds the arteries, forming a periarteriolar lymphoid sheath (PALS) populated mainly by T lymphocytes. Clusters of B-lymphocytes in the PALS form primary follicles occupying a more peripheral position. Dendritic cells surrounding the B cells act as antigen

presenting cells to B cells upon antigenic challenge and the primary follicles thus develop features of secondary follicles like the germinal center [1, 4]. A summary of the major cells of the immune system has been described in Fig1.

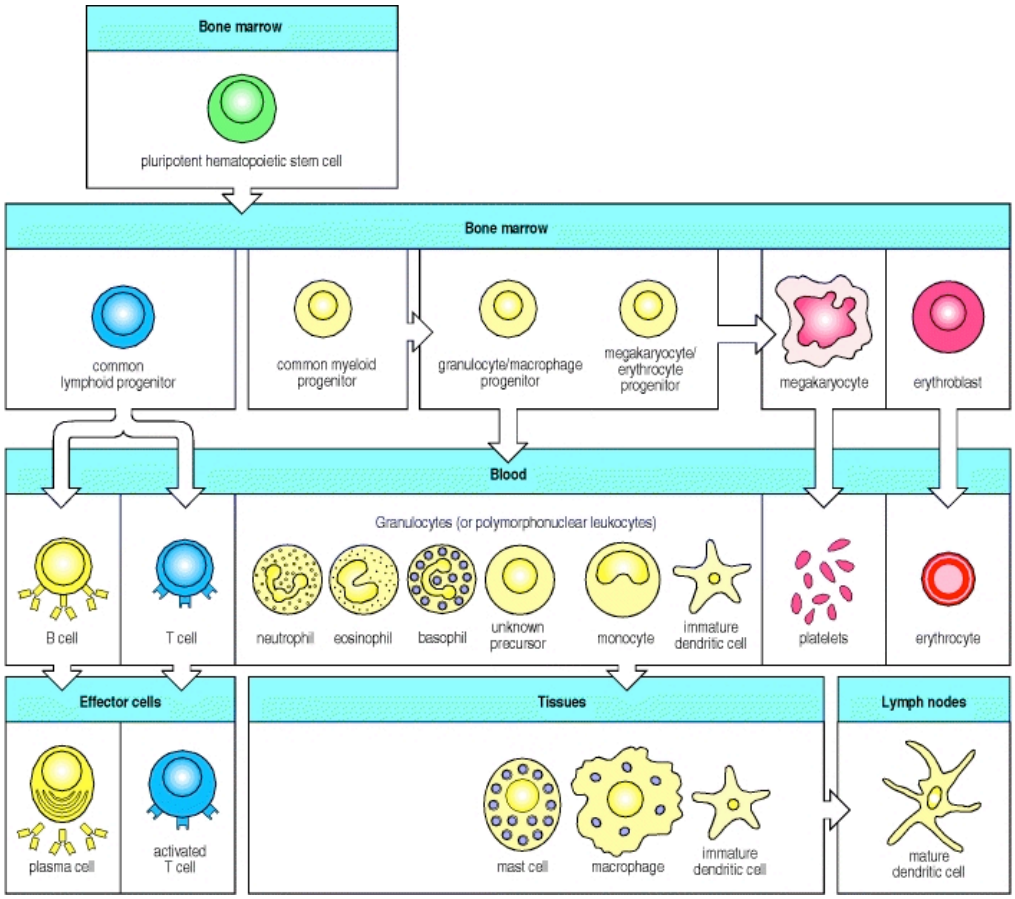


Fig 1. All the cellular elements of blood, including the lymphocytes of the adaptive immune system, arise from hematopoietic stem cells in the bone marrow. *Taken from Charles Janeway (Immunobiology Immune system of Health and disease 2001)*

1.2.1. TNF family of ligands and receptors.

A complex network of hormones, chemical mediators and cytokines maintains, regulates and connects a variety of cell types and enables a cross talk between various organs that form an organism. Cytokines are signaling molecules used extensively in intercellular communication.

Cytokines can be classified as proteins, peptides, or glycoproteins and include a large and diverse family of regulators produced throughout the body by cells of diverse embryological origin. Ligands and receptors of the tumour necrosis factor (TNF) superfamilies are examples of signal transducers with a role to play principally in the development, regulation and overall homeostasis of the immune system but also in other biological functions like inflammation, cell death or development of ectodermal appendages (Reviewed in [5-7]). TNF was first identified as a factor with antitumor activity [8] [9]. It was used in the treatment of soft tissue sarcomas and melanomas and was demonstrated to have potent antitumor activity with an acceptable safety profile [10-12]. The tumor necrosis factor (TNF) superfamily is composed of 19 ligands and 29 receptors.

1.2.2. Structural features of TNF ligands and receptors.

The ligands are Type II transmembrane proteins (i.e. intracellular N terminus and extracellular C terminus), with a conserved C-terminal domain termed the 'TNF homology domain' (THD). The THD is a 150 amino acid long sequence that assembles as trimers through a conserved network of aromatic and hydrophobic amino acid residues, has a sequence identity of ~20–30% between family members and is responsible for receptor binding. Atomic level structures are available among others for the THD of TNF [13],[14], LTα [15], CD40L [16] and TRAIL, RANKL [17], OX40L [18], EDA1, EDA2 [19-22]. The ligands are expressed as membrane-bound proteins, but soluble forms can be generated by proteolytic processing by various proteases. Metalloproteases of the ADAM (a disintegrin and metalloproteinase domain) family act on TNF and RANKL [23, 24], matrilysin acts on Fas ligand (FasL) [25], and members of the subtilisin-like furin family act on BAFF, EDA, TWEAK and APRIL-members of the TNF family [26, 27]. The event of processing is relevant for the physiological activity of some ligands like EDA [26, 28], whereas some ligands are functional only as membrane bound ligands like FasL cleavage [29]. For some ligands, like for TNF-related apoptosis-inducing ligand (TRAIL), the importance of processing remains unclear

[30]. The cleavage of BAFF seems to be important to generate soluble BAFF, which mainly is required for early development of mature naïve B cells by signaling via BAFFR [31]. THDs share a virtually identical tertiary fold and associate to form trimeric proteins. The THDs are β-sandwich structures containing two stacked β-pleated sheets, each formed by five anti-parallel β strands that adopt a classical 'jelly-roll' topology.

Trimeric THDs are ~60 Å in height and resemble bell-shaped, truncated pyramids with variable loops protruding out of a compact core of conserved anti-parallel β strands [19, 21, 22]. The trimer is assembled such that one edge of each subunit (strands E and F) (illustration in Fig 2) is packed against the inner sheet of its neighbour, forming large and mostly hydrophobic interfaces, resulting in a very stable interaction [13, 21, 22]. CD40L contains a single disulfide bridge linking some of the loops (CD and EF loop) [13, 32]. Similar disulfide links are predicted to occur in FasL, LIGHT, TL1A, CD30L and CD27L, whereas TWEAK, EDA, APRIL and BAFF have a predicted disulfide bridge between β strands E and F. In TRAIL, a single cysteine residue (Cys230) in the EF loop is involved in the coordination of a Zn(II) ion, with each monomer contributing to one coordination position; the fourth coordination position is occupied by an internal solvent molecule or a chloride counter-ion [24–26]. This metal-binding site is unique so far in the TNF family, and affects the stability and bioactivity of TRAIL [26,28,29]. Incomplete Zn coordination, and formation of partially oxidized, disulfide-linked species of TRAIL, have been suggested to account for its hepatotoxicity [33].

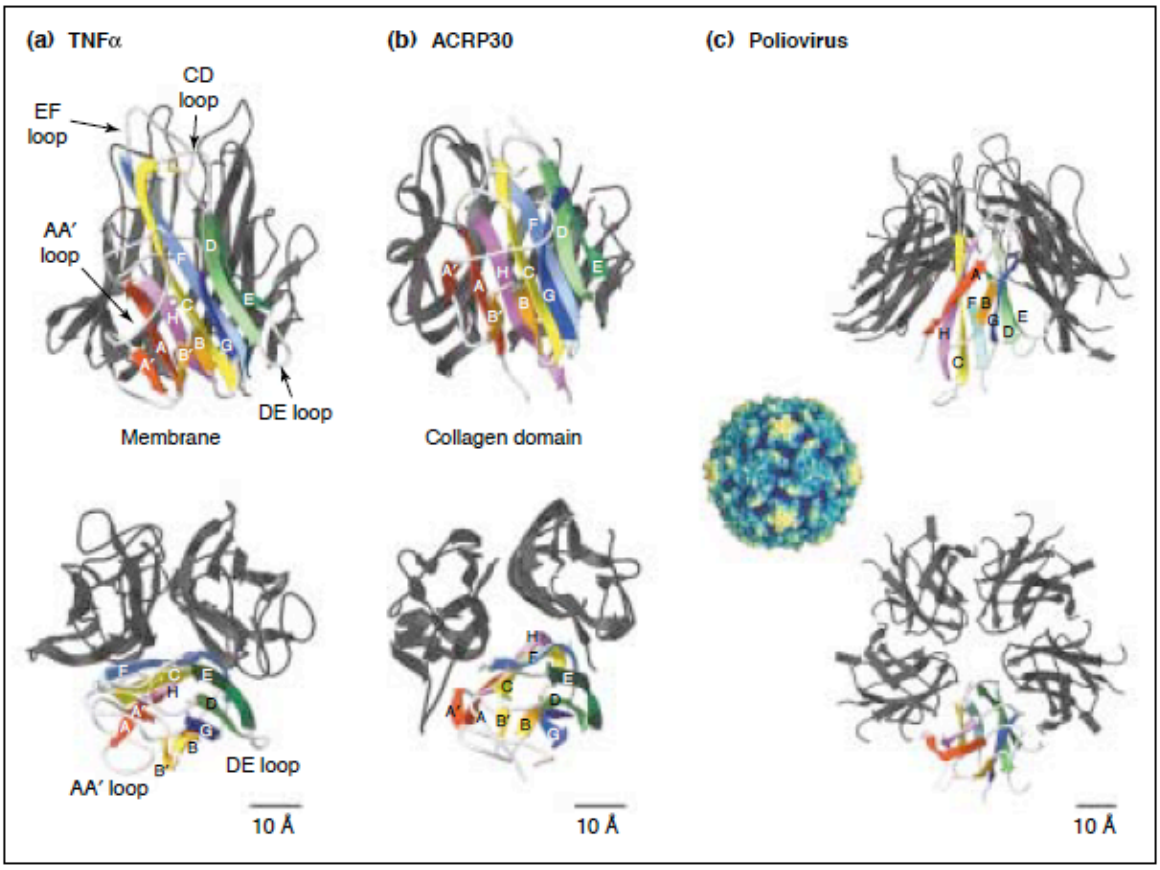


Fig 2. Structures of TNFSF ligands. *Taken from Bodmer et al. (TIBS 2002).*

The TNF receptors are mostly Type I transmembrane proteins with an extracellular N terminus consisting of cysteine-rich domains (CRDs), which are pseudo-repeats typically containing six cysteine residues engaged in the formation of three disulfide bonds. The number of CRDs in a given receptor varies from one to four, except in the case of CD30 where the three CRDs have been partially duplicated in the human but not in the mouse sequence. Based on distinct structural modules that allows sequence comparison between TNF receptors, a classification has been introduced by Naismith and Sprang [34]. Each module type is designated by a letter (A, B, C and N for crystallized modules, and X for modules of unknown structure), and by a numeral indicating the number of disulfide bridges it contains. (Illustration in figure 2). A typical CRD is usually composed of an A1–B2 or A2–B1 module or, less frequently by a different pair of modules. A1 modules are 12–27 amino acids long, consist of three short β strands linked by turns, and contain a

single disulfide bridge connecting strands 1 and 3, yielding a characteristic C-shaped structure. A2 modules contain a second disulfide bridge linking the second and third strands without affecting the overall structure. B modules are 21–24 amino acids long and comprise three anti-parallel strands adopting an S-shaped fold like a paper clip. In this case, the fold is constrained by two entangled disulfide bridges linking strands 1 and 3 in B2 modules. The first disulfide bridge is replaced by a hydrogen bond in B1 modules [35]. The structure of A and B modules is also reflected at the level of the primary sequence by the conservation of a few non-cysteine residues. Other modules are less frequent. So far, the N-terminal N1 modules have been found only in the TRAIL receptors, in which they precede the first A1–B2 CRD. Structurally, the N1 module resembles the second half of a B module [19, 20, 22]. The fourth CRD of TNF-R1 contains an A1–C2 module pair, in which the cysteine connectivity of C2 is different from that of a B2.

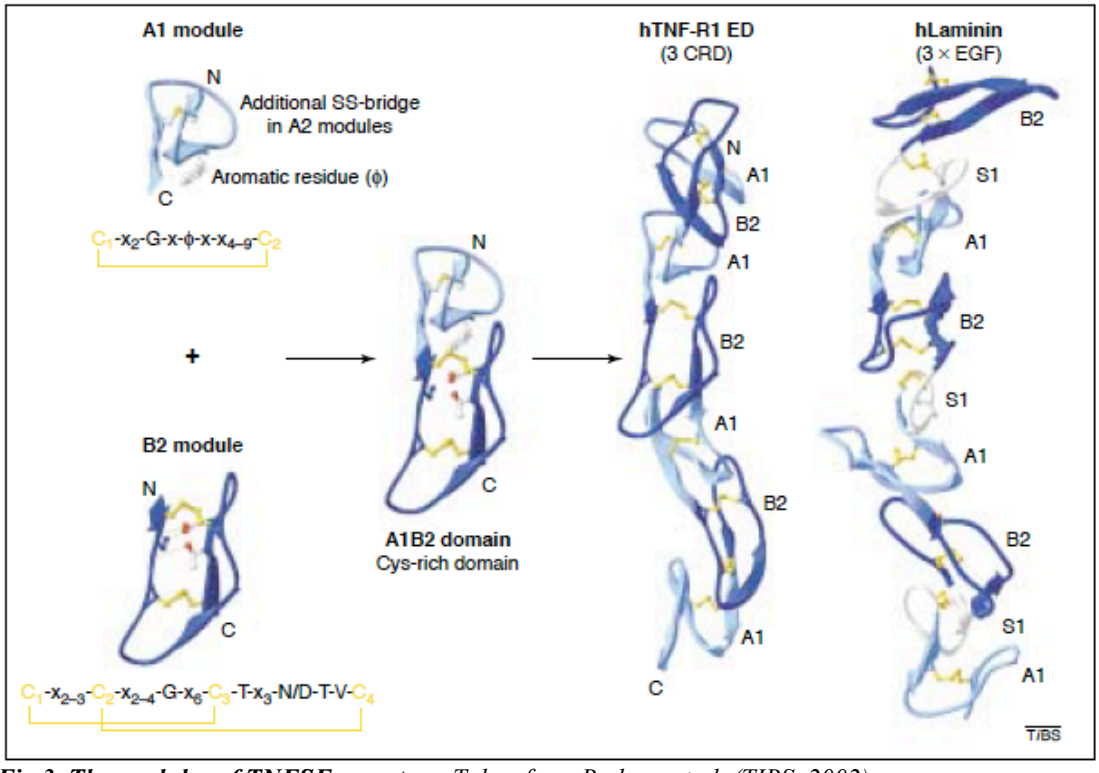


Fig 3. The modules of TNFSF receptors. *Taken from Bodmer et al. (TIBS, 2002).*

TACI, BCMA and Fn14 also contain putative A1–C2 CRDs. Four unrelated modules of unknown structure that are found in DR3, GITR, BAFFR and viral CrmC have been collectively designated

as ‘module X’. The later described BAFF receptor (BAFFR) [36] contains a single X2 module whose sequence resembles an A module entangled with the beginning of a B module. TNF receptors are often viewed as monomers, principally because they appear in this form in crystal structures of ligand–receptor complexes. However, TNF-R1 has also been crystallized as both head-to-head and head-to-tail dimers [35], and there is genetic and experimental evidence that Fas, TNF-R1 and CD40 exist as preformed oligomers within the plasma membrane [37]. Self-association of the receptors depends on an N-terminal pre-ligand association domain (PLAD) that includes the first CRD and that is not directly involved in ligand binding.

1.2.3. Ligand Receptor Interaction.

The first crystal structure of a TNF ligand (LT**α**) bound to its cognate receptor (TNF-R1) [15] followed by the structure of TRAIL bound to TRAILR2 [19, 20, 32] and then further ligand receptor complexes indicated that the basic signaling unit is a trimeric ligand bound to three monomeric receptors. The receptor ligand interactions could be of two major types: large elongated receptors contact ligands at the monomer-monomer interface, whereas small, compact receptors make most contacts with a single monomer of the trimeric ligand. There are promisquities in the ligand-receptor interactions, with some of the ligands and receptors having more than one binding partners (e.g. both TNF and LTα bind to TNFR1; BAFF binds to BCMA, TACI and BAFFR; APRIL share TACI and BCMA with BAFF) (Reviewed in [5],[6]). On the other hand, EDA1 and EDA2 that differ by only two amino acids bind respectively to EDAR and XEDAR with a strict specificity [38]. The receptor HVEM (Herpes Virus Entry mediator) interacts with LIGHT and LTα. As an illustration of the existence of non TNF ligands, the viral glycoprotein D hijacks HVEM to allow cellular entry of Herpes Simplex Virus [39]. Regarding signaling, receptors are perhaps pre-assembled at the cell surface in the absence of ligand. For Fas, this preferentially requires the PLAD (pre-ligand assembly) domain at the N-terminus of the receptor [37]. The PLAD

is believed to mediate self-association and to increase its responsiveness to ligand-mediated signals. Ligand receptor interactions seem to be conserved across species. Most of the human and mouse TNFSF ligands cross-react with the TNFSF receptors [40].

1.2.4. Signaling pathways and physiological importance of TNF proteins.

Most TNF ligands can be expressed by cells of the immune system. Exceptions are TL1A, which is expressed by endothelial cells, and EDA1, which is expressed, in embryonic skin and in some ectodermal appendages. In contrast, TNF receptors are expressed by a wide variety of cells within and outside of the immune system (Reviewed in [6]). The type of signal transmitted by a ligand depends largely on the intracellular moieties of the TNF receptors and their ability to recruit various adapter and effector proteins. Some TNF family ligands can mediate cell death (FasL, TRAIL) by engaging caspase-activating receptors containing an intracellular death domain. Other ligands can lead to cell survival or differentiation (CD40L, BAFF, CD27L, 4-1BBL, OX40L, RANKL) or promote inflammatory reactions (TNF, RANKL) by activating signaling pathways such as those leading to activation of the transcription factor NF-**κ**B or to the activation of mitogen-activated protein kinases (MAPKs) [6]. Of note, RANKL is essential for the formation of bone-resorbing osteoclasts [41] and EDA1 for the correct formation of ectodermal appendages such as hair, teeth and sweat glands [26, 28].

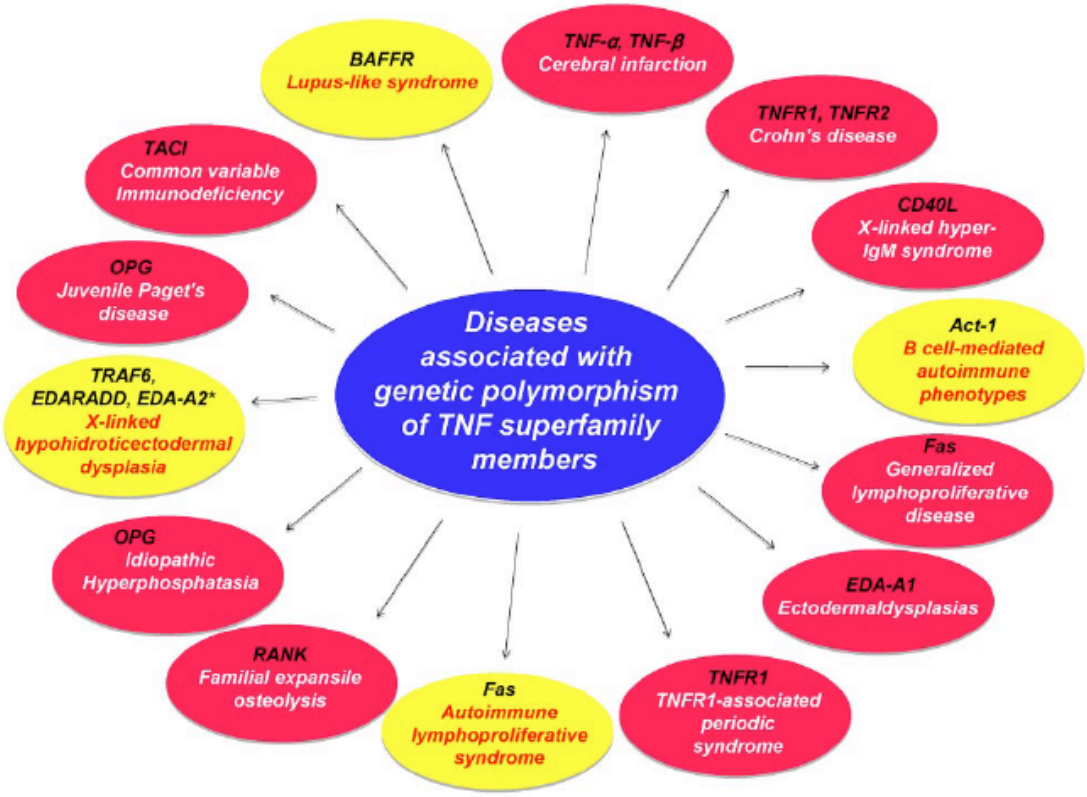


Fig 4. Diseases caused by TNF family members. *Taken from review by Aggarwal et al. (Blood 2012) (*EDA-A1 is involved in X-linked hypohidroticectodermal dysplasia and not EDA-A2)*

Fig 5. Table of TNFSF gene knockout phenotype of mice. *Taken from Review* by *Aggarwal et al. (Blood 2012)*

Table 2. Effect of gene knockout on the phenotype of TNF superfamily, receptors, and receptor-associated proteins

Gene	Phenotype
Cytokine	
TNF-β (LT-α)	Defects in secondary lymphoid organ development; disorganized splenic microarchitecture[61]
TNF-α	No phenotypic abnormalities in LN; lack splenic primary B-cell follicles; disorganized FDC networks and germinal centers[62]
LT-β	Defects in organogenesis of the lymphoid system; lymphocytosis in the circulation and peritoneal cavity; lymphocytic infiltrations in lungs and liver[81]
OX40L	Defective T-cell responses[82]
CD40L	Defective T-cell and IgG responses; hyper-IgM syndrome[82]
FasL	Impaired activation-induced T-cell death; lymphoproliferation; autoimmunity[82]
4–1BBL	Defective T-cell responses[82]
TRAIL	Delayed regression of retinal neovascularization[64]
RANKL	Osteopetrosis; growth retardation of limbs, skull, and vertebrae; chondrodysplasia[83]
APRIL	Normal immune system development [84]
	Impaired IgA class switching[85]
BAFF	Impaired B-cell maturation[86]
	Low Ig serum levels; block in B-cell development at the T1 stage; absence of T2, mantle, and follicular zone B cells in the LN and spleen[87]
EDA-A1	Ectodermal dysplasias[88]*
EDA-A2	Impaired development of hair, eccrine sweat glands, and teeth[89]
	Multifocal myodegeneration[90]
Receptor	
TNFR1	Resistant to low levels of LPS; increased susceptibility to *Listeria* monocytogenes infection[91]
	Impaired oval cell proliferation; reduction of tumorigenesis[92]
TNFR2	Increased sensitivity to bacterial pathogens; decreased sensitivity to LPS; reduced antigen-induced T-cell apoptosis[82]
LT-βR	Absence of LN, PP; defective GC formation[82]
OX40	Defective T-cell responses[82]
CD40	Defective Ig class switching and GC formation causing immunodeficiency[82]
Fas	Impaired activation-induced T-cell death; lymphoproliferative syndrome; autoimmunity[93]
	Accumulation of autoreactive B cells in T cell-rich zones; production of autoantibodies[94]
	Resistant to suppression by high doses of antigen and to apoptosis in mature $CD4^+$ T cells[95]
CD27	Defective T-cell responses[82]
CD30	Impaired follicular GC responses; reduced recall-memory Ab responses[96]
4–1BB	Enhanced T-cell response but normal T-cell development[97]
	Reduced number of NK and NKT cells; resistance to LPS-induced shock syndrome[98]
	Increased number of myeloid progenitor and mature DCs; impaired DC function[99]
	Reduced atherosclerosis in hyperlipidemic mice[100]
DR5	Normal development with an enlarged thymus[66]
RANK	Osteopetrosis; absence of osteoclasts and LN; PP present; abnormal B-cell development[82]
OPG	Osteoporosis; arterial calcification[82]
FN14	Reduced proliferative capacity; altered myotube formation[101]
	Reduced neurogenesis in the subventricular zone[102]
	Reduced LPC numbers; attenuated inflammation; cytokine production[103]
TACI	Increased B-cell accumulation; splenomegaly[104]
BAFFR	Reduced late transitional and follicular B-cell numbers; devoid of marginal zone B cells; reduced CD21 and CD23 surface expression[105]
DR3	Impaired negative selection and anti–CD3-induced apoptosis[106]
GITR	Abolished anti–CD3-induced T-cell activation[107]
EDAR	Abnormal tooth, hair, and sweat gland formation[82]
XEDAR	No different than wild-type littermates[90]
TROY	No apparent defects in skin appendages[108]
DR6	Enhanced $CD4^+$ T-cell expansion and Th2 differentiation; enhanced splenic GC formation[109]
	Impaired JNK activity; T-cell differentiation[110]
	$CD4^+$ T-cell proliferation; Th differentiation[111]
NGFR	Decreased sensory neuron innervation; impaired heat sensitivity[112]
Receptor-associated proteins	
TRAF1	Normal lymphocyte development[113]
	Attenuation of atherosclerosis[114]
TRAF2	Died prematurely; elevated sTNF levels; hypersensitivity to TNF-induced cell death[115]
TRAF3	Postnatal lethality; defect in T-dependent immune responses[116]
TRAF5	Defect in proliferation; up-regulation of surface molecules CD23, CD54, CD80, CD86, and Fas after CD40 stimulation[117]

Fas indicates fibroblast-associated; FN14, fibroblast growth factor-inducible immediate-early response gene 14; GITR, glucocorticoid-induced TND receptor; EDAR, EDA receptor; XEDAR, X-linked ectodysplasin receptor; TROY, TNFRSF expressed on the mouse embryo; NGFR, nerve growth factor receptor; FAN, factor associated with neutral SMase activation; FLICE, FADD-like IL-1β-converting enzyme; Apaf-1, *apoptotic* protease activating factor-1; EDARADD, ectodysplasin-A receptor-associated adapter protein; LN, lymph node; FDC, follicular dendritic cell; LPS, lipopolysaccharide; PP, Peyer patches; GC, germinal center; and sTNF, serum TNF.

*Study performed in human, others in mouse model.

1.3.1. Structural forms of BAFF and APRIL, two homologs in the TNF superfamily.

Most members of the BAFF family were discovered through genomic homology searches [27, 42, 43]. Since some family members were discovered simultaneously by several laboratories, they possess multiple names (Reviewed in [44, 45]). The gene encoding BAFF is located on human chromosome 13q34 and mouse chromosome 8. BAFF is a Type II, 285 amino acid-long membrane-bound protein, which can be released as a soluble trimeric ligand upon proteolytic processing at a furin consensus site. In humans, exon 1 of BAFF gene codes for the transmembrane domain and its flanking regions, exon 2 for the furin processing site, and exons 3–6 for the TNF homology domain (THD). The receptors bind to the THD. At neutral or basic pH, 20 trimers of soluble recombinant human BAFF associate into a 60-mer virus-like structure, which irreversibly dissociates into trimers at acidic pH, or when fused to N-terminal extensions such as a myc tag [46, 47]. An extended loop, known as the "Flap", that is unique to BAFF in the TNF family is responsible for the association of the BAFF trimers [46]. BAFF 60-mer is a biologically active entity that can bind receptors and is moderately more active than trimers as found in *in vitro* assays [46, 48]. In recent past it was found that TACI among the BAFF and APRIL receptors gets solely activated by oligomerised BAFF/APRIL or recombinant 60-mer [49], however the physiological importance of this finding remains unknown. Endogenously produced BAFF 60-mer has been detected in supernatants of an histiocytic cell line [48] and in the sera of BAFF transgenic mice [49]. The gene for mouse BAFF contains an additional exon encoding a stretch of 30 amino acids located between the furin site and the THD. This extension may possibly interfere with 60-mer formation in the mouse, but may be not. A dominant negative form of BAFF (delta BAFF) has been described resulting from a splice variant in which exon 4 is skipped [50, 51]. This results in a molecule with a cryptic N-glycosylation site that arrests any trimer containing the mutant BAFF peptide (s) to be secreted [52]. Mice transgenic for delta BAFF exhibit reductions in peripheral B cell numbers and

impaired humoral responses [50] indicating towards a possible inhibitory role. BAFF is highly conserved across species. More than 75% homology is shared by chicken and duck than with murine and human BAFF [53-55]. The cross reactivity is also evident by the fact that recombinant human or murine BAFF are both effective on both chicken and mouse B cells [53-55]. BAFF polymorphisms have been found in mice but they do not show any phenotypic significance. BAFF has several features unique to itself in the TNF superfamily (Reviewed in [44]). First, the axis involved with trimerization is shortened resulting in a somewhat flatter molecule than other TNF ligands. In addition, BAFF can interact with magnesium, and has an exceptionally deep and acidic cleft that in part explains its unique affinity for the comparatively basic BAFFR receptor, as well as its lower affinity for BCMA when compared to APRIL [56, 57].

(Reviewed in [44, 45, 58]) The gene encoding APRIL is found on chromosome 17p13.3 in humans and chromosome 11 in the mouse [59]. The nomenclature of APRIL A Proliferation Inducing Ligand comes from its ability to induce proliferation in transfected fibroblasts. [60, 61]. The 250 amino acid APRIL molecule shares only 20–30% homology with most other TNF family members; but displays nearly 50% homology with BAFF in the TNF-homology domain [49, 62, 63]. APRIL interacts with BCMA and TACI, but not with BAFFR. The structure of the APRIL gene is similar to that of BAFF though APRIL does not form 60-mers, but possesses residues close to the furin processing site in exon 3 that are crucial for binding to glycosaminoglycans. APRIL binds sulfated glycosaminoglycans at sites independent from those used to bind other receptors [64, 65]. Upon splicing between exon 1 and 3 a membrane bound uncleavable APRIL can be generated. The physiological significance of the binding to glycosaminoglycans is unclear but is believed to oligomerise APRIL in the extracellular matrix or at the surface of syndecan-positive cells and increases its accessibility to receptor TACI which also interacts with syndecans [66], or to intracellular BCMA [67] upon syndecan internalization.

Another splice variant of APRIL lacking exon 3, known as APRIL β, is considered to be a

homologue of delta BAFF that also lacks the first sheet of the THD [59], and this may also regulate APRIL activity in a dominant-negative manner [50]. Another splicing event of intron in exon 6 results in APRIL γ having a truncation of four amino acids at the C-terminal and is replaced by a single residue, but this isoform has not been further studied [59]. ESTs of murine APRIL have not shown similar sequences. Unlike human APRIL mouse APRIL exist as two variants differing by a single amino acid (Ala120) resulting from usage of two splice acceptor sites three nucleotide apart at the beginning of exon 4. Both isoforms can bind TACI and BCMA, but the shorter form in can also weakly interact with mouse BAFFR [40], but this may not be of physiological relevance.

(Reviewed in [44, 45, 58]) APRIL gene has some interesting features. The TNF homology domain encoding region β sheets B to H is separated in two exons, whereas most TNF family members APRIL, BAFF, EDA and TWEAK use a single exon to encode this domain. Finally, the gene encoding APRIL lies 3' of a TNF family member named TNF Weak Inducer of Apoptosis (TWEAK) [68, 69]. This particular organization of TWEAK and APRIL genes is conserved from mice to humans, indicating a possible functional relationship. Interestingly, messenger RNA for a fusion product of the TWEAK and APRIL genes resulting from the splicing between exon 6 of TWEAK and exon 2 of APRIL in humans and between exon 7 of TWEAK and exon 1 of APRIL in mice has been reported (termed TWE-PRIL) in T cells and various cell lines. The resulting TWEAK-APRIL fusion proteins in both species contain the complete THD of APRIL, indicating that TWE-PRIL could bind BCMA and TACI, but the processing event to yield an active soluble form would be similar to pathways characteristic of TWEAK [69]. Interestingly, the TWEAK receptor, Fn14, bears striking resemblance to BCMA both in terms of structure and TRAF binding capacity.

1.3.2. Cells and tissues expressing BAFF and APRIL.

(Reviewed in [70-72]) Distinct cell lineages produce BAFF. It is constitutively produced by radiation resistant cells possibly stromal cells [73, 74] and also produced upon induction by cells of myeloid origin such as monocytes, macrophages, dendritic cells. Oher cell types like activated T cells, malignant B cells and CD34+ cells from cord blood [75-83], have shown BAFF expression. In patients with multiple myeloma bone marrow derived cells Bone marrow derived cells of patient with multiple myeloma show BAFF expression. Cytotrophoblast cells in the placenta [84] or even astrocytes also express BAFF [85]. To date, mechanisms regulating BAFF expression in these cells are not fully understood. Cytokines like IL10, IFN-γ increase BAFF expression in various cell types such as monocytes, macrophages and dendritic cells. Resting monocytes constitutively express a low level of membrane-bound BAFF but expression is up-regulated by IFN-γ and IL-10 [86]. IL-4 also inhibits the up regulation of BAFF expression in monocytes stimulated with IL-10, but not with IFN-γ [87]. In myeloid cells, the binding of immune complexes increased BAFF processing in a manner that was dependent on the expression of high-affinity Fc receptor for IgG (FcγRI) [88]. BAFF expression can be induced by bacterial components like LPS and peptidoglycan that can also modulate BAFF secretion by macrophages, dendritic cells and, upon LPS stimulation only, monocytes [75, 86, 87]. Similar to BAFF, APRIL is expressed by monocytes, macrophages, dendritic cells , T cells, tumor infiltrating neutrophils [68, 75, 80, 83, 86]. APRIL is also produced by non-immune cells like epithelial cells and osteoclasts [60, 89]. APRIL expression in macrophages and dendritic cells is up-regulated by IFN-γ and IFN-γ treatment [75, 86]. LPS also induces APRIL expression in both macrophages and dendritic cells [75, 86]. In addition, APRIL expression is also induced upon CD40 ligation on dendritic cells. CD40 ligation also stimulates BAFF secretion from dendritic cells [86]. Similar to IL-4, activation with PMA/ionomycin is a negative regulator of BAFF expression [80]. In addition, acetylcholine receptor-pulsed DCs express reduced levels of BAFF [90]. Absence of suppressor of cytokine signaling-1 (SOCS-1) results in

the aberrant expression of BAFF in dendritic cells [91]. Neutrophils have been shown to synthesize and secrete the highest levels of BAFF, particularly following GCSF and IFN-stimulation [79]. However, BAFF is released from neutrophils after intracellular processing, rather than processing from the cell membrane like in myeloid cells [79]. In addition another source of endogenous BAFF production could be neoplastic B cells for example in B cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM) cells [76, 78, 84, 92, 93]. Epstein-Barr virus (EBV)-infected B cells have also been shown to express BAFF [94]. Whether normal B cells express BAFF is uncertain. Most reports agree that normal B cells do not express BAFF [84, 94], however, Kern et al. reported otherwise [92].

1.3.3. BAFF, APRIL and the receptors in B cell survival.

BAFF and APRIL play key roles in regulating the behavior of most mature B cell subsets. The signaling of BAFF and APRIL at different stages of B cell development is much dependent on the expression pattern of its receptors. BAFFR and then TACI are the first expressed among immature B cells in the bone marrow, followed by an increase in their expression through the transitional stages. In the pre-immune follicular and marginal zone pools, BAFFR and TACI reach relatively high and constant levels [95]. BAFF plays a major role in the differentiation of MZ B cells expressing uniformly high levels of BAFFR and TACI [96, 97]. Oligomeric BAFF is particularly effective at inducing marginal zone B cells and expression of CD23 surface markers [31].

BAFFR provides the most crucial signal for the survival of all pre-immune B cell subsets from the transitional stage onwards as was observed by the profound B cell deficiency in the A/WySnJ mouse [58, 98-103], a characteristic that came across as a single autosomal trait arising from a 400 bp insertion in the intracellular tail of BAFFR [96]. Ectopic BAFF expression or exogenous BAFF administration yields B cell hyperplasia and humoral autoimmune manifestations [42, 58, 104, 105]. Both BAFF and BAFFR knockout mice display primary B cell deficiencies resembling the

pattern of the A/WySnJ [101, 106]. The transitional stage during B cell development determines the fate of a B cell, whether or not it will be selected positively or negatively. The availability of BAFF, along with the BCR signals within the competing cohort of emerging cells, decides the thresholds for negative and positive selection of cells. This is demonstrated in several studies using transgenic systems [74, 107, 108], showing that when BAFF is non-limiting, self-reactive clonotypes, instead of dying at the transitional stage, survive and mature. BAFF over expression does not rescue cells deleted at the immature stages, suggesting BCR signals, and not BAFFR, are indespensible at these stages. This may reflect the onset of BAFF receptor expression per se, but a more and more emerging studies indicate that differentiation-dependent changes in intra-cellular signaling systems that involve cross-talk between the BCR and BAFFR may have some effect [128–132].

(Reviewed in [58]) Antigen-experienced cells which are less well characterized than the pre immune B cells can also be influenced by BAFF during its development and subsequent maintenance. Competing naïve cells are more dependent on B cell survival factors which are limiting among them than antigen-experienced cells. The axis of BAFF-BAFFR mediated signaling plays a less critical role here. Instead the axis shifts towards APRIL-TACI or APRIL-BCMA, the later two receptors found on both memory and plasma cells, the cell types, especially memory cells must persist to confer protective immunity on a longer term. Hence different downstream signaling systems of TACI and BCMA dominate. B cell responses are generally categorized as either as T-dependent (TD) or T independent (TI), depending on the presence or absence of cognate T cell help in initiating the response. T-independent antigens can be of two types. TACI has been shown to be upregulated dramatically on B cells following encounter with either TI-1 or TI-2 antigens [109] and this has been correlated with increase in number of antibody forming cells [110], but its precise significance is unclear. Some studies suggest that it could play a role in controlling cell cycle regulation [110]. A role of BAFF in regulation of cell cycle entry has been indicated, though the receptor mediating this effect was not mentioned [111].

(Reviewed in [58]) BAFF and APRIL can play a role in the establishment and evolution of germinal center reactions. The mechanism of action remain obscure. BAFFR up regulation could be by BCR engagement as well as concomitant CD40 ligation [112]. TD stimulation can result in TACI elevation as well, although but to a lesser extent than seen with TI antigens. The general view is that BAFFR is not required for the generation of GCs [113, 114]. Thus, even though not severe but, GCs generated in the absence of appropriate BAFF or BAFFR signaling are small, comparatively short-lived, and fewer in number. Follicular dendritic cells (FDC) networks do not mature in germinal center when BAFF and APRIL are blocked [115]. This may reflect a loss of FDC supporting signals secondary to the decreased number of B cells in the GC. High levels of TACI are found in rapidly proliferating short-lived plasma cells generated during either TI or TD responses, while BCMA is up regulated in long-lived plasma cells. Recent studies support a role for BCMA in maintaining long-lived plasma cell (LLPC) survival [116]. BCMA binds to both BAFF and APRIL, and is sensitive to the simultaneous absence of both the cytokines [117]. Osetoeclasts serves as a localized source of high levels of APRIL in bone marrow niches and is consumed by the long lived plasma cell for survival [118]. Resident bone marrow accessory cells express high amount of proteoglycans [64], where long lived plasma cells co-exist indicating a network of APRIL, proteoglycans and BCMA expressing LLPC. The downstream effects of BCMA signaling in long-lived plasma cells are not yet extensively characterized, owing to the technical limitations of maintaining these rare populations of B cells *in vitro*. However, B cell and plasma cell lines reveal classical NF-**κ**B activation, the upregulation of a number of proteins associated with antigen presentation and co-stimulation, and increased IL-2 production following BCMA ligation [119]. Memory B cells show an increased level of TACI, but inspite of this, memory cells seem to be independent for their needs of BAFF or APRIL [117].

1.3.4. Signaling pathways downstream of BAFF, APRIL and their receptors.

(Reviewed in [71],[72],[58]) BAFF receptor (BAFFR/BR3) is the first of the BAFF/APRIL receptors to be expressed on primary B cells. It engages various signaling cascades utilizing different downstream adapter molecules. The other important signal on B cells at this early cell stage comes from the BCR which employs mainly Src and tyrosine kinases activated upon receptor oligomerisation to initiate primary and secondary message systems. This involves the formation of a primary signaling complex leading to Ca^2+ sensitive responses, recruitment of the MALT/Bcl-10/CARMA complex, and classical NF-**κ** B activation [120]. In contrast, stimulation of BAFFR leads to the degradation of TRAF3 in a TRAF2-dependent manner. As TRAF3 is normally involved in the constitutive degradation of NF-**κ**B inducing kinase (NIK), BAFFR-mediated TRAF3 elimination rescues NIK expression. NIK potently activates the alternative non-classical NF-κB2 pathway that degrades p100 into p52. This is of likely relevance *in vivo* as the B cell compartments of B cell-specific NIK-deficient (*aly/aly*), IKKα -null and NF-**κ**B2 null mice are reduced, and as B splenocytes from these mice are unresponsive to BAFF and present impaired survival *ex vivo* [121-123]. Mice with basal IKKα activity but impaired responses to further activation of this kinase (IKKα-AA) display an intermediate phenotype, with B cells partially unresponsive to BAFF [105, 121, 124]. Conditional deletion of TRAF3 results in unrestricted p100 processing and enhancement of BAFF mediated survival [125, 126]. Conversely, mice with constitutive expression of p52 have more B cells [127]. At the most fundamental level, it appears that each receptor exerts differential effects through the independent engagement of different NF-**κ**B systems. However, downstream integration seems to be a likely outcome given that p100, the major substrate for BAFFR signaling, is a target of the classical pathway [128].

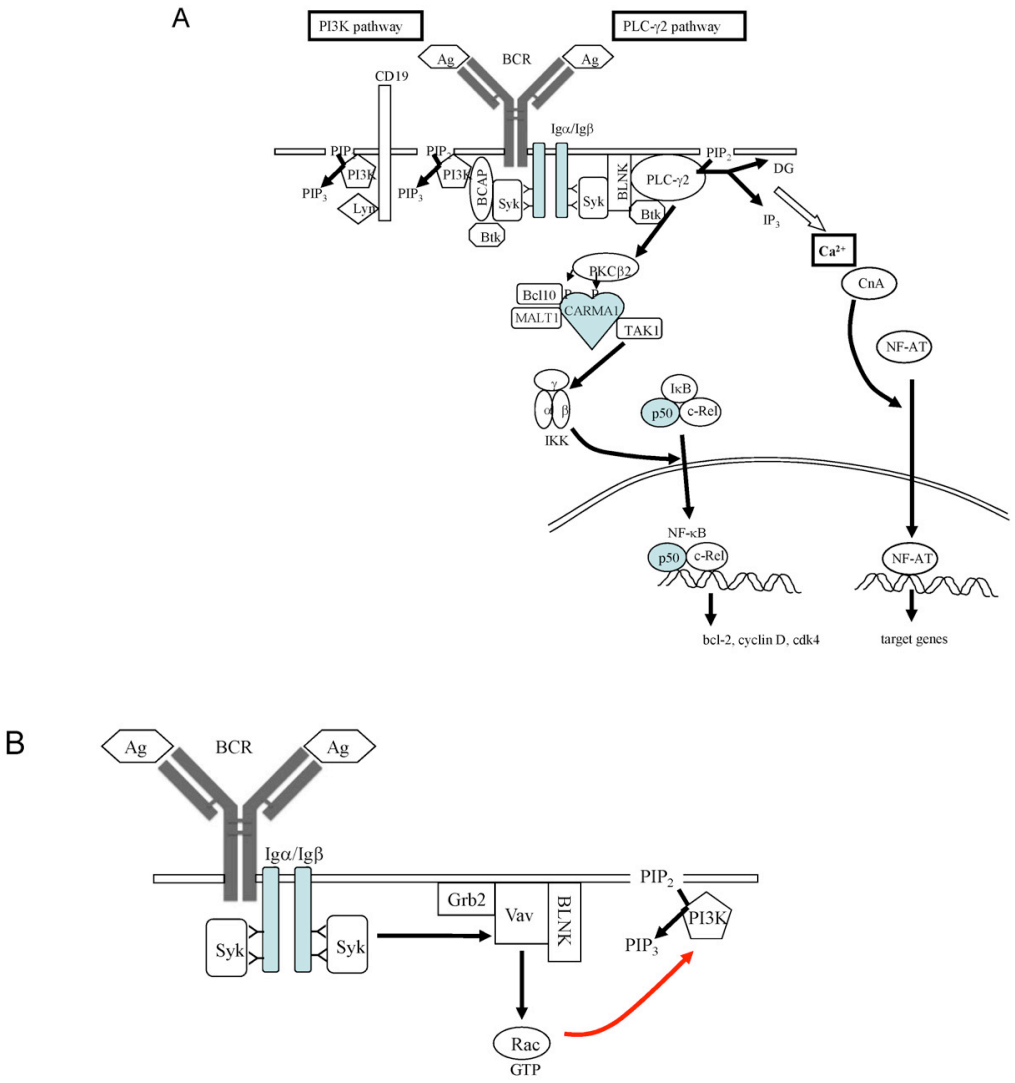


Fig 6. Signaling downstream of BCR. *Taken from review* by *Kurosaki et al. (Molecular Immunology 2011).*

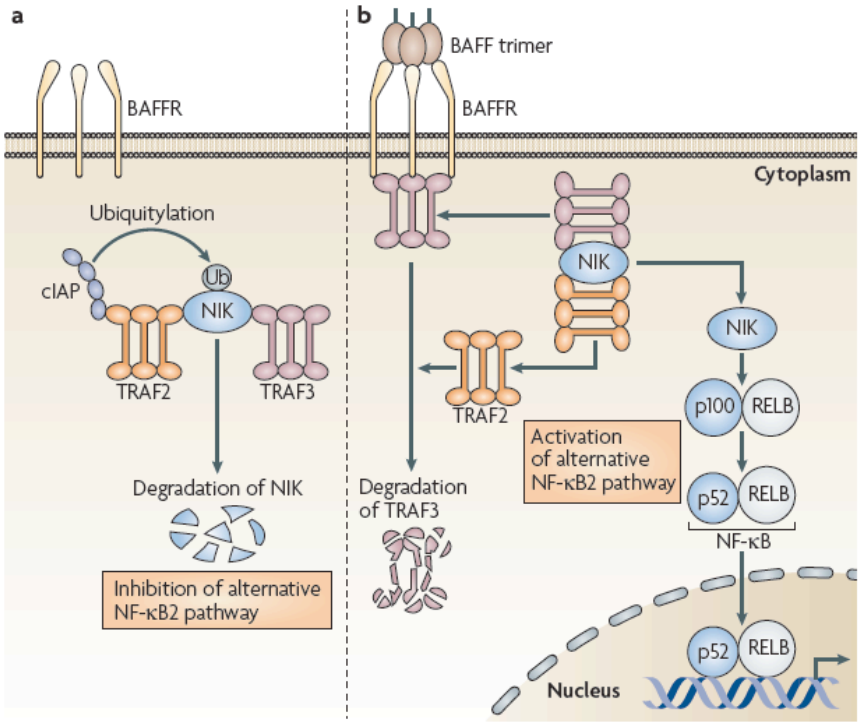


Fig 7. NF-κB signaling downstream of BAFFR. *Taken from review by Mackay et al. (Nature reviews 2009)*

Taken together, these data indicate that the non-canonical NF-κB pathway is a key downstream element of BAFF signals. However, the canonical NF-κB pathway can also mimic BAFF signaling, as inactivation of NEMO in B cells, and to a lesser extent NF-κB1, also leads to B cell deficiency, whereas expression of constitutively active IKKβ enlarges the peripheral B cell compartment and promotes survival of B cells ex vivo, which do not display further survival increase in response to BAFF. The sole presence of a functional BCR is not sufficient to allow mature B cells to survive, as observed in BAFFR-deficient B cells. Likewise, the expression of BAFFR alone does not allow for the survival of mature B cells in which the BCR has been conditionally deleted [129].

There are signaling pathways other than NF κB downstream of BAFF. BAFF-mediated survival has been linked to upregulation of anti-apoptotic proteins like members of the Bcl-2 family, increased expression of anti-apoptotic proteins, to integrin-mediated localization of B cells in the marginal zone and to T cell-independent antibody class switching (Reviewed in [130]).

Other than B cell survival, BAFF could play a role in glycolysis, protein synthesis and cell growth, in part through the activation of the protein kinase mammalian target of rapamycin (mToR; also known as FRAP1) in the context of mToR complex 1 as shown in Fig 8. The resulting cascade of signaling events lead to cell growth or cell survival (Reviewed in [130]).

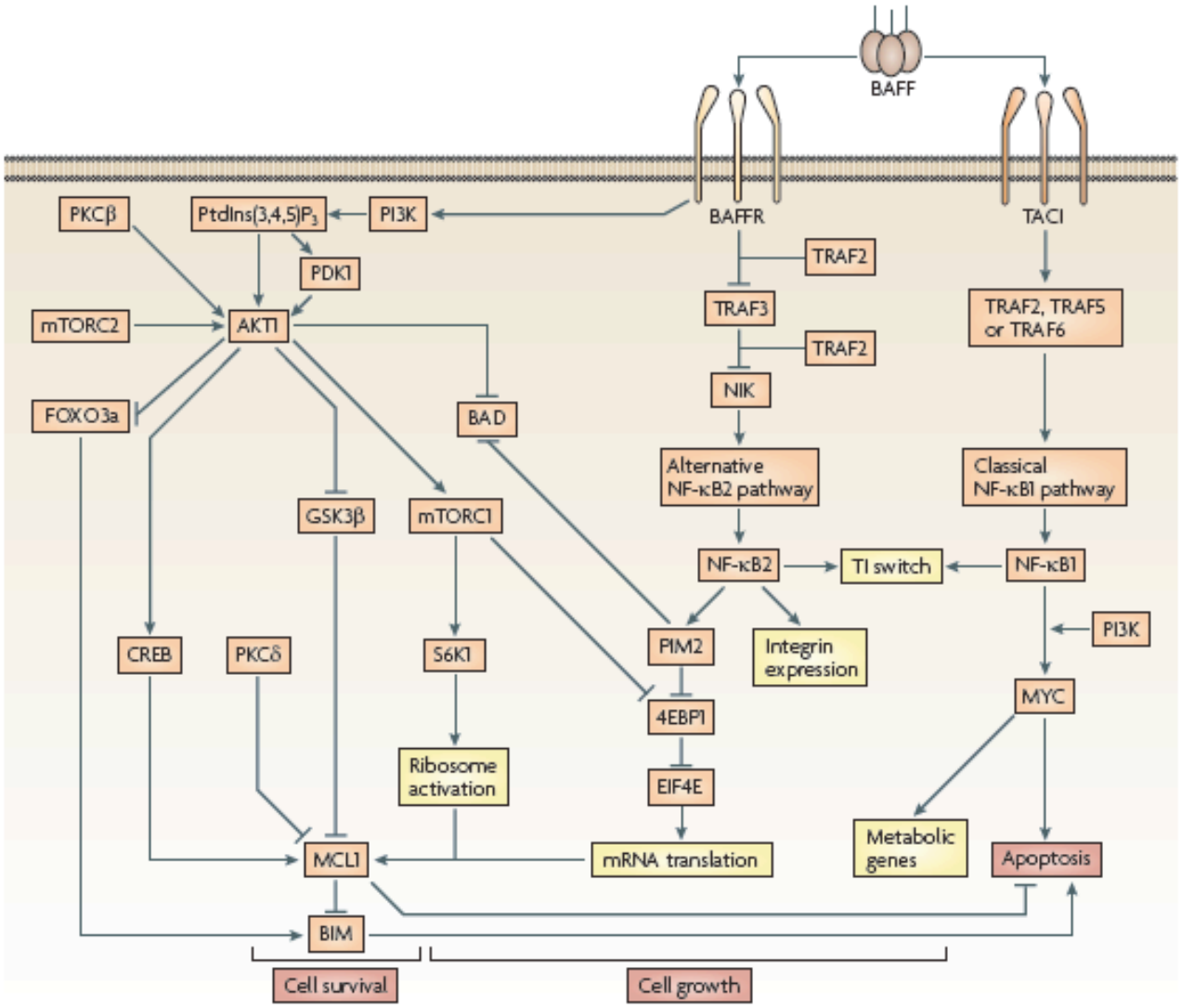


Fig 8. Signaling pathways downstream of BAFF receptors; *Taken from review by Mackay et al. (Nature Reviews 2009)*

A trimeric BAFF or APRIL binds to three different receptors to generate a signal but for productive signaling a binding is not enough (Reviewed in [130]). For example, binding of APRIL to TACI is ineffective unless it is further multimerized, by binding to heparin sulfate proteoglycans (HSPG) [64, 131]. Only BAFF 60-mer but not trimers signal through TACI, but both forms of BAFF can initiate BAFFR-mediated effects [49]. Data obtained from *in vitro* studies show that TACI requires multimeric ligands like BAFF 60-mers, HSPG-bound APRIL or membrane-bound ligands, to signal but not soluble, trimeric ligands.

Trimeric intracellular adapter proteins TNF receptor-associated factors (TRAFs) bind several receptors, including BCMA, TACI and BAFFR (Reviewed in [130]). The stability of interaction between these TRAF molecules and receptors are enhanced by avidity effects upon binding TRAF trimer binds to three receptors held in the correct geometry by the ligand [132] as affinity of TRAF to a monomeric receptor is low. Signaling via TACI to activate NF-κB transcription factor require TRAF2 and TRAF6 binding and these molecules held in close proximity[133] This possible state can be obtained when at least six receptors are recruited. Signaling via BAFFR involves the activation of the non-canonical NF-κB pathway, which selectively recruits TRAF3 for the purpose of degrading it, thereby minimizing the need for higher order receptor clustering to obtain an effect [125, 134, 135]. These facts for intracellular signaling perhaps serve the basis for the hypothesis that oligomeric ligands are necessary to induce signaling via TACI because of the downstream molecular requirements.

TACI is a strong stimulator of the classical NF-κB1 pathway unlike BAFFR which is a strong activator of the alternative NF-κB2 pathway and weak activator of the classical NF-κB1 pathway in primary B cells, (Reviewed in [130]). TACI signaling is important for specific B cell types [110]. TACI has a dual role to play in the regulation of mouse B cell development. Excess number of B cells have been found in TACI- deficient mice indicating the negative effect of TACI on the size of the B cell compartment *in vivo* (Reviewed in [130]) whereas on the other hand, TACI is required for efficient T cell-independent type II humoral immune responses. The expression of TACI is on innate B cells (including marginal zone and B1 cells) that are involved in T cell-independent type II immune responses. This hints towards the possibility that TACI provides positive signals for the maturation or survival of plasmablasts derived from these cells [110]. There is evidence of a tight connection between TLR activation and TACI expression. Some studies have shown that following BCR ligation, BAFF and APRIL receptor expression levels change suggesting that overall BAFF

receptor profiles and thus ligand sensitivity can be altered by activation cues [109]. BAFF receptor expression can be altered by TLR stimuli, but unlike BCR ligation, TACI expression is preferentially increased by TLR9 and TLR4 signals. Although both of these TLRs act through MyD88-dependent mechanisms to increase TACI expression, their downstream mediators and their target B cell subsets are different. Interestingly, only TLR4 relies on c-Rel and p50 to increase TACI expression, whereas TLR9 does not. Furthermore, although all follicular and marginal zone B cells up-regulate TACI in response to TLR9 stimulation, only marginal zone B cells and a subset of follicular B cells respond to TLR4. This study shows that BAFF and APRIL enhance viability among quiescent and BCR-stimulated B cells. However, although viability among TLR stimulated B cells is enhanced by BAFF but not APRIL, suggests that TACI but not BAFFR may share survival promoting pathways with TLRs. A latest study show that TACI may participate in immunoglobulin class switching by activating B cells through adaptor Myd88 downstream of the TLR pathway [136].

The phenotype of $BCMA^{-/-}$ mice is not so severe. They are generally healthy, but the survival of the long-lived bone marrow plasma cells is impaired in these mice [116]. BCMA can also promote the antigen-presenting function of B cells [119], although TACI and BAFFR can also participate in BAFF mediated up-regulation of MHC class II expression [49]. BCMA also signals via the classical NF-κB pathway, a TRAF5-, TRAF6-, NIK-. and IKK-dependent pathway [137]. BCMA, though known to be a type III transmembrane protein, has been found in a human malignant myeloma cell line not on the cell surface, but in a perinuclear structure that partially overlaps the Golgi apparatus [138]. Upon transient or stable transfection, BCMA could be found located on the cell surface, as well as in a perinuclear Golgi-like structure. Apart from the NF-κB pathway, overexpression of BCMA activates Elk-1, the c-Jun N-terminal kinase, and the p38 mitogen-activated protein kinase. Co-immuno-precipitation experiments performed in transfected cells showed that BCMA associates with TNFR-associated factor (TRAF) 1, TRAF2, and TRAF3.

Analysis of deletion mutants of the intra-cytoplasmic tail of BCMA showed that the 25-aa protein segment, from position 119 to 143, conserved between mouse and human BCMA, is essential for its association with the TRAFs and the activation of NF-kappa B, Elk-1, and c-Jun N-terminal kinase. BCMA belongs structurally to the TNFR family. Its unique TNFR motif corresponds to a variant motif present in the fourth repeat of the TNFRI molecule [138].

1.3.5. BAFF and APRIL in pathological conditions.

The involvement of BAFF in the pathogenesis of autoimmune diseases is well studied by BAFF overexpression in mice models, which leads to autoimmune disease mimicking rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and primary Sjogren's Syndrome (pSS) [42, 139, 140], as well as predisposition to B cell lymphoma increase in occurrence of B cell lymphoma [141]. In humans, an increased serum level of BAFF was reported in patients with RA and SLE [142], but the more consistent findings concerned pSS [42, 140, 143-145] with an increase in BAFF level reported in all reported findings of patients with pSS [140, 146-148]. Studies have tried to establish the correlation between the serum level of BAFF and serum level of immunoglobulins and titers of autoantibodies and the major cell types influenced by BAFF involved in these pathologies [149, 150].

It was mostly believed that BAFF is produced by cells of myeloid origin like monocytes, macrophages, dendritic cells, and neutrophils as well as unidentified non bone marrow–derived radiation-resistant cells, until some studies reported astrocytes as producers of BAFF and established a role of BAFF in B cell survival in multiple sclerosis and primary CNS lymphoma [85, 151]. The same group also reported the presence of BAFF in Wegener's granulomatosis. Other than BAFF, BAFF receptors have been also found up-regulated in multiple sclerosis. BAFF and APRIL being identified as major players in B lymphocyte survival led to the investigation of their role in

the pathogenesis of hematological B cell malignancies. It was found that they are produced by leukemic cells as well as the tumor microenvironment or both [152]. In addition to increased serum levels of BAFF and APRIL, the receptors of these ligands were also found up-regulated in various non Hodgkin's lymphoma and multiple myelomas apart from being present in lymphoproliferative disorders [78, 84, 93, 153-155]. In a much aggressive form of B-CLL BAFF and APRIL can evade apoptosis in an autocrine manner [156]. Some studies also indicate that BAFF and APRIL can be produced by nurse-like cells, promoting lymphocytic leukemia in a paracrine manner [92]. APRIL is also implicated in multiple myeloma [157] and in promoting B1 cell related neoplasms [158].

1.4.1. First discovery of heteromers in the TNF family.

TNF family ligands have been described to usually assemble as homotrimers. The first proof of existence of heteromeric complex formation among the TNF ligands came from the characterization of Lymphotoxin alpha-lymphotoxin beta complexes. Like many other TNF ligands, lymphotoxin alpha forms both soluble and surface-bound complexes in humans. LTα was originally believed to be functionally identical to TNF. Interestingly, biochemical characterization of a surface form of LT-a showed that it was a complex of two proteins [159, 160]. Surface LTα is does not retain the transmembrane region like TNF but is attached to the surface by forming a complex with surface LTβ [161, 162]. The predominant form of membrane bound LTα on human cells is believed to be most likely a trimer with an $LT\alpha_1\beta_2$ stoichiometry, whereas the $LT\alpha_2\beta_1$, form is a relatively minor component. LTβ-R a receptor for surface LTα has been identified and binds to $LT\alpha_1\beta_2$ [163]. Trimeric LTα binds to TNFR1 and TNFR2 i.e. the 55–60- and 75–80-kDa forms of TNF receptors (referred to in the original paper as TNF-R55 and TNF-R75 [164]. The minor form of surface LT with $LT\alpha_2\beta_1$ composition can bind to both TNFRs. The LTβR is structurally related to both TNFR1 and TNFR2, yet does not recognize either soluble LTα or TNF. The phenotypic differences between LTα- and LTβ-null mice led to the conclusion that the LT and TNF systems

are distinct. In general, the hLTα gene is expressed solely by lymphocytes and not by monocytes [165]. The expression of both LTα and LTβ in cell lines is dramatically induced following cell activation whereas LTβ gene transcription may be more constitutive, at least as revealed by analysis of primary murine splenocytes [166, 167]. LTα-deficient mice were found to lack lymph nodes [168], the spleen did not show the clear demarcation between T and B cell-rich regions, and germinal center formation was impaired [169, 170]. Furthermore, since the administration of soluble LTβR-Ig, but not TNF-R55-Ig treatment, during development could mimic much of the phenotype of the LTα- null mouse, it led to the conclusion that the surface LTαβ complex is involved in lymph node organogenesis and splenic organization [171]. Surface LTαβ plays a role in maintenance of splenic organization in the mature system as demonstrated by the use of the soluble LTβR to inhibit LTαβ function in normal adult mice [172]. Surface LTαβ complex possibly does not account for all the developmental functions of LTα, administration of LTβR-Ig, led to inhibition of LT function during development while it did not affect the development of mesenteric lymph nodes [171]. LTβ-deficient mice also possess mesenteric and cervical lymph nodes [173]. Expression of the LTα gene in transgenic mice under the control of the rat insulin promoter led to the formation of lymph nodes in unusual locations, indicating that LTα is involved in lymph node neo-organogenesis [174]. The fundamental signaling process follows the pattern of the TNF family where trivalent ligand engages together two or more receptors. The arrangement of the intracellular domains of the receptors are perhaps altered upon oligomerisation by the extracellular domains which is in turn translates into some further signal [175]. In the case of $LT\alpha_1\beta_2$ on a cell surface, the three receptor binding clefts are not equivalent and therefore elucidating which receptors bind to which cleft serves as an important explanation of how signaling can occur. Efforts to produce recombinant soluble LT heteromeric complexes allowed the characterization of key aspects of this unique structure [176].

1.4.2. BAFF and APRIL heteromers.

Elevated serum levels of BAFF have been associated with several pathologies, including autoimmune diseases. While dosing the amount of BAFF and APRIL in sera of patients with systemic immune-based rheumatic disease, researchers obtained different results depending on the anti-BAFF antibodies used in an ELISA assay [177]. They developed and characterized various monoclonal and polyclonal antibodies to measure elevated levels of the ligands BAFF, APRIL or BAFF-APRIL heteromers. These antibodies allowed the affinity purification of BAFF-APRIL heteromers produced from mammalian expression systems. The purified heteromers could then be tested for their biological activity by heteromer-induced proliferation on primary B cells. The activity could be inhibited by TACI-Ig but not BCMA-Ig or BAFFR-Ig. Based on this and other results confirming interaction based on co-expression in mammalian cell lines they concluded that BAFF-APRIL form heteromers and can be detected in serum and can be differentially recognized by adequate antibodies. However these studies could not highlight on the various stoichiometric forms of the heteromers and their receptor binding specificities.

More recently, a study [178] described the generation of recombinant BAFF APRIL heteromers using a novel trimerization domain [179] [180]. After proteolytic processing, untagged BAFF APRIL heteromers was obtained for further biochemical characterization. Owing to the limitation of their method of production, the heterotrimers had a predominant stoichiometry of 2 APRIL to 1 BAFF and a very small fraction of 1APRIL to 2 BAFF. The heterotrimers were less-potent inducers of B-cell proliferation than were BAFF or APRIL. They developed a bead based immunoassay and using the recombinant heterotrimers as a reference of quantification they measured endogenous heterotrimers in sera of patients with autoimmune diseases. Their studies indicate that serum levels of heterotrimers increased as disease state worsened as observed with respect to clinical parameters usually measured in SLE.

1.5. BAFF/APRIL inhibitors.

(Reviewed in [181-184]) In certain autoimmune diseases blockade of BAFF leading to some improvement in disease prognosis has been achieved. Like in SLE, which is a systemic autoimmune disease in which the loss of tolerance to nucleic acids and their binding proteins, results in the generation of autoantibodies that initiate tissue-damaging inflammation. Current treatments for SLE are suboptimal with significant side effects. A human antibody targeting BAFF and overall B cell survival named Belimumab has been approved by the FDA and has been reported to have modest efficacy in phase II clinical trial. Mouse models of autoimmune diseases have shown the impact or BAFF/APRIL inhibition both at early and late stages of the disease [185, 186]. Using soluble receptors of BAFFR-Ig and TACI-Ig (Atacicept) it was observed that the onset of the disease could be delayed effectively but reversal of established disease is more difficult to achieve and depends on the amount of concomitant systemic inflammation [187]. All therapeutic strategies however are inefficient to prevent both autoantibody formation as well as its deposition in kidneys, though they are effective in B cell depletion resulting in reduction of secondary lymphoid organ size and thereby reduction in total number of T and dendritic cells. In Lyn-deficient mouse [188], there is evidence that BAFF inhibition directly inhibits T cell activation, an effect that has not been observed in the other SLE strains. By contrast, in MRL/lpr mice, autoantibody producing plasma cells that are mostly generated in extra follicular foci are highly dependent on BAFF and APRIL and serum IgG autoantibody levels plummet within 1-2 weeks of receiving TACI-Ig; this is associated with a marked decrease in renal immune complex deposition and improved survival. T cell activation and interstitial nephritis are not affected by TACI-Ig in this strain [189]. These studies highlight the heterogeneity of responses to BAFF and BAFF/APRIL inhibition in multiple murine models of SLE and suggest that there may be subsets of humans that respond better to BAFF inhibition than others. Both Belimumab and Atacicept or TACI-Ig have been used for the treatment of rheumatoid arthritis [190, 191] (Reviewed in [181]). In Phase II studies Belimumab

had a modest effect on disease activity whereas no effect was observed in Phase II studies with TACI-Ig. In a phase II study, moderate, but not high or low doses of a different anti-BAFF antibody (LY2127399; Eli Lilly) that blocks both soluble and membrane BAFF, had beneficial effects in RA similar to that of TNF blockers (M Genovese, abstract 1923 presented at American College of Rheumatology Meeting, Philadelphia, 2009). A clinical trial of LY2127399 began in 2009 and is ongoing. The clinical efficacy of Belimumab, as evaluated by the SLE responder index, was demonstrated at week 52 in two large phase III clinical trials (BLISS-52 and BLISS-76), as well as by a decrease in severe flares and steroid-sparing effects [192]. Mechanistic studies in humans have shown that, as predicted by the mouse physiology, and based on its selective inhibition of BAFF, Belimumab depletes naive and transitional B cells within the first 6 months of treatment and depletes IgM+ memory B cells and IgM producing plasma cells with delayed kinetics but has no effect on class switched memory B cells even after 2 years of treatment [193],[194]. The effect of drug on T cell activation pathways and on monocytes remains to be determined. An important difference between the mouse and human/primate studies is that the kinetics of B cell depletion takes much longer in humans and is associated with delayed shrinkage of lymphoid organs [194]. This is consistent with the apparently delayed onset of action of Belimumab.

BAFF overexpression has been detected in the brains of mice and patients with multiple sclerosis [195] and TACI-Ig had a beneficial effect in a mouse model of MS, though a phase II study of Atacicept for MS had to be terminated because of disease worsening (www.clinicaltrials.gov). It raises questions about the beneficial effects of B cell depletion in MS using anti-CD20 antibodies. Whether the negative effect of Atacicept was due to a decrease in Type I IFN (which is used for treatment in MS and increases serum BAFF levels) [151], or alterations in other cytokines such as IFN-γ or IL-10 remains unexplained.

2. Materials and methods

Mice

Mice were handled according to Swiss Federal Veterinary Office guidelines, under the authorization of the Office Vétérinaire Cantonal du canton de Vaud. 6-8 weeks-old C57Bl/6 mice were purchased from Harlan. TACI $^{-/-}$, TACI $^{-/-}$ x BCMA $^{-/-}$ and BAFF $^{-/-}$ in the C57Bl/6 background (kindly provided by Martin Scott and Susan Kalled, BiogenIdec, Boston) were bred in the animal facility of the Department of Biochemistry of the University of Lausanne.

Plasmids and plasmid construction.

Plasmids used were form the collection of group Schneider and are identified by the prefix ps followed by a number. Some of these plasmids, especially mammalian expression vectors for Fc-ligands, Flag-ligands, Receptor-Fc and receptor-GPI have been published [40], and details for others can be found in group Schneider's plasmid register (available upon request). Plasmids produced in the frame of this work were constructed according to standard molecular biology techniques and are briefly described here. Plasmid maps are provided in Annex A. Plasmids for the expression of single chain TNF family members were constructed on a modular basis and comprise a) a N-terminal sequence that can be i) a signal peptide followed by a Flag tag, ii) a signal peptide followed by the Fc portion of human IgG1, excluding the stop codon or iii) the intracellular domain, transmembrane domain and part of the stalk of human BAFF, but excluding the TNF homology domain; b) the TNF homology domain of a TNF family ligand, excluding the Stop codon (Mono 1); c) a linker with sequence GGGGS; d) the TNF homology domain of a TNF family ligand, excluding the Stop codon (Mono 2); e) a linker with sequence GGGGS; f) the TNF homology domain of a TNF family ligand with its Stop codon (Mono 3); g) optionally, an IRES-EGFP sequence. Typically, Mono 1 was first inserted in the vector of interest as a PstI/BamHI (or SalI/BamHI) fragment. Mono 2 was then inserted in the BamHI site 3' of Mono 1 as a BglII/BamHI fragment. Its insertion in the correct orientation was checked by restriction. Note that

BglII and BamHI generate identical overhangs that can be ligated together without reconstituting either a BamHI or a BglII site. Mono 3 was then inserted after Mono 2 as a BamHI/EcoRI fragment, followed by an IRES-EGFP in an EcoRI/XbaI cassette. The following plasmids were generated: Fc-BAFF-BAFF-BAFF (ps2680), Fc-APRIL-BAFF-BAFF IRES-GFP (ps2883), Fc-BAFF-APRIL-APRIL IRES-GFP (ps2877), Fc-APRIL-APRIL-APRIL IRES-GFP (ps2879), Flag-BAFF-BAFF-BAFF IRES-GFP (ps2890), Flag-APRIL-BAFF-BAFF IRES-GFP (ps2886), Flag-BAFF-APRIL-APRIL IRES-GFP (ps2889), Fc-APRIL-APRIL-APRIL IRES-GFP (ps2876). Representative plasmid maps are provided in annex A.

Expression plasmids for full-length mBAFF with mutation E247K (ps2583) or E278K (ps2575) were prepared from a plasmid encoding full-length WT mBAFF (ps694). Plasmid maps are provided in annex A.

Construction of a knock-in vector.

A BAC clone containing the murine BAFF gene from the 129 mouse strain was purchased from Gene Service Cambridge (BAC BMQ-135D21) (ps2769). A 5663 bp SpeI fragment containing mBAFF exons 5 and 6 was excised from ps2769 and cloned in a modified pBluescript vector (ps2793) to yield plasmid ps2806. A PCR fragment containing the target mutation E247K was amplified by double PCR using ps2806 as template, cut with ApaI and cloned in a vector containing an ApaI cloning site to yield plasmid ps2839. A NeoR cassette flanked by LoxP recombination sites, kindly provided by Dr. Edith Hummler (Transgenic Animal Facility, University of Lausanne) (ps2787), was excised NotI/XbaI, blunted with Klenow enzyme, and cloned in the EcoRV site of ps2839 to yield plasmid ps2841. Finally, the ApaI fragment of ps2841 was cloned in ps2806 to yield the targeting vector, ps2846, whose map is provided in annex A.

mBAFF sequences located on the 5'- and 3'-edges of the targeting vector were amplified by PCR (using plasmids ps2806 and ps694 as templates, respectively) and cloned EcoRI/BamHI in pBluescript to yield ps2559 and ps2851. Maps are shown in Annex A.

Antibodies and recombinant proteins

Unless specifically mentioned, recombinant proteins have been produced in house. The following proteins and antibodies have been used [applications are indicated in square brackets]: Flag-mBAFF standard (Alexis APO-50N-040) [ELISA], anti-Flag M2 antibody (Sigma F3165) [WB], biotinylated anti-Flag M2 mouse IgG1 (Sigma F9291) [ELISA], mouse anti-human IgG Fc(gamma) fragment-specific (Jackson 209-005-139 or 209-005-098) [ELISA], horse radish peroxydase (HRP)-coupled streptavidin (Jackson 016-030-084 or eBioSciences) [ELISA], HRP-coupled donkey anti-human Ig (H+L) (Jackson 709-036-149) [ELISA], HRP-coupled goat anti-human IgG (H+L) (Jackson 109-035-003) [WB], phycoerythrin (PE)-coupled goat anti-human IgG (SAB #2040-09) [FACS], PE-coupled goat anti-rat IgG (H+L) (SAB #3010-09) [FACS]. HRP-coupled anti-mouse [WB]. Rat IgG2a anti-TRAIL-R3 mAb572 [40] [FACS], rat IgM anti-human BAFF Buffy-2 [43] [WB] and mouse IgG1 anti-human APRIL Aprily 2 [64] [WB] were produced in house. Goat Fab'2 anti-mouse IgM μ chain (Jackson 115-006-020) [B cell stimulation]. Blocking rat IgG anti mBAFFR 9B9 was a kind gift from Antonius Rolink [196] [B cell stimulation]. Rat IgG1 anti mBAFF 5A8 (Alexis ALX-804-158) [ELISA] and biotinylated rat anti mBAFF IgG1 1C9 (Alexis ALX-804-157B) [ELISA]. Polyclonal goat anti-mouse BAFF antibody (R&D AF2106) [WB].

Other reagents

Reagents are listed with: Material; provider; catalogue number. [Methyl-3H]-thymidine (20 Ci/mmol); PerkinElmer; NET027. [alpha-32P]-deoxycytidine 5'-triphosphate (dCTP, 3000 Ci/mmol, 10 μCi/μl); Hartman; SCP-205. Phenazine methosulfate (PMS); Sigma; P9625. 3-[4,5-dimethlythiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt (MTS); Promega; G1111. Lysozyme; Sigma Aldrich; L7651 G418 sulfate; Calbiochem; 345812. Pen/strep; Invitrogen; 15140-122**.** Polyfect; Qiagen; 301-105. SIGMA*FAST* OPD tablet set (*o*-phenylenediamine dihydrochloride (OPD) and urea hydrogen peroxide tablets, Sigma, P9187). Pre-

stained protein ladder for Western blot; NEB; P7703S, Peptide-N glycanase F (PNGaseF); (500'000 units/ml); NEB; P0704. Dual luciferase reporter assay system, Promega; E1910 (containing 5 x concentrated passive lysis buffer, 50 x concentrated Stop and Glow solution to be reconstituted with Stop and Glow buffer just before use, and luciferase assay substrate to reconstitute, aliquot in 0.5 ml black tubes and store at -70°C). Liquemine (heparin solution, 5000 IU/ml); Roche Pharma;B01AB01, TaKaRa LA polymerase (5U/ml); TaKaRa RR002. B cell isolation kit (negative selection); Miltenyi; 130-090-862.

Cells and cell lines.

Human embryonic kidney (HEK) 293T cells were grown in DMEM Glutamax supplemented with 10% FCS. Chinese hamster ovary (CHO) cells and stable CHO transfectants were grown in DMEM-F12 (Gibco #31331) supplemented with 2% FCS and 0.2% $NaHCO_3$. Jurkat or Fas-deficient Jurkat (JOM2; a kind gift of Olivier Micheau, University of Dijon, France) cells lines expressing receptor:Fas fusion proteins (Jurkat-hBCMA:Fas-2309 cl13 [49], JOM2-hBAFFR:Fas-2308 cl21, JOM2-hBAFFR:Fas-2308 cl11.1, JOM2-mBAFFR:Fas-2922 cl7, JOM2-hTACI:Fas-2455 cl112) were cultured in RPMI supplemented with 10% FCS. Primary mouse B splenocytes were cultured in RPMI supplemented with 10% FCS and 0.5 mM β-mercaptoethanol. All media contained 5 μg/ml each of penicillin and streptomycin.

Establishment and screening of stable CHO clones.

Stably transfected CHO cells were generated as follow: 2 x 10^5 cells were seeded in a well of a 6-well plate in 3 ml medium and left to attach overnight. The next day, medium was aspirated and replaced with 1.5 ml fresh medium. 1.5 μg of plasmid of interest was mixed with 100 μl medium (no FCS, no antibiotics) and 10 μl Polyfect for 5 min at room temperature (RT), after which time 600 μl of complete medium was added and the mixture transferred to cells. 8 h later, supernatant was removed, cells were washed with PBS and 3 ml complete medium was added. 48h later, cells

were selected with medium containing 500 µg/ml of G418. After 2 passages in selection medium, resistant cells were cloned by inoculating 6 cm culture dishes with 4 ml of cells diluted 1/4000, 1/800 and 1/300. When clones were clearly discernable and big enough, 12 of them (or sometimes more) were randomly selected, except in cases where plasmids contained an IRES-GFP, in which case green clones were identified by fluorescence microscopy. Clones were detached with a pipette mounted with a bend tip and transferred in wells of 96-well plates. Once clones became confluent, they were maintained for two additional weeks with bi-weekly passages. 20 µl of 2 weeks-old supernatants were analyzed for the presence of the protein of interest by western blot analysis with anti-Flag or anti-hFc. In some cases, proteins of interest were detected by ELISA. The best clones were amplified and frozen.

Production of recombinant proteins

Production by transient transfections:

293 T cells were transfected with the calcium phosphate method. Briefly, confluent 293T cells were diluted 1/8 in 10 cm culture plates in 8 ml of DMEM 10% FCS and transfected 8 to 24 h later. For transfection, 7 µg of plasmid of interest and 1 µg of an EGFP expression plasmid (ps515) were mixed with 50 µl of 2.5 M $CaCl_2$ and water was added to 500 µl. 500 µl of 2 x HeBS solution (50 mM HEPES free acid, 280 mM NaCl, 1.5 mM $NaHPO_4$, pH to 7.05) was added dropwise while vortexing and added within 1 min to 293T cells. Cells were washed 16 h later and cultured either in serum-free OptiMEM medium or in DMEM, 10% FCS. Supernatant were harvested after 7 days. When required, supernatants in OptiMEM were concentrated using centrifugal concentrators with cutoff at 30 or 10 kDa (Millipore, #UCF801024).

Production from stably transfected CHO clones:

Stable cell lines of interest were amplified and cultured according to the following scheme: 1 confluent 10 cm plate in 8 ml medium was used to inoculate two 16 cm plates in 30 ml medium, which themselves were used, once confluent, to inoculate one 2 L roller bottle in 1 L medium.

Roller bottles were incubated for 14 days at 37°C under rotation at 3.5 rotations per minute, after which time cells were decanted, supernatant was centrifuged at 3000 rpm in 50 ml tubes, filtered at 0.22 μm, supplemented with 0.05% NaN_3 and kept at 4°C until purification.

Affinity purification of Flag- and Fc-tagged proteins

Fc-tagged proteins were purified from stable cell supernatants using 1 or 5 ml HiTrap Protein A-Sepharose columns (GE Healthcare), and Flag-tagged proteins were purified on 1 ml anti-Flag M2-Agarose columns (M2-Agarose gel was from Sigma, and HiTrap columns were prepared by GE Healthcare). Columns were equilibrated in 50 mM sodium-phosphate buffer pH 7.05 (buffer A), and supernatants loaded overnight with a peristaltic pump. Columns were washed with 5 volumes of buffer A and eluted with 50 mM Na-citrate pH 2.7 (buffer B). In some cases, for Fc-tagged proteins, elution was performed with a 30 to 70% gradient of buffer B over 10 column volumes, followed by a wash with buffer B using an AktaPrime liquid chromatography system (GE Healthcare). Eluted fractions of interest were neutralized with 1/10 volume of 1 M Tris-HCl pH 9, concentrated and their buffer exchanged for PBS. When required, the flow through was loaded again onto the column until depletion of the protein of interest. Eluted fractions of interest were pooled, proteins were concentrated and buffer exchanged to PBS with a centrifugal concentrator. The proteins were then sterilized by filtration at 0.22 μm on low protein binding filters (Millex-GV, SLGV013SL). Protein concentration was determined by A_{280} using extinction coefficient calculated for the mature protein sequence using the Protein Analysis Toolbox option of the MacVector progam, assuming that all Cys residues appear as half-cystines. All columns were stored in 20% EtOH, immersed in 20% EtOH. In some cases, 0.5 M NaCl was added to both buffer A and B, without much incidence on the entire process.

Gel permeation chromatography.

Purified proteins were concentrated to 200 to 300 µl, loaded onto a Superdex-200 gel permeation chromatography column (GE Healthcare) equilibrated in PBS and eluted at 0.5 ml/min in PBS with 1 ml fraction collection and online UV monitoring at 280 nm. Fractions of interest were pooled, dosed and sterilized by filtration as required. A similar protocol was used for serum-free, concentrated cell supernatant in OptiMEM.

Protein precipitation.

500 µl of fractions eluted from the Superdex-200 column were supplemented with 20 µl of 1 mg/ml lysozyme and precipitated for 10 min on ice with 50 µl of 60% ice-cold trichloracetic acid (final conc ~5%). Samples were centrifuged at 13000 rpm for 5 min at 4°C in a tabletop centrifuge. Supernatant were discarded, tubes were briefly spun again and any supernatant remnants removed. Pellets were dissolved in reducing SDS-PAGE sample buffer for western blot analysis. When sample buffer turned yellow, an adequate volume of 1 M Tris-HCl pH9 was added to neutralize acid.

ELISA

A generic method is described first, followed by specific protocols. ELISA were performed in 96 well Nunc maxisorp plates coated with proteins or antibodies in 100 µl of 50 mM $NaHCO_3$ pH 9 (carbonate buffer) or in PBS. Plates were saturated with 350 µl of 4% powdered skimmed milk in PBS, 0.5% Tween-20 (block buffer) for 1 h, unless mentioned otherwise. Plates were washed 4 times with PBS, 0.05% Tween-20 (wash buffer). Ligands, purified or in cell supernatants, were adjusted to 100 µl with 0.4% milk in PBS 0.05% Tween-20 (incubation buffer) and incubated for 1 h at 37°C unless stated otherwise. Plates were washed 4 times with wash buffer. Revealing reagents in 100 µl of incubation buffer were added as required for 1 h at 37°C, in two steps if required (*e.g.* biotinylated antibody followed by HRP-coupled streptavidin). Plates were washed 4 times with

wash buffer between each step and after the last incubation. Peroxydase activity was revealed with 100 µl of OPD solution, and the reaction was stopped after colour development by the addition of 50 µl of 2 N HCl. Absorbance was monitored at 490 nm with an ELISA reader.

Detection of Flag-tagged ligands.

Plates were coated with receptor-Fc fusion proteins at 1 µg/ml in carbonate buffer, followed by addition of Flag-tagged ligands in cell supernatant (usually 100 µl supernatant in DMEM-FCS or 20 µl in OPTIMEM plus 80 µl incubation buffer) and revelation with biotinylated anti-Flag M2 antibody at 0.5 µg/ml and HRP-conjugated streptavidin at 1/4000.

Detection of Fc-tagged ligands.

Plates were coated with mouse anti-human antibody at 5 µg/ml in carbonate buffer, followed by Fc-tagged ligands in cell supernatants and revelation with HRP-coupled donkey anti-human antibody at 1/8000.

Detection of heteromers.

Plates were coated with mouse anti-human antibody at 5 µg/ml in carbonate buffer, followed by supernatants containing Flag-ligands and Fc-ligands and revelation with biotinylated anti-Flag M2 antibody and HRP-conjugated streptavidin.

Detection of mouse BAFF.

Plates were coated overnight at RT with rat anti-mouse BAFF 5A8 at 3 µg/ml in PBS and blocked for 2 h. mBAFF-containing samples or purified Flag-mBAFF were then added for 3 h at RT. Bound mBAFF was detected with biotinylated rat anti-mouse BAFF 1C9 at 2 µg/ml in incubation buffer for 2 h at RT, followed by HRP-conjugated streptavidin.

Immunoprecipitations

0.5 to 1 ml of DMEM, 10% FCS supernatants of transfected 293T cells were mixed with 10 µl of a 50% slurry of Protein A-Sepharose beads (GE Healthcare) or M2-Agarose beads (Sigma) and incubated for 1 h at 4°C on a rotating wheel. Samples were centrifuged for 1 min at 5000 rpm in a

tabletop centrifuge, beads were recovered with a wide-opening (cut) tip, loaded on micro-columns, washed with 4 x 200 µl of PBS and eluted with 15 µl of 50 mM Na-citrate pH 2.7. The eluate was neutralized and denatured with 5 µl of Tris 1M pH 9 and 10 µl of 3x concentrated reducing SDS-PAGE sample buffer followed by heating for 3 min at 95°C.

Enzymatic deglycosylation of proteins

Immunoprecipitated, neutralized proteins were heated for 5 min at 95°C in PNGaseF denaturation buffer (1% of 2-mercaptoethanol and 0.5% of SDS). After cooling, samples were supplemented with 1% of NP40 and 50 mM of sodium phosphate pH 7.5 and digested overnight with or without 500 Units of PNGaseF. Subsequently, SDS-PAGE sample buffer was added and proteins analyzed by western blotting.

Western blotting.

SDS-PAGE and Western blotting were performed according to standard procedures. Briefly, samples were resolved by electrophoresis at 200 Volts for 50-60 min on 12% or 15% polyacrylamide gels. A pre-stained protein ladder was used as molecular weight marker. Proteins were transferred by electrophoresis to nitrocellulose membranes for 1 h at 100 Volts. Membranes were stained briefly with Ponceau red, rinsed with deionized water, photocopied, saturated for 15 min in block buffer (5% powdered skimmed milk in PBS, 0.5% Tween-20), probed with primary antibodies (Table 1) for 1 h at RT or overnight at 4°C in the presence of 0.05% azide, washed for 3 x 15 min in PBS, 0.1% Tween-20, probed with secondary antibodies (Table 1) in milk for 1 h, washed and revealed with ECL reagent (GE Healthcare). In some instances, after revelation, blots were quenched by incubation overnight with sodium azide at 0.05% and H_2O_2 at 10%, rinsed with wash buffer and reprobed with adequate primary and secondary reagents. For detection, the co-immunoprecipitated ligand was detected first, and the immunoprecipitated one second.

Table 1: Antibodies used for Western blot

Antigen	Primary antibody (concentration or dilution)	Secondary antibody (dilution)
Flag-tagged protein	Anti-Flag M2 (1 µg/ml)	HRP-anti-mouse (1/5000)
Fc-tagged proteins	HRP-anti-human (1/5000)	---
hBAFF	Buffy-2 (1 µg/ml)	HRP-anti-rat H+L (1/5000)
hAPRIL	Aprily2 (1 µg/ml)	HRP-anti-mouse (1/5000)
mBAFF	Goat polyclonal –R &D (0.5µg/ml)	HRP- anti-goat (1/5000)

Cytotoxic assay.

BAFF or APRIL preparations were titrated with 2-fold dilutions in 50 µl of RPMI, 10% FCS in 96-wells cell culture plates. 50 µl of a confluent culture of receptor:Fas reporter cells (*i.e.* about 30'000 to 50'000 cells per well) were added, and plates were incubated for 16 h at 37°C, 5% CO_2. In some cases, anti-Flag M2 antibody was added at a final concentration of 1 µg/ml. After incubation, 20 µl of a 1:20 (v/v) mixture of PMS (0.9 g/ml in PBS) and MTS (2 mg/ml in PBS) was added to cells. After colour development (typically 2-5 h), A_{490} was monitored with an ELISA reader.

NF-κB reporter assay.

Confluent 293T cells were seeded in 96-wells culture plates at a 1/8 dilution in 100 µl of DMEM, 10% FCS and left to adhere overnight. Medium was aspirated and replaced with 50 µl of fresh medium. Cells were transfected by adding 25 µl of a transfection mix, prepared as follows (quantities for 1 well): 0.65 µl of a plasmid mix containing a total of 70 ng plasmid (7.5 ng of NF-κB luciferase reporter plasmid (ps1614), 7.5 ng of Renilla luciferase normalization plasmid (ps1615), 7.5 ng of an EGFP tracer plasmid (ps515), 25 ng of a full-length hBCMA expression plasmid (ps1303), the rest with empty PCR3 plasmid (ps015)), plus 3.25 µl DMEM without any

supplement, plus 0.35 µl of PolyFect. The mix was incubated for 5 min at RT before addition of 20.7 µl of complete DMEM and addition to cells. 8 h later, medium was exchanged for fresh complete DMEM containing titrations of ligands of interest at the indicated concentrations. After 24 h, cells were washed with 200 µl of PBS, lyzed in 50 µl of 1 x concentrated passive lysis buffer for 15 min on a shaking table. 25 µl of lysate was transferred to a black 96-well plate (PerkinElmer, 6005270), 15 µl of firefly luciferase substrate was added and luminescence was monitored in a TopCount-NTX luminometer (Packard). 15 µl of Stop and Glow solution was then added and Renilla luciferase-generated luminescence was recorded again. Ratio of firefly to Renilla luciferase activities were used to calculate the NF-κB fold-increase relative to unstimulated cells transfected with empty plasmid in place of BCMA.

Flow cytometry.

Receptor-ligand interaction assay.

293T cell were co-transfected with the receptor-GPI of interest [hBAFFR (ps1589), hTACI (ps897), hBCMA (ps1467), hTNFR1 (ps1426), hEDAR (ps1431), hXEDAR (ps1432), hNGFR (ps1456), hKDR (ps1476), hDR6 (ps689), hTROY (ps1434), hRELT (ps1433), Mock (ps015)] and an EGFP tracer (ps515) [40]. Cells were detached by pipetting, transferred into round-bottomed 96-well plates (about 350 µl/well corresponding to 2-3 x 10^5 cells) and centrifuged for 5 min at 1200 rpm. Medium was aspirated, cells were resuspended in 50 µl of FACS buffer (PBS, 5% FCS) containing 0.1 µl of Liquemine and ligands of interest at the indicated concentrations, and incubated for 20 min on ice. 200 µl of FACS buffer was then added, cells were spun and resuspended in a) 50 µl of PE-coupled goat anti-human IgG at 1/500 in FACS buffer for 20 min on ice (for Fc-ligands) or b) 50 µl of M2-biotin at 1/500 in FACS buffer for 20 min on ice followed by a wash and staining with 50 µl of PE-coupled streptavidin at 1/500 in FACS buffer for 20 min on ice. After staining, cells were resuspended in 200 µl of FACS buffer and analyzed on a FACS scan

flow cytometer (BD Bioscience) using the Cell Quest program. Data was analyzed with the FlowJo application (Tree Star).

Analysis of mature murine B cells.

100,000 of total mouse splenocytes (prepared as described under "isolation of mouse B splenocytes", but before MACS separation) were stained with anti-CD93-APC (AA4.1) at a concentration of 1/500 and anti-CD19 PECy5.5 (eBioscience 45-0193-80) at a concentration of 1/500 in FACS buffer (PBS + 5% FBS). Cells were analyzed using the LSR II and SORP.

Isolation of mouse B splenocytes.

Spleens were homogenized in 8 ml of complete RPMI using a tight fitting Dounce homogenizer. Cells were transferred in a tube, and medium was added to 12 ml. Cells were centrifuged for 5 min at 1200 rpm (260 x *g*), resuspended in 3 ml of AKC buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 1 mM Na_2-EDTA, pH 7.3) and incubated for 2 min on ice to lyse red blood cells. 6 ml of PBS, 2% FBS was added by spleen-equivalent. Cells were centrifuged, supernatant was discarded, the pellet was resuspended in 5 ml of PBS, 2% FCS, cells were filter cells through a 100 µm cell strainer and counted using a cell counter (Becton Dickinson). Cells were washed and resuspended at 10^8 cells/ml in PBS, 2% FBS. A MACS B cell negative selection kit was used for isolation of B cells. Cells were added in 40 µl of MACS buffer/10^7 cells to which 10 µl of biotin beads/10^7 cells were added and incubated at 4 °C for 15 min. This was followed by addition of 30 µl of MACS buffer/10^7 cells to which 20 µl of anti-biotin beads/10^7 cells were added and incubated at 4°C for another 15 min. Cells were washed with 1-2 ml of MACS buffer/ 10^7 cells. In the meantime MACS LS column were equilibrated on a MACS magnet with 3 ml of MACS buffer. Cells were resuspended with MACS up to 500 µl for 10^8 cells and loaded onto the LS column. The column was washed with 3 ml of MACS buffer three times. Cells were spinned, counted and used for B cell proliferation assay. Generally B cell yield was 30-50% of the total splenocytes.

[3H]-thymidine incorporation assay

Ligands of interest were serially diluted in 200 µl of B splenocyte proliferation medium (RPMI 10% FCS, 0.5 mM β-mercaptoethanol, antibiotics) supplemented with 2 µg/ml of anti-µ chain antibody. 100'000 splenic B cells purified by MACS with the B cell negative selection kit were added per well in 200 µl of medium. When required, anti-Flag M2 antibody or anti-BAFF-R 9B9 antibody were added at a final concentration of 1 µg/ml and 0.25 µg/ml, respectively. Cells were grown for 48 h, after which time 1 µCi of tritiated thymidine was added per well and cells were cultured for an additional 18 h. Cells were harvested on white OptiPlate 1536 (Perkin Elmer 6004290) with a 96-well plate harvester (Packard). Plates were dried before the addition of 50 µl of scintillator MicroScint TM 40 (Perkin Elmer 6013641). Radioactivity was counted with a Top Count-NTX liquid scintillation counter (Packard).

ES cells screen by PCR

Mrs. Anne-Marie Mérillat at the Transgenic Animal Facility of UNIL electroporated embryonic stem (ES) cells with the targeting construct, performed the selection, cloned cells and extracted genomic DNA from replicate cultures in six 96-well plates.

Positive clones were screened by PCR using primers JT7164 (5'-CCTTCTATCGCCTTCTTGAC-3') and JT7165 (5'-GTGGAACAGATAAGGTGCCT-3') under the following conditions:

0.8 µl of ES cell gDNA diluted 1/15 in H_2O
1.8 µl of 10x TaKaRa PCR buffer
2.88 µl of TaKaRa 2 mM each dNTP solution
0.029 µl JT7164 at 100 µM
0.029 µl JT7165 at 100 µM
0.11 µl TaKaRa LA polymerase (5 U/ml)
H_2O to 18 µl

In some cases, an additional primer (JT7162 3'-ACCCTGTTCCGATGTATTCA-5') was added in the reaction (to amplify the WT allele).

PCR amplification program:

1.- 3 min at 95°C
2.- 30 sec at 95°C
3.- 30 sec at 64°C (with 0.5°C decrease per cycle)
4.- 2 min at 68°C (cycle 19 x to point 2.-)
5.- 30 sec at 95°C
6.- 30 sec at 54°C
7.- 2 min at 68°C (cycle 29 x to point 5.-)
8.- 7 min at 72°C
9.- Store at 10°C

Amplified DNA products were analyzed on agarose gels stained with ethidium bromide.

Screen of ES cells by Southern blot

30 µl of genomic DNA (from one well of a 96 well plate) was digested O/N with 40 U of HindIII restriction enzyme in a final volume of 40 µl in the presence of 1 mM spermidine. Samples were mixed with 8 µl of TAE loading buffer, loaded on a 1% TAE agarose gel and submitted to electrophoresis at 140 V for several hours in TAE buffer (40 mM Tris-Acetate at ~pH8.5, 1 mM EDTA). One lane with standards was cut and stained independently with GelRed (Biotium), while the remaining of the gel was treated under gentle agitation for 10 min in 0.25 N HCl, then 30 min in 0.4 M NaOH, then 20 min in neutralizing buffer (500 mM Tris-HCl pH 7.2, 1.5 M NaCl, 1 mM EDTA). Hybond membranes were wet in water, then in 20 x SSC buffer (0.3 M Na_3Citrate, 3 M NaCl). The gel was placed upside-down on a plexiglass plate covered with 2 layers of blotting paper dipping in 20 x SCC buffer. The paper and the side of the agarose gel were covered with a plastic film and the gel covered with the Hybond membrane, then 2 layers of wet blotting paper, 2 layers of dry blotting paper, a bunch of absorbing paper, a plate and a weight. After blotting O/N, membranes were recovered and autocrosslinked with UV at 1200 J. Membranes were wet in 25 mM sodium phosphate pH 7.2, 1 mM EDTA and added together with 15 ml of pre-hybridization solution (5.25 ml of 20% SDS, 7.5 ml of 1 M sodium phosphate pH 7.2, 150 µl 0.5 M EDTA, 750 µl of 5% powdered skimmed milk, 1.35 ml H_2O) in a hybridization tube and incubated for 30 min

or more at 65°C in a rotating oven. The radioactive probe was prepared as follows: 18 µl of a HindIII/BamHI fragment of ps2859 at 4.4 ng/µl was heated for 5 min at 95°C. After cooling, 3 µl of hexanucleotide mix (Roche, 11008404001), 3 µl of dNTP at 0.5 mM each (except dCTP), 5 µl of [32P]-dCTP at 10 µCi/µl and 1 µl of Kleenow (2U/µl) (Roche 11008404001) were added and the tube was incubated for 30 min at 37°C. A ProbeQuant G-50 column (GE Healthcare, 28917924) was dried by centrifugation (2 min, 3000 rpm in a tabletop centrifuge). The labelled probe was mixed with 20 µl of ProbeQuant buffer, loaded onto the column and centrifuged for 2 min at 3000 rpm. The eluate, containing the labelled probe with about 2/3 incorporation, was denatured for 5 min at 95°C, then added to the membrane in pre-hybridization buffer and incubated O/N at 65°C in the rotating oven. The membrane was then washed at RT with wash solution (40 mM sodium phosphate pH 7.2, 1% SDS, 5 mM EDTA pH 8), washed at 42°C for 20 min in 20 ml wash solution, and for 30 min at 50°C in 20 ml wash solution. The membrane was wrapped in Saran and exposed for 16 h to 7 days at -80°C between two amplifying screens. The membrane was then stripped in 0.2 M NaOH for 15 min at RT under agitation, followed by 25 mM sodium phosphate pH 7.2, 1 mM EDTA for 15 min at RT. The membrane was re-hybridized with a NeoR-specific probe as described above for the 5' BAFF probe, except that washing steps in wash solution were performed at 50 and 55°C.

3. Results

3.1. A screen for heteromers in the TNF family of ligands.

3.1.1. Aims and objectives of research.

TNF family ligands assemble as homotrimeric ligands, which is the basic unit for signal transduction through their cognate receptors. However there is also evidence of heteromer formation within TNF family ligands. The first heteromer was observed between LTα and LTβ and the predominant form of this heteromer with a $LT\alpha_1\beta_2$ stoichiometry was found to specifically bind to LTβR and provide essential signals for lymph node formation (described in section 1.3.1). In addition, recent reports also suggested the formation of heteromers between BAFF and APRIL (described in section 1.3.2). These data raise the intriguing possibility that other heteromers may exist among TNF family ligands. The aim of the study was therefore to screen for novel interaction partners among TNF family ligands, with the potential of identifying novel receptor specificities.

Some TNF family receptors are orphan, *i.e.* have no identified ligand. NGFR is a typical TNF receptor family member with no known TNF ligand. Instead, NGFR binds low affinity ligands of the neutrophin family (NGF, BDNF and neurotrophins), and shares these ligands with a family of high-affinity tyrosine receptor kinases (TrkA, B and C) [197] that are unrelated to TNF receptors. NGFR also associates with the membrane proteins Lingo and NogoR to regulate axonal growth and neuronal apoptosis [198]. The existence of a *bona fide* TNF ligand for NGFR cannot be excluded at the moment. The TNF family contains three additional orphan receptors, TROY, RELT and DR6. These receptors may function in a ligand-independent manner, or may interact with ligands or protein outside the TNF family. Thus, TROY appears to associate with Lingo to mediate signal transduction [199]. It is unlikely that the orphan receptors have a cognate TNF ligand that has not

yet been identified, but it is possible that these receptors may be specific for a heteromeric TNF ligand. The aim of the study was also to test if newly identified heteromers would specifically bind an orphan receptor.

3.1.2. Screen to identify heteromers in the TNF family.

A screen was carried out to detect heteromers in the TNF family. For this purpose, Flag-tagged or Flag ACRP30 tagged [200] and Fc-tagged ligands were co-expressed in mammalian 293T cells, and culture supernatants were screened by ELISA or Western blotting for the presence of Flag-ligands, Fc-ligands, and heteromers thereof (Fig.10). This approach detected the interaction of most TNF ligands with themselves, and of LTα with LTβ. However, no interaction was seen between BAFF and APRIL, and some positive controls gave inconclusive or negative results (e.g. TRAIL or CD30L with themselves), suggesting that the screen was not fully reliable (Fig10). In addition, the number of false positives was relatively high, as judged by the number of heteromers that were detected in one direction only (Flag-ligand X with Fc-ligand Y, but not vice-versa). Despite these limitations, the following heteromers were detected: APRIL-EDA1, APRIL-EDA2, EDA1-EDA2, LIGHT-FasL, LIGHT-LTα and LIGHT-LTβ (Fig 11). Data of all ELISA or immunoprecipitation assays have been described in Fig. 10.1,2,3,4.

Some of these interactions were additionally confirmed by co-immunoprecipitation (Fig 12).

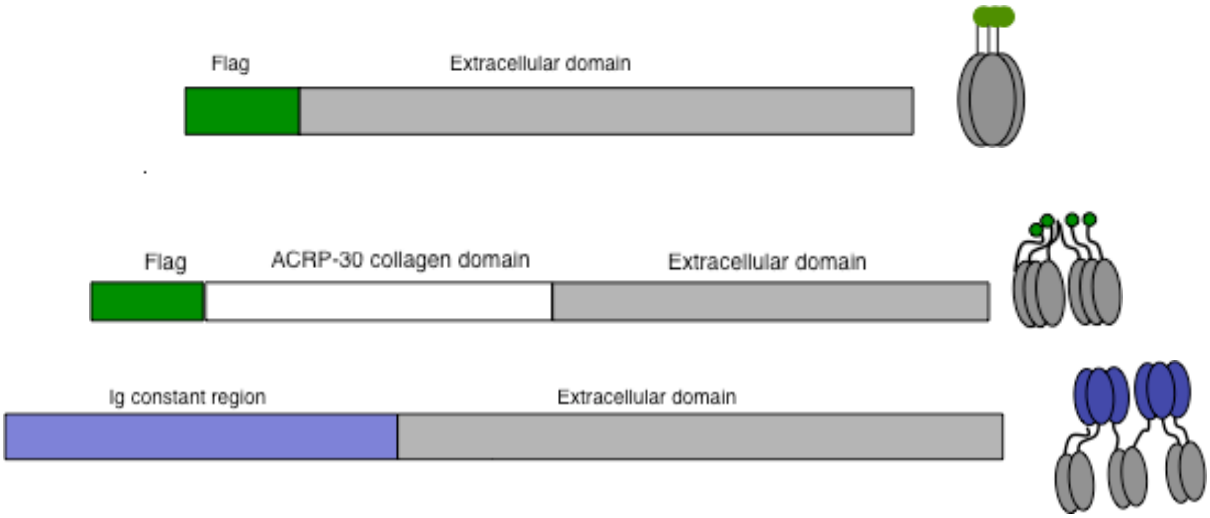


Fig 9. Recombinant TNF ligands

Schematic representation of Flag tagged ligands, Flag ACRP tagged ligands and Fc tagged ligands produced in mammalian cells. The extracellular segment containing the TNF homology domain is fused either to the Flag sequence or the constant region of human IgG1(Fc portion). The ACRP sequence was used in some ligands. It allows for the

hexamerisation and detection by ELISA (using cognate receptors) by strengthening the binding avidity.

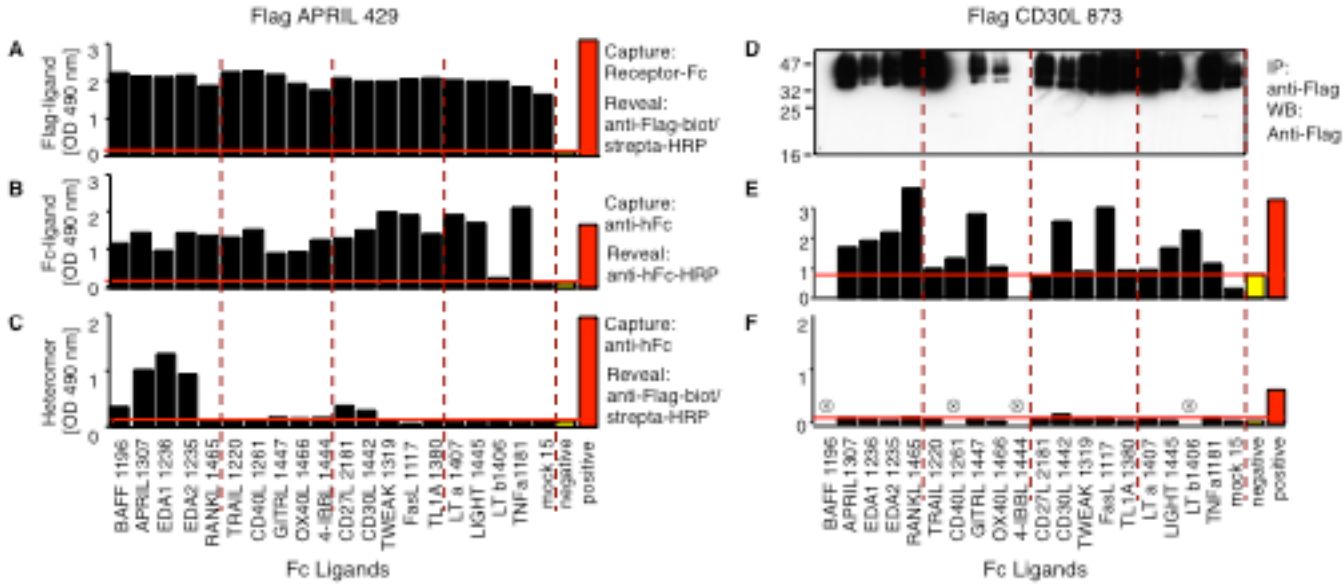


Fig 10.1. ELISA screen for the detection of heteromers in the TNF family.

Cell culture supernatants of 293T cells co-transfected with either Flag-hAPRIL (plasmid number 429) or Flag-hCD30L (plasmid number 873) and 19 different Fc-ligands (the numbers indicating the plasmid numbers used in the lab) were analyzed by ELISA. A. Flag-hAPRIL was captured with hBCMA-Fc and revealed with biotinylated anti-Flag followed by HRP-coupled streptavidin. B. Fc-ligands were captured with anti-Fc, and revealed with a second, HRP-coupled anti-Fc. C. Fc-ligands were captured with anti-Fc, and Flag-tagged ligands in the heteromer, if present, were revealed with anti-Flag. D. Flag-hCD30L was immunoprecipitated with anti-Flag, and revealed by Western blotting with anti-Flag. Panels E and F are like B and C. Positive controls are suitable Flag-ligand e.g. Flag-hBAFF in panel A, purified Fc-hFasL at 1 µg/ml in panels B and E or Flag-hLTα / Fc-hLTβ in panels C and F. A circled cross indicates that at least one ligand in the transfection was not expressed.

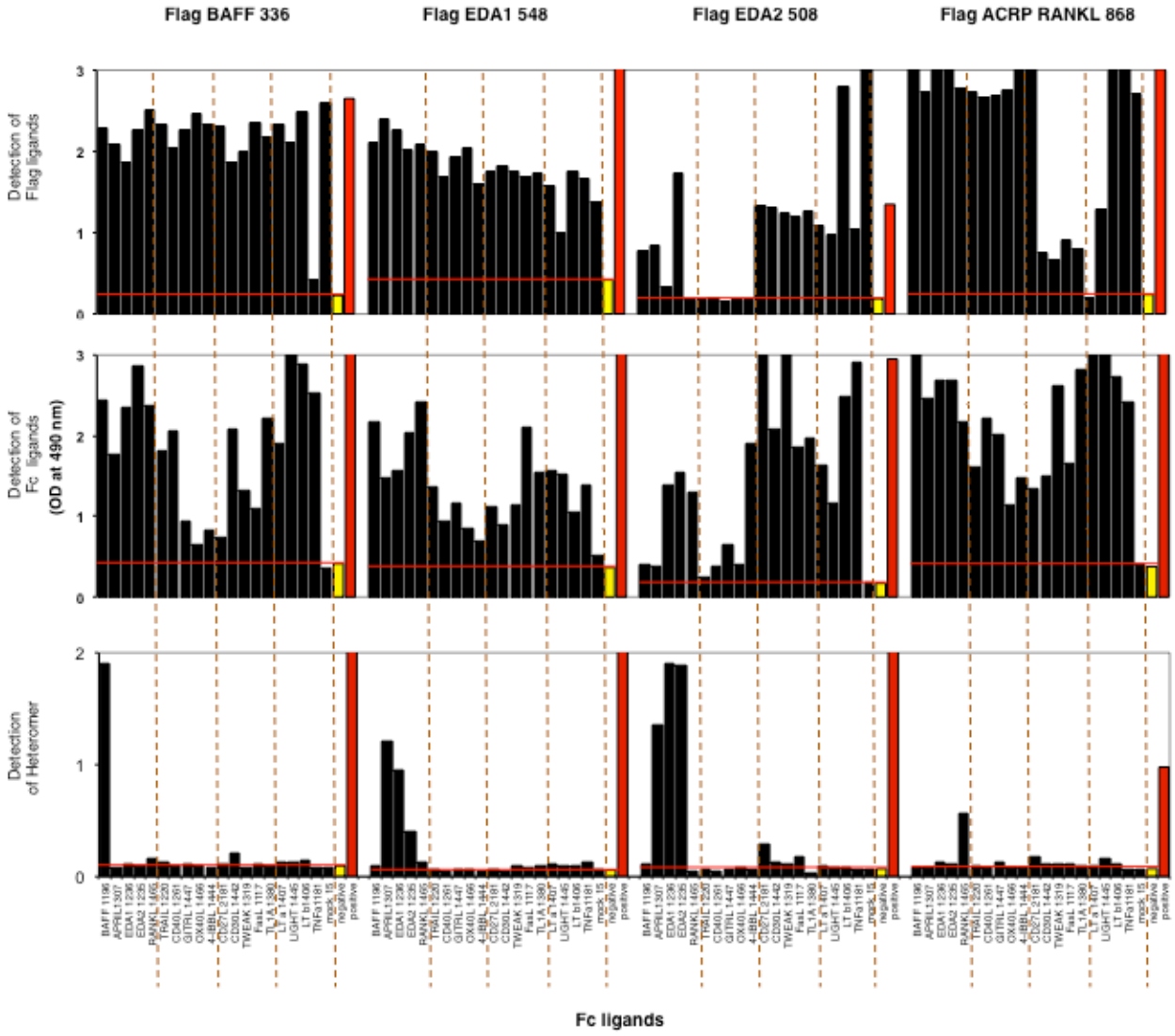


Fig 10.2. Screen for the detection of heteromers in the TNF family.

Cell culture supernatants of 293T cells co-transfected with respective Flag or Flag-ACRP tagged ligands (with indicated plasmid numbers) on top of the panel and 19 diferent Fc ligands (the plasmid numbers are as per designated in the lab) were analysed by ELISA to detect the expression of Flag and Fc ligands and to detect the heteromer formation as in Fig 10.1. Red line indicates limit of the negative control and red star indicates absence of ligands either not expressed or not done (ND).

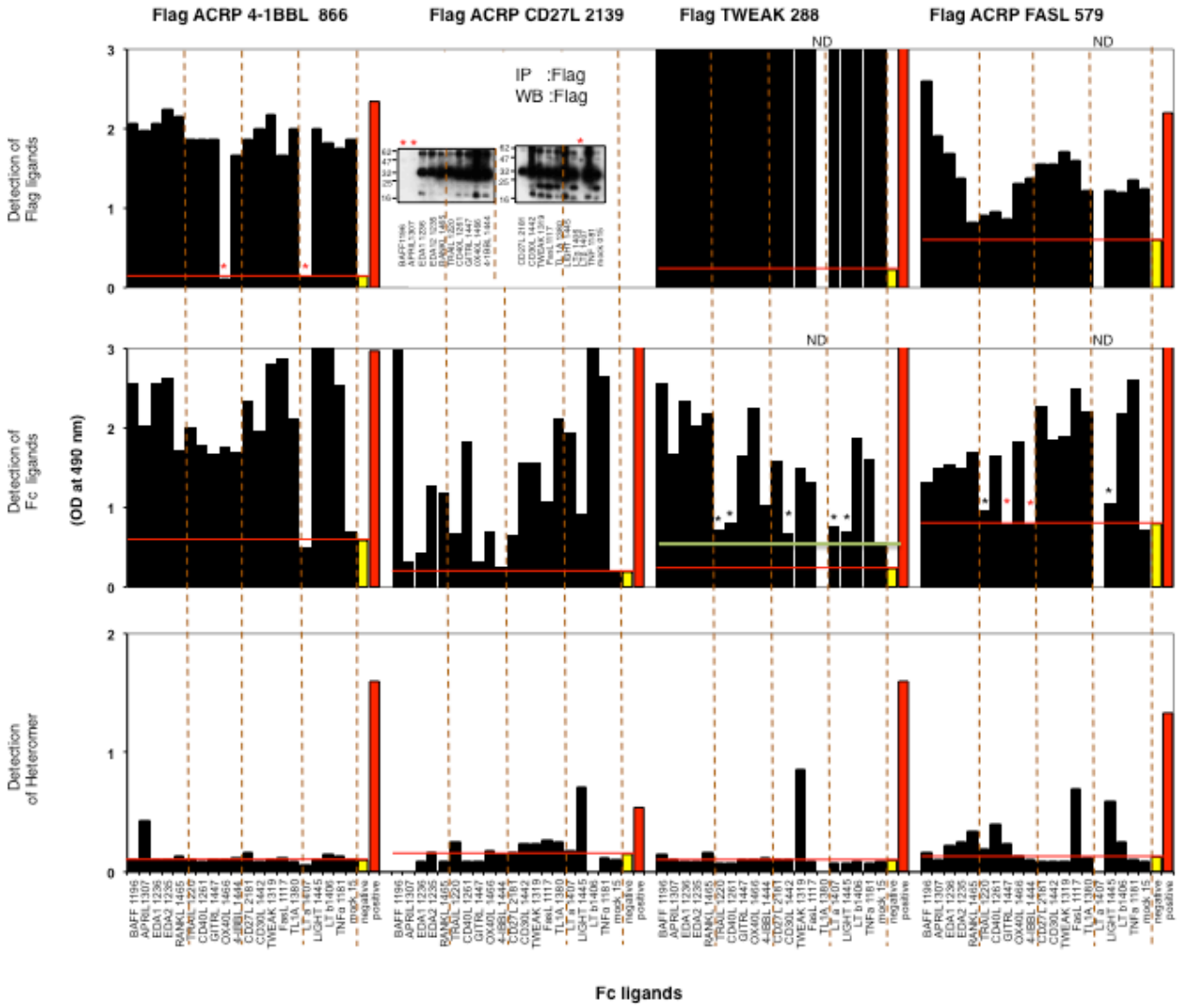


Fig 10.2. Screen for the detection of heteromers in the TNF family.

Cell culture supernatants of 293T cells co-transfected with respective Flag or Flag-ACRP tagged ligands (with indicated plasmid numbers) on top of the panel and 19 diferent Fc ligands (the plasmid numbers are as per designated in the lab) were analysed by ELISA and coimmunoprecipitation to detect the expression of Flag and Fc ligands and to detect the heteromer formation as in Fig 10.1. Red line indicates limit of the negative control, green line indicates the limit of the signal obtained from the empty vector control (as in some cases negative control is higher or lower than the mock 015 value). The red star indicates absence of ligands that are not expressed , Not done is indicated as ND and black star indicates possible minimal expression.

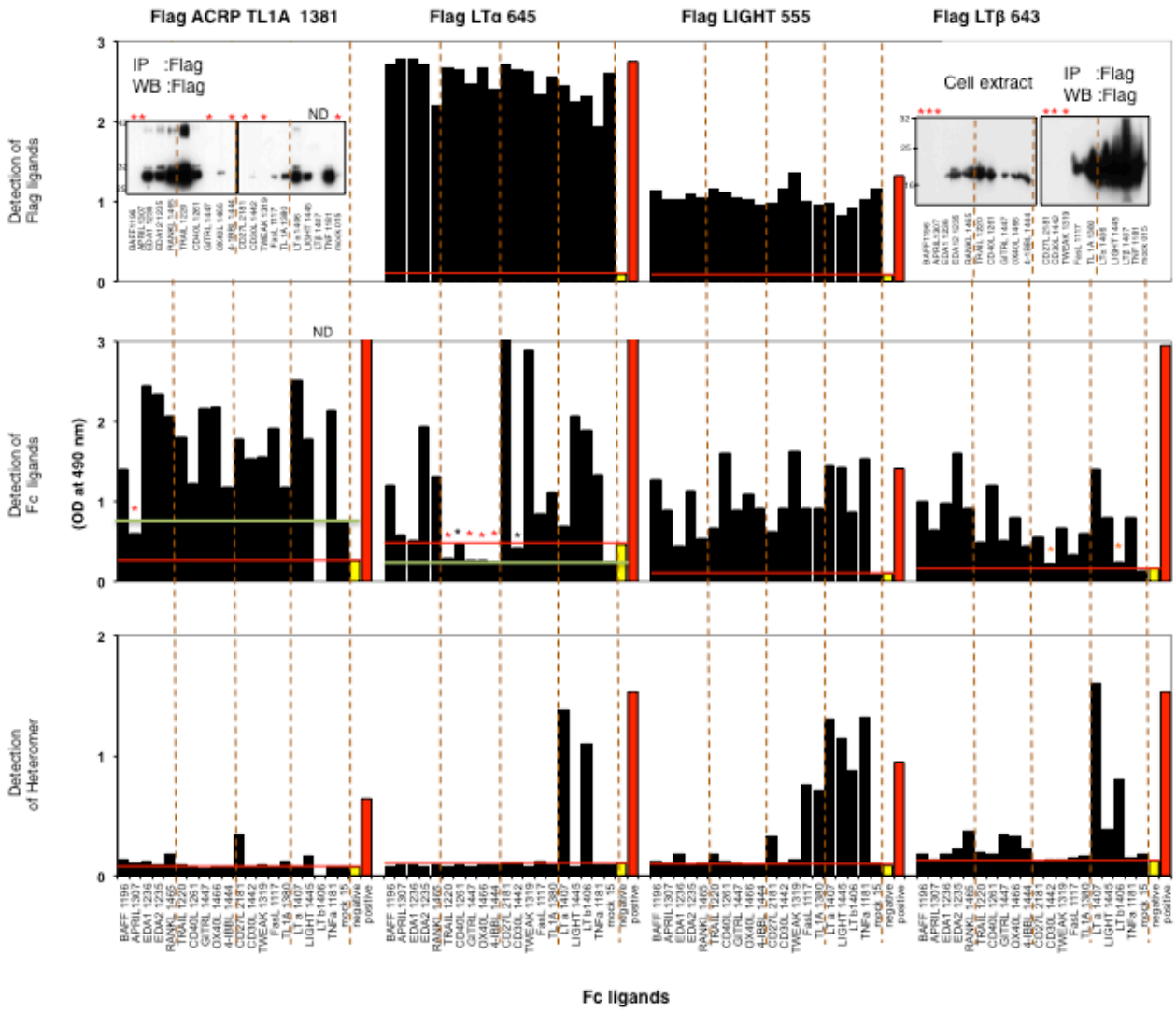


Fig 10.3. Screen for the detection of heteromers in the TNF family.

Cell culture supernatants of 293T cells co-transfected with respective Flag or Flag-ACRP tagged ligands (with indicated plasmid numbers) on top of the panel and 19 diferent Fc ligands (the plasmid numbers are as per designated in the lab) were analysed by ELISA and coimmunoprecipitation to detect the expression of Flag and Fc ligands and to detect the heteromer formation as in Fig 10.1. Red line indicates limit of the negative control, green line indicates the limit of the signal obtained from the empty vector control (as in some cases negative control is higher or lower than the mock 015 value). The red star indicates absence of ligands that are not expressed , Not done is indicated as ND and black star indicates possible minimal expression.

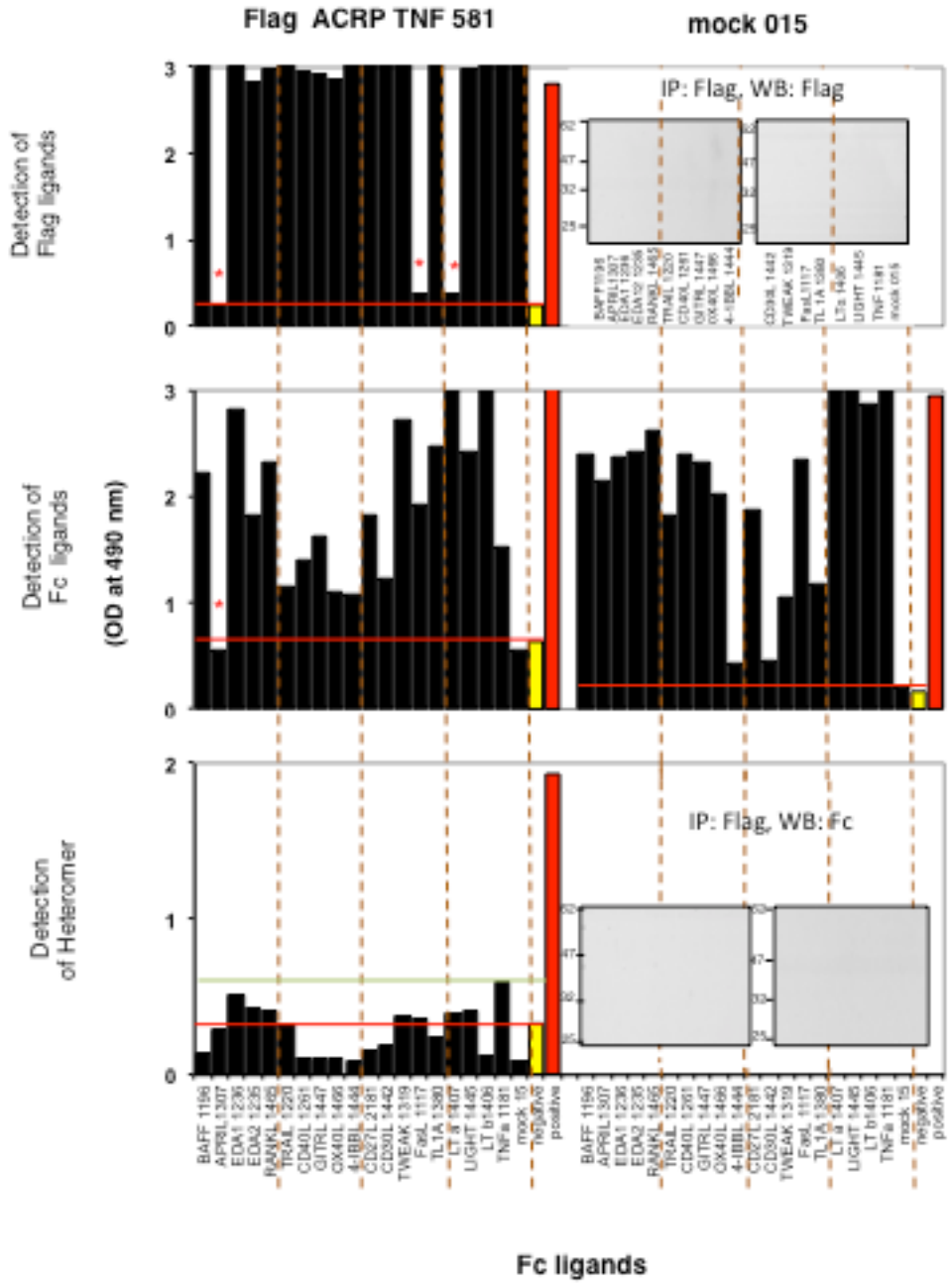


Fig 10.4. Screen for the detection of heteromers in the TNF family.

Cell culture supernatants of 293T cells co-transfected with respective Flag or Flag-ACRP tagged ligands (with indicated plasmid numbers) on top of the panel and 19 diferent Fc ligands (the plasmid numbers are as per designated in the lab) were analysed by ELISA and coimmunoprecipitation to detect the expression of Flag and Fc ligands and to detect the heteromer formation as in Fig 10.1. Red line indicates limit of the negative control, green line indicates the limit of the signal obtained from the empty vector control (as in some cases negative control is higher or lower than the mock 015 value). The red star indicates absence of ligands that are not expressed , Not done is indicated as ND and black star indicates possible minimal expression.

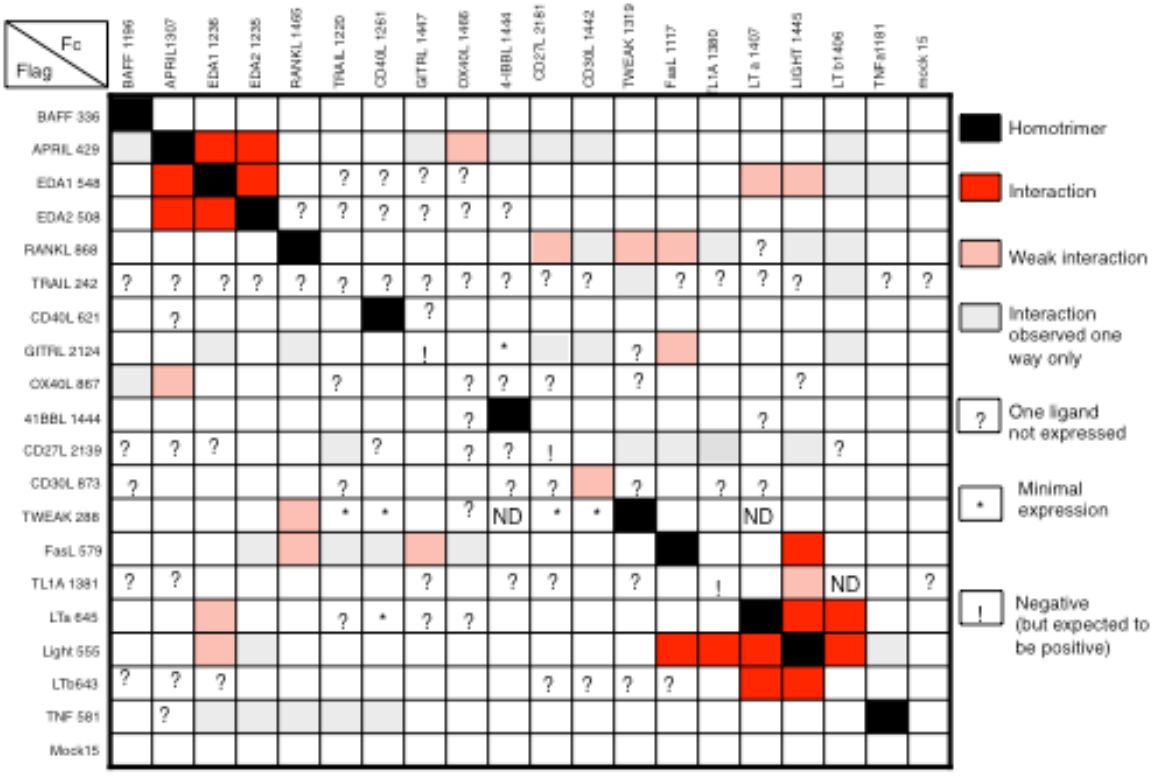


Fig 11. Summary of interactions detected in the ELISA-based screen for heteromers.

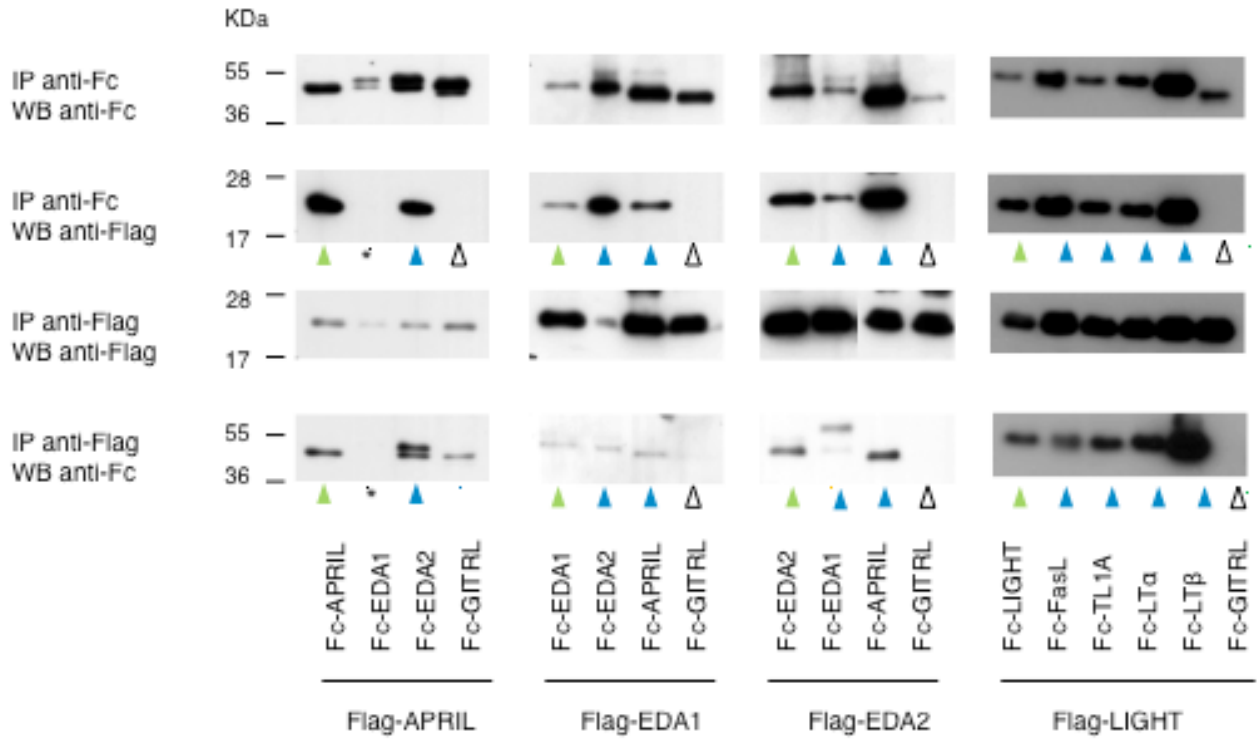


Fig 12. Co-immunoprecipitation of Flag-ligands and Fc-ligands to confirm the heteromers.

The indicated Flag-tagged and Fc-tagged ligands were co-transfected in 293T cells. Cell supernatants were immunoprecipitated with anti-Flag or protein A (anti-Fc) beads and revealed by western blot with anti-Flag or anti-Fc antibodies, as indicated. The green traingles represent formation of the expected homomer (positive control). The blue traingles indicate formation of the heteromer. The open triangles indicate no heteromer formation (negative control). The black star indicates that the immunoprecipiatation did not work while the blue star indicates the signal on the IP anti-Flag and WB anti-Fc may be non-specific and it needs to be repeated (with a pre-clearing step).

Co-immunoprecipitations (Fig. 12) were done to confirm the strong interactions as marked in red in Fig. 11 (summary of the screen). The ligands, which showed the interactions by ELISA, were re-expressed in 293 T cells and supernatants were immunoprecipitated for either Flag or Fc followed by immunoblotting. As seen on the blots, interactions between Flag-APRIL and Fc-EDA1 showed some discrepancies. Upon immunoprecipitation of Flag and anti Fc the respective ligands were shown to be present by the western blot anti-Flag or anti-Fc, but their putative interaction partner co-IP along with them were not revealed. It is possible that the level of Flag APRIL in this transfection was low enough to sufficiently co-IP EDA1 along with it and vice-versa. However, immunoprecitpations of Flag EDA1 with the Fc-ligands APRIL, EDA1, EDA2 and with the negative control of Fc-GITRL clearly indicate an interaction. Also, the interaction of EDA2 with all its above-mentioned partners worked in both the directions. For interaction of LIGHT with FasL, TL1A, LTα and LTβ, co-immunoprecipitation was performed in one direction only, but indeed revealed some association between these ligands.

3.1.3. Screen for novel receptor specificity.

Newly identified heteromers were tested for their putative binding to orphan receptors (Fig 13). For this purpose, a FACS based assay was used as described [40]. This assay requires no ligand or receptor-specific antibodies and no protein purification. The extracellular domains of the receptors are fused to the C-terminal portion of TRAIL-R3, containing 5 repeats of a 15 amino acid sequence followed by a GPI anchor addition signal that targets the receptor to the membrane. This portion can be recognized by a monoclonal antibody (rat anti-hTRAIL-R3, LEIA), that allows monitoring of surface expression of the fusion receptors by FACS (Fig. 13B). Fusing receptors to a glycolipid anchor circumvents problems associated with use of full-length receptors like induction of cell death, intracellular retention or secretion in the case of OPG and DcR3. The receptor-expressing cells co-express EGFP, while non-transfected EGFP-negative cells serve as internal negative controls.

Cell supernatants of the heteromeric combination of ligands containing Flag and Fc ligands were used to stain the receptors. The heteromers showed no binding to any of the orphan receptors (Fig. 13A) although orphan receptors are expressed (Fig. 13B). The activity for some of the ligands were confirmed by staining on their cognate receptors (Fig. 14) while this was not confirmed for the rest of the series though it is unlikely that the ligands are not active.

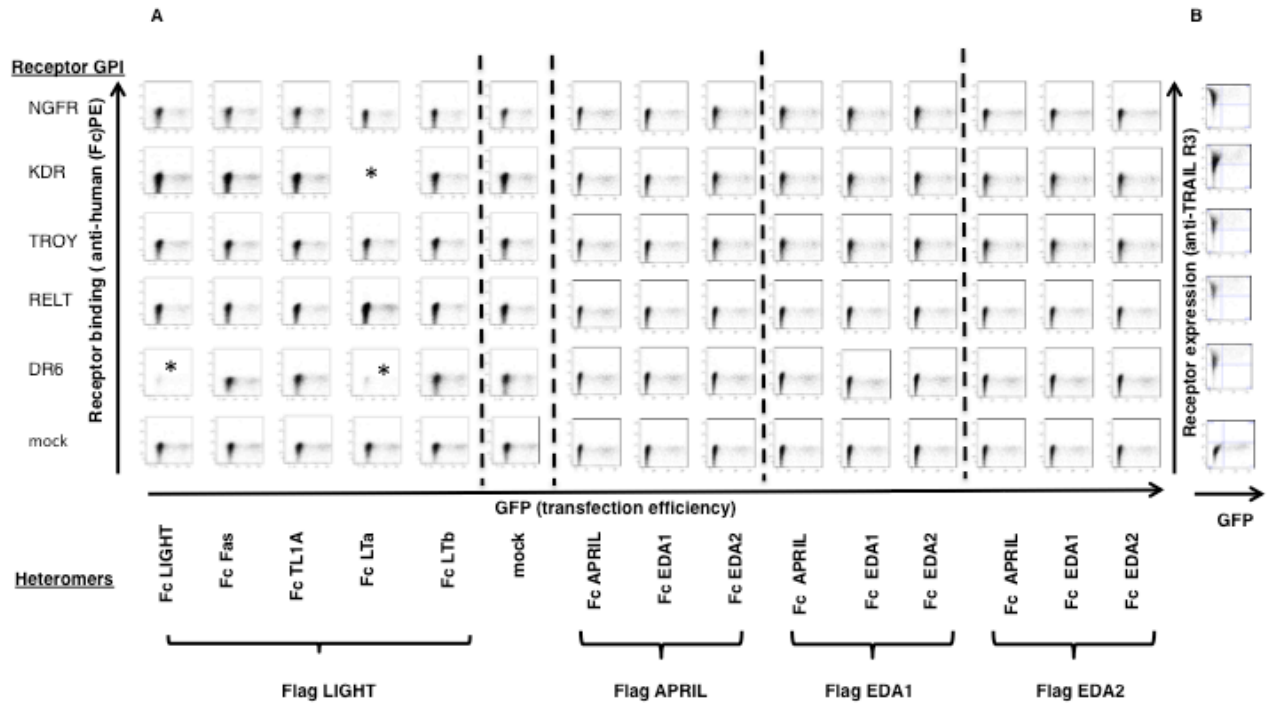


Fig 13. Interaction of heteromers with orphan receptors

*Human receptor-GPI's are expressed in 293T cells A. Binding of homotrimers to orphan receptors. Binding of ligands to receptors was revealed with anti-human (Fc) PE-antibody B. staining with anti-h TRAIL3 (rat) antibody indicating receptor expression and GFP expression indicating transfection efficiency. * Problem in staining/detection.*

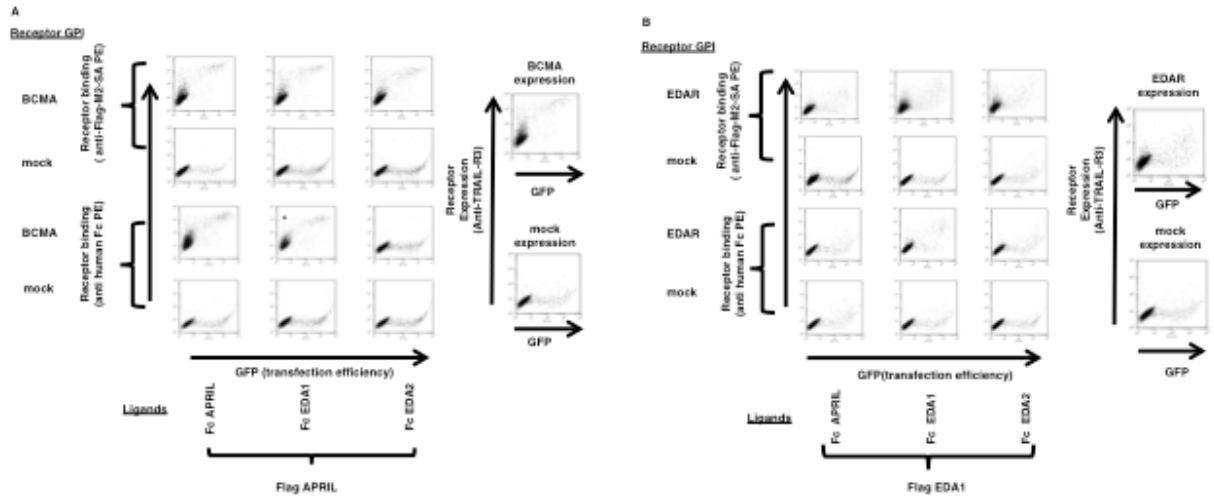


Fig 14. Interaction of heteromers with cognate receptors

Human receptor GPI's were expressed in 293T cells with GFP like in Fig 13 and stained with supernatants of ligands as indicated. Binding was detected with biotinylated anti-Flag (M2) antibody followed by PE coupled Streptavidin or anti human Fc-PE. Receptor expression is shown by staining with anti-TRAILR3 antibody. A. Staining of Flag APRIL-Fc APRIL, Flag APRIL-Fc EDA1, Flag APRIL-Fc EDA2 on BCMA and empty vector control B. Staining of Flag

*EDA1-Fc APRIL, Flag EDA1- Fc EDA1, Flag EDA1-Fc EDA2 on EDAR and empty vector. * Binding of Flag APRIL-Fc EDA1 revealed by Fc staining on BCMA was unexpected. This staining was not repeated for confirmation.*

3.2. Characterisation of BAFF and APRIL heteromers.

3.2.1. Aims and objectives of research.

As described in section 1.3.2, a few studies reported heteromer formation between the closely related ligands BAFF and APRIL. BAFF and APRIL heteromers were found in sera of patients with autoimmune diseases. The aim of the present study is to further characterize these heterotypic interactions with respect to receptor binding and signaling ability.

3.2.2. Single chain recombinant heteromers.

3.2.2.1. Validation of single chain method.

Based on the results of the screen (section 3.1.2), it seemed to be difficult to study BAFF and APRIL interactions using tagged ligands. Based on the studies of Klaus Pfizenmaier [201] single chain trimers were designed (Fig. 15A). The goal was to produce defined heterotrimers that can be used to test their binding to desired receptors or their activity for functional assays. In a single chain trimer, short peptides link the N- and C-termini of adjacent monomers that are spatially close one to each other (Fig. 15B). A Flag tag was attached to the N-terminus in place of the trans-membrane segment of the natural protein.

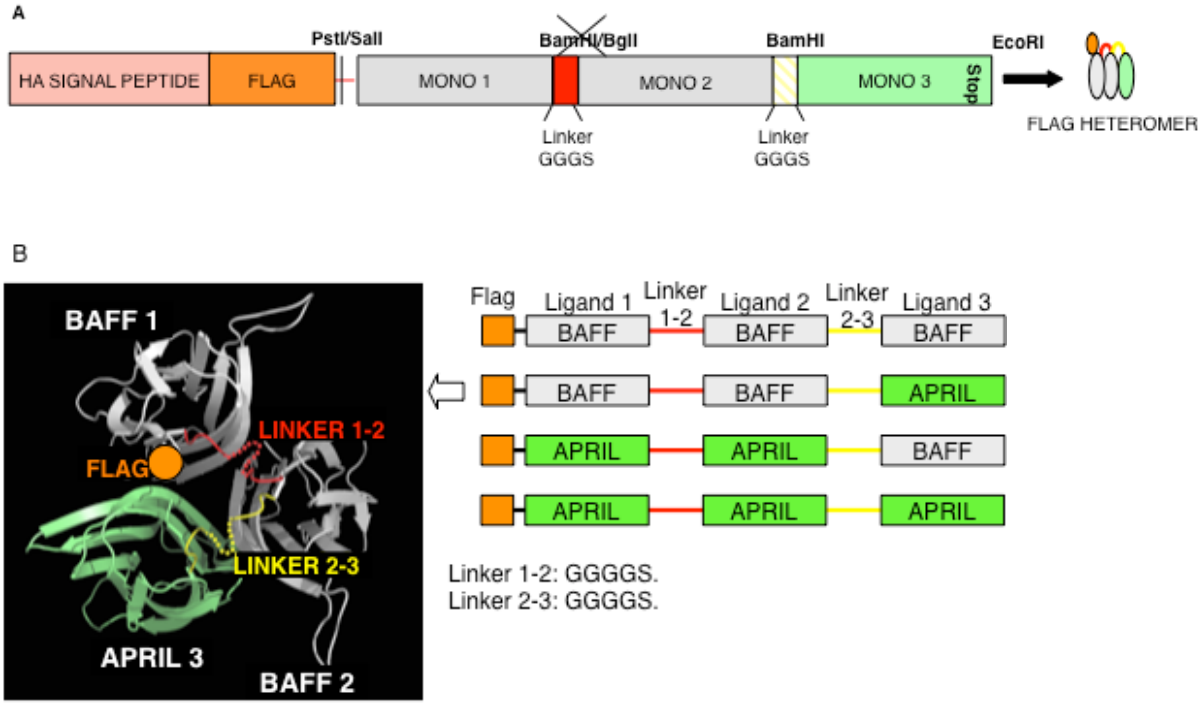


Fig 15. Single chain trimers Cloning and structure.

A. Molecular cloning strategy of single chain trimers. Flag-tagged vector with HA leader sequence was used as backbone. THD's of TNF ligands were cloned using the indicated restriction enzymes in a cloning cassette with linkers connecting the monomers. B. Predicted structure of single chain homotrimers and heterotrimers of BAFF and APRIL

As the C-termini of ligands can play a structural role in the THD, addition of linkers can potentially interfere with ligand folding, expression, secretion and/or function. Therefore single chain LTαβ heteromers were designed as positive controls to validate this strategy. Flag-tagged single chain LT-[ααα], LT-[ααβ], LT-[ββα] and LT-[βββ] were all expressed and secreted (Fig. 16 C) as seen by immunoblotting of cell supernatants. Interestingly, single chain heteromers displayed the expected receptor binding specificities, with LT-[ααα] binding to TNFR1 but not LTβR, and LT-[ββα] binding to LTβR (Fig 16 E).

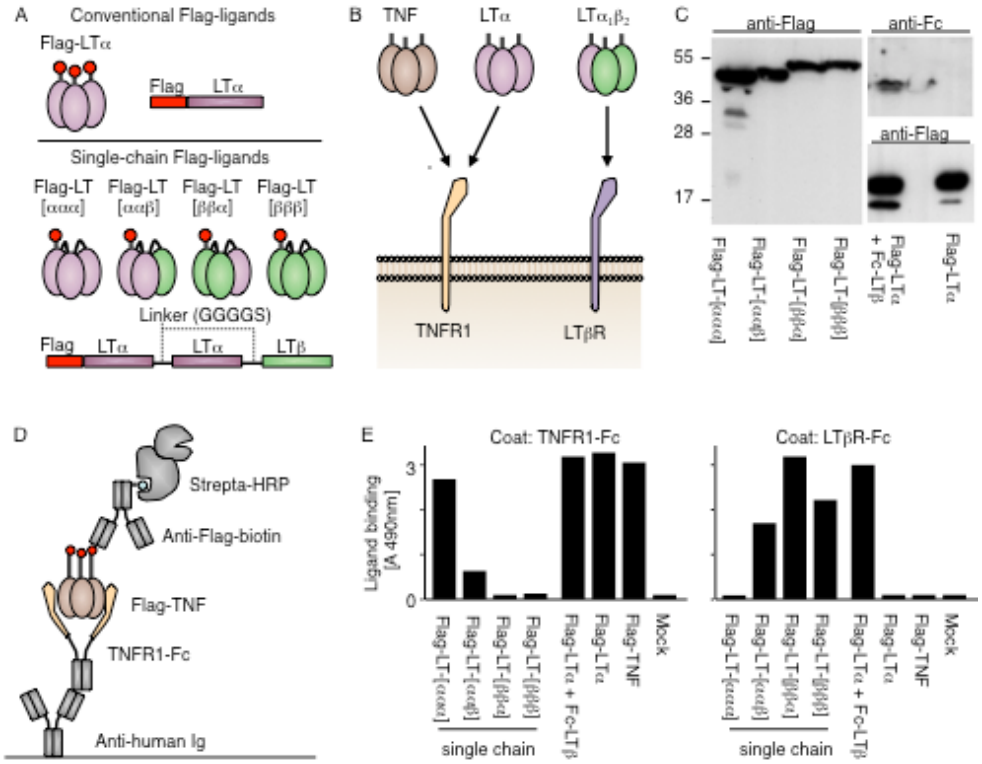


Fig 16. Validation of the single-chain heteromer strategy with lymphotoxin α and β.

A. Cartoon showing conventional Flag-ligands (three individual Flag-tagged monomers associated via non-covalent interactions) and single-chain Flag-ligands (the C-terminus of monomer 1 is linked by a short peptide sequence to monomer 2, that itself is linked to monomer 3). It is noteworthy that the cartoon reflects the close proximity of the N- and C-termini of adjacent ligands. B. Receptor-binding specificity of hTNF, hLTα and of the hLTαβ heteromer. C. Supernatant of 293T cells transiently transfected with single chain lymphotoxin homotrimers (Flag-hLT- [ααα] and -[βββ]) or heterotrimers (Flag-hLT- [ααβ] and -[ββα]), or with conventional Flag-hLTα ± Fc-hLTβ were analyzed by

western blotting with anti-Flag and anti-Fc antibodies. D. Experimental strategy used in panel E to monitor receptor-ligand interactions by ELISA to measure binding of the indicated ligands to hTNFR1-Fc (left) or hLTβR-Fc (right). Ligand binding was revealed with an anti-Flag antibody.

3.2.2.2. Size characterization of single chain ligands

Binding of an heteromer to a receptor could be explained in two ways: either a heterotrimeric ligand really forms and displays the observed receptor-binding specificities, or the proteins form larger structures containing both homotrimeric ligands each of which can bind to its cognate receptor(s).

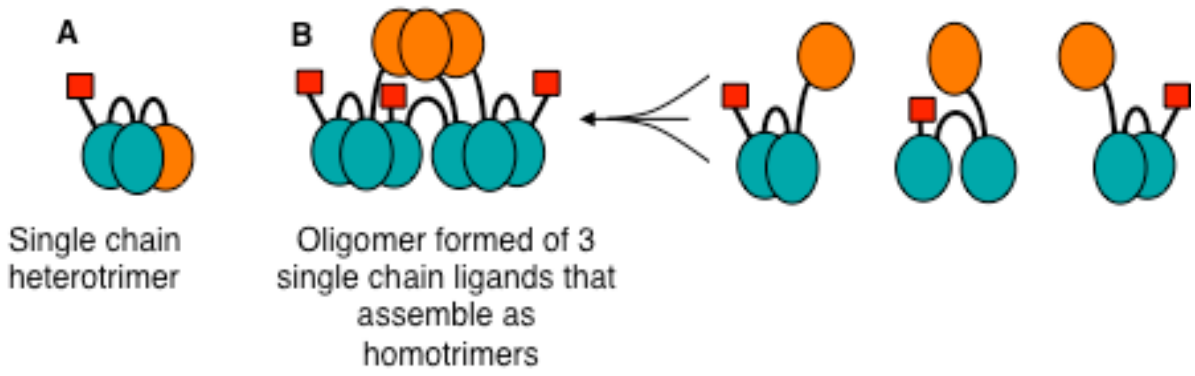


Fig 17. Schematic representation of how single chain ligands may assemble. *A. Single chain heterotrimer. B. Putative larger oligomer containing two homotrimers of the first ligand and one homotrimer of the second ligand.*

In order to reduce false positives coming from homotrimeric ligands formed in larger aggregates, single chain heteromers were fractionated by size exclusion chromatography, and eluted fractions were tested for their content of Flag-tagged protein by immunobloting and for their receptor-binding specificity by ELISA. An irrelevant heteromer of BAFF and TNF was used as control. For all ligands, a peak at around 40 kDa was identified, which most probably corresponds to homotrimers or heterotrimers. Most ligands, including conventional and single chain Flag-TNF, also displayed higher molecular weight material called. The trimeric fraction of TNF and of single

chain TNF bound its cognate receptor, as expected (Fig 18 A and B). Regarding the receptor-binding activity of the negative control BAFF-BAFF-TNF, it was exclusively found in oligomers, suggesting that this heteromer is indeed inactive and that the receptor binding is due to homotrimers of TNF and BAFF present in the oligomers (Fig. 18 C).

Single chain APRIL-APRIL-BAFF heterotrimer bound BCMA (Fig 18 D). The later result indicates that the preparation of APRIL-BAFF heteromers of defined stoichiometry should be possible.

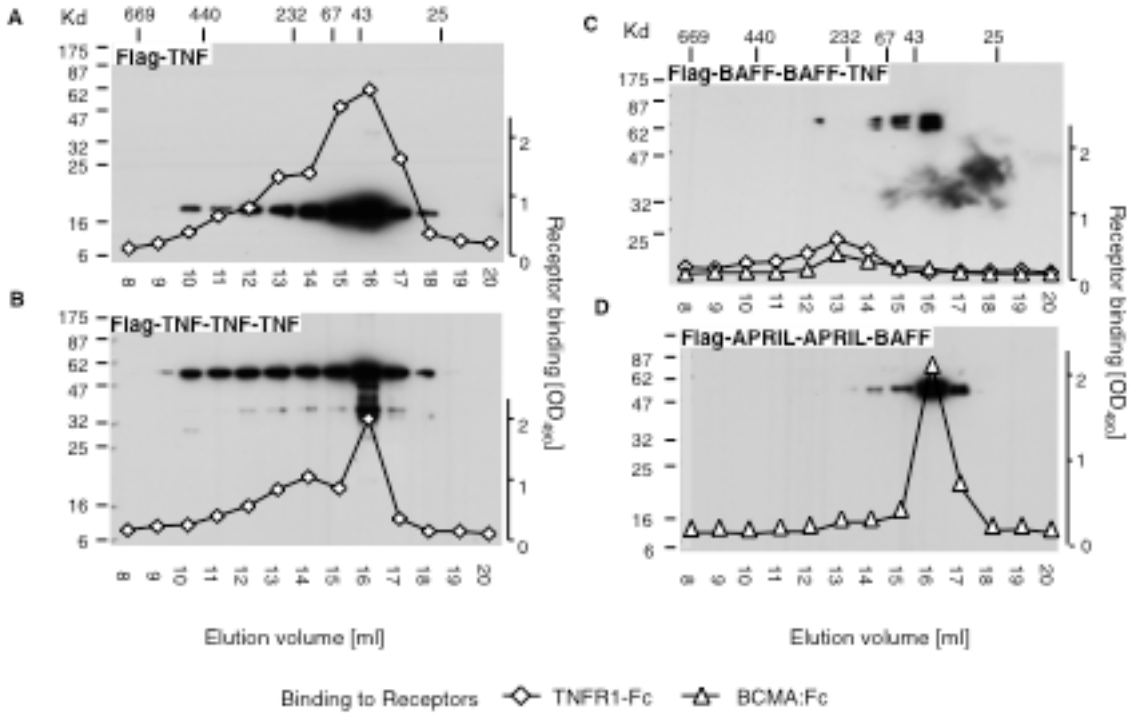


Fig 18. Gel permeation chromatography of conventional and single chain Flag-ligands reveals the presence of both homo and heterotrimers and of higher molecular weight oligomers. *Concentrated serum-free supernatants of transfected 293T cells (TNF and BAFF-TNF) or purified BAFF-APRIL heteromers were loaded onto a Superdex-200 gel filtration column and eluted with PBS. Fractions of 1 ml were collected, of which one half was precipitated with 5% ice-cold trichloroacetic acid and analyzed by immunoblot with anti-Flag. The other half of the fraction was used to monitor receptor-binding by ELISA. A. Conventional Flag-TNF. B. Single chain Flag-TNF. C. Single chain Flag-BAFF-BAFF-TNF heteromer. D. Single chain APRIL-APRIL-BAFF heteromer. Sizes of SDS-PAGE molecular weight (in kDa) are indicated on the left, and those of size exclusion chromatography on the top of the figures. Open diamonds: binding of Flag-ligands to TNFR1-Fc by ELISA [OD490]. Open triangles: binding of Flag-ligands to BCMA-Fc.*

3.2.2.3. Production of recombinant BAFF APRIL heteromers.

Upon validation of the concept, the next step was to express single chain BAFF and APRIL in all stoechiometries. Single chain ligands were first cloned in a Flag vector and then subcloned into a Fc vector. The dimeric Fc tag should in theory incorporate two single chain ligands in the same molecule, which may be a signaling advantage for receptors known to respond to oligomerized ligand (see section 1.2.4). The ligands were thereafter expressed in mammalian cells and analysed by immunoblotting using anti-Flag, anti-Fc, anti-APRIL or anti-BAFF antibodies (Fig 19).

It was not always easy to get equal protein expression levels in different transfections. To circumvent this problem and to improve expression efficiency, stable clones in CHO cells were established. In an attempt to increase production efficiency, recombinant ligands were expressed from plasmids containing an IRES-GFP to help for selection of the best producers based on the fluorescence of GFP (Fig. 20).

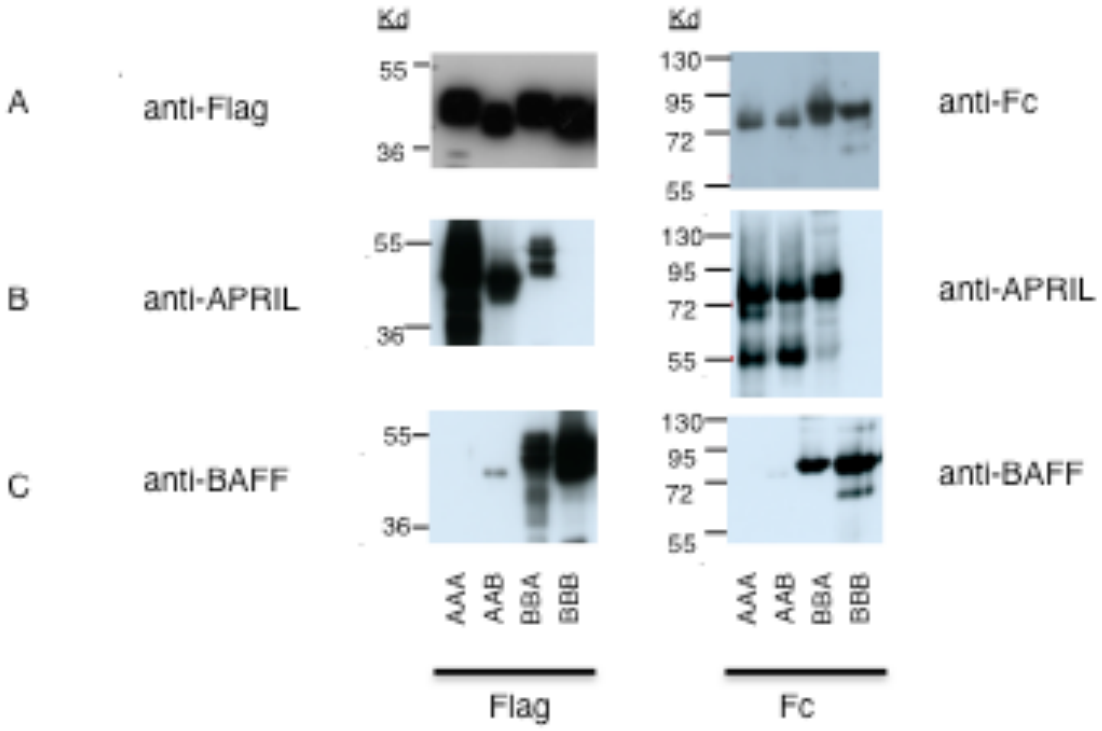


Fig 19. Single chain homotrimers and heteromers of BAFF and APRIL .

Cell supernatant of Flag and Fc-tagged single chain homo and heteromers of BAFF and APRIL expressed in 293T cells. Expression is analysed by western blot using A. anti-Flag (M2) or anti-human Fc, B. anti-APRIL (Aprily2) and C. anti-BAFF (Buffy2) antibodies.

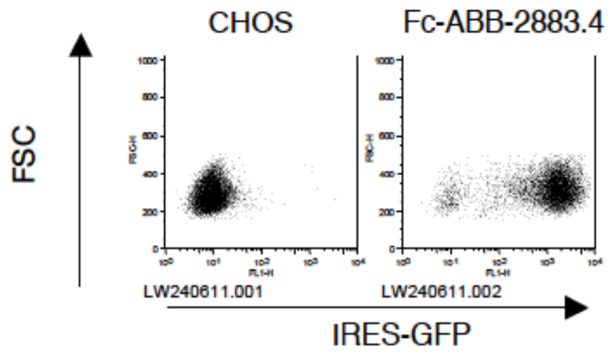


Fig 20. Stable CHO cells transfected with Fc-ABB in an IRES-GFP-containing vector. *Untransfected CHO cells are shown as control. A GFP-positive clone producing Fc-ABB is shown on the right. A proportion of GFP-negative cells was often present, even after sub-cloning, and may represent dead cells having lost their GFP.*

3.2.2.4. Expression and purification of single-chain BAFF, APRIL and BAFF-APRIL heteromers.

Expression plasmids for Flag- and Fc-tagged BAFF, APRIL and BAFF-APRIL heteromers were constructed, usually with GFP co-expression thanks to an internal ribosomal entry site, and used for the generation of stable CHO cell lines. Clones were established either randomly, or by 2 or 3 rounds of FACS sorting to select green cells. Proteins were affinity purified with anti-Flag or Protein A, followed by size fractionation on a Superdex-200 gel permeation chromatography column. All samples contained various ratios of high molecular weight aggregates and lower molecular weight species eluting in fraction 15 for single chain Flag-ligands and in fraction 12 for

single chain Fc-ligands (Fig. 21). Yield obtained in the “small” molecular weight fractions are summarized in Table 1.

S200-purified single-chain ligands were analyzed by Western blot. As expected, single-chain heteromers reacted with both anti-BAFF and anti-APRIL antibodies, in addition to the anti-tag antibody (Fig. 22).

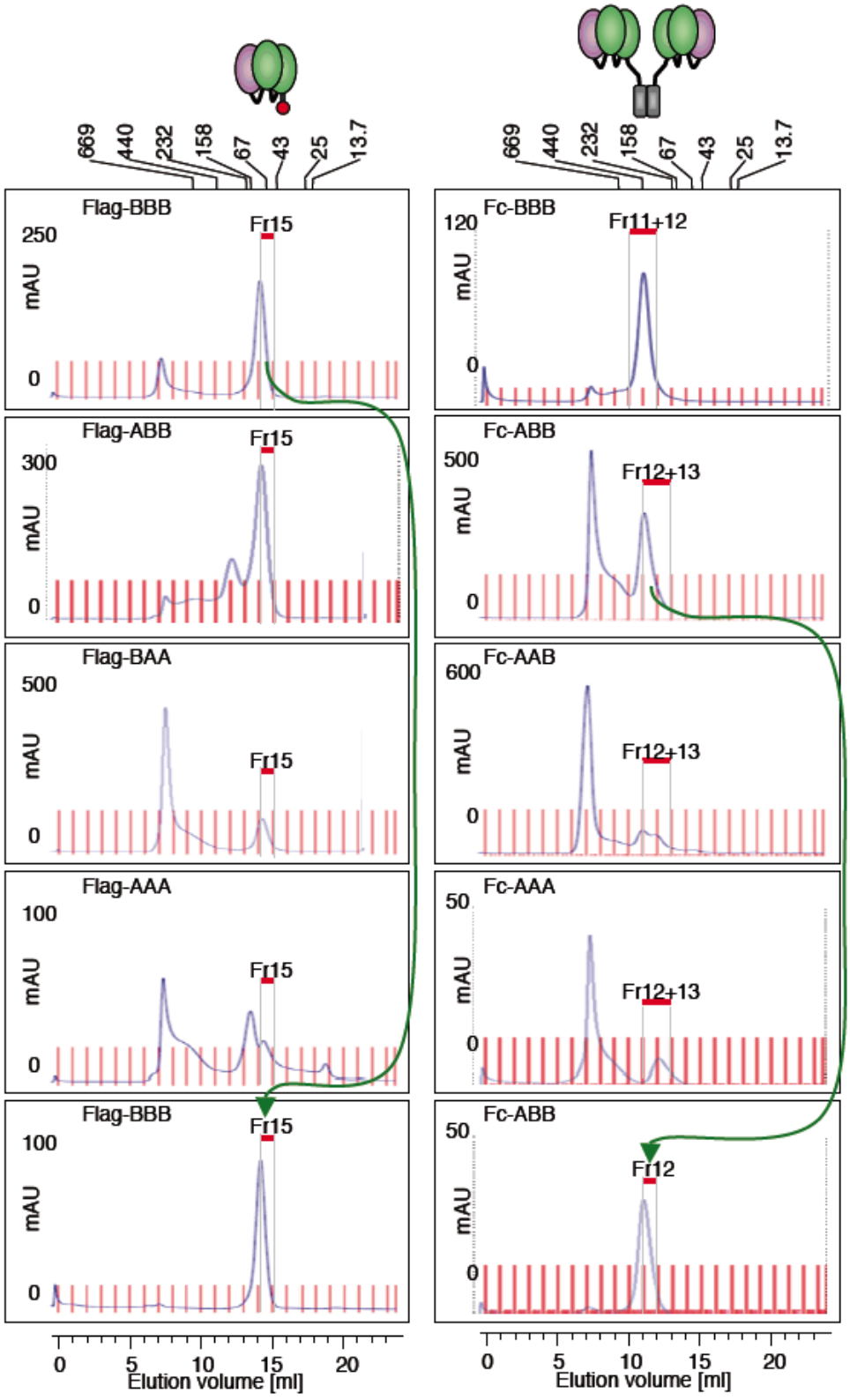


Fig 21. Gel filtration profiles of single-chain BAFF and APRIL heteromers. *Elution profiles of affinity-purified Flag-tagged (left) and Fc-tagged (right) single-chain ligands from a Superdex-200 column. Fractions collected for subsequent characterization of the proteins are indicated. For Flag-hBBB and Fc-hABB, the indicated fractions were re-analyzed.*

Table 1. Purification yields after affinity purification and S200 size fractionation (for 1 L of cell supernatant).

	Flag				Fc			
Single-chain ligand	AAA	ABB	BAA	BBB	AAA	ABB	BAA	BBB
Yield in S200 fraction(s) of interest	40 µg	160 µg	400 µg	220 µg	<10 µg	200 µg	600 µg	160 µg

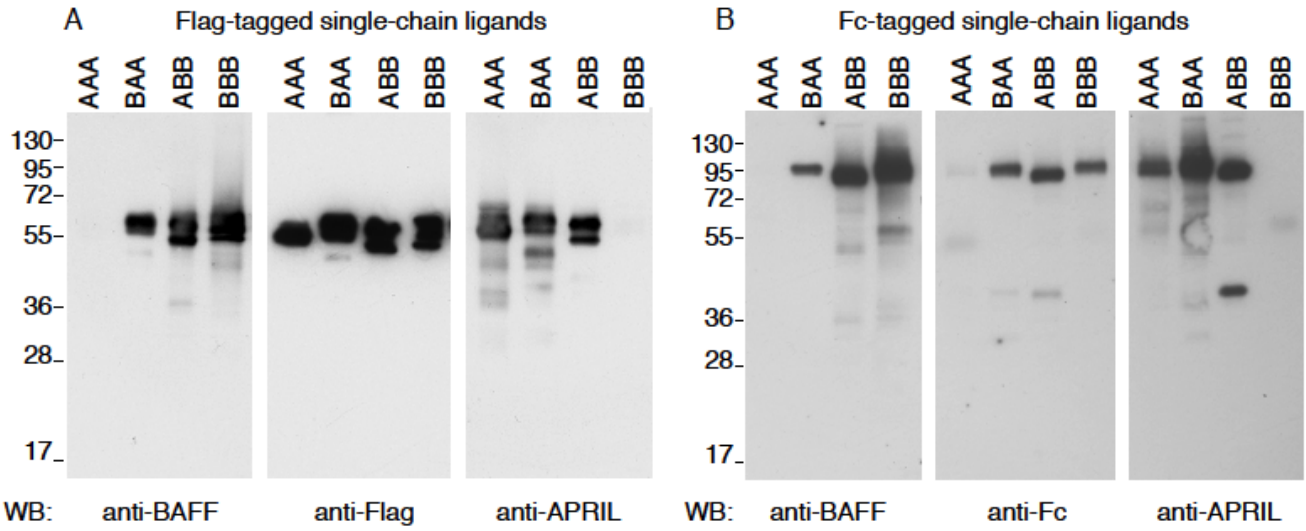


Fig 22. Western blot analysis of purified, size-fractionated Flag- and Fc-tagged single-chain ligands.

A. 200 ng of single-chain, Flag-tagged hAPRIL (A) and hBAFF (B) homo- and heteromers eluting in Fr15 of the Superdex-200 column (see Fig. 20) were analyzed by western blotting with the indicated antibodies.

B. 100 ng of single chain, Fc-tagged single-chain ligands eluting around fractions 12 (see Fig 20) were analyzed under reducing conditions as in A). The real amount of Fc-hAAA loaded was obviously less than 100 ng.

Table 2. Molecular weight estimations for single-chain BAFF and APRIL heteromers

		MW [kDa] Theoric	N-link Theor	MW [kDa] Theoric + N-linked	Approximative MW [kDa] by WB DTT	Approximative MW [kDa] by S200	WB/S200 ratio
ps2879	Flag-AAA	49.3	6	64.3	58	86	1.5
ps2889	Flag-BAA	50	5	62.5	59.8	93	1.6
ps2886	Flag-ABB	50.9	4	60.9	58.9	101	1.7
ps2890	Flag-BBB	51.8	3	59.3	58	96	1.7
ps2876	Fc-AAA	75.2	7	92.7	97.5	272	2.8
ps2877	Fc-BAA	76	6	91	97.5	335	3.4

ps2883	Fc-ABB	76.9	5	89.4	92.4	509	5.5
ps2680	Fc-BBB	78	4	88	100.3	509	5.1

Single chain ligands migrated by SDS-PAGE with sizes of about 60 kDa and 100 kDa for Flag and Fc-tagged ligands, which are higher than the predicted molecular masses of about 50 and 75 kDa. For Flag-tagged ligands, the difference is most probably due to N-linked glycosylation. The difference of about 40 kDa between Flag-tagged and Fc-tagged ligands can however not be entirely explained by the presence of the 25 kDa Fc portion. We hypothesize that the rod-like structure (PQPQPKPQPKPEPEGS) that we have introduced between the Fc and the single-chain ligand could be responsible for this peculiar migration. Single-chain Flag-tagged ligands migrated on S200 with a molecular weight 1.5 to 1.7 bigger than the expected one (Table 2). We find it unlikely that these molecules would assemble as dimers, and favour the hypothesis that the proteins have either an elongated shape and/or that the numerous and voluminous N-linked glycans account for this size difference. Regarding Fc-tagged AAA and BAA ligands, their apparent sizes correspond relatively well to the sum of the 50 kDa dimeric Fc plus 2 Flag-tagged single-chain ligands. Fc-tagged ABB and BBB are however bigger than expected and might represent dimers of the structure schematized above the S200 profiles in Fig. 21.

3.2.3. Receptor binding specificity of BAFF-APRIL heteromers.

Flag- and Fc-tagged single chain ligands post S200 were analysed for binding to the BAFF and APRIL receptors. The receptors used were either in the form of a receptor-Fc, where the binding for Flag ligands were detected by ELISA using a biotinylated anti-Flag (M2) antibody (Fig 23), or expressed as receptor-GPI in HEK 293T cells, where the binding of any ligand can be revealed with appropriate anti-tag secondary reagents. A heteromer consisting of two molecules of BAFF and one molecule of APRIL bound to all three receptors BAFFR, TACI and BCMA and hence retains the

properties of BAFF for binding to BAFFR, whereas a heteromer consisting of one molecule of BAFF and two molecules of APRIL had a binding pattern resembling that of APRIL, with binding to TACI and BCMA only, but not BAFFR. It therefore seems that at least two monomers of the same kind in an assembled trimer are required to bind a specific receptor.

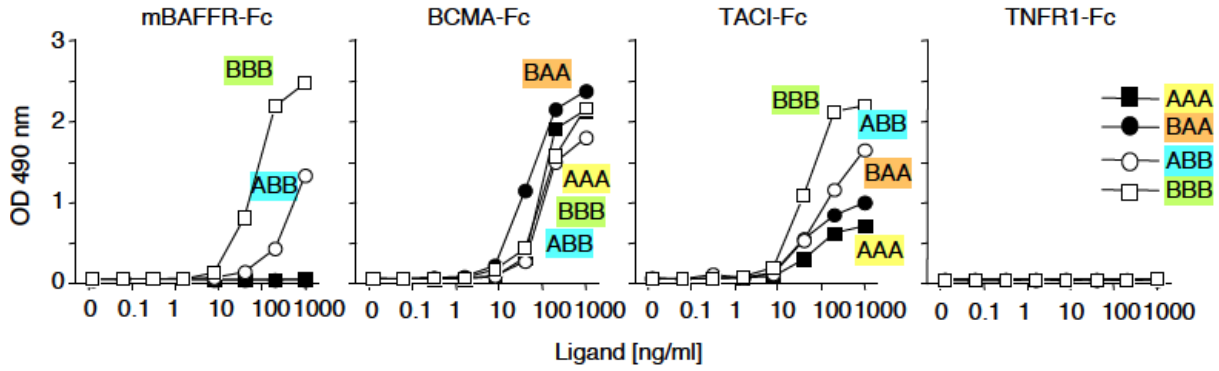


Fig 23. Binding specificity of Flag-tagged BAFF and APRIL single chain heteromers to plate-bound receptors. *The binding of Flag-tagged single-chain hBAFF and hAPRIL heteromers to the indicated human receptors-Fc was monitored by ELISA, according to the principle shown in Fig16.*

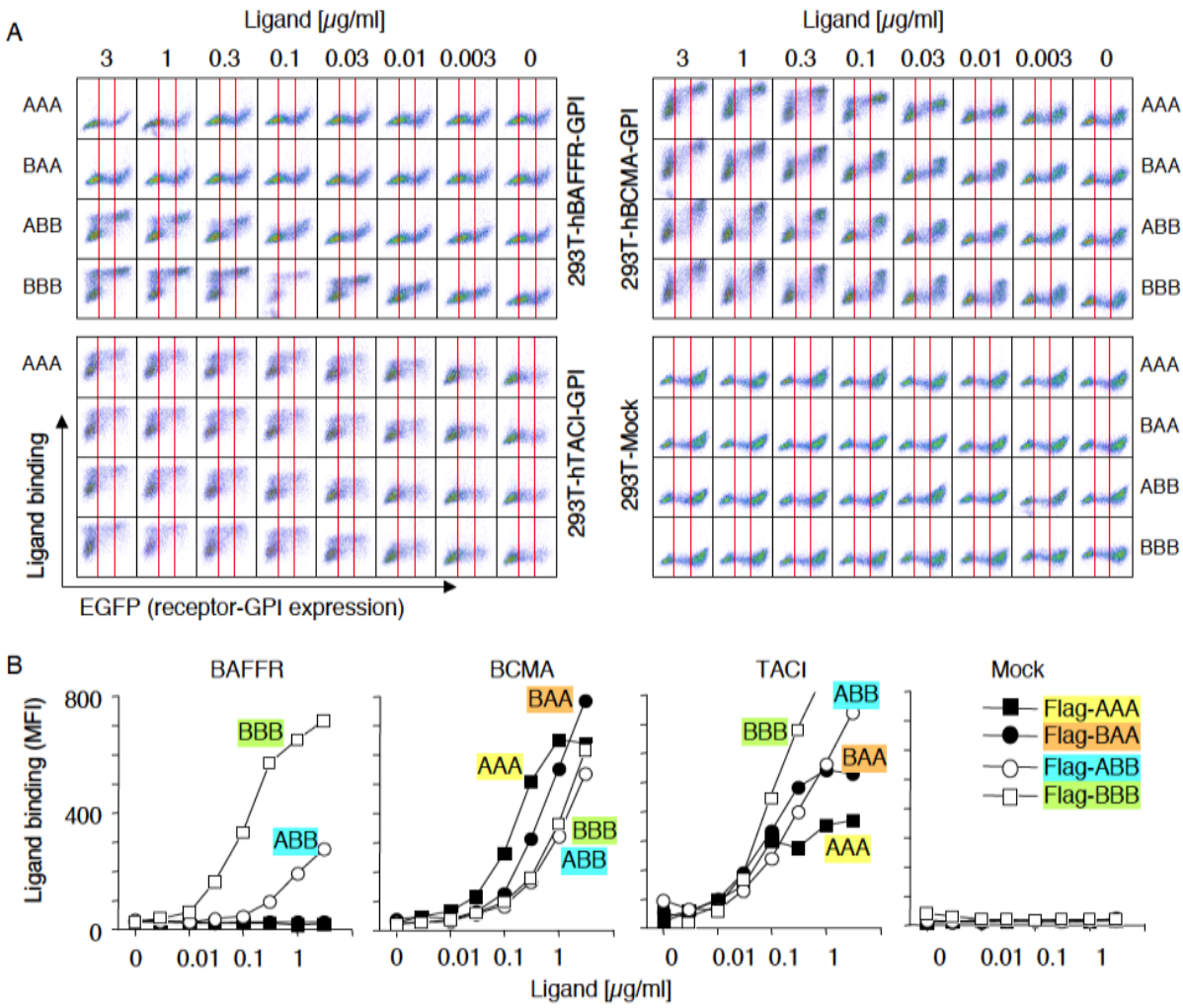


Fig 24. Binding specificity of Flag-tagged BAFF and APRIL single chain heteromers to receptors expressed at the cell surface.*293T cells were co-transfected with an EGFP expression vector and the indicated receptor-GPI expression vectors. Cells were stained with single-chain hBAFF-APRIL heteromers and appropriate PE-coupled secondary reagents and analyzed by FACS.*

A. Scattergrams showing concentration-dependent ligand binding to various GPI-anchored receptors. EGFP expression approximately reflects receptor expression (x-axis; log scale from 10^0 to 10^4). Ligand binding is detected with PE (y-axis; log scale from 10^0 to 10^4). B. Graphic representation of the data shown in A

3.2.4.1. Signaling of BAFF APRIL heteromers on reporter cell lines.

The recombinant ligands were shown to bind to their cognate receptors both expressed as a receptor-Fc or receptor-GPI. To gain insight into the signaling abilities of these ligands via these

receptors, they were tested on a surrogate cell death assay in which oligomerisation-dependent apoptotic Fas pathway can be initiated by BAFF and APRIL. For this purpose Jurkat (Fas-positive or Fas-deficient) cell lines expressing a fusion protein consisting of the extracellular domain of BCMA or TACI or BAFFR fused to the transmembrane and intracellular domain of Fas were used (as described in [49]). Some of the cell lines are responsive to only oligomerised ligands. A cell viability assay was performed using the Flag BAFF APRIL heteromers (in presence of anti-Flag used for cross-linking) in a dose-dependent manner on these cell lines.

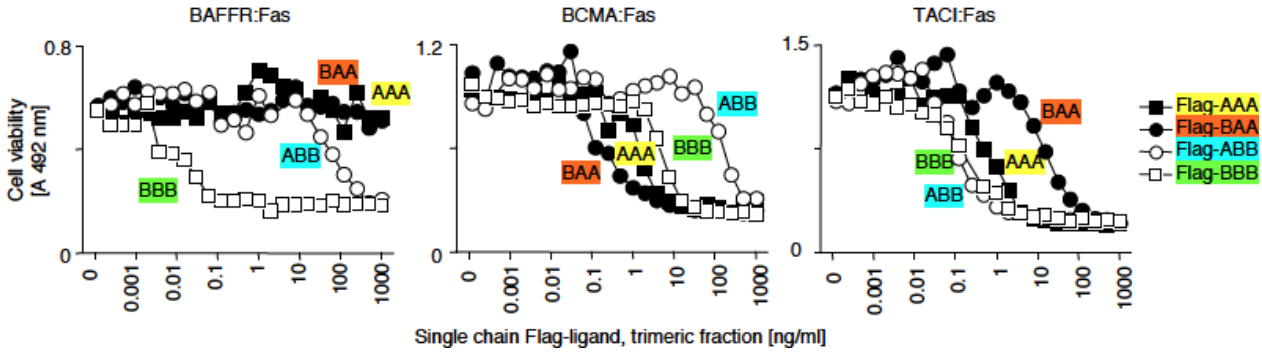


Fig 25. Activity of Flag-tagged BAFF and APRIL single-chain heteromers on receptor:Fas expressing reporter cell lines. *Jurkat cells stably transduced with the indicated version of receptor:Fas fusion proteins were incubated with increasing concentrations of Flag-tagged single-chain ligands in the presence of a fixed (1 µg/ml) concentration of anti-Flag M2 antibody. Triggering of the fusion receptors induces the apoptotic Fas pathway that kills cells. Cell death was measured by the PMS/MTS assay.*

Due to limitation of gel filtration fractionated purified Fc ligands at the time of the assay, this assay was performed with non-fractionated Fc ligands, which showed similar results as Flag tagged fractionated ligands on reporter cell lines.

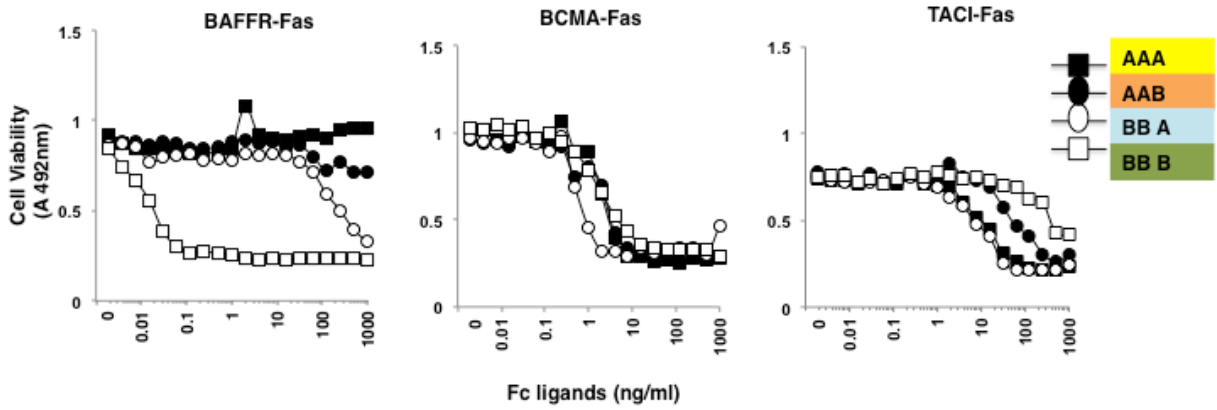


Fig 26. Activity of Fc-tagged BAFF and APRIL single-chain heteromers on receptor:Fas expressing reporter cell lines. *Reporter cell lines as shown in Fig 25 were incubated with increasing concentrations of Fc-tagged single-chain ligands. Cell death was measured by the PMS/MTS assay.*

The results were consistent with the receptor binding data. APRIL-APRIL-APRIL and BAFF-APRIL-APRIL signaled via BCMA and TACI while BAFF-BAFF-BAFF and APRIL-BAFF-BAFF signaled via all three receptor-Fas cell lines. An interesting observation was that APRIL-BAFF-BAFF showed much less activity than BAFF-BAFF-BAFF on BAFFR-Fas cells. This could perhaps indicate that at least two molecules in an assembled trimer, though sufficient to bind to its cognate receptors, is not enough to generate an active signal.

3.2.4.2. Signaling of BAFF APRIL heteromers on primary cell lines.

Although the signaling abilities of the BAFF APRIL heteromers could be assessed on reporter cell lines, these remain an artificial set up not providing a clearer view of the signaling abilities of these heteromers on each of these receptors. To determine this, ligands were tested on primary B cells in a thymidine-incorporation assay, in which primary B splenocytes of WT or TACI-ko mice were stimulated with anti-B cell receptor antibodies and co-stimulated with single chain BAFF and/or APRIL proteins.

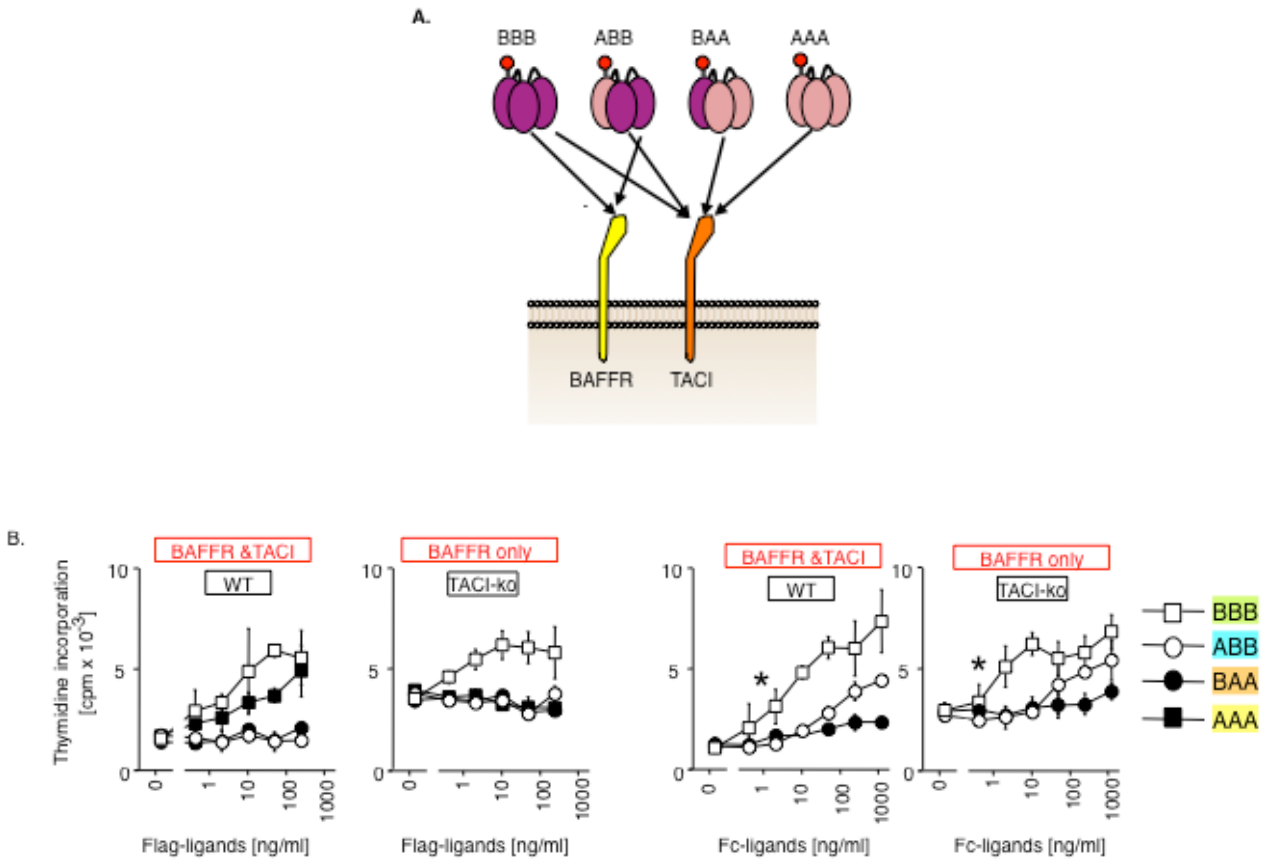


Fig 27. Activity of single chain BAFF and APRIL heteromers on primary murine B cells of WT and TACI-ko mice. *A. Schematic representation of binding of single chain BAFF-APRIL homomers and heteromers on the respective receptors on murine B cells. B. Murine B cells from WT mice and TACI $^{-/-}$ mice were cultured under BCR-stimulating conditions with increasing concentrations of Flag and Fc ligands. For the Flag ligands a fixed (1 µg/ml) concentration of anti-Flag M2 antibody was used as a crosslinker. After 48 hours, cells were pulsed for 16 hours with thymidine, harvested, and counted. Proliferation of primary B cells was measured by thymidine incorporation. The black squared boxes on top of the graphs indicate the genotype of the mice and the red boxes indicate the receptors on splenocytes *Purified S200 fractionated Fc AAA was not available for this assay.*

MACS isolated WT splenocytes from adult C57Bl6 mice were stimulated with the various single chain ligands as indicated in Fig 27. As primary B cells do not express BCMA, WT B cells provide a picture of signaling via BAFFR and TACI. For all the Flag-tagged single-chain ligands, an anti-Flag antibody was used as a cross-linker. To assess the signaling via BAFFR alone, proliferation assay was performed on TACI $^{-/-}$ B cells. As BAFFR $^{-/-}$ cells produce very little B cells, an anti-

mouse BAFFR blocking antibody 9B9 (kindly provided by Antonius Rolink) was used to selectively block BAFFR, resulting in a TACI-only situation.

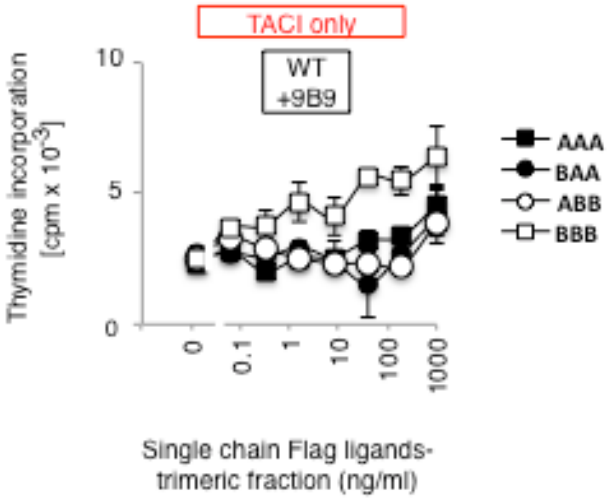


Fig 28. Activity of single chain Flag-tagged BAFF and APRIL heteromers via TACI on primary murine B cells. *Purified murine B cells from WT mice were cultured under BCR-stimulating conditions stimulated by Flag ligands in presence of anti-Flag antibody for a proliferation assay using thymidine as described in Fig 27. Cells were additionally incubated with 9B9 mBAFFR blocking antibody at 0.25 µg/ml. The black squared box on top of the graph indicates the genotype of the mice and the red box indicates the receptor on splenocytes.*

As seen in Fig 27, the Flag-tagged homomers of BAFF-BAFF-BAFF and APRIL-APRIL-APRIL signal better on WT and TACI $^{-/-}$ cells as compared to the heteromers. Proliferation via BAFFR (TACI $^{-/-}$ splenocytes) in the presence of Fc-tagged ligands, showed stronger signals than with Flag - ligands. The stronger signal could be due to the efficiency of Fc as a cross-linker than anti-Flag antibody. Both Flag and Fc BAFF-BAFF-BAFF showed strongest signaling as compared to the other ligands on TACI $^{-/-}$ splenocytes.

3.2.4.3. BCMA-induced NF-κB luciferase reporter assay.

Signaling via TACI and BAFFR could be measured on primary B cells obtained from mouse splenocytes. However signaling via BCMA was not possible by this method, as primary B

splenocytes express little or no BCMA. This receptor is indeed expressed later during B cells development and found on differentiated B cells like plasma cells and plasmablasts, which are difficult to work with. As a surrogate to measure signaling via BCMA, a NF-κB luciferase reporter assay was performed in HEK 293T cells expressing full-length BCMA.

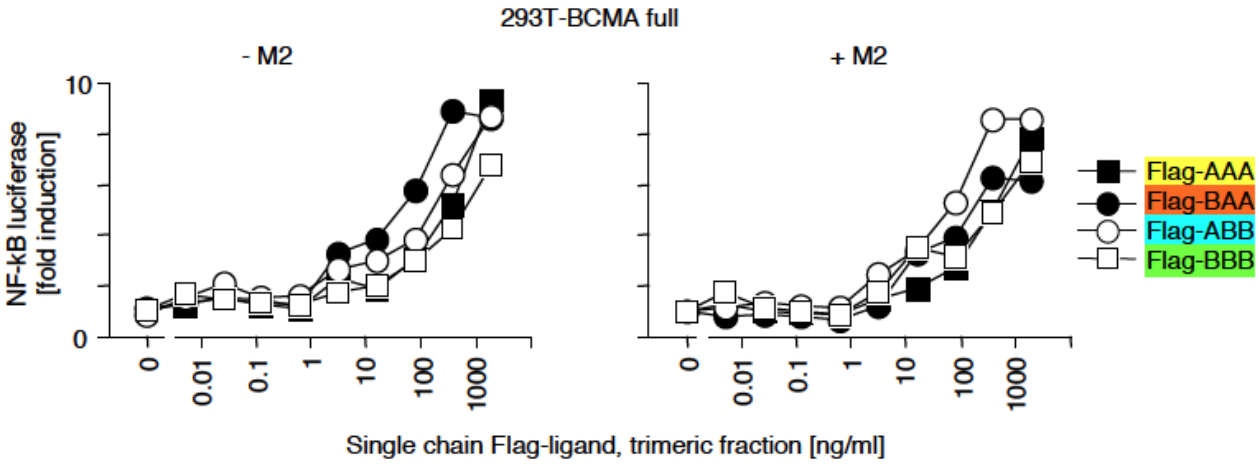


Fig 29. Signaling via BCMA in a NF-κB luciferase reporter assay

293 T cells transiently co-transfected with full-length human BCMA and NF-κB reporter plasmids were stimulated with purified Flag-ligands at the indicated concentration in the presence or absence of anti-Flag antibody (M2). NF-κB activity is shown as fold induction compared to BCMA-transfected, but non-stimulated cells (concentration zero).

All Flag-ligands were able to induce BCMA signaling as observed by the NF-κB activity on HEK 293 T cells. All the homomers and heteromers showed similar EC_{50} of around 100 ng/ml, indicating that they have similar signaling capacities via BCMA.

3.2.5. APRIL-BAFF-BAFF versus BAFF-BAFF-BAFF.

APRIL-BAFF-BAFF showed weak signaling as compared to BAFF-BAFF-BAFF on reporter Fas cell line and also on primary splenocytes. This lead to the hypothesis that it may act as an inhibitor

of BAFF signaling via BAFFR. In order to demonstrate this, a competition assay was performed between Flag-BAFF-BAFF-BAFF and Flag-APRIL-BAFF-BAFF on BAFFR:Fas reporter cell lines by a PMS-MTS cell viability assay as well as a proliferation assay by thymidine incorporation on primary B cells isolated from TACI-BCMA double knock out mice.

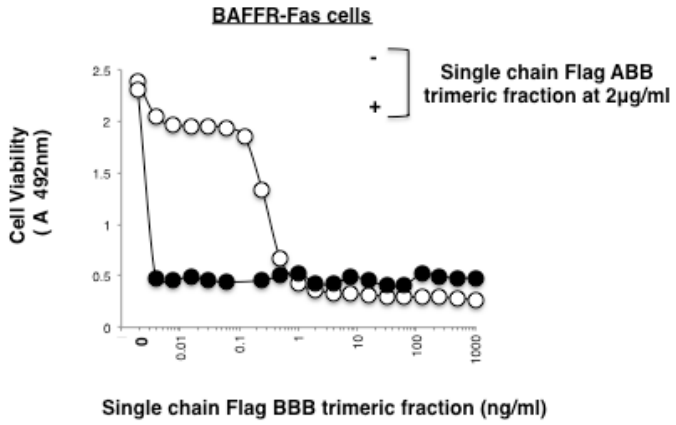


Fig 30. Competition of Flag-APRIL-BAFF-BAFF with Flag-BAFF-BAFF-BAFF trimeric fraction on BAFFR:Fas expressing reporter cell line (2308-21). *Cells were incubated overnight with Flag BAFF-BAFF-BAFF at the indicated concentration with or without Flag APRIL-BAFF-BAFF added at a constant concentration of 2 µg/ml. Cell viability was measured the following day using PMS-MTS.*

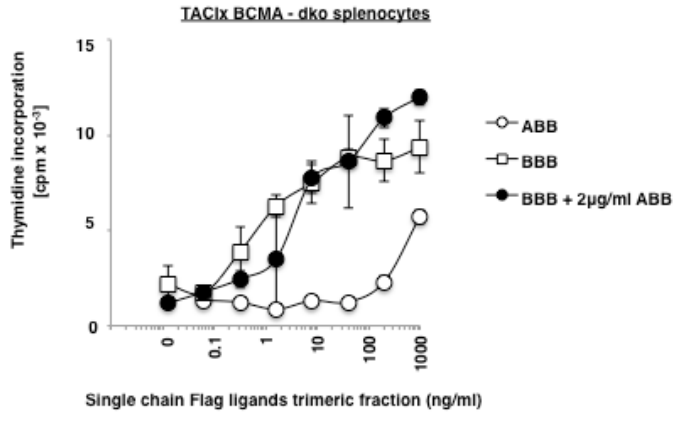


Fig 31. Competition of Flag-APRIL-BAFF-BAFF with Flag-BAFF-BAFF-BAFF trimeric fraction on TACI $^{-/-}$x BCMA $^{-/-}$splenocytes expressing BAFFR only*. MACS isolated murine B cells from spleen of a TACI$^{-/-}$BCMA$^{-/-}$ mouse*

were cultured under BCR stimulating conditions in the presence of Flag-BAFF-BAFF-BAFF or Flag-APRIL-BAFF-BAFF at indicated concentrations in the presence of anti-Flag antibody (M2). Cells were also incubated with Flag-BAFF-BAFF-BAFF at increasing concentration with a constant concentration of Flag-APRIL-BAFF-BAFF of 2 µg/ml. Proliferation was measured by thymidine incorporation after 72 h.

The assay on reporter cell lines as shown in Fig 30 shows the sensitivity of Flag-BAFF-BAFF-BAFF on these BAFFR-Fas cells upto around 1ng/ml. The sensitivity of these cell lines to BAFF mediated killing was enhanced in presence of Flag APRIL-BAFF-BAFF at 2 µg/ml. This effect was perhaps mediated by the presence of Flag APRIL-BAFF-BAFF alone at 2 µg/ml independent of Flag BAFF-BAFF-BAFF. Including an additional control of killing in response to Flag APRIL-BAFF-BAFF alone in a dose dependent manner and at a constant concentration of 2 µg/ml would confirm the fact if Flag APRIL-BAFF-BAFF can act via BAFFR better at a higher concentration. This would confirm that APRIL-BAFF-BAFF is a weak binder of BAFFR as compared to BAFF-BAFF-BAFF (as has already been shown in Fig 23 and 24) and it signals efficiently only at higher concentrations. The proliferation assay on B cells show Flag-BAFF-BAFF-BAFF signals via BAFFR both in presence and absence of the Flag-APRIL-BAFF-BAFF added in excess at a constant concentration. B cells also show some signal for proliferation with Flag APRIL-BAFF-BAFF at the starting concentration of 1µg/ml. This is much in contrast with the earlier experiments (Fig 27) where no signal is seen on WT and TACI $^{-/-}$ cells. Based on results obtained in this particular experiment signaling via BAFFR in presence of APRIL-BAFF-BAFF at 2 µg/ml alone should be expected (black squares at 0.1 ng/ml of BAFF-BAFF-BAFF). It is possible that the signal obtained in presence of APRIL-BAFF-BAFF at 1µg/ml could be an aberrant signal. Hence the data obtained is not sufficient enough to draw conclusions. It would be necessary to repeat this experiment (done once) to comment further on the putative inhibitory role of heteromer on homotrimer signaling.

3.2.6. *In vivo* effect of APRIL-BAFF-BAFF on B cell restoration.

So far, the *in vitro* assays of binding on receptor-Fc and receptor-GPI and signaling measured on reporter Fas cells, and *ex vivo* on splenocytes from various receptor knockout mice indicated that APRIL-BAFF-BAFF binds and signals weakly as compared to BAFF-BAFF-BAFF. Our next attempt was to confirm these results *in vivo*. The primary signals for the development of B cells are mediated by BAFFR. Our objective would be to dose BAFF $^{-/-}$ mice with Fc-BAFF-BAFF-BAFF and Fc-APRIL-BAFF-BAFF and look for B cell restoration.

In order to do this assay, so far we have set up the conditions for B cell restoration in BAFF $^{-/-}$ mice and optimized the necessary conditions for rescue of the phenotype. We injected a BAFF $^{-/-}$ mouse intraperitoneally with 100 μg of Fc-BAFF per injection three times at an interval of 7 days. The mouse was sacrificed on day 18. Spleen and lymph nodes were isolated and cell were stained with antibodies against mature and immature B cell surface markers (CD19, CD93) and analysed by flow-cytometry. WT and untreated BAFF $^{-/-}$ mice were used as controls (Fig 32). In addition total number of cells were also counted. This treatment successfully but partially restored mature B cells in a BAFF $^{-/-}$ mouse as compared to an untreated knock out mouse as has been shown in (Fig. 32). There was a clear increase in the percentage of CD19 B cells in the Fc-BAFF treated BAFF $^{-/-}$ mouse both in the spleen and in lymph nodes. These conditions for the *in vivo* B cell restoration assay could be used to assess the *in vivo* activity of Fc-BAFF-BAFF-BAFF and Fc-APRIL-BAFF-BAFF.

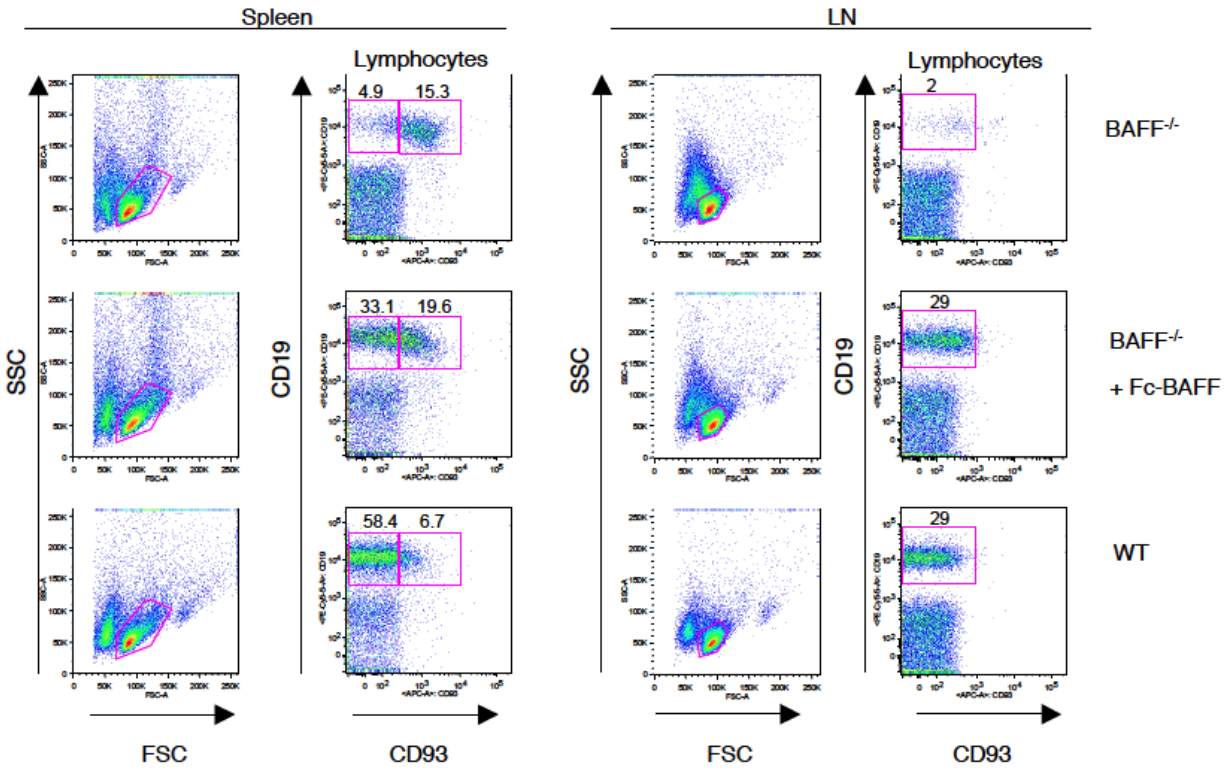


Fig 32. Set up of the in vivo B cell rescue assay with administration of Fc-BAFF in BAFF $^{-/-}$ mice

Adult C57Bl6 mice WT, untreated BAFF -/- and BAFF -/- injected i.p with 100 µg of Fc hBAFF at Day 0,7,14, analysed on Day 18 . Spleen and set of six lymph nodes (inguinal, axillary, brachial) were isolated. Splenocytes (post RBC lysis) and lymphocytes were stained with anti-CD19 and anti-CD93 antibodies and analysed on LSRII.

3.3. BAFF 60-mer deficient knock-in Mouse.

3.3.1. Aim

As described in section 1.3.4, some TNF family receptors respond better to oligomerised ligands. Both BAFF and APRIL present characteristic properties of oligomer formation, though their functional relevance and endogenous existence remain unclear. However receptors like TACI and perhaps BCMA too can be activated by oligomerised BAFF or APRIL [49] and also oligomerised BAFF may play a role in marginal zone B cell formation [31]. BAFF 60-mer has been shown to form upon over expression and has also been crystallized [46, 48]. Therefore, BAFF 60-mer could possibly be a biologically active form of soluble BAFF.

The aim of this project was first to make a mutant murine BAFF that is unable to form a BAFF 60-mer but that retains its ability to form a trimers and second to make a knock-in mouse with this mutant to assess the physiological role of BAFF 60-mer.

3.3.2. Identification of a Flap mutation in mouse BAFF.

For the purpose of identifying the role of BAFF 60-mer *in vivo*, the aim was to create a knock-in mouse in which BAFF cannot associate as a 60-mer. The first step in this procedure was to identify a mouse BAFF mutant that trimerizes and retains normal binding to receptors, but that cannot associate as 60-mer. For human BAFF, it has been shown convincingly that an H218A mutation in the flap region fulfills all of these requirements [48]. However, results obtained previously in our laboratory suggested that mutation of the corresponding mouse residue (H242A) was insufficient to abolish 60-mer formation. The crystal structure of BAFF 60-mer suggests that several residues beside His^{218} might be important for ligand assembly through flap-flap interactions. In particular, Glu^{254} of one BAFF 3-mer contacts Lys^{252} of another 3-mer, and vice-versa (Glu^{278} and Arg^{276} in mouse BAFF). The same is true for the pair Glu^{223} and Lys^{216} (Glu^{247} and Lys^{240} in mouse BAFF). We reasoned that mutations E278K and E247K should generate electrostatic repulsion with Arg^{276} and Lys^{240}, respectively.

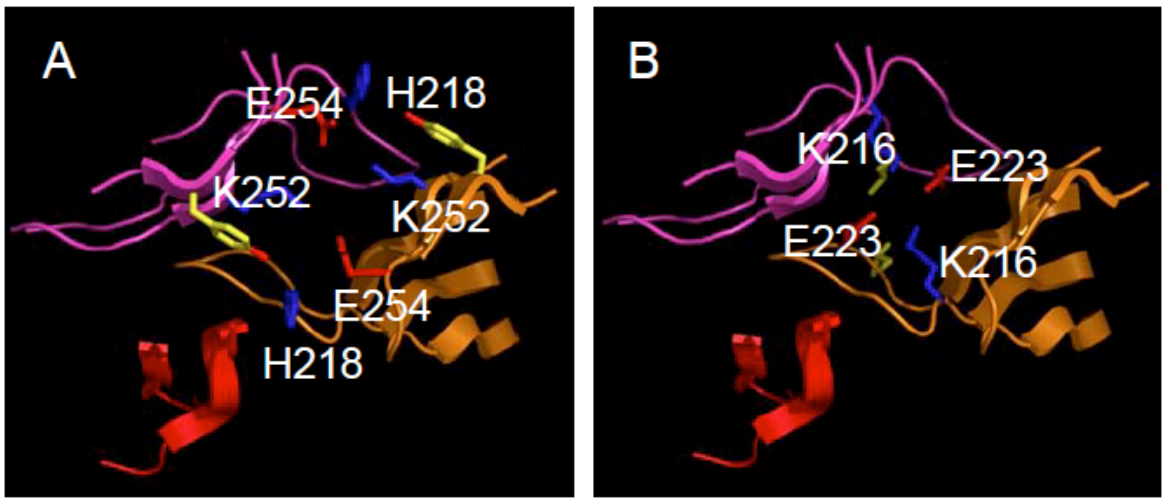


Fig 33. Structural details of the Flap-Flap interaction region of the human BAFF 60-mer. *(a) Electrostatic interactions between Glu254 and Lys252. (b) Electrostatic interactions between Glu223 and Lys216, which takes place underneath those of Glu254 and Lys252. Structural coordinates are from pdb accession number 1OTZ.*

Full length cDNAs of mouse BAFF WT, H242A, E247K and E278K (see plasmid maps in Annex

A), and of human BAFF WT and H218A as controls, were expressed in 293T cells (Fig. 34) using buffered medium, to avoid disruption of 60-mers because of medium acidification. Indeed, BAFF 60-mer is stable at a neutral or alkaline pH but dissociates into trimer at acidic pH [48]. Processed BAFF was recovered in culture supernatants (Fig. 34).

Mutant E278K was not well expressed for reasons that remain to be determined. WT mouse BAFF and mutants E247K and H242A were dosed by sandwich ELISA (capture with mAb 5A8, and detection with mAb 1C9) and by receptor binding ELISA (capture with BCMA-Fc, TACI-Fc or BAFFR-Fc, and detection with mAb 1C9). Results indicate that mAb 1C9 recognizes mouse BAFF also when it is bound to a receptor, but that it cannot recognize the H242A mutant, which presumably has a destroyed epitope (Fig. 34B). Recognition of mutant E247K by mAb 1C9 may also be partially affected, because amounts of E247K BAFF that gave a signal as intense as WT BAFF by immunoblot (revealed with a polyclonal anti-BAFF antibody) was recognized 4-fold less efficiently by 1C9 in the sandwich and receptor-binding ELISAs (Fig. 34 B,C,D). Taking this into account, it can be concluded that there is no obvious difference between WT and E247K mouse BAFF regarding their binding to receptors.

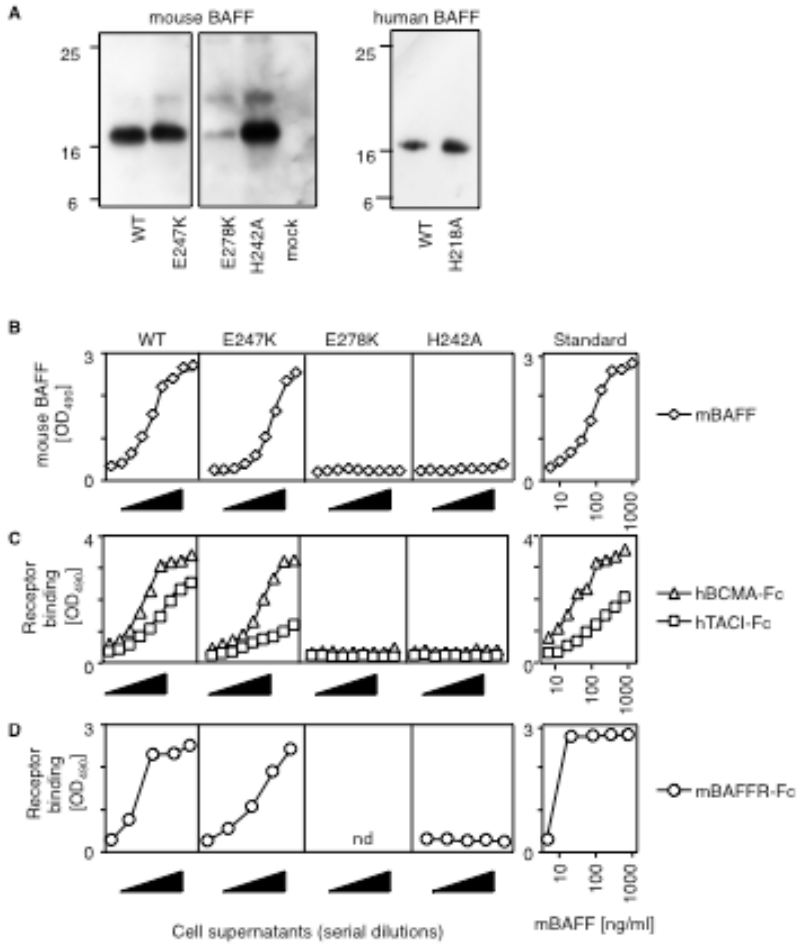


Fig 34. Mouse BAFF mutant E247K is expressed and binds BAFF receptors. *293T cells were transfected with various full-length BAFFs. Conditioned serum-free supernatants were analyzed for their BAFF content. A. Immunoblot of mouse BAFF revealed with polyclonal goat-anti-mouse antibody 852 (from R&D) and Immunoblot of human BAFF revealed with the rat mAb Buffy-2. 20 µl of supernatant were loaded on the gel. B. Mouse BAFFs were dosed by sandwich ELISA, using mAb 5A8 to capture and biotinylated mAb 1C9 to reveal. Purified recombinant Flag mouse BAFF was used as a concentration standard. C. Mouse BAFFs were dosed for their binding to hBCMA-Fc and hTACI-Fc. D. Same as C but binding to mBAFFR-Fc. In panels B, C and D 50 µl of supernatants were used (and 2-fold dilutions).*

3.3.3. E247K Flap mBAFF mutant is not functional as an oligomer.

Upon identification of a suitable Flap mutant, the next step was to determine whether mouse BAFF E247K was impaired in its ability to form 60-mers. When analyzed by gel permeation chromatography, 60-mers were readily detected in the positive control (WT human BAFF) but not in its H218A mutant, as expected (Fig. 34A). WT mouse BAFF formed comparatively little high molecular weight oligomers, and these oligomers were still present in H242A BAFF and, to a lesser extent, in E247K BAFF, making it difficult to draw conclusions regarding BAFF 60-mer (Fig. 34B). Therefore a functional assay was used for the detection of BAFF 60-mer, based on a reporter cell line. To enhance the production of total mouse BAFF, WT and E247K mutant Flag-mBAFF were expressed in 293 T cells grown in complete rather than serum-free medium. The expression and concentration was determined by immunoblotting using Flag mBAFF purified protein as a standard (Fig. 36A). Both proteins were expressed almost equally. Crude supernatants were then concentrated, adjusted to similar concentrations and dosed on reporter BAFFR:Fas cell lines that were sensitive to oligomer alone and to both oligomers and trimers (Fig. 36B,C). Supernatants containing WT mouse BAFF displayed some toxicity towards the oligomer-sensitive reporter cells (presumably due to the presence of BAFF 60-mer), whereas those containing the E247K mutant did not. Both WT and E247K mutant mBAFF killed the oligomer- plus trimer-sensitive cell lines indicating that the mutant cannot associate as an oligomer but can form trimers.

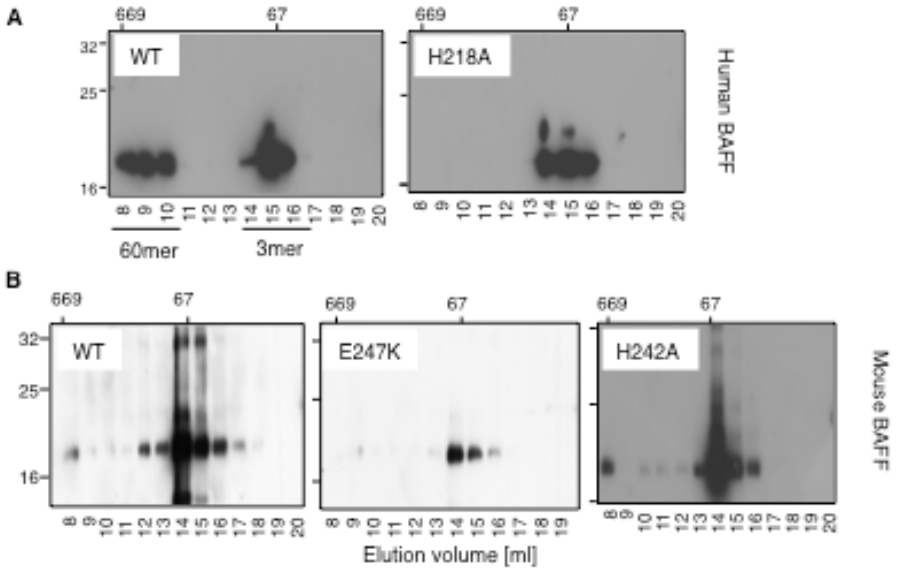
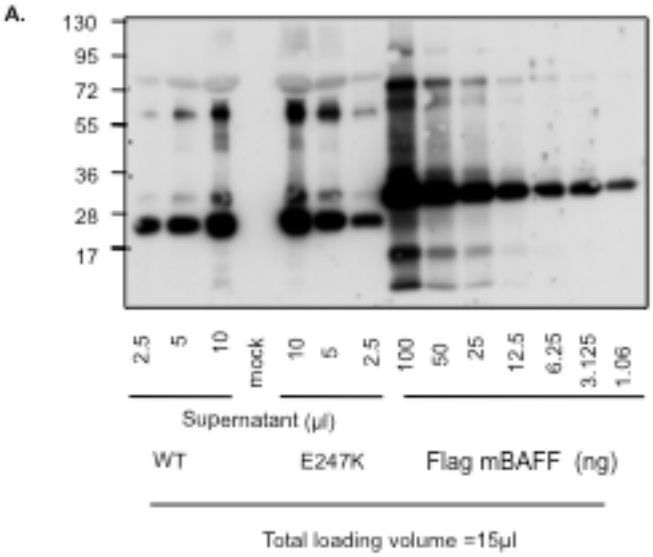


Fig 35. Mouse BAFF forms high molecular weight oligomers less efficiently than human BAFF. *BAFF-containing supernatants of 293T cells transfected in serum-free medium were concentrated 20 x, and analyzed by size exclusion chromatography. Mouse BAFF was revealed by immunoblot with polyclonal anti-BAFF antibody 852, and human BAFF with mAb Buffy-2. A. WT human BAFF. B. Human BAFF H218A. C. WT mouse BAFF. D. Mouse BAFF E247K. E. Mouse BAFF H242A. The elution position of molecular weight standards (in kDa) are indicated at the top of the figure, and the migration position of molecular weight standards for SDS-PAGE are shown on the left.*



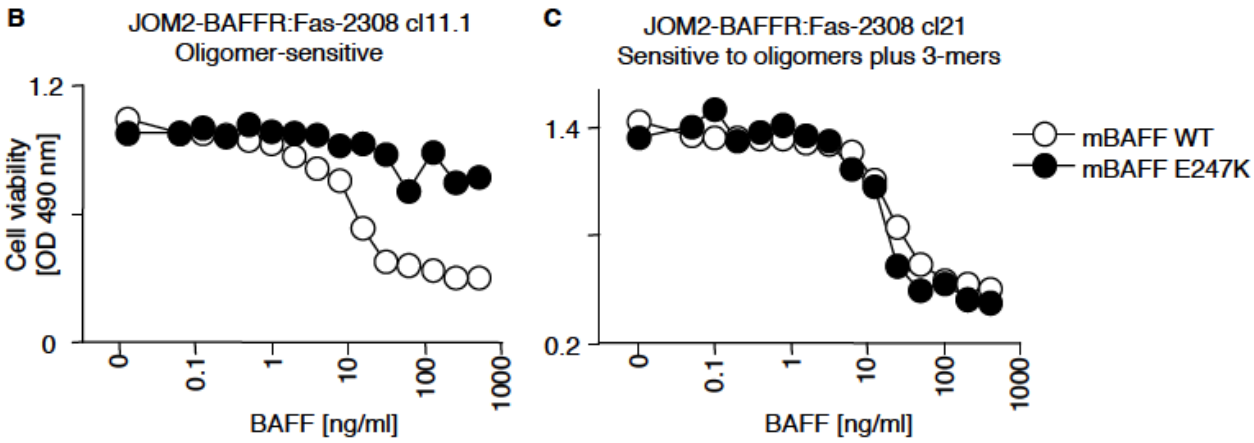


Fig 36. Mutant E247K mouse BAFF does not form active oligomers. *293T cells were transfected in complete medium with full length WT mBAFF and mBAFF E247K, and naturally processed BAFF was recovered in supernatants. A. Processed BAFF was quantified by immunoblot with a polyclonal goat anti-mouse BAFF antibody, using purified Flag-mouse BAFF as a standard. B. Oligomer-sensitive and C. Oligomer- plus 3-mer sensitive BAFFR:Fas reporter cell lines were dosed with WT and E247K mBAFF and tested for cell viability using the PMS/MTS assay.*

3.3.4.1. Generation of a Knock-In vector for E247K mutant mBAFF.

To make the knock-in mouse deficient for BAFF-60mer, E247K mutant BAFF was selected as a target. A knock-in vector was constructed from a BAC clone containing the genomic BAFF sequence as a template. The principle of the knock-in method relies on the homologous recombination and thereby the exchange between a fragment of the genomic mouse BAFF region of interest with the fragment containing the mutation in the knock-in vector. The knock-in vector containing the fragment and mutation of interest for the mouse BAFF must meet some criteria, with at least 1 kb of unmodified sequence 3' of the mutation and 3 kb 5' of the mutation. Briefly, a 5.6 kb SpeI fragment (Fig. 37) was chosen for the homologous recombination and was extracted from the BAC clone containing the genomic BAFF sequence. This 5.6 kb region contains exon 6, which is the target for introducing the mutation, flanked by a 4.5 kb region on its 5' and a 1.1 kb region on its 3'. The SpeI fragment

was subcloned into a modified pBlueScript vector (without ApaI and BamHI sites). Using the BAC as a template, a E247K mutation with an additional silent mutation introducing a HindIII site was introduced by double PCR. This fragment of about 1500 bp was delimited by 2 ApaI sites. It was sublconed into a suitable intermediate vector, for the purpose of introducing the neomycin resistance cassette in the natural EcoRV present in the intro 3' of exon 6. The ApaI fragment now containing the target mutation and NeoR cassette was cloned into the vector containing the SpeI genomic fragment and this served as the final knock-in vector.

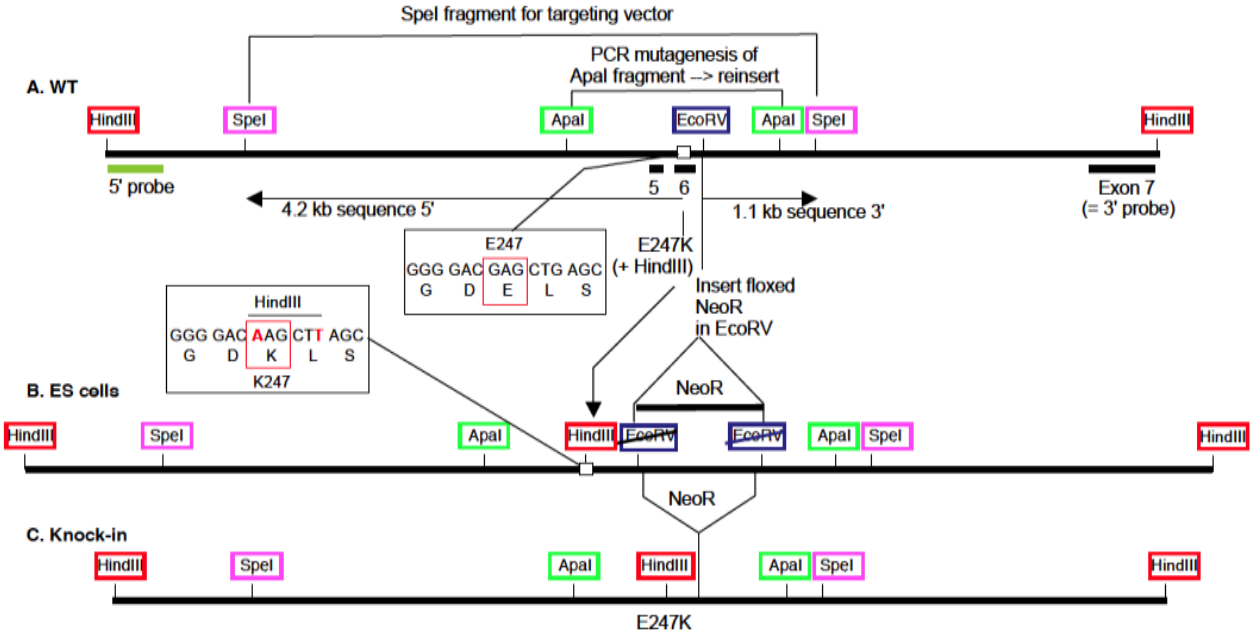


Fig 37. Strategy for the construction of BAFF E247K knock-in.

A. Hind III fragment of the WT mouse BAFF allele containing exons 5, 6 and 7. The HindIII fragment contains an SpeI fragment used for targeting. The SpeI fragment contains an ApaI fragment used to introduce the target mutation E247K in the targeting vector. The ApaI fragment contains an EcoRV site used to insert the floxed NeoR cassette. B. Predicted genomic sequence of a correctly recombined allele in ES cells. Mutation E247K creates a novel HindIII site, while the EcoRV site has been disrupted by insertion of the NeoR cassette.

C. Predicted genomic sequence of a knock-in mouse after removal of the NeoR cassette (by crossing with a Cre-expressing mouse).

3.3.4.2. Southern Blot probes for the detection of knock-in transgene.

The knock-in vector, after electroporation into ES cells of 129 mice and successful recombination will yield the desired ES cells (containing a mutant allele and a WT allele) and confer cells with resistance to G418. After selection, clones need to be verified for the correct insertion of the mutant first by PCR and then by southern blot. Probes that would allow distinguishing recombined from WT alleles were therefore designed 5' and 3' of the targeted sequence. Genomic DNA isolated from embryonic stem cells (ES cells) of 129 mice were digested with a selection of restriction enzymes, then revealed by southern blot with the 5' or 3' probe. Both probes gave clean signals while revealing the predicted fragment, as depicted in Fig. 38B. It should be noted that the molecular weight marker band at 1.6 kb contains some repetitive sequence giving background with any labelled probe.

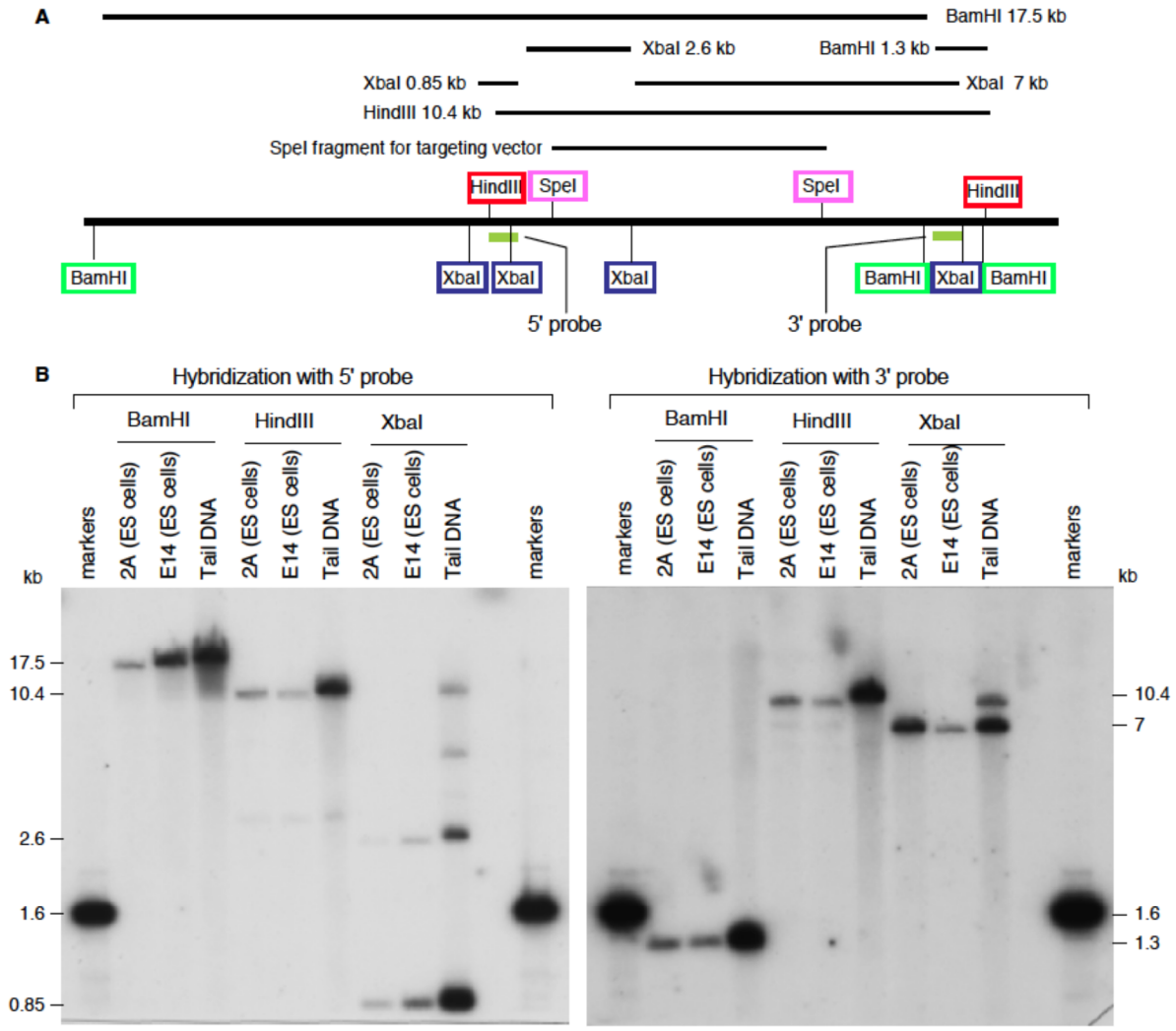


Fig 38. Southern blot of wild type gDNA for the characterization of 3' and 5' probes.

A. Schematic representation of the portion of interest of the mouse BAFF gene, with indication of the relevant restriction sites, location of the 5'- and 3'-probes, location of the SpeI fragment that will be used to construct the targeting vector, and predicted restriction fragments that could hybridize with one or both of the probes.

B. Southern blot of 3 different samples of WT genomic DNA digested with restriction enzymes BamHI, HindIII or XbaI, and revealed with 32P-labelled 5' (left) or 3' (right) probes.

ES cells were electroporated with the SpeI fragment of the targeting vector. Genomic DNA extracted from G418-resistant clones were screened by PCR for the identification of correct recombination events. For this purpose, one of the primer was in the sequence of the NeoR and the second was 3' of the targeting sequence. Therefore, an amplification product of the expected site can only be obtained in case of a correct recombination event, and not in case of

a random insertion (Fig. 39A). A fair number of positive clones were identified (Fig 39B). Genomic DNA of these clones were analyzed by Southern blot with the BAFF 5' probe. Positive clones give the WT band at 10.4 kb plus a shorter, recombined band at 5.6 kb. This short band is also a signature for the presence of the K247E mutation that introduces a HindIII site (Fig 39C). Results were confirmed by hybridization with a probe specific for the NeoR cassette, to exclude an additional random insertion of the transgene. Several ES clones were found positive and further amplified. Amplified clones were confirmed by PCR (Fig. 39D) and 4 of them were selected for injections (boxed in Fig. 39D). The process is now ongoing.

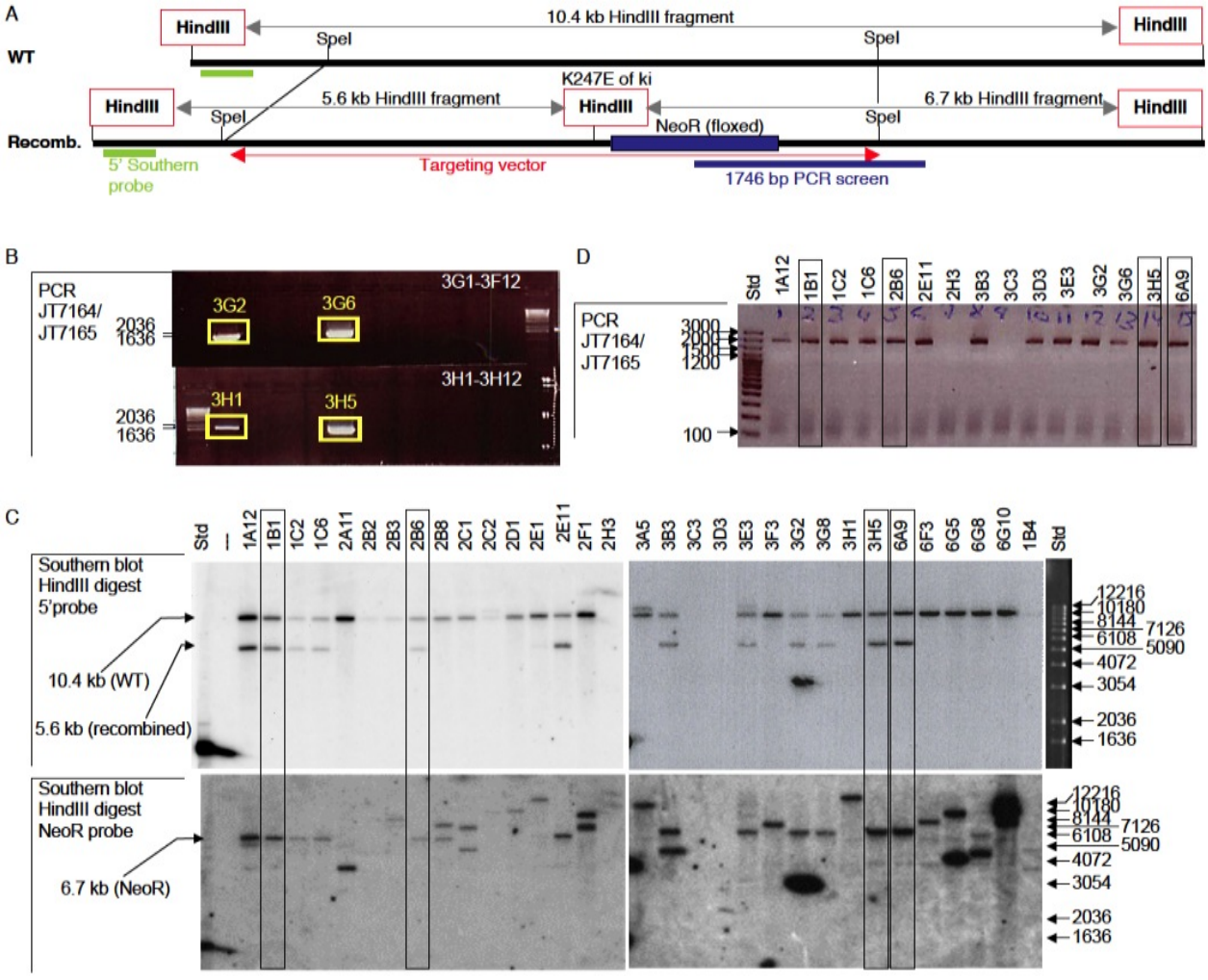


Fig 39. Screen for correct recombination events in ES cell clones.

A. Scheme of the WT and recombined BAFF alleles in the region of interest. The targeting vector is a SpeI fragment of BAFF genomic DNA, where the K247E mutation was introduced in exon 6, generating a novel HindIII site, and a floxed NeoR cassette was introduced in intron 6-7. The position of the 1746 bp sequence amplified with oligos JT7164 (in the NeoR cassette) and JT7165 (in the BAFF gene, 3' of the targeted sequence) and of the 5' Southern blot probe (5' of the targeted sequence) are shown. Relevant HindIII fragments generated by digestion of genomic DNA are also indicated (10.4 kb for WT, 5.6 kb and 6.7 kb for a recombined allele.

B. Representative result of the primary PCR screen of ES cell clones (one quarter of plate N°3), showing positive amplification for 4 clones, 3 of which turned out to be true positives.

C. Southern blot analysis of clones found positive in the primary screen, hybridized with the 5' probe (top panels) followed by the NeoR probe (bottom panel). The 4 clones selected for generating knock-in animals are boxed. The ladder shown on the right was cut from the gel before transfer, and stained for DNA.

D. 12 ES clones that were positive by Southern blot (plus 3 clones that gave no signal by Southern) were amplified and confirmed to be positive by PCR after amplification.

4. Discussion and future perspectives

4.1.1. Novel interaction partners in the TNF family of ligands.

TNF ligands form homotrimers owing to their structural properties. Homotrimer formation is a fundamental requirement for receptor binding and receptor oligomerisation for adequate signal transduction. Formation of a trimer thus decides the fate of the signaling event via the receptor. LTα has been shown to form soluble homotrimers that bind to TNFR1. It forms a surface bound complex with LTβ that binds to LTβ-R. Formation of LTα-LTβ heterotrimers have been found to play a crucial role in lymph node formation. The discovery of six new interaction partners in the TNF family ligands in our study was therefore interesting. It is interesting to notice that the interacting partners are very closely related and can be classified as a sub-family on the basis of structural similarity. APRIL interacts with EDA1 and EDA2, EDA1 interacts with EDA2 and LIGHT interacts with FasL, TL1A, LTα, LTβ. APRIL and EDA1 are structurally similar as much as LIGHT is similar to FasL, TL1A, LTα, LTβ.

The screening method undertaken to identify interaction partners had its own limitations. Some of the ligands in the combinations did not express themselves. Hence no conclusion could be reached about their heteromer forming abilities. Some interactions were not consistent and also showed very faint signal as compared to the negative controls. In some combination of Flag and Fc ligands signal was observed whereas the same ligand with the tags other way around yielded no signal. For example Flag-APRIL and Fc-BAFF interacted but not Flag-BAFF and Fc-APRIL (Fig 11) making it unclear if a real heteromer forms or not. But in spite of these limitations seven positive hits could be identified. These pair showed strong interaction signal (in red in Fig 11) and overcame all the limitations of the screening method.

It would be interesting to study if they arise in the same cell or tissue and whether the formation of these heteromers would be possible post translationally as well. The ratio between the homo versus heterotrimer formation would provide an indication of the predominant species providing some clues about its physiological relevance.

However the study of heteromers *in vivo* remains technically challenging owing to their tendency to exchange among themselves [202]. Isolation of homogenous species of heteromers would be difficult. A recombinant form of heterotrimer may solve the purpose to an extent though the reliability of the study would depend on the yield of recombinant heteromers to be used for biochemical characterisation. To further validate these interactions, the need would be to use untagged forms of these ligands in co-transfection studies. This would also demand the characterisation of suitable antibodies meeting various criteria; capable of recognizing such an interaction pair and also be sensitive enough for heteromer detection. The physiological importance of these interactions remains unexplored in our studies.

4.1.2. Novel receptor specificity of heteromers.

Within the TNF family of receptors several receptors are still found to be orphan i.e. they do not have a binding partner within the TNF family of ligands. It was hypothesized that the novel interaction partners within the TNF ligands identified in this study may have some novel receptor-binding specificity with respect to these orphan receptors. Hence the various combinations containing the interaction partners were used in an assay to test their binding specificities. So far, none of the novel interaction partners exhibited binding to these orphan receptors, though further confirmatory tests proving the activity of the ligands (shown for some of the heteromers in annexure C for binding to cognate receptors) still needs to be carried out with adequate controls. Based on the current observation, if it holds true that the orphan receptors do not bind the heteromers, this could imply that there may be other candidates outside the TNF family receptors for the interacting ligands or they might bind to their cognate receptors and act as an activator or inhibitor or play redundant roles like the homotrimers. For e.g. LIGHT-FasL may bind to HVEM and Fas respectively. A species of $LIGHT_2FasL$ may bind to HVEM better than Fas while a species of $FasL_2LIGHT$ may bind to Fas better than HVEM and can generate or block the signaling via these receptors.

The orphan receptors may actually have no binding partner within the TNF family ligands even if they have been classified as TNFSF receptors. Nerve growth factor receptor (NGFR) Nerve Growth Factor and other ligands outside the TNF family and plays a role not only in the nervous system [203, 204]but also in the immune system [205-210]. TROY is another orphan receptor, which has been shown to bind to ligand outside the TNF family [199, 211-215]. It is possible that these receptors might induce signaling in a ligand-independent manner, though the deficiency of some of them in mice have not exhibited any prominent phenotype [216, 217].

4.2. BAFF APRIL heteromer characterization.

4.2.1.1. Single chain ligands.

Use of short peptide linkers (GGGGS) for the trimerisation of TNF homology domains have already been described before [201] as it prevents the dissociation of monomers and increases the activity of trimers linked in a single chain. However the method provides potential caveat. The linkers joining the C and N termini of the ligands could interfere with protein folding and its subsequent activity. Thus this method had potential limitation of interference with activity. Thus arose the need to validate this method and check if it would yield active forms of trimers. One way to validate this method was to check the expression and secretion of homotrimers and subsequently the binding to their cognate receptors. Another aspect to check was the ability of a single chain heteromer to mimic its naturally occurring form. Single chain LTα-LTβ homotrimers as well as heteromers were thus constructed. All the four single chain ligands expressed and could be efficiently secreted. They showed binding to their cognate receptors TNFR1 and LTβR respectively. This was a validation of the proof of concept of the single chain method and it could be utilized further for BAFF-APRIL heteromer characterisation. The advantage of this method was that it could yield a homogenous species of heteromers.

Although BAFF-APRIL heteromers could be produced, they had some limitations in their production method. As described before this method utilizes the linker, which is in close proximity to the C termini of the ligands. The combination of APRIL-BAFF-BAFF proved to be better in terms of expression and activity. It is possible that changing the order in which the linkers were inserted and the ligands were cloned had an impact on the overall folding and activity of the proteins. It is however difficult to comment that if the physiological activity of 2BAFF 1APRIL will be closer to the BAFF-BAFF-APRIL or APRIL-BAFF-BAFF recombinant ligand.

4.2.1.2. Production of BAFF-APRIL single chain heteromers.

As the single chain method have been described previously [201] and also have been validated in our studies (LTα-LTβ heteromers), it was thus utilized to produce single chain BAFF-APRIL heteromers using Flag tag and Fc tag. Though this method met the desired objective, there were limitations of production of these heteromers in large amounts. It could be reasoned that although this method works for ligands like TNF and LTα very well, it has some limitations for BAFF-APRIL heteromer production. The yield of heteromers produced from established stable clones of CHO cells remained low. This could be due to the efficiency of the stable clone or because of the cloning method linking BAFF-APRIL in a particular sequence in a single chain, or both. To improve the efficiency of production and obtain a larger yield for further biochemical characterization, the order in which BAFF-APRIL were linked was modified. A variant of BAFF-BAFF-APRIL and APRIL-APRIL-BAFF was cloned by reversing the C terminal third ligand to the N-terminal position, resulting in APRIL-BAFF-BAFF and BAFF-APRIL-APRIL. To improve the efficiency of stable clones the IRES-GFP was inserted in the plasmid and it proved to be a useful way of selecting the best clones in terms of brightest GFP. However these efforts did not prove to drastically increase the yield of production of these heteromers and it was necessary to upscale the method for further production of heteromers.

4.2.2. Size characterization of single chain heteromers.

The rational behind adapting to the single chain method was to obtain a homogenous species of heteromers, which could be characterized exclusive of any homotrimeric ligands. However this method had the potential of generating higher order aggregates (as described in section 3.2.2.2) consisting of a mixture of both and hence it was necessary to perform a size

exclusion chromatography using gel filtration to exclude this possibility. As described before (section 3.2.2.2 and 3.2.2.4) the Flag-tagged heteromers produced by transient transfection and fractionated from crude supernatant mostly folded and formed trimers and eluted at the fraction with the correct size, while there were more aggregation formed in purified ligands as seen by the presence of proteins in the higher molecular weight fractions. But these could be only aggregation caused due to the method of purification and not necessarily a mixture of homo and heteromers contributing to misleading results.

4.2.3. Receptor binding specificity and signaling abilities of heteromers.

The most sought after and obvious candidates to screen for the receptors of BAFF-APRIL heteromers were BAFFR, TACI, BCMA. The latest group reporting the presence of BAFF-APRIL heteromers [178] studied the binding affinities of these heteromers and homomers using surface plasmon resonance experiments. They found that only BAFF bound to BAFFR while the heteromers bound to TACI and BCMA. They also measured the dissociation constant of the heteromers as compared to APRIL and BAFF with respect to TACI and BCMA and BAFFR. The biological activity of the recombinant heterotrimers were assessed *in vitro* doing a TACI-Jurkat NF-kB assay and looking at proliferation signals on primary B cells. The binding of 2 APRIL and 1 BAFF to TACI, BCMA but not BAFFR in our studies were consistent with previous studies on BAFF-APRIL heteromer. As expected the single chain BAFF-APRIL heteromers could bind to these receptors as shown in Fig. 23 by ELISA (receptor-Fc's) or flow cytometric analysis in Fig. 24 (receptor-GPI's). All the heteromers and homomers showed similar EC50 on TACI-Fc and BCMA-Fc while on TACI-GPI the EC50 was bit lesser than on BCMA-GPI (as measured by the MFI) suggesting stronger binding to TACI. However the fact that receptor Fc's are dimeric and receptor GPI's may also oligomerise on the surface of cells contributing to a somewhat blurry picture about their precise binding affinities, must be taken into account. In both the previous studies describing

BAFF-APRIL heteromer formation, either the stoichiometry of these heteromers could not be determined [218] or only one of them $APRIL_2BAFF$ was defined owing to their limitations of recombinant protein production. $BAFF_2APRIL$ was thus not well characterized so far. In our studies we found that 2 BAFF and 1 APRIL can bind to all 3 BAFF-APRIL receptors namely BAFFR, TACI and BCMA. APRIL-BAFF-BAFF also shows binding to BCMA, TACI as good as BAFF-APRIL-APRIL, APRIL-APRIL-APRIL and also BAFF-BAFF-BAFF. However binding to BAFFR is many fold reduced for as compared to BAFF-BAFF-BAFF. This result could be explained by the fact that binding to BAFFR is facilitated primarily by BAFF and presence of one molecule of APRIL weakens the binding.

Furthermore the signaling on BCMA:Fas Jurkat reporter cell lines and NF-κB reporter assay in 293 T cells show similar signaling capacities of all the four ligands. On TACI-Fas cells Flag BAFF-APRIL-APRIL shows much less signaling as compared to the other three ligands, which is in contrast with the binding data on TACI-Fc and TACI-GPI. The signaling ability of APRIL-BAFF-BAFF and BAFF-APRIL-APRIL on BAFFR-Fas cells seem to be consistent with the ELISA and FACS data. BAFF-APRIL-APRIL neither binds nor signals via BAFFR, whereas signaling via BAFFR is many fold reduced by APRIL-BAFF-BAFF as compared to BAFF-BAFF-BAFF.

On primary splenocytes, results obtained for the signaling by the homo and heteromers had some differences with the data obtained on receptor Fc's or GPI and reporter Fas cells . On WT splenocytes all single chain Flag tagged homomers signaled better compared to heteromers while for the Fc heteromers the signal was stronger than Flag tagged ligands but still less than the Fc-homomers. Flag-BAFF-APRIL-APRIL signaled weaker as compared to Flag-APRIL-APRIL-APRIL on WT splenocytes (Fig. 27) which was in contrast to binding data on TACI-GPI, TACI-Fc and also signaling via TACI-Fas reporter cell lines, but on TACI only cells (+9B9 Fig. 28) both displayed similar signals. On BAFFR only cells (TACI ko splenocytes) the results obtained were consistent for all the ligands. On TACI only cells

(so far done with only fractionated trimeric Flag ligands) Flag BAFF-BAFF-BAFF showed strongest signals as compared to the rest. This raises the question whether heteromers play a role in signaling via BAFFR or TACI at all?

4.2.4. Is APRIL-BAFF-BAFF is it an inhibitor or an activator of BAFFR mediated signals in B cells?

This brings to the question concerning the physiological relevance of these heteromers and their possible role in autoimmune diseases. The first group reporting the formation of heteromers [218] had assessed the signaling capacity of BAFF-APRIL heteromers on a B cell proliferation assay. They found that the activity could be inhibited by TACI-Ig but not BCMA-Ig or BAFFR-Ig and they hypothesized that perhaps the predominant form of heteromers in sera would be of $APRIL_2BAFF$ stoichiometry. However the second group reporting the presence of BAFF-APRIL heteromers [178] could produce recombinant heteromers with a predominant stoichiometry of 2 APRIL to 1 BAFF and a very small fraction of 1APRIL to 2 BAFF as confirmed by Western blot and size exclusion chromatography. Signaling was similar to that of APRIL in the *in vitro* TACI-Jurkat assay. Atacicept and BCMA-Ig neutralized the activity of BAFF APRIL, and heterotrimers in the TACI-Jurkat assay. BAFFR-Ig only neutralized the activity of BAFF. The heterotrimers were less-potent inducers of B-cell proliferation than were BAFF or APRIL, as evidenced by the higher EC50 values for heterotrimers than those of BAFF or APRIL in the primary human B cell-proliferation assay. They developed a bead based immunoassay and using the recombinant heterotrimers as a reference of quantification they measured endogenous heterotrimers in sera of patients with autoimmune diseases. Their studies indicate an increase in serum levels of heterotrimers correlated with disease prognosis as indicated by clinical parameters used in SLE. These heteromers had very little activity on B cell proliferation on primary human B cells.

As per our studies, we found that APRIL-BAFF-BAFF signals weakly via BAFFR as compared to BAFF-BAFF-BAFF. This raises the question of whether they could they act as an inhibitor of homomeric BAFF with respect to BAFFR signaling.

As described in section 3.2.5 we performed a competition assay on both reporter cell line as well as on primary B cells expressing BAFFR. As per our results in Fig. 30 and 31 a conclusive comment on the inhibitory role of APRIL-BAFF-BAFF on BAFF-BAFF-BAFF signaling via BAFFR cannot be drawn yet and needs to be repeated. But with the trends shown so far based on the binding data (ELISA and receptor GPI) APRIL-BAFF-BAFF may be a weak binder of BAFFR and activate signaling only at higher concetration.

4.2.5. Potential effect of heteromer signaling via TACI or BCMA?

As seen in Fig 26 and 27 on primary splenocytes the heteromers signal weaker than the homomers. But the results obtained on receptor GPI or receptor Fc do not show weak binding. In addition to weak signaling via primary B cells also on TACI-Fas reporter cells Flag APRIL-BAFF-BAFF shows less potent effect than the rest of the ligands. TACI is a receptor that signal well via oligomerised ligands as described previously [49]. So it is possible that oligomerised heteromers if they exist will signal via TACI while trimeric heteromers may signal weakly or inhibit signaling of homomers. To understand if they would activate or inhibit signaling via TACI one could do the competition assay with APRIL-APRIL-APRIL or BAFF-BAFF-BAFF on TACI only cells. A limitation of this step may be the usage of the blocking antibody 9B9, which provides a higher background and may make interpretation of results difficult.

Based on the current results with all the available reagents it seems all the homo and heteromers signal equally well via BCMA. Both in presence or absence of anti-Flag used as a cross linker the signal obtained was same. Therefore it is possible that the heteromers have a physiological importance with respect to BCMA signaling. Both trimeric as well as oligomerised forms of heteromers, if they exist, can signal via BCMA. During pathological conditions the heteromers are found in high levels in sera as reported previously [218] correlated with disease progression [178]. It is possible that most of the effects of the heteromers in these conditions are a result of BCMA-mediated signaling.

4.2.6. BAFF-APRIL heteromer characterization in animal models: Requirement for powerful tools.

Although we could purify and study recombinant BAFF-APRIL heteromers, the significance for their co-existence along with the commonly found BAFF and APRIL homomers in the autoimmune diseases remain to be further explored. Whether they aggravate the disease or their presence is beneficial remains unclear. *In vivo,* studies in mouse models of these autoimmune diseases could provide a better or clearer picture of what is possible in the human counterpart. But these endeavours are much dependent on novel and powerful tools to study these heteromers. We could produce human single chain heteromers. Our limitation for endogenous detection was lack of antibodies, which could detect these naturally occurring heteromers. Alternatively a BAFF-APRIL specific antibody could be produced which would serve as an important tool. For studies in mouse models, it would be necessary to construct single chain mouse BAFF-APRIL heteromers, characterise antibodies specific to them and thereby set up an ELISA and other detection methods. Measuring the levels of heteromers in animal models during disease progression would also serve as an important indication towards its possible biological role.

4.3.1 BAFF 60-mer deficient Knock-In mouse.

TACI has been shown to be specifically activated by oligomerised BAFF or APRIL [49]. In addition, BAFF has been overexpressed and crystallised to form an oligomeric soluble BAFF consisting of twenty trimers (60mer) [46, 48] and its presence has been detected in BAFF-transgenic mice [49]. These findings raise a possibility that BAFF-60mer may be a physiologically relevant species of BAFF having a role to play in TACI activation.

Our studies aimed at identifying a mutant BAFF which retains its capacity to form trimers but does not form 60-mer. We targeted the extra loop in BAFF called the Flap region unique to the TNF family, required for association of BAFF trimers and holds the 60-mer together and introduced mutations in full-length mouse BAFF. We identified some amino acid residues in the flap region which could possibly disrupt the 60-mer formation. Based on our hypothesis of disrupting salt bridge formation between oppositely charged amino acids like glutamate and lysine, we made few mutants and expressed them in 293T cells. We checked their expression and secretion. Based on their ability to bind to all BAFF receptors as seen by ELISA and their capacity to signal via reporter cell lines responsive specifically to only an oligomeric BAFF and oligomeric plus trimeric BAFF we selected a E247K mutant for further studies.

Our aim was to introduce this mutation in a WT mouse. Thereby we adopted the strategy of a Knock-In mouse deficient for 60-mer but sufficient for trimer. Using molecular biology methods we constructed the Knock-In vector with a neomycin resistance gene and transfected ES cells for the production of the recombinant genome with the point mutation. Our probes designed for the detection of the transgene by Southern blot as well as the primers designed for the screening of the mutant BAFF proved to be efficient. The screening of the ES cells so far has also been successful. The rest of the process as mentioned before is currently ongoing.

4.3.2. Role of BAFF-60mer *in vivo.*

To address the question about the possible role of BAFF-60mer *in vivo* the Knock-In mouse would be evaluated for a primarily TACI-deficient phenotype as the role of BAFF-60mer so far seems to be TACI mediated. TACI is a receptor of BAFF and APRIL, which seems to play a dual role in B cell survival. On one hand in *in vitro* assays TACI induces B cell proliferation on primary mouse B cells, while TACI-deficient mice have increased number of B cells indicating that it might act as a negative regulator of B cell survival. Some unpublished data indicate that signaling via TACI may up-regulate some death receptors like Fas, which lead to cell death. TACI has also been implicated in the T independent type II response and class switch recombination in a cross talk with TLR's. TACI-deficiency leads to impaired TI II response marked by impaired class switching and an increase in unswitched Ig's. In absence of BAFF-60mer, a potential activator of TACI *in vivo,* it is possible that these TACI-mediated responses would be impaired and would serve as a read out of the Knock-In mice. It is also possible that BAFF-60mer has a redundant role to play in activation of TACI and in its absence, oligomerised APRIL whose physiological presence is still in question may compensate for BAFF-60mer deficiency and activate TACI. Another possibility is that membrane-bound BAFF, whose functional relevance has been addressed in furin mutant BAFF-Tg mice but whose existence in WT conditions has not been identified yet, can also serve as a form of oligomerised surface-bound BAFF capable of activating TACI.

One question that still remains to be addressed is the physiological existence of BAFF 60-mer, as in a healthy condition it is impossible to detect its presence. It is only upon over-expression in mammalian cells and in BAFF-Tg mice one could find a small fraction of total BAFF. Thus in BAFF 60-mer-deficient mice, the validation of its existence becomes challenging and one has to rely only on TACI-mediated effects as read-outs. To develop

methods that would allow for the detection of endogenous BAFF 60-mer would prove to be useful. As has been described before, there are some already available reporter cell lines (Jurkat BAFF receptor-Fas cell lines), which exclusively signals via oligomerised BAFF. Improvement of the sensitivity of these cell lines could improve the chances and possibilities of BAFF 60-mer detection.

BAFF 60-mer in BAFF-Tg mice forms a very minor fraction of total BAFF. Also in healthy mice, although BAFF trimer can be detected, its level is obviously less than in BAFF-Tg mice. One could hypothesize that perhaps the major species of soluble BAFF detected in circulating sera is perhaps a trimer and BAFF 60-mer which may arise in a healthy condition exist in a very low level perhaps beyond the limit of detection. It is also possible that perhaps BAFF-60mer is a species which remains bound to surface receptors and thus is not found in circulation. In a BAFF 60-mer-deficient condition, it is possible that the majority of surface-bound BAFF will be absent. But to detect surface bound BAFF provides another challenge of characterizing tools like antibodies, which can recognize BAFF 60-mer bound to its surface receptors.

If TACI activation were BAFF 60-mer-dependent, it would be interesting to see if levels of TACI expression (either the mRNA or the protein) are dependent upon BAFF-60mer ligation. This could be already tested on a WT mouse and if necessary can be used as a read out for the 60-mer Knock-In mice.

As it is possible that BAFF 60-mer arises only in pathological conditions or in conditions with high levels of BAFF, as seen in BAFF-Tg mice, disease models of mice mainly with autoimmune diseases with an elevated level of BAFF could be used to assess the presence of 60-mer and, if verified, the Knock-In mice could be induced to develop such disease conditions in order to detect the presence of BAFF 60-mer. Alternatively, a BAFF-Tg mouse which shows endogenous BAFF 60-mer can be developed having a E247K Flap mutant which would disrupt the 60-mer but not the trimer. In such a case, one limitation would be

that high BAFF levels might not provide a clear physiological status. It may also be possible that high levels of BAFF trimer compensate for the effect of a 60-mer.

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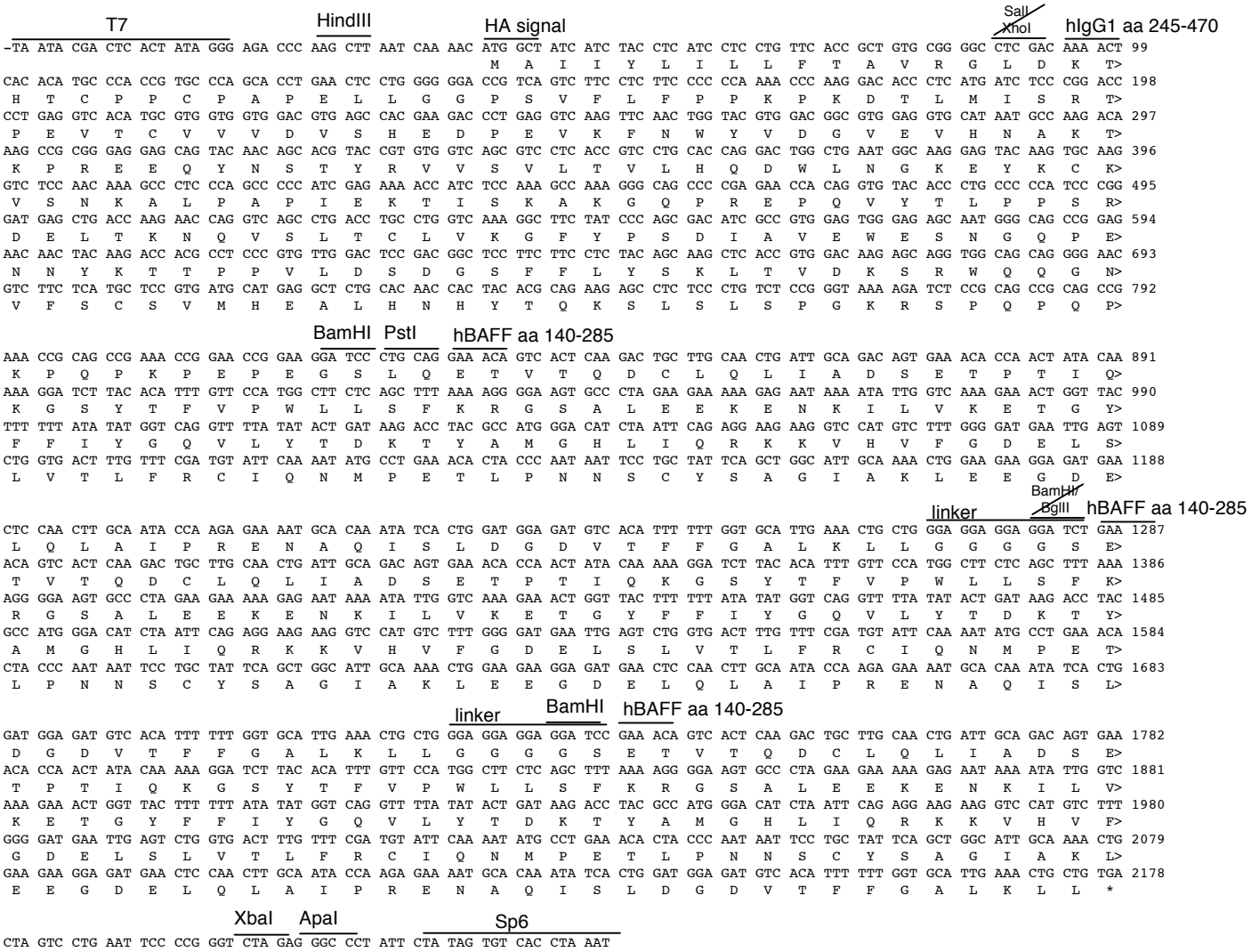
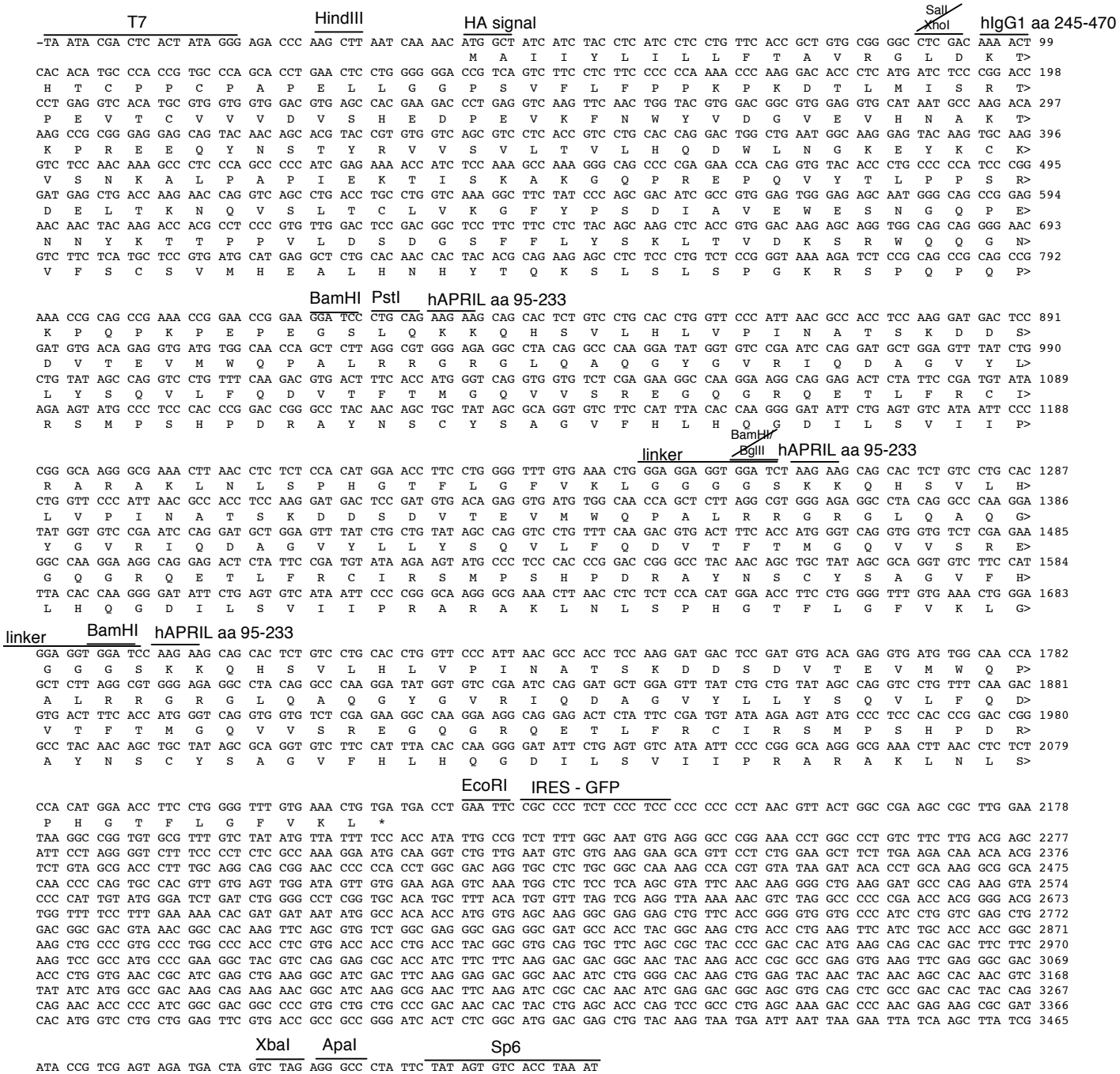
ps2680 **Fc hBAFF mono1 hBAFF mono2 hBAFF mono3 in PCR3**



ps2876 **Fc hAPRIL mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3**



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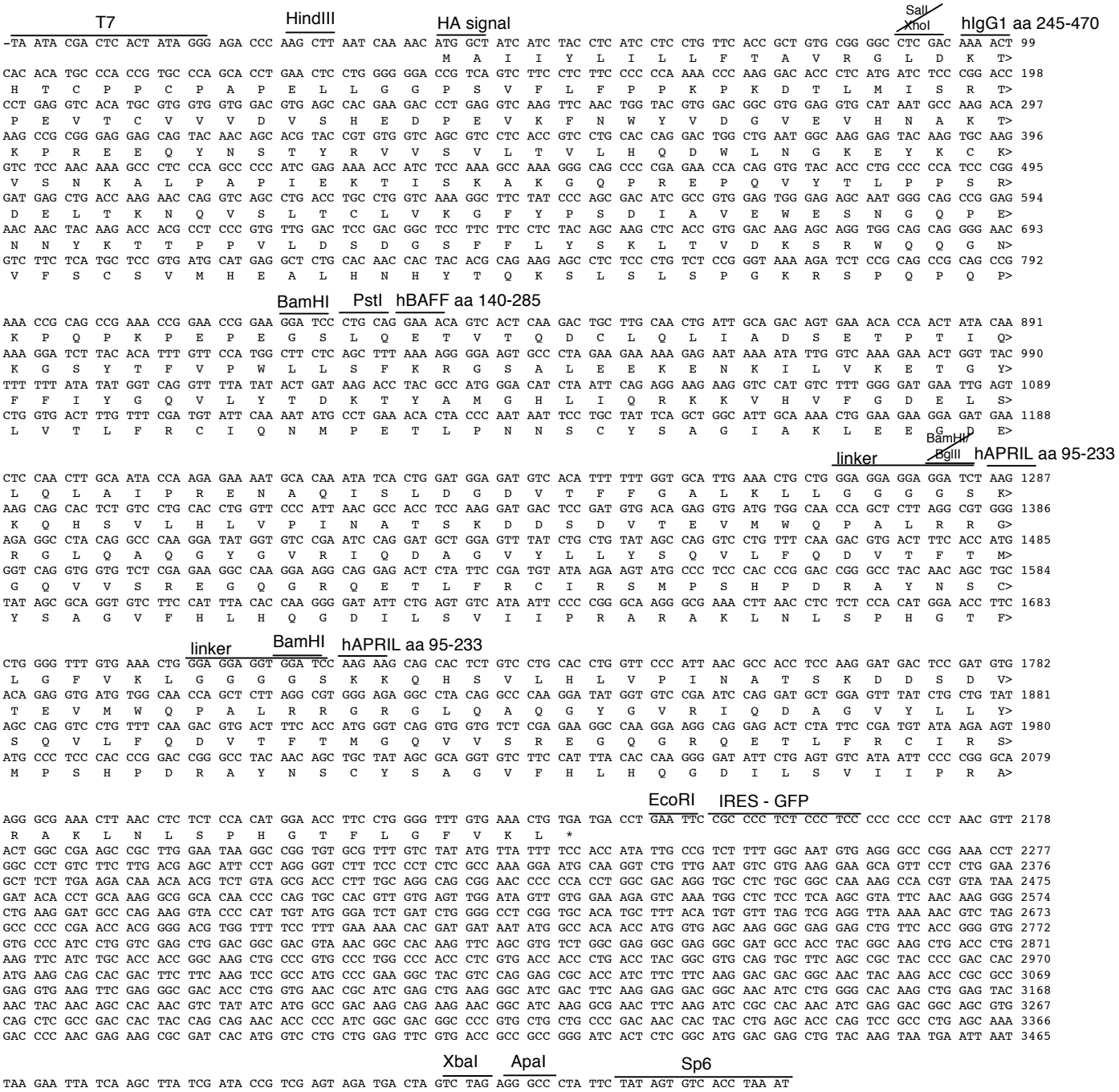
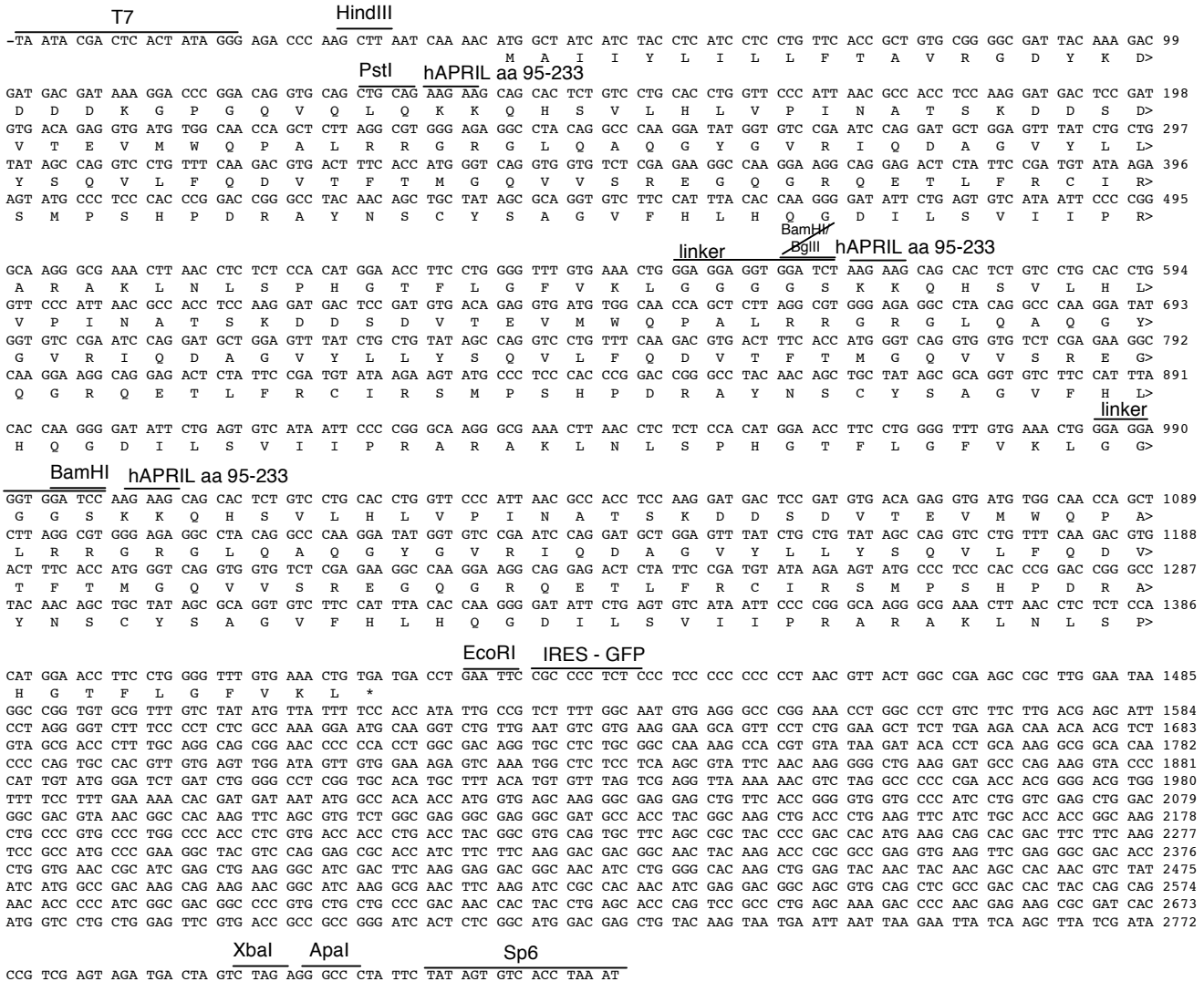
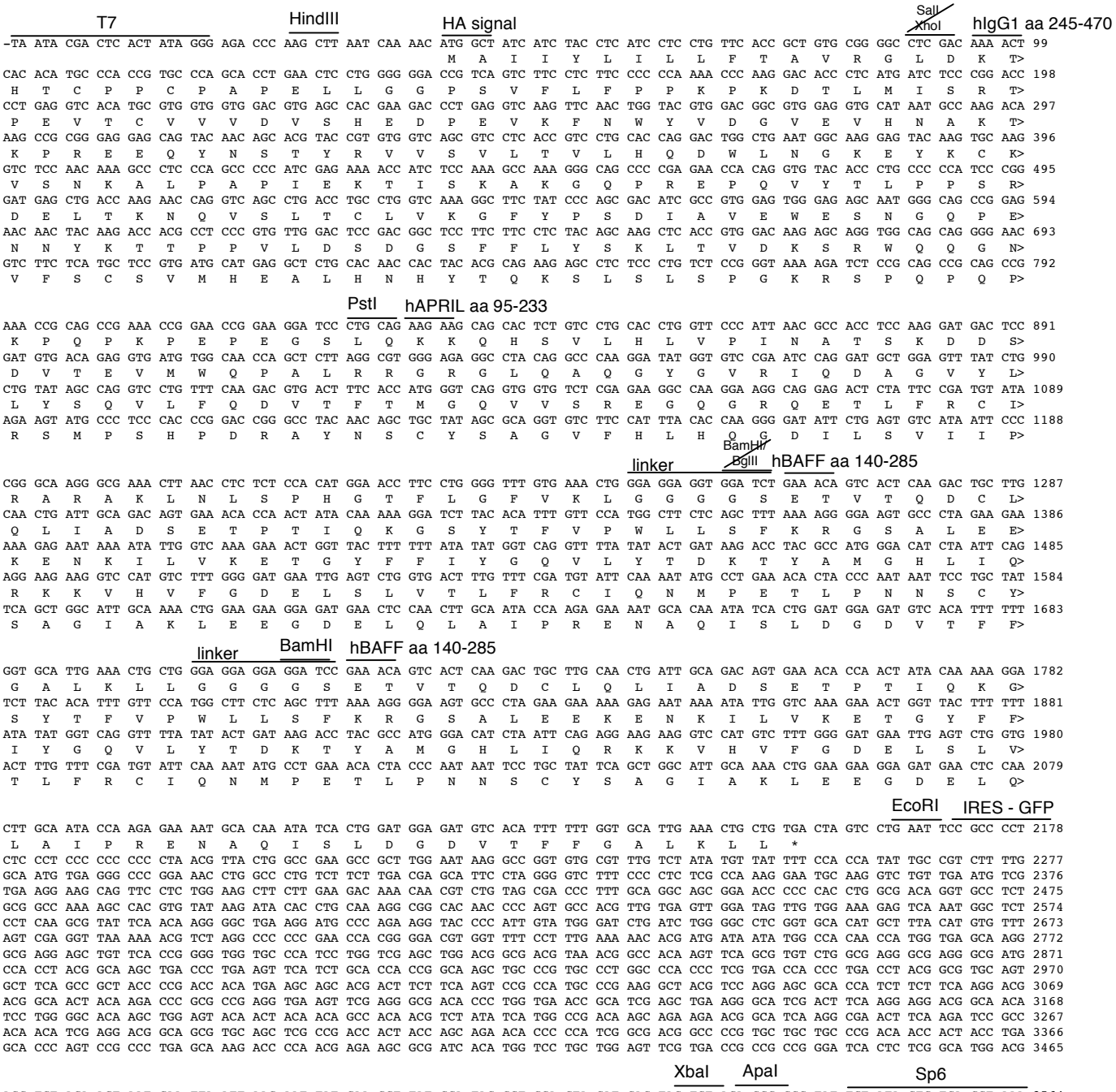


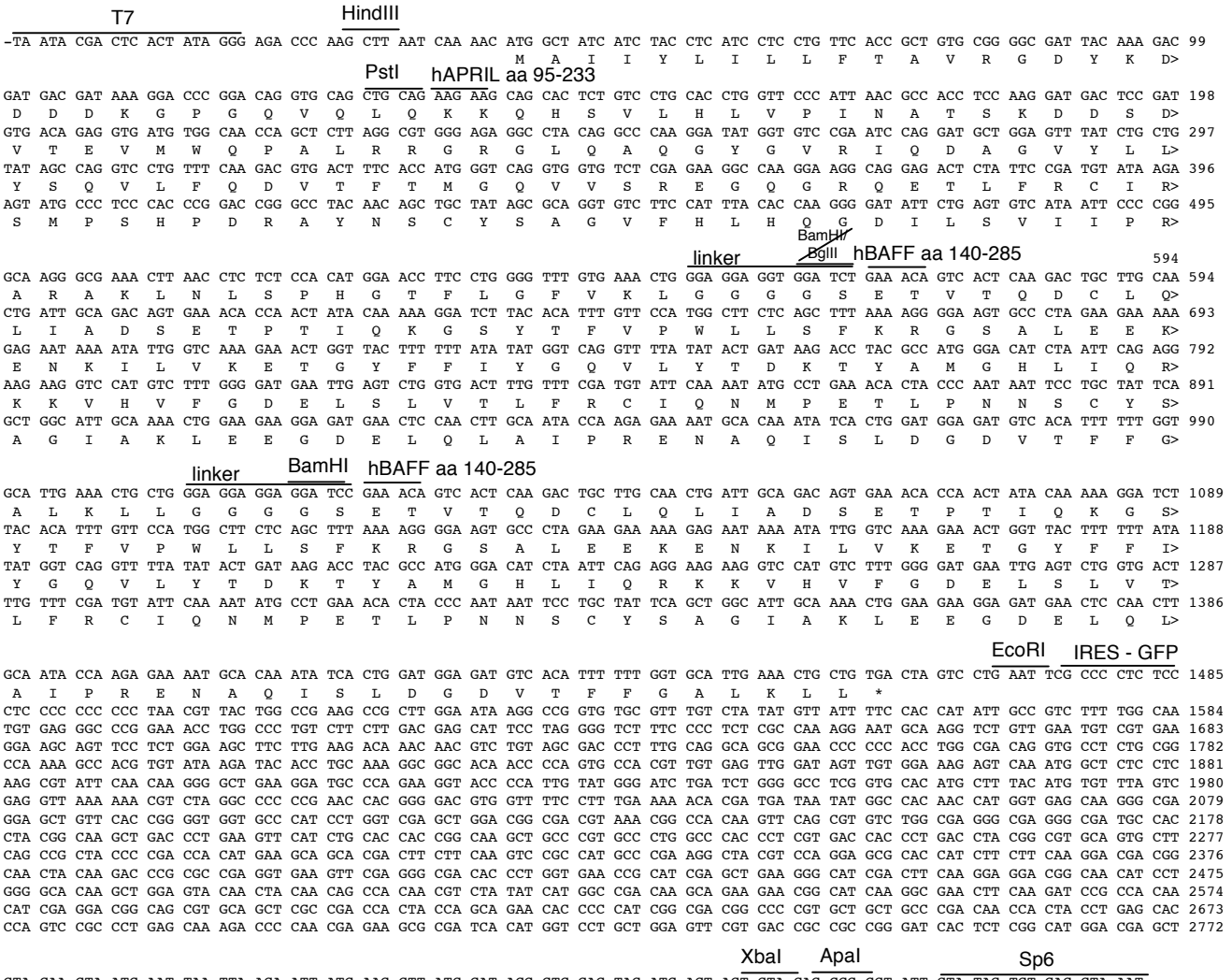
ps2879 **Flag hAPRIL mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3**



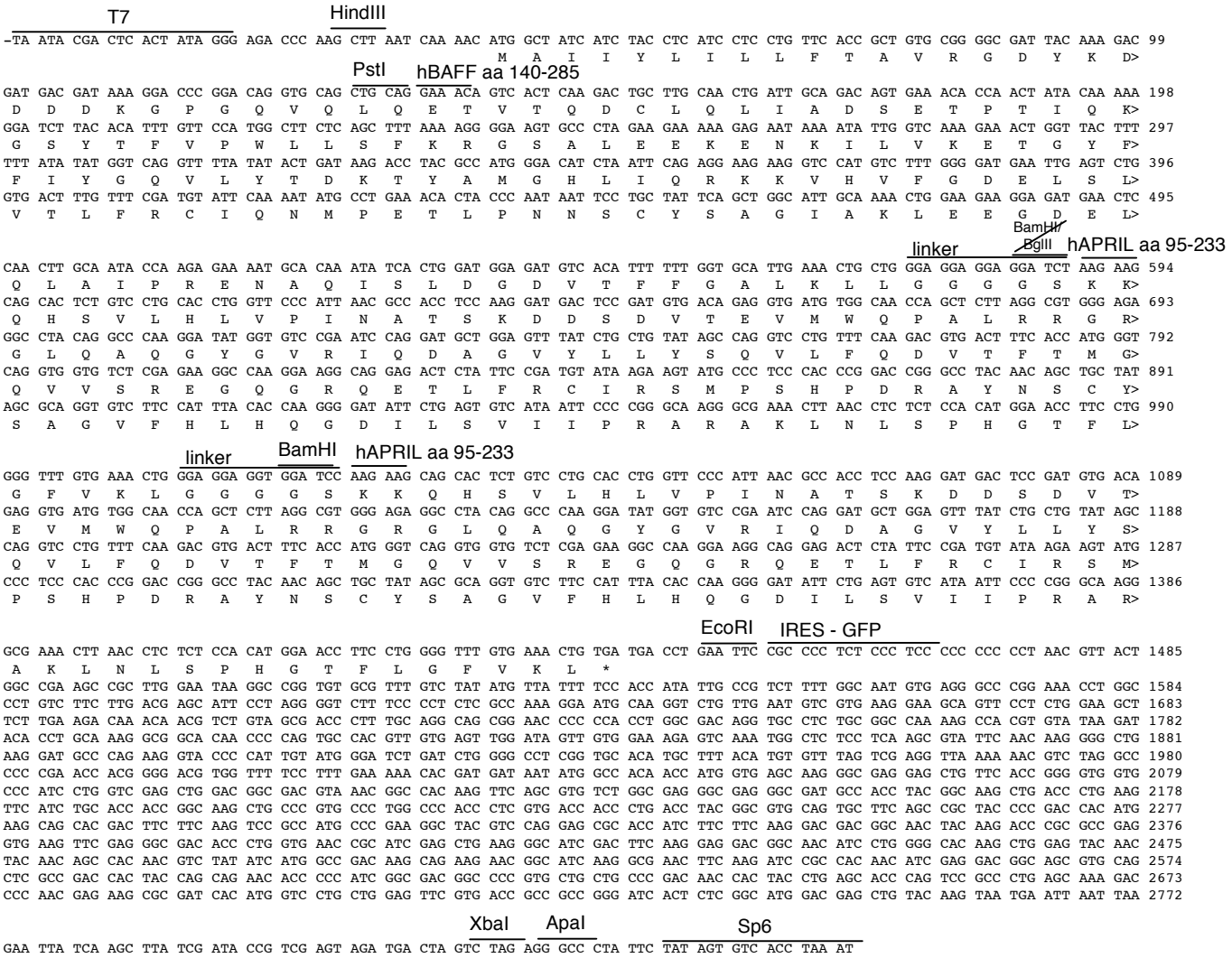
ps2883 **Fc hAPRIL mono1 hBAFF mono2 hBAFF mono3 IRES GFP in PCR3**



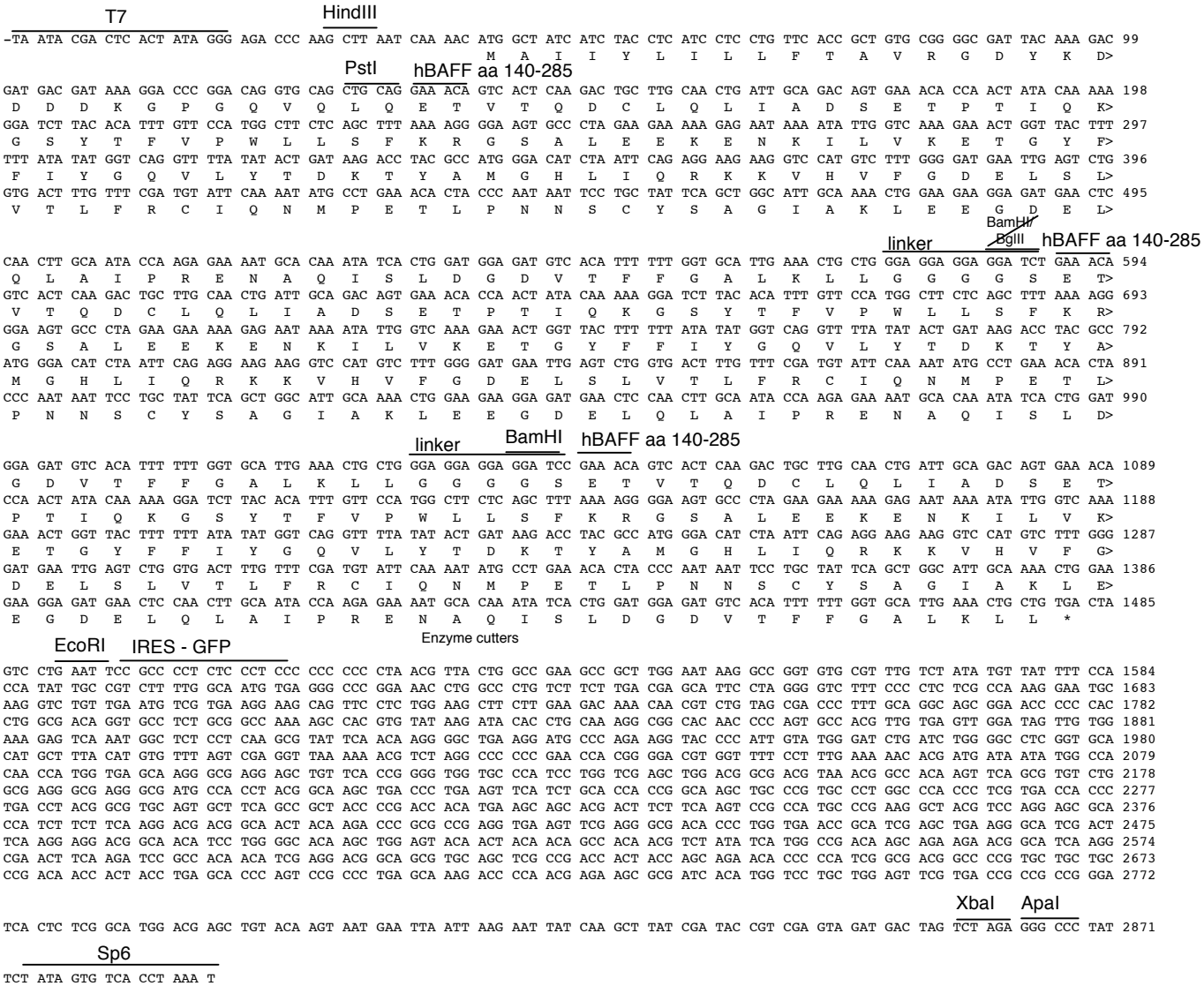
ps2886 **Flag hAPRIL mono1 hBAFF mono2 hBAFF mono3 IRES GFP in PCR3**



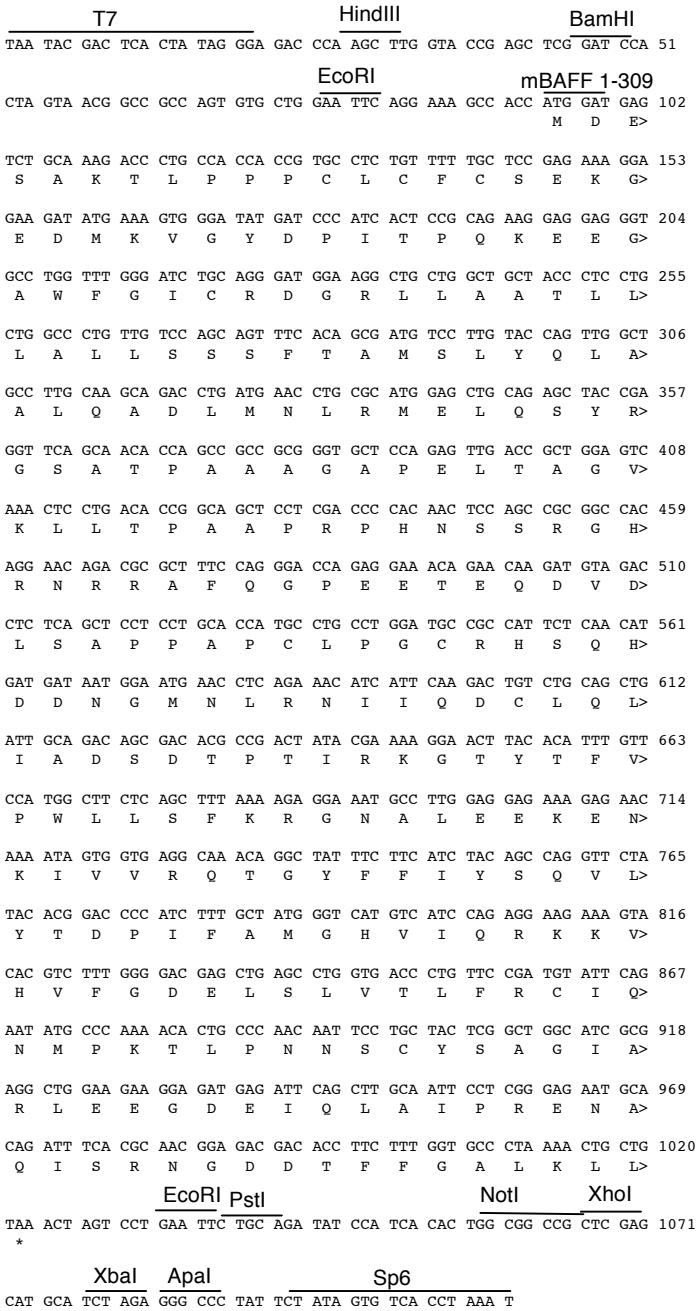
ps2889 **Flag hBAFF mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3**



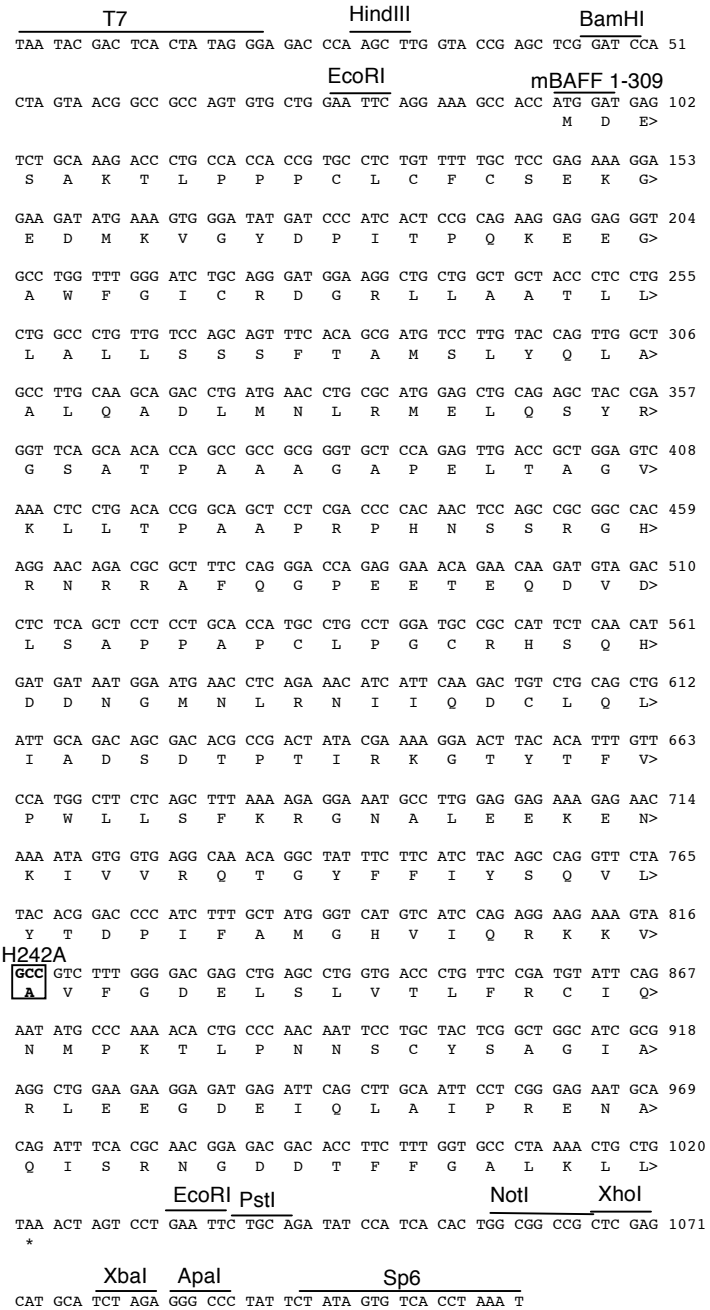
ps2890 **Flag hBAFF mono1 mono2 mono3 IRES GFP in PCR3**



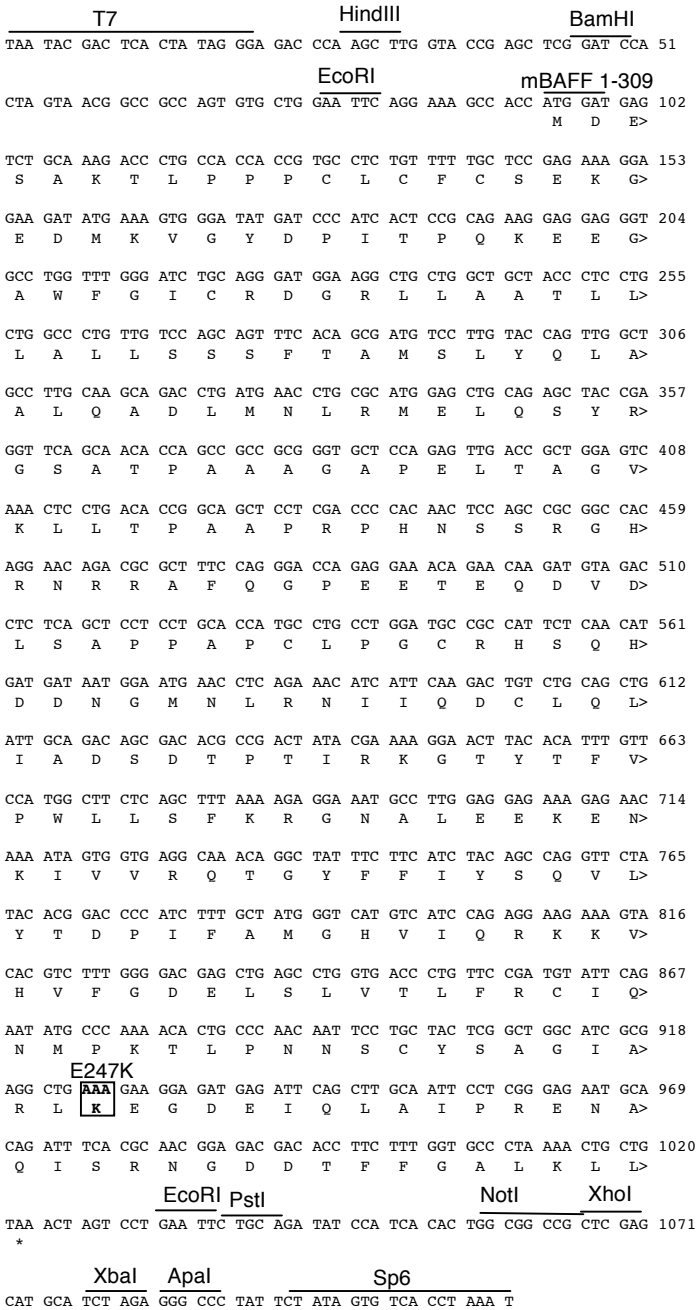
ps694 **m BAFF full in PCR3**



ps2355 **m BAFF H242A full in PCR3**



ps2575 **m BAFF E278K full in PCR3**



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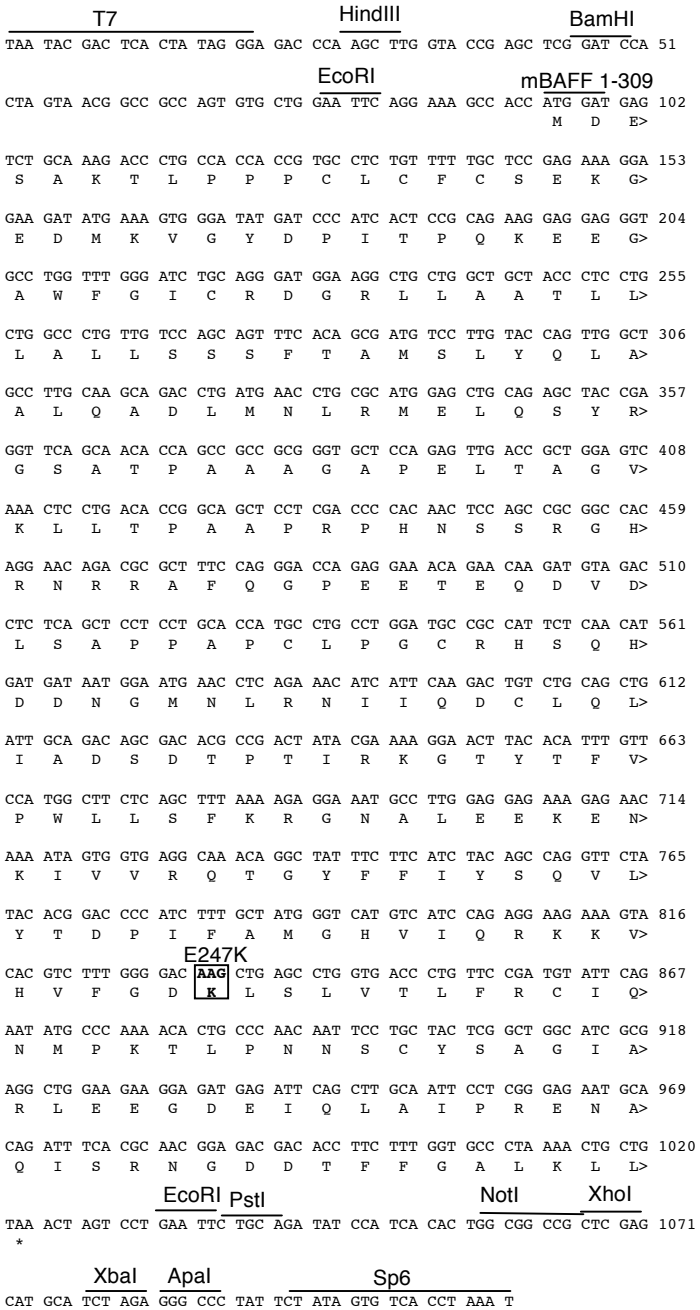

ps2859 **m BAFF 5' probe for E247K knock-in in pBluescript**

pBluescript
TAATACGACT CACTATAGGG CGAATTGGGT ACCGGGCCCC CCCTCGAGGT

HindIII mBAFF 5' sequence
CGACGGTATC GATAAGCTTG TTGCATAACA AATGATATCC AAAGATGGAG 100
TCGTCTACTT CAGTTCTACA TTTTTCCTTC GTGGCTGAAA TGAAAATGAC
TTTGGAGAGT TGACCCTTTA TGAAGCCGCT TAGCTACATC CATTTGTTAG 200
TTTTGCTGGG TTGAATATCT GTTCCATAGA TGACTGCTTT GTGTGACAAC
AAACGGTTCT ACTTTACTAC TTTCAGTTGG ATGCCTTTCC TCCGGTTCAC 300
TTTGTATGCT GGCTGCTGTA GCTGGAATTC CAGTGCAGCA CTGGGCAGCA
GTAATAAAAA TCCTGTCCTG CACCCACCAA GGAGCATTGC ATCTACTTAC 400
ATGATTAATG TTTGTTATGG AGTTTCTTGT GGTTTCTGGC TTCAGGACCA
GGGTGATTCT AGCTTCACGT CATGAGTTAA TAACTATTCT GGTTTCTAGA 500
AGAATTTGAG GAAGTTATTG GAAGTGTCTT GTTTATATGT CACCATCCTG
GTTCCCTTCA GTTGCATAAA CATGTTTAAT AGTTCCATGT CTGGCATTTT 600

BamHI BamHI pBluescript
GGGGGATGGT TCCTGTTAAG GGGATCCCTG CAGCCCGGGG GATCCACTAG
TTCTAGAGCG GCCGCCACCG CGGTGGAGCT CCAGCTTTTG TTCCCTTTAG 700
TGAGGGTTAA TT

ps2871 **m BAFF 3' probe (in exon 7) for E247K knock-in in pBluescript**

pBluescript
TAATACGACT CACTATAGGG CGAATTGGGT ACCGGGCCCC CCCTCGAGGT

HindIII mBAFF 3' sequence
CGACGGTATC GATAAGCTTG CATCGCGAGG CTGGAAGAAG GAGATGAGAT 100
TCAGCTTGCA ATTCCTCGGG AGAATGCACA GATTTCACGC AACGGAGACG
ACACCTTCTT TGGTGCCCTA AAACTGCTGT AACTCACTTG CTGGAGTGCG 200
TGATCCCCTT CCCTCGTCTT CTCTGTACCT CCGAGGGAGA AACAGACGAC
TGGAAAAACT AAAAGATGGG GAAAGCCGTC AGCGAAAGTT TTCTCGTGAC 300
CCGTTGAATC TGATCCAAAC CAGGAAATAT AACAGACAGC CACAACCGAA
GTGTGCCATG TGAGTTATGA GAAACGGAGC CCGCGCTCAG AAAGACCGGA 400
TGAGGAAGAC CGTTTTCTCC AGTCCTTTGC CAACACGCAC CGCAACCTTG
CTTTTTGCCT TGGGTGACAC ATGTTCAGAA TGCAGGGAGA TTTCCTTGTT 500
TTGCGATTTG CCATGAGAAG AGGGCCCACA ACTGCAGGTC ACTGAAGCAT

BamHI
TCACGCTAAG TCTCAGGATT TACTCTCCCT TCTCATGCTA AGTACACGGA 600

pBluescript
TCCACTAGTT CTAGAGCGGC CGCCACCGCG GTGGAGCTCC AGCTTTTGTT
CCCTTTAGTG AGGGTTAATT

ps2846 **Targeting vector for mBAFF E247K knock-in in pBS**