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

DAS Dolon

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UNIL | Université de Lausanne Faculté de biologie et de médecine

Département de biochimie

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

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présentée à la

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

par

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

Lausanne, le 6 décembre 2012

pour Le Doyen de la Faculté de Biologie et de Médecine

Christian Widmann Prof.

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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 $\alpha 1\beta 2$ 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 BAFF₂APRIL, like BAFF, binds to all BAFF receptors - namely BAFFR, TACI and BCMA -, while APRIL₂BAFF 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 $\alpha 1\beta 2$ 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 BAFF₂APRIL, comme BAFF, lie tous les récepteurs de BAFF - c'est-à-dire BAFFR, TACI et BCMA -, alors qu'APRIL₂BAFF 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 \rightarrow TLR4), bacterial flagellin, lipoteichoic acid, lipoproteins and peptidoglycan (\rightarrow TLR1,-2,-6), mannose residues, N-formylmethionine, fungal glucans, endogenous heat shock proteins, extracellular matrix molecules, and nucleic acid variants associated with viruses (vRNA \rightarrow TLR3, unmethylated cytosinguanosin dinucleotide (CpG) \rightarrow TLR9, dsRNA) and bacteria (bacterial DNA, unmethylated cytosinguanosin dinucleotide (CpG) \rightarrow 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].

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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 10¹³ 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 $\sim 20-30\%$ between family members and is responsible for receptor binding. Atomic level structures are available among others for the THD of TNF [13],[14], LT α [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 LT α 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 LT α . 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-κB 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)

Gene	Phenotype
Cytokine	
TNF-β (I T-γ)	Defects in secondary lymphoid organ development: disorganized splenic microarchitecture ⁶¹
TNF-a	No observations and generating in LN: lack solaric primary R-cell follicles: dispresentation of the convertes and germinal conters ⁶²
17-8	Defects in organonenesis of the lymphoid system: lymphocytosis in the circulation and peritoneal cavity: lymphocytic infiltrations
210	in lungs and liver ⁸¹
OX40L	Defective T-cell responses ⁸²
CD40L	Defective T-cell and IgG responses; hyper-IgM syndrome ^{go}
FasL	Impaired activation-induced T-cell death; lymphoproliferation; autoimmunity ⁸²
4–1BBL	Defective T-cell responses ¹²²
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 ⁹⁵
BAFF	Impaired B-cell maturation ⁹⁶
	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
	apontosis [®]
LT-BB	Absence of I.N. PP' defective GC formation ⁹²
0X40	Defactive T-cell resonance
CD40	Defective la class switching and GC formation causing immunodeficiency ⁸²
Fas	Impaired activation-induced T-cell death: lumphonolificrative sundrome: autoimmunity/9
1 45	Inspared advantation in advantation is collising a collision advantation of autonantiation and
	Desistanto o autoreación la biola deses of antinon ana to anostesis in matura COU+1 a celle%
0027	
CD27	Detective Freeinesponses-
4 188	Impaired lowing of responses, reduced recammentory AD responses**
4-100	Enhanced incent response out normal incent developments
	Reduced humber of KK and KKT cers, resistance to LF of hubber shock syndromers
	Increased number of myeloid progenitor and mature DCs, impaired DC function**
225	Heduced atheroscierosis in hyperlipidemic mice ***
DH5	Normal development with an enlarged thymus
RANK	Osteopetrosis; absence of osteoclasts and LN; PP present; abnormal B-cell development ^{ac}
OPG	Osteoporosis; arterial calcification ^{az}
FN14	Reduced proliferative capacity; altered myotube formation to a
	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 ⁹²
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	3 · · · · · · · · · · · · · · · · · · ·
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

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

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-18-converting enzyme; Apaf-1, apoptotic protease activating factor-1; EDARADD, ectodysplasin-A receptor-associated adapter protein; LN, lymph node; FDC, follicular dendritic cell; LPS, lipoplysaccharide; 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 membranebound 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-y 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 (FcyRI) [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-KB 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-kB inducing kinase (NIK), BAFFR-mediated TRAF3 elimination rescues NIK expression. NIK potently activates the alternative non-classical NF-kB2 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), IKKa -null and NF-kB2 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 IKKa activity but impaired responses to further activation of this kinase (IKKa-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- κB 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).

Rac GTP



Fig7.NF-кBsignalingdownstreamofBAFFR.TakenfromreviewbyMackayetal.(Nature reviews 2009)

Taken together, these data indicate that the non-canonical NF- κ B pathway is a key downstream element of BAFF signals. However, the canonical NF- κ B pathway can also mimic BAFF signaling, as inactivation of NEMO in B cells, and to a lesser extent NF- κ B1, also leads to B cell deficiency, whereas expression of constitutively active IKK β 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 κ B 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- κ B 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- κ B 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 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. LT α 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 LT α is does not retain the transmembrane region like TNF but is attached to the surface by forming a complex with surface LT β [161, 162]. The predominant form of membrane bound LT α 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. LT β -R a receptor for surface LT α has been identified and binds to LT $\alpha_1\beta_2$ [163]. Trimeric LT α 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 LT β R is structurally related to both TNFR1 and TNFR2, yet does not recognize either soluble LT α or TNF. The phenotypic differences between LT α - and LT β -null mice led to the conclusion that the LT and TNF systems

are distinct. In general, the hLTa gene is expressed solely by lymphocytes and not by monocytes [165]. The expression of both $LT\alpha$ and $LT\beta$ in cell lines is dramatically induced following cell activation whereas LTB gene transcription may be more constitutive, at least as revealed by analysis of primary murine splenocytes [166, 167]. LTa-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 LTBR-Ig, but not TNF-R55-Ig treatment, during development could mimic much of the phenotype of the LT α - null mouse, it led to the conclusion that the surface LT $\alpha\beta$ complex is involved in lymph node organogenesis and splenic organization [171]. Surface $LT\alpha\beta$ plays a role in maintenance of splenic organization in the mature system as demonstrated by the use of the soluble LT β R to inhibit LT $\alpha\beta$ function in normal adult mice [172]. Surface LT $\alpha\beta$ complex possibly does not account for all the developmental functions of LTa, administration of LTBR-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 LT α gene in transgenic mice under the control of the rat insulin promoter led to the formation of lymph nodes in unusual locations, indicating that $LT\alpha$ 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 Fcligands, 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 Sall/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 BgIII and BamHI generate identical overhangs that can be ligated together without reconstituting either a BamHI or a BgIII 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 IRES-GFP (ps2879), Flag-BAFF-BAFF IRES-GFP (ps2890), Flag-APRIL-BAFF-BAFF IRES-GFP (ps2886), Flag-BAFF-APRIL-APRIL IRES-GFP (ps2889), Fc-APRIL-BAFF-BAFF IRES-GFP (ps2886), Flag-BAFF-APRIL-APRIL IRES-GFP (ps2889), Fc-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]: FlagmBAFF 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% NaHCO₃. 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: 2×10^5 cells were seeded in a well of a 6well 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 μ g/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 μ l 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 CaCl₂ 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 NaHPO₄, 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 μ m, supplemented with 0.05% NaN₃ 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 A₂₈₀ 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 μ l, 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 μ l of fractions eluted from the Superdex-200 column were supplemented with 20 μ l of 1 mg/ml lysozyme and precipitated for 10 min on ice with 50 μ l 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 μ l of 50 mM NaHCO₃ pH 9 (carbonate buffer) or in PBS. Plates were saturated with 350 μ l 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 μ l 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 μ l 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 μ l of OPD solution, and the reaction was stopped after colour development by the addition of 50 μ l 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 μ g/ml in carbonate buffer, followed by addition of Flag-tagged ligands in cell supernatant (usually 100 μ l supernatant in DMEM-FCS or 20 μ l in OPTIMEM plus 80 μ l incubation buffer) and revelation with biotinylated anti-Flag M2 antibody at 0.5 μ g/ml and HRP-conjugated streptavidin at 1/4000.

Detection of Fc-tagged ligands.

Plates were coated with mouse anti-human antibody at 5 μ g/ml in carbonate buffer, followed by Fctagged 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 μ g/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 μ g/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 μ g/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 μ l 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 μ l of PBS and eluted with 15 μ l of 50 mM Na-citrate pH 2.7. The eluate was neutralized and denatured with 5 μ l of Tris 1M pH 9 and 10 μ l 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 H₂O₂ 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.

Antigen	Primary antibody	Secondary antibody
	(concentration or dilution)	(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)

Table 1: Antibodies used for Western blot

Cytotoxic assay.

BAFF or APRIL preparations were titrated with 2-fold dilutions in 50 µl of RPMI, 10% FCS in 96wells 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% CO₂. 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), A₄₉₀ 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 μ l of DMEM, 10% FCS and left to adhere overnight. Medium was aspirated and replaced with 50 μ l of fresh medium. Cells were transfected by adding 25 μ l of a transfection mix, prepared as follows (quantities for 1 well): 0.65 μ l of a plasmid mix containing a total of 70 ng plasmid (7.5 ng of NF- κ B 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 μ l DMEM without any

supplement, plus 0.35 μ l of PolyFect. The mix was incubated for 5 min at RT before addition of 20.7 μ l 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 μ l of PBS, lyzed in 50 μ l of 1 x concentrated passive lysis buffer for 15 min on a shaking table. 25 μ l of lysate was transferred to a black 96-well plate (PerkinElmer, 6005270), 15 μ l of firefly luciferase substrate was added and luminescence was monitored in a TopCount-NTX luminometer (Packard). 15 μ l 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- κ B 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 μ l/well corresponding to 2-3 x 10⁵ cells) and centrifuged for 5 min at 1200 rpm. Medium was aspirated, cells were resuspended in 50 μ l of FACS buffer (PBS, 5% FCS) containing 0.1 μ l of Liquemine and ligands of interest at the indicated concentrations, and incubated for 20 min on ice. 200 μ l of FACS buffer was then added, cells were spun and resuspended in a) 50 μ l of M2-biotin at 1/500 in FACS buffer for 20 min on ice followed by a wash and staining with 50 μ l of PE-coupled streptavidin at 1/500 in FACS buffer for 20 min on ice.

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 NH₄Cl, 1 mM KHCO₃, 1 mM Na₂-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 μ l of MACS buffer/10⁷ cells to which 10 μ l of biotin beads/10⁷ 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 μ l for 10⁸ 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 μ l of B splenocyte proliferation medium (RPMI 10% FCS, 0.5 mM β -mercaptoethanol, antibiotics) supplemented with 2 μ g/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 μ l of medium. When required, anti-Flag M2 antibody or anti-BAFF-R 9B9 antibody were added at a final concentration of 1 μ g/ml and 0.25 μ g/ml, respectively. Cells were grown for 48 h, after which time 1 μ Ci 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 μ l 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 H₂O
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)
H₂O 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 Na₃Citrate, 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 μ l of 5% powdered skimmed milk, 1.35 ml H₂O) 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 LT α and LT β and the predominant form of this heteromer with a LT $\alpha_1\beta_2$ stoichiometry was found to specifically bind to LT β R 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 Flagligands, Fc-ligands, and heteromers thereof (Fig.10). This approach detected the interaction of most TNF ligands with themselves, and of LT α with LT β . 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-LT α and LIGHT-LT β (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





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 μ g/ml in panels B and E or Flag-hLT α / Fc-hLT β 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).



Fc ligands

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.





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.



Fc ligands



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 immunoprecipitatation 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, immunoprecipitations 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, LT α and LT β , 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.





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 LT $\alpha\beta$ heteromers were designed as positive controls to validate this strategy. Flag-tagged single chain LT-[$\alpha\alpha\alpha$], LT-[$\alpha\alpha\beta$], LT-[$\beta\beta\alpha$] and LT-[$\beta\beta\beta$] 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-[$\alpha\alpha\alpha$] binding to TNFR1 but not LT β R, and LT-[$\beta\beta\alpha$] binding to LT β R (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, hLTa and of the hLTa β heteromer. C. Supernatant of 293T cells transiently transfected with single chain lymphotoxin homotrimers (Flag-hLT- [aaa] and - [$\beta\beta\beta$]) or heterotrimers (Flag-hLT- [aa β] and -[$\beta\beta\alpha$]), or with conventional Flag-hLTa ± Fc-hLT β were analyzed by
western blotting with anti-Flag and anti-Fc antibodies. D. Experimental strategy used in panel E to monitor receptorligand interactions by ELISA to measure binding of the indicated ligands to hTNFR1-Fc (left) or hLT β R-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 receptorbinding 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 receptorbinding 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.



Binding to Receptors - TNFR1-Fc - BCMA:Fc

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 Flagtagged (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.

Flag Fc BAA BBB ABB BAA BBB Single-chain ligand AAA ABB AAA 40 µ g Yield in S200 fraction(s) of interest <10 µg 160 µg 400 µg 220 µg $200 \mu g$ 600 µg 160 µg В А Fc-tagged single-chain ligands Flag-tagged single-chain ligands AA ABB BBB ABB BBB 888 888 ABB 388 BAA AAA AAA 8 ¥ BAA ABB BBB ¥ ₹ BA BA ₿₿ **B** B 130-130-95-72-95-72-55-55-36-36-28_ 28_ 17_ 17_ WB: anti-BAFF anti-Flag anti-APRIL WB: anti-BAFF anti-Fc anti-APRIL

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

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]	Approximative	Approximative	
		MW [kDa]	N-link	Theoric +	MW [kDa]	MW [kDa]	WB/S200
		Theoric	Theor	N-linked	by WB DTT	by \$200	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 μ g/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 μ g/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.



trimeric fraction (ng/ml)

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 μ g/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-кВ 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-KB luciferase reporter assay

293 *T* cells transiently co-transfected with full-length human BCMA and NF- κ B reporter plasmids were stimulated with purified Flag-ligands at the indicated concentration in the presence or absence of anti-Flag antibody (M2). NF- κ B 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- κ B activity on HEK 293 T cells. All the homomers and heteromers showed similar EC₅₀ of around 100 ng/ml, indicating that they have similar signaling capacities via BCMA.

3.2.5. APRIL-BAFF-BAFF versus 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.



Single chain Flag BBB trimeric fraction (ng/ml)

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 μ g/ml. Cell viability was measured the following day using PMS-MTS.



Single chain Flag ligands trimeric fraction (ng/ml)

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 μ g/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 60mer 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 His²¹⁸ might be important for ligand assembly through flap-flap interactions. In particular, Glu²⁵⁴ of one BAFF 3-mer contacts Lys²⁵² of another 3-mer, and vice-versa (Glu²⁷⁸ and Arg²⁷⁶ in mouse BAFF). The same is true for the pair Glu²²³ and Lys²¹⁶ (Glu²⁴⁷ and Lys²⁴⁰ in mouse BAFF). We reasoned that mutations E278K and E247K should generate electrostatic repulsion with Arg²⁷⁶ and Lys²⁴⁰, 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 10TZ.

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 μ l 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 μ l 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 trimersensitive 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. BAFFcontaining 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.



Total loading volume =15µl



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 Creexpressing 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 Spel 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. LT α has been shown to form soluble homotrimers that bind to TNFR1. It forms a surface bound complex with LT β that binds to LT β -R. Formation of LT α -LT β 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, LT α , LT β .

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 LIGHT₂FasL may bind to HVEM better than Fas while a species of FasL₂LIGHT 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 ligands expressed and could be efficiently secreted. They showed binding to their cognate receptors TNFR1 and LT β R 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-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 (LT α -LT β 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 LTa 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 APRIL₂BAFF was defined owing to their limitations of recombinant protein production. BAFF₂APRIL 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.

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 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 APRIL₂BAFF 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 or 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 60mer, as in a healthy condition it is impossible to detect its presence. It is only upon overexpression 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 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

TZ HindIII																													Sal					
			T7						Hir	ndIII				HA	sigr	nal													Xho	ol l	hlg	G1 a	aa 24	5-470
-TA	ATA	CGA	CTC	ACT	ATA	GGG	AGA	CCC	AAG	CTT	AAT	CAA	AAC	ATG	GCT	ATC	ATC	TAC	CTC	ATC	CTC	CTG	TTC	ACC	GCT	GTG	CGG	GGC	CTC	GAC	AAA	ACT	99	
														М	A	I	I	Y	L	I	L	L	F	т	Α	v	R	G	L	D	K	T>		
CAC	ACA	TGC	CCA	CCG	TGC	CCA	GCA	CCT	GAA	CTC	CTG	GGG	GGA	CCG	TCA	GTC	TTC	CTC	TTC	CCC	CCA	AAA	CCC	AAG	GAC	ACC	CTC	ATG	ATC	TCC	CGG	ACC	198	
Н	т	С	Р	Р	С	Р	A	Р	Е	L	L	G	G	Ρ	s	v	F	L	F	Р	Р	K	Р	K	D	т	L	М	I	s	R	T>		
CCT	GAG	GTC	ACA	TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	297	
P	E	V	T	C	V	V	V	D	V	S	Н	E	D	P	E	V	K	F	N	W	Y	V	D	G	V	E	V	H	N	A	K	-T>	200	
AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC	CGT	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC	TGG	CTG	AAT	GGC	AAG	GAG	TAC	AAG	TGC	AAG	396	
CTTC	P	R NAC	5 777	E CCC	Q	I CCA	IN CCC	5	ALC N	I CAC	R 777	NCC	V Amc	5	V 777	L	.T.	v ccc		п ссс	CC N	D C D D	W CCD		CIIIC	G mac	NCC	CTTC	I CCC	CCA	тсс	CCC	405	
V	s	N	K	Δ	T.	P	Δ	P	T	F	K	T ACC	T	s	K	Δ	K	G	O	P	R	F	D	CAG 0	V	v	T	T.	P	P	s	R>	495	
GAT	GAG	CTG	ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC		GGC	TTC	TAT	222	AGC	GAC	ATC	GCC	GTG	GAG	TGG	GAG	AGC	ААТ	GGG	CAG	CCG	GAG	594	
D	E	т.	т Т	ĸ	N	0	v	s	т.	т Т	C	т.	v	K	G	F	v	P	s	D	т	Δ	v	E	W	E	s	N	G	0	P	E>		
AAC	AAC	TAC	AAG	ACC	ACG	ССТ	ccc	GTG	TTG	GAC	тсс	GAC	GGC	TCC	TTC	TTC	СТС	TAC	AGC	AAG	СТС	ACC	GTG	GAC	AAG	AGC	AGG	TGG	CAG	CAG	GGG	AAC	693	
N	N	Y	K	т	т	Р	Р	v	L	D	s	D	G	s	F	F	L	Y	s	K	L	т	v	D	K	s	R	W	0	0	G	N>		
GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CTG	TCT	CCG	GGT	AAA	AGA	TCT	CCG	CÂG	CCG	CAG	CCG	792	
v	F	s	С	s	v	М	Н	Е	А	L	н	N	Н	Y	т	Q	К	s	L	s	L	s	Р	G	к	R	s	Р	Q	Р	Q	P>		
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									Da			u	nB		aa	140-	285																	
AAA	CCG	CAG	CCG	AAA	CCG	GAA	CCG	GAA	GGA	TCC	CTG	CAG	GAA	ACA	GTC	ACT	CAA	GAC	TGC	TTG	CAA	CTG	ATT	GCA	GAC	AGT	GAA	ACA	CCA	ACT	ATA	CAA	891	
K	Р	Q	Р	K	Р	Е	Р	Е	G	s	L	Q	Е	т	v	т	Q	D	С	L	Q	L	I	A	D	s	Е	т	Ρ	т	I	Q>		
AAA	GGA	TCT	TAC	ACA	TTT	GTT	CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGG	GGA	AGT	GCC	CTA	GAA	GAA	AAA	GAG	AAT	AAA	ATA	TTG	GTC	AAA	GAA	ACT	GGT	TAC	990	
K	G	S	Y	т	F	v	Р	W	L	L	S	F	K	R	G	S	A	L	Е	Е	K	Е	N	K	I	L	V	K	Е	т	G	¥>		
TTT	TTT	ATA	TAT	GGT	CAG	GTT	TTA	TAT	ACT	GAT	AAG	ACC	TAC	GCC	ATG	GGA	CAT	CTA	ATT	CAG	AGG	AAG	AAG	GTC	CAT	GTC	TTT	GGG	GAT	GAA	TTG	AGT	1089	
F	F'	L	Y	G	Q	V	고	Y	T	D	K	T	Y	A	M	G	Н	L	1 mcc	Q	R	K	K	V	Н	V	F.	G	D	E	L	S>	1100	
CTG	GTG	ACT	TTG	TTT	CGA	TGT	ATT	CAA	AA'I'	ATG	CCT	GAA	ACA	CTA	CCC D	AAT	AAT	TCC	TGC	TAT	TCA	GCT	GGC	ATT	GCA	AAA	CTG T	GAA	GAA	GGA	GAT	GAA	1188	
ц	v	1	ц	г	ĸ	C	T	Q	IN	м	P	Б	T	ц	P	IN	IN	5	C	T	5	A	G	1	А	к	ц	Б	Б	Bam	шř	E~		
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CTC	CAA	CTT	GCA	ATA	CCA	AGA	GAA	AAT	GCA	CAA	ATA	TCA	CTG	GAT	GGA	GAT	GTC	ACA	TTT	TTT	GGT	GCA	TTG	AAA	CTG	CTG	GGA	GGA	GGA	GGA	TCT	GAA	1287	
L	Q	L	A	I	Ρ	R	Е	N	А	Q	I	s	L	D	G	D	v	т	F	F	G	А	L	K	L	L	G	G	G	G	s	E>		
ACA	GTC	ACT	CAA	GAC	TGC	TTG	CAA	CTG	ATT	GCA	GAC	AGT	GAA	ACA	CCA	ACT	ATA	CAA	AAA	GGA	TCT	TAC	ACA	$\mathbf{T}\mathbf{T}\mathbf{T}$	\mathbf{GTT}	CCA	TGG	CTT	CTC	AGC	$\mathbf{T}\mathbf{T}\mathbf{T}$	AAA	1386	
т	v	т	Q	D	С	L	Q	L	I	А	D	S	Е	т	Ρ	т	I	Q	K	G	s	Y	т	F	v	Ρ	W	L	L	s	F	K>		
AGG	GGA	AGT	GCC	CTA	GAA	GAA	AAA	GAG	AAT	AAA	ATA	TTG	GTC	AAA	GAA	ACT	GGT	TAC	TTT	TTT	ATA	TAT	GGT	CAG	GTT	TTA	TAT	ACT	GAT	AAG	ACC	TAC	1485	
R	G	s	A	L	Е	Е	K	Е	N	K	I	L	v	K	Е	т	G	Y	F	F	I	Y	G	Q	v	L	Y	т	D	К	т	Υ>		
GCC	ATG	GGA	CAT	CTA	ATT	CAG	AGG	AAG	AAG	GTC	CAT	GTC	TTT	GGG	GAT	GAA	TTG	AGT	CTG	GTG	ACT	TTG	TTT	CGA	TGT	ATT	CAA	AAT	ATG	CCT	GAA	ACA	1584	
A	М	G	н	L	I	Q	R	K	K	v	Н	v	F	G	D	E	L	s	L	V	т	L	F	R	С	I	Q	N	М	Р	Е	T>		
CTA	CCC	AAT	AAT	TCC	TGC	TAT	TCA	GCT	GGC	ATT	GCA	AAA	CTG	GAA	GAA	GGA	GAT	GAA	CTC	CAA	CTT	GCA	ATA	CCA	AGA	GAA	AAT	GCA	CAA	ATA	TCA	CTG	1683	
Г	Р	N	N	S	С	¥	s	A	G	Ţ	A	K	Г	Е	Е	G		Е	Г	Q	Г	A	Ţ	Р	R	Е	N	A	Q	Ţ	S	Τ>		
													linl	ker		Barr	ηΗΙ	hB	AFF	aa	140-	285												
GAT	GGA	GAT	GTC	ACA	TTT	TTT	GGT	GCA	TTG	AAA	CTG	CTG	GGA	GGA	GGA	GGA	TCC	GAA	ACA	GTC	ACT	CAA	GAC	TGC	TTG	CAA	CTG	ATT	GCA	GAC	AGT	GAA	1782	
D	G	D	v	т	F	F	G	А	L	K	L	L	G	G	G	G	s	Е	т	v	т	Q	D	С	L	Q	L	I	А	D	s	E>		
ACA	CCA	ACT	ATA	CAA	AAA	GGA	TCT	TAC	ACA	TTT	GTT	CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGG	GGA	AGT	GCC	CTA	GAA	GAA	AAA	GAG	AAT	AAA	ATA	TTG	GTC	1881	
т	Р	т	I	Q	К	G	s	Y	т	F	v	Ρ	W	L	L	s	F	К	R	G	s	А	L	Е	Е	К	Е	N	К	I	L	V>		
AAA	GAA	ACT	GGT	TAC	TTT	$\mathbf{T}\mathbf{T}\mathbf{T}$	ATA	TAT	GGT	CAG	\mathbf{GTT}	TTA	TAT	ACT	GAT	AAG	ACC	TAC	GCC	ATG	GGA	CAT	CTA	ATT	CAG	AGG	AAG	AAG	GTC	CAT	GTC	$\mathbf{T}\mathbf{T}\mathbf{T}$	1980	
K	Е	т	G	Y	F	F	I	Y	G	Q	v	L	Y	т	D	K	т	Y	А	М	G	Н	L	I	Q	R	K	K	v	Н	V	F>		
GGG	GAT	GAA	TTG	AGT	CTG	GTG	ACT	TTG	TTT	CGA	TGT	ATT	CAA	AAT	ATG	CCT	GAA	ACA	CTA	CCC	AAT	AAT	TCC	TGC	TAT	TCA	GCT	GGC	ATT	GCA	AAA	CTG	2079	
G	D	E	L	s	L	v	т	L	F	R	С	I	Q	N	М	Р	E	т	L	Ρ	N	N	s	С	Y	s	А	G	I	А	K	L>		

G D E L S L V T L F R C I Q N M P E T L P N N S C Y S A G I A K L> GAA GAA GGA GAT GAA CTC CAA CTT GCA ATA CCA AGA GAA AAT GCA CAA ATA TCA CTG GAT GGA GAT GTC ACA TTT TTT GGT GCA TTG AAA CTG CTG TGA 2178 E E G D E L Q L A I P R E N A Q I S L D G D V T F F G A L K L L * <u>Xbal</u> Apal <u>Sp6</u>

CTA GTC CTG AAT TCC CCG GGT CTA GAG GGC CCT ATT CTA TAG TGT CAC CTA AAT



(ps2876) Fc hAPRIL mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3

																													Sal	~			
			T7						Hir	ndIII				HA	sigr	nal													Xho	n l	hlg	G1 a	aa 245-47
-TA	ATA	CGA	CTC	ACT	ATA	GGG	AGA	CCC	AAG	CTT	AAT	CAA	AAC	ATG	GCT	ATC	ATC	TAC	CTC	ATC	CTC	CTG	TTC	ACC	GCT	GTG	CGG	GGC	CTC	GAC	AAA	ACT	99
CAC	ACA	TGC	CCA	CCG	TGC	CCA	GCA	сст	GAA	CTC	CTG	GGG	GGA	M	A TCA	I GTC	I TTC	Y CTC	L TTC	I	L CCA	L AAA	F	T AAG	A GAC	V ACC	R CTC	G ATG	L ATC	D TCC	K CGG	T> ACC	198
Н	т	С	Р	Р	С	Р	A	Р	Е	L	L	G	G	Р	s	v	F	L	F	Р	Р	К	Р	К	D	т	L	М	I	s	R	T>	
CCT	GAG	GTC	ACA	TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	297
P AAG	ECCG	V CGG	T GAG	GAG	V CAG	V TAC	V AAC	AGC	V ACG	S TAC	H CGT	E GTG	D GTC	AGC	E GTC	V CTC	K ACC	F' GTC	N CTG	W	Y CAG	V GAC	D TGG	G CTG	V AAT	EGGC	V AAG	H GAG	N TAC	A AAG	K TGC	'T'> AAG	396
к	Ρ	R	Е	Е	Q	Y	N	s	т	Y	R	v	v	s	v	L	т	v	L	Н	Q	D	W	L	N	G	К	Е	Y	К	С	К>	
GTC	TCC	AAC	AAA	GCC	CTC	CCA	GCC	CCC	ATC	GAG	AAA	ACC	ATC	TCC	AAA	GCC	AAA	GGG	CAG	CCC	CGA	GAA	CCA	CAG	GTG	TAC	ACC	CTG	CCC	CCA	TCC	CGG	495
GAT	GAG	CTG	ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	GGC	TTC	TAT	ccc	AGC	GAC	ATC	GCC	GTG	GAG	v TGG	I GAG	AGC	AAT	GGG	CAG	CCG	GAG	594
D	Е	L	т	К	N	Q	v	S	L	т	С	L	v	К	G	F	Y	Ρ	s	D	I	А	v	Е	W	Е	s	N	G	Q	Р	E>	
AAC	AAC	TAC	AAG	ACC	ACG	CCT	CCC	GTG	TTG	GAC	TCC	GAC	GGC	TCC	TTC	TTC	CTC	TAC	AGC	AAG	CTC	ACC	GTG	GAC	AAG	AGC	AGG	TGG W	CAG	CAG	GGG	AAC	693
GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CTG	TCT	CCG	GGT	AAA	AGA	TCT	CCG	CAG	CCG	CAG	CCG	792
v	F	S	С	s	v	М	Н	Е	А	L	Н	N	Н	Y	т	Q	К	s	L	s	L	s	Ρ	G	к	R	s	Р	Q	Ρ	Q	P>	
									Bar	nHI	Pst	I	hAF	RIL	aas	95-2	33																
AAA	CCG	CAG	CCG	AAA	CCG	GAA	CCG	GAA	GGA	TCC	CTG	CAG	AAG	AAG	CAG	CAC	TCT	GTC	CTG	CAC	CTG	GTT	CCC	ATT	AAC	GCC	ACC	TCC	AAG	GAT	GAC	TCC	891
K Chrr	P	Q	P	K	P	E	P	E	G	S	L	Q CCm	K	K	Q	H	S	V		H	L	V CC ^m	P	I	N	A	Т	S	K	D	D Tram	S>	990
D	V	T	E	V	M	W	Q	P	A	L	R	R	G	R	G	L	Q	A	Q	G	Y	G	V	R	I	Q	D	A	G	V	Y	L>	550
CTG	TAT	AGC	CAG	GTC	CTG	TTT	CAA	GAC	GTG	ACT	TTC	ACC	ATG	GGT	CAG	GTG	GTG	TCT	CGA	GAA	GGC	CAA	GGA	AGG	CAG	GAG	ACT	CTA	TTC	CGA	TGT	ATA	1089
L AGA	Y AGT	S ATG	Q CCC	V TCC	L CAC	F	Q GAC	D CGG	V GCC	T TAC	F AAC	T AGC	M TGC	G TAT	Q AGC	V GCA	V GGT	S GTC	R TTC	E	G TTA	Q CAC	G CAA	R GGG	Q GAT	E ATT	T CTG	L AGT	F GTC	R ATA	С	I> CCC	1188
R	s	М	Р	s	Н	Р	D	R	А	Y	N	s	С	Y	s	A	G	v	F	Н	L	Н	Q	G	D	I	L	s	v	I	I	P>	
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CGG	GCA	AGG	GCG	AAA	CTT	AAC	CTC	TCT	CCA	CAT	GGA	ACC	TTC	CTG	GGG	TTT	GTG	AAA	CTG	GGA	GGA	GGT	GGA	TCT	AAG	AAG	CAG	CAC	TCT	GTC	CTG	CAC	1287
R	A	R	A	K	L	N	L	S	P	H	G	Т	F	L	G	F	V	K	L	G	G	G	G	S	K	K	Q	Н	S	V	L	H>	1206
CTG L	V	P	I	AAC N	A	ACC	S	AAG K	D	GAC	S	D	V	ACA T	GAG	GTG V	M	W	CAA 0	P	A	L	AGG R	R	GGG	R	GGC	L	CAG O	A	0 0	GGA G>	1380
TAT	GGT	GTC	CGA	ATC	CAG	GAT	GCT	GGA	GTT	TAT	CTG	CTG	TAT	AGC	CAG	GTC	CTG	TTT	CĀA	GAC	GTG	ACT	TTC	ACC	ATG	GGT	CAG	GTG	GTG	TCT	CGA	GAA	1485
Y	G	V	R	I	Q	D	A	G	V	Y	L	L	Y	S	Q	V	L	F	Q	D	V	Т	F	T	M	G TTATT	Q	V	V	S	R	E>	1584
G	Q	G	R	Q	E	T	L	F	R	C	I	R	S	M	P	s	Н	P	D	R	A	Y	N	S	C	Y	S	A	G	V	F	H>	1304
TTA	CAC	CAA	GGG	GAT	ATT	CTG	AGT	GTC	ATA	ATT	CCC	CGG	GCA	AGG	GCG	AAA	CTT	AAC	CTC	TCT	CCA	CAT	GGA	ACC	TTC	CTG	GGG	TTT	GTG	AAA	CTG	GGA	1683
Г	н	Q Dama	G		1	г	s	V	T	T	Р	R	A	R	A	K	Г	N	г	s	Р	н	G	т	F.	Г	G	F.	v	K	Г	G>	
nker		Bam	HI —	hAF	<u>'R</u> IL	aa	95-2	233																									
GGA	GGT	GGA	TCC	AAG K	AAG K	CAG	CAC H	TCT	GTC	CTG T.	CAC H	CTG T.	GTT V	CCC	ATT	AAC	GCC	ACC T	TCC	AAG	GAT	GAC	TCC	GAT	GTG V	ACA T	GAG	GTG V	ATG M	TGG	CAA	CCA P>	1782
GCT	CTT	AGG	CGT	GGG	AGA	GGC	CTA	CAG	GCC	CAA	GGA	TAT	GGT	GTC	CGA	ATC	CAG	GAT	GCT	GGA	GTT	TAT	CTG	CTG	TAT	AGC	CAG	GTC	CTG	TTT	CAA	GAC	1881
A	L	R	R	G	R	G	L	Q	A	Q	G	Y	G	V	R	I	Q	D	A	G	V	Y	L	L	Y	S	Q	V	L	F	Q	D>	1000
V	T	F	T	M	GGT	Q	V	V	S	R	E	GGC	Q	GGA	R	Q	E	T	L	F	R	C	I	R	S	M	P	S	H	P	D	R>	1980
GCC	TAC	AAC	AGC	TGC	TAT	AGC	GCA	GGT	GTC	TTC	CAT	TTA	CAC	CAA	GGG	GAT	ATT	CTG	AGT	GTC	ATA	ATT	CCC	CGG	GCA	AGG	GCG	AAA	CTT	AAC	CTC	TCT	2079
A	Y	N	S	C	Y	s	A	G	v	F	н	L	Н	Q 	G	D	I	L	S	V	I	I	Р	R	A	R	A	ĸ	L	Ν	L	s>	
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CCA D	CAT	GGA	ACC	TTC	CTG	GGG	TTT F	GTG	AAA	CTG	TGA *	TGA	ССТ	GAA	TTC	CGC	CCC	TCT	CCC	TCC	CCC	CCC	CCT	AAC	GTT	ACT	GGC	CGA	AGC	CGC	TTG	GAA	2178
TAA	GGC	CGG	TGT	GCG	TTT	GTC	r TAT	v ATG	TTA	TTT	TCC	ACC	ATA	TTG	CCG	TCT	TTT	GGC	AAT	GTG	AGG	GCC	CGG	AAA	CCT	GGC	CCT	GTC	TTC	TTG	ACG	AGC	2277
ATT	CCT	AGG	GGT	CTT	TCC	CCT	CTC	GCC	AAA	GGA	ATG	CAA	GGT	CTG	TTG	AAT	GTC	GTG	AAG	GAA	GCA	GTT	CCT	CTG	GAA	GCT	TCT	TGA	AGA	CAA	ACA	ACG	2376
TCT CAA	GTA CCC	CAG	ACC TGC	CAC	TGC	AGG GTG	CAG AGT	TGG	AAC ATA	GTT	GTG	GAA	GGC AGA	GAC	AGG	TGC	CTC	TGC	GGC	CAA	AAG GTA	TTC	AAC	GTA AAG	TAA GGG	GAT	ACA AAG	GAT	GCA GCC	AAG	GCG	GCA GTA	24/5 2574
CCC	CAT	TGT	ATG	GGA	TCT	GAT	CTG	GGG	CCT	CGG	TGC	ACA	TGC	TTT	ACA	TGT	GTT	TAG	TCG	AGG	TTA	AAA	AAC	GTC	TAG	GCC	ccc	CGA	ACC	ACG	GGG	ACG	2673
TGG	TTT	TCC	TTT	GAA	AAA	CAC	GAT	GAT	AAT	ATG	GCC	ACA	ACC	ATG	GTG	AGC	AAG	GGC	GAG	GAG	CTG	TTC	ACC	GGG	GTG	GTG	CCC	ATC	CTG	GTC	GAG	CTG	2772
AAG	CTG	CCC	GTG	CCC	TGG	CCC	ACC	CTC	GTG	ACC	ACC	CTG	ACC	TAC	GGC	GTG	CAG	TGC	TTC	AGC	CGC	TAC	CCC	GAC	CAC	ATG	AAG	CAG	CAC	GAC	TTC	TTC	2970
AAG	TCC	GCC	ATG	ccc	GAA	GGC	TAC	GTC	CAG	GAG	CGC	ACC	ATC	TTC	TTC	AAG	GAC	GAC	GGC	AAC	TAC	AAG	ACC	CGC	GCC	GAG	GTG	AAG	TTC	GAG	GGC	GAC	3069
ACC TAT	CTG ATC	GTG ATC	AAC	CGC	ATC	GAG	CTG	AAG	GGC	ATC	GAC	TTC	AAG	GAG TTC	GAC	GGC ATC	AAC	ATC	CTG	GGG ATC	CAC	AAG	CTG	GAG	TAC	AAC	TAC	AAC	AGC	CAC	AAC TAC	GTC	3168 3267
CAG	AAC	ACC	ccc	ATC	GGC	GAC	GGC	CCC	GTG	CTG	CTG	ccc	GAC	AAC	CAC	TAC	CTG	AGC	ACC	CAG	TCC	GCC	CTG	AGC	AAA	GAC	CCC	AAC	GAG	AAG	CGC	GAT	3366
CAC	ATG	GTC	CTG	CTG	GAG	TTC	GTG	ACC	GCC	GCC	GGG	ATC	ACT	CTC	GGC	ATG	GAC	GAG	CTG	TAC	AAG	TAA	TGA	ATT	AAT	TAA	GAA	TTA	TCA	AGC	TTA	TCG	3465
							Xb	bal	A	pal					Spe	6																	
АТА	CCG	TCG	AGT	AGA	TGA	СТА	GTC	TAG	AGG	GCC	СТА	TTC	ТАТ	AGT	GTC	ACC	ТАА	AT															

(ps2877)

)Fc hBAFF mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3

																													Sal	\checkmark			
			Τ7						Hir	ndill				HA	sigr	nal													Xho	bl	hlg	<u>G1</u> a	aa 245-470
-TA	ATA	CGA	CTC	ACT	ATA	GGG	AGA	CCC	AAG	CTT	AAT	CAA	AAC	ATG	GCT	ATC	ATC	TAC	CTC	ATC	CTC	CTG	TTC	ACC	GCT	GTG	CGG	GGC	CTC	GAC	AAA	ACT	99
CAC	707	TCC	CCA	CCC	TCC	CCA	CCA	CCT	GAA	CTC	CTTC	ccc	CCA	M	A	I	I	Y	L	I	L	L	F	T	A	V	R	G ATC	L	D	K	T>	108
H	т	C	P	P	C	P	A	P	E	T.	L L	G	GGA	P	S	V	F	L L	F	P	P	K	P	K	D	T	L L	M	T	s	R	T>	190
CCT	GAG	GTC	ACA	TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	ССТ	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	297
Р	Е	v	т	С	v	v	v	D	v	s	н	Е	D	Ρ	Е	v	К	F	N	W	Y	v	D	G	v	Е	v	Н	N	А	К	T>	
AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC	CGT	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC	TGG	CTG	AAT	GGC	AAG	GAG	TAC	AAG	TGC	AAG	396
K	P	R	Е	E	Q	Y	N	S	T	Y	R	V	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K	С	K>	405
V	s	N	K	A	L	P	A	P	I	E	K	T	I	s	K	A	K	G	0	P	R	E	P	0	V	Y	T	L	P	P	s	R>	495
GAT	GAG	CTG	ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	GGC	TTC	TAT	ccc	AGC	GAC	ATC	GCC	GTG	GÂG	TGG	GAG	AGC	AAT	GGG	CAG	CCG	GAG	594
D	Е	L	т	К	N	Q	v	s	L	т	С	L	v	К	G	F	Y	Р	s	D	I	А	v	Е	W	Е	s	N	G	Q	Ρ	E>	
AAC	AAC	TAC	AAG	ACC	ACG	CCT	CCC	GTG	TTG	GAC	TCC	GAC	GGC	TCC	TTC	TTC	CTC	TAC	AGC	AAG	CTC	ACC	GTG	GAC	AAG	AGC	AGG	TGG	CAG	CAG	GGG	AAC	693
N	N	Y	K	T	T	P	P	V	L	D	S	D	G	S	F	F	L	Y	S	K	L	T	V	D	K	S	R	W	Q	Q	G	N>	702
V	F	S	C	S	V	M	H	E	A	L	H	N	H	Y	T	0	K	S	L	s	L	S	P	G	K	R	s	P	0	P	0	P>	192
									Ram	пНI	Ps	sti i	۱BAI	FF a	a 14	10-2	85												-		-		
۵۵۵	CCG	CAG	CCG	ممم	CCG	GAA	CCG	GAA	GGA	TCC	CTG		GAA		GTC	<u>аст</u>	CAA	GAC	TGC	ጥጥG	CAA	CTG	<u>አ</u> ጥጥ	GCA	GAC	AGT	GAA	ACA	CCA	۵CT	ልሞል	CAA	891
K	P	0	P	K	P	E	P	E	G	S	L	Q	E	T	v	T	0	D	C	L	0	L	I	A	D	S	E	T	P	T	I	Q>	001
AAA	GGA	TCT	TAC	ACA	TTT	GTT	CCA	TGG	CTT	CTC	AGC	TTT	ААА	AGG	GGA	AGT	GCC	CTA	GAA	GAA	AAA	GAG	AAT	AAA	ATA	TTG	GTC	AAA	GAA	ACT	GGT	TAC	990
К	G	s	Y	т	F	v	Ρ	W	L	L	s	F	К	R	G	s	А	L	Е	Е	K	Е	N	K	I	L	v	K	Е	т	G	Υ>	
TTT	TTT	ATA	TAT	GGT	CAG	GTT	TTA	TAT	ACT	GAT	AAG	ACC	TAC	GCC	ATG	GGA	CAT	CTA	ATT	CAG	AGG	AAG	AAG	GTC	CAT	GTC	TTT	GGG	GAT	GAA	TTG	AGT	1089
r CTG	r GTG	ACT	I TTG	G TTT	CGA	V TGT	АТТ	CAA	ААТ	ATG	ССТ	GAA	ACA	CTA	CCC	ААТ	н ААТ	тсс	TGC	U TAT	TCA	GCT	GGC	V ATT	GCA	v AAA	r CTG	GAA	GAA	GGA	GAT	GAA	1188
L	v	т	L	F	R	С	I	Q	N	М	Р	Е	т	L	Р	N	N	s	С	Y	s	А	G	I	A	К	L	Е	Е	Bamk	-uP	E>	
																											link	er		Bgll	ĥ,	APR	IL aa 95-233
CTC	CAA	СТТ	GCA	ата	CCA	AGA	GAA	аат	GCA	CAA	ΔТΔ	тса	CTG	GAT	GGA	GAT	GTC	ACA	ጥጥጥ	ጥጥጥ	GGT	GCA	TTG	ΔΔΔ	CTG	CTG	GGA	GGA	GGA	GGA	тст	AAG	1287
L	Q	L	A	I	P	R	E	N	A	Q	I	S	L	D	G	D	v	т	F	F	G	A	L	K	L	L	G	G	G	G	S	K>	1207
AAG	CAG	CAC	TCT	GTC	CTG	CAC	CTG	\mathbf{GTT}	CCC	ATT	AAC	GCC	ACC	TCC	AAG	GAT	GAC	TCC	GAT	GTG	ACA	GAG	GTG	ATG	TGG	CAA	CCA	GCT	CTT	AGG	CGT	GGG	1386
K	Q	Н	S	V	L	Н	L	V	Р	I	N	A	Т	S	K	D	D	S	D	V	Т	Е	V	М	W	Q	Р	A	L	R	R	G>	1.105
AGA	GGC	CTA	CAG	GCC	CAA	GGA	TAT	GGT	GTC	CGA	ATC	CAG	GAT	GCT	GGA	GTT	TAT	CTG	CTG	TAT	AGC	CAG	GTC	CTG	TTT	CAA	GAC	GTG	ACT T	TTC	ACC	A'I'G M>	1485
GGT	CAG	GTG	GTG	TCT	CGA	GAA	GGC	CAA	GGA	AGG	CAG	GAG	ACT	CTA	TTC	CGA	TGT	ATA	AGA	AGT	ATG	ccc	TCC	CAC	CCG	GAC	CGG	GCC	TAC	AAC	AGC	TGC	1584
G	Q	v	v	s	R	Е	G	Q	G	R	Q	Е	т	L	F	R	С	I	R	s	М	Р	s	Н	Р	D	R	А	Y	N	s	C>	
TAT	AGC	GCA	GGT	GTC	TTC	CAT	TTA	CAC	CAA	GGG	GAT	ATT	CTG	AGT	GTC	ATA	ATT	CCC	CGG	GCA	AGG	GCG	AAA	CTT	AAC	CTC	TCT	CCA	CAT	GGA	ACC	TTC	1683
Y	s	A	G	v	F	Н	L	Н	Q	G	D	I	L	s	v	I	I	Р	R	Α	R	Α	K	L	N	L	s	Ρ	Н	G	т	F>	
						link	er	I	Bam	HI	hAF	PRIL	aa	95-2	33																		
CTG	GGG	TTT	GTG	AAA	CTG	GGA	GGA	GGT	GGA	TCC	AAG	AAG	CAG	CAC	TCT	GTC	CTG	CAC	CTG	\mathbf{GTT}	CCC	ATT	AAC	GCC	ACC	TCC	AAG	GAT	GAC	TCC	GAT	GTG	1782
L	G	F	V	K	L	G	G	G	G	S	K	K	Q	Н	S	V	L	Н	L	V	Р	I	N	A	Т	S	K	D	D	S	D	V>	
ACA T	GAG	GTG	A'I'G M	'TGG W	CAA	CCA	GCT	CTT	AGG	CGT	GGG	AGA	GGC	CTA T.	CAG	GCC	CAA	GGA	'T'A'T V	GGT	GTC	CGA	ATC	CAG	GAT	GCT	GGA	GT"F	T'A'I' V	CTG T.	CTG T.	T'A'T V>	1991
AGC	CAG	GTC	CTG	TTT	CAA	GAC	GTG	ACT	TTC	ACC	ATG	GGT	CAG	GTG	GTG	TCT	CGA	GAA	GGC	CAA	GGA	AGG	CAG	GAG	ACT	CTA	TTC	CGA	TGT	ATA	AGA	AGT	1980
s	Q	v	L	F	Q	D	v	т	F	т	М	G	Q	v	v	s	R	Е	G	Q	G	R	Q	Е	т	L	F	R	С	I	R	S>	
ATG	ccc	TCC	CAC	CCG	GAC	CGG	GCC	TAC	AAC	AGC	TGC	TAT	AGC	GCA	GGT	GTC	TTC	CAT	TTA	CAC	CAA	GGG	GAT	ATT	CTG	AGT	GTC	ATA	ATT	ccc	CGG	GCA	2079
М	Р	s	Н	Р	D	R	A	Y	N	S	С	Y	S	А	G	v	F	н	L	н	Q	G	D	I	L	S	v	I	I	Р	R	A>	
																					Eco	RI	IR	ES -	GFF	>							

AGG GCG AAA CTT AAC CTC TCT CCA CAT GGA ACC TTC CTG GGG TTT GTG AAA CTG TGA TGA CCT GAA TTC CCC CCC TCT CCC TCC CCC CCC CCT AAC GTT 2178 А К L Ν L S Р H G т F L G F v K L ACT GEC CGA AGC CGC TTG GAA TAA GEC CGG TGT GCG TTT GTC TAT ATG TTA TTT TCC ACC ATA TTG CCG TCT TTT GGC AAT GTG AGG GCC CGG AAA CCT 2277 GGC CCT GTC TTC TTG ACG AGC ATT CCT AGG GGT CTT TCC CCT CTC GCC AAA GGA ATG CAA GGT CTG TTG AAT GTC GTG AAG GAA GCA GTT CCT CTG GAA 2376 GCT TCT TGA AGA CAA ACA ACG TCT GTA GCG ACC CTT TGC AGG CAG CGG AAC CCC CCA CCT GGC GAC AGG TGC CTC TGC GGC CAA AAG CCA CGT GTA TAA 2475 GAT ACA CCT GCA AAG GCG GCA CAA CCC CAG TGC CAC GTT GTG AGT TGG ATA GTT GTG GAA AGA GTC AAA TGG CTC TCC TCA AGC GTA TTC AAC AAG GGG 2574 CTG AAG GAT GCC CAG AAG GTA CCC CAT TGT ATG GGA TCT GAT CTG GGG CCT CGG TGC ACA TGC TTT ACA TGT GTT TAG TCG AGG TTA AAA AAC GTC TAG 2673 GCC CCC CGA ACC ACG GGG ACG TGG TTT TCC TTT GAA AAA CAC GAT GAT AAT ATG GCC ACA ACC ATG GTG AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG 2772 GTG CCC ATC CTG GTC GAG GTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCT GGC GAG GGC GAT GCC ACC TAC GGC AAG CTG ACC CTG 2871 AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC TGG CCC ACC CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC CAC 2970 ATG AAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC 3069 GAG GTG AAG TTC GAG GGC GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC ATC CTG GGG CAC AAG CTG GAG TAC 3168 AAC TAC AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC AAG CAG AAG AAG GGC ATC AAG GCG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC GTG 3267 CAG CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CTG CCC GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA 3366 GAC CCC AAC GAG AAG CGC GAT CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GTG TAC AAG TAA TGA ATT AAT 3465

Xbal Apal

Sp6

TAA GAA TTA TCA AGC TTA TCG ATA CCG TCG AGT AGA TGA CTA GTC TAG AGG GCC CTA TTC TAT AGT GTC ACC TAA AT



Flag hAPRIL mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3

HindIII **T**7 -TA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT AAT CAA AAC ATG GCT ATC ATC TAC CTC ATC CTC TC ACC GCT GTG CGG GGC GAT TAC AAA GAC 99 Pstl hAPRIL aa 95-233 т А v R G D Y Κ GAT GAC GAT AAA GGA CCC GGA CAG GTG CAG CTG CAG AAG AAG CAG CAC TCT GTC CTG CAC CTG GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT 198 G Ρ G Q v Q L Q к К Q Н s v L Н L GTG ACA GAG GTG ATG TGG CAA CCA GCT CTT AGG CGT GGG AGA GGC CTA CAG GCC CAA GGA TAT GGT GTC CGA ATC CAG GAT GCT GGA GTT TAT CTG CTG 297 R G v G V R Е м W 0 Р А T. R R G T. Q A Q Y G т O D А G Y T. T.2 TAT AGC CAG GTC CTG TTT CAA GAC GTG ACT TTC ACC ATG GGT CAG GTG GTG TCT CGA GAA GGC CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA 396 0 D т М G 0 v v s Е G 0 G R 0 Е R S 0 т. F v F R т L F C R> Y S Q V L F Q D V T F T M G Q V V S R E G Q G R Q E T L F R C I R> AGT ATG CCC TCC CAC CCG GAC CGG GCC TAC AAC AGC TGC TAT AGC GCA GGT GTC TTC CAT TTA CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG 495 S H Р D R A Y N S С Y SAG v F HLHQGDILSV Bamb#/ М Ρ I I Р R> hAPRIL aa 95-233 GCA AGG GCG AAA CTT AAC CTC TCT CCA CAT GGA ACC TTC CTG GGG TTT GTG AAA CTG GGA GGA GGT GGA TCT AAG AAG CAG CAC TCT GTC CTG CAC CTG 594 A R A K L N L S P H G T F L G F V K L G G G G S K K Q H S V L H L> GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT GTG ACA GAG GTG ATG TGG CAA CCA GCT CTT AGG CGT GGG AGA GGC CTA CAG GCC CAA GGA TAT 693 А т s к D D s D v т Е v М W Q Ρ А LRR G R G Q А Q G GGT GTC CGA ATC CAG GAT GCT GGA GTT TAT CTG CTG TAT AGC CAG GTC CTG TTT CAA GAC GTG ACT TTC ACC ATG GGT CAG GTG GTG TCT CGA GAA GGC 792 v 0 D G Y T. Y s Q T. Q D т F т М G s R А T. 0 CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA AGT ATG CCC TCC CAC CCG GAC CGG GCC TAC AAC AGC TGC TAT AGC GCA GGT GTC TTC CAT TTA 891 0 G R O E т L F R C I R S M P S H P D R A Y N S C Y S A G V F Н T.> linker CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG GCA AGG GCG AAA CTT AAC CTC TCT CCA CAT GGA ACC TTC CTG GGG TTT GTG AAA CTG GGA GGA 990 H Q G D I L S V I I P R A R A K L N L S P H G T F L G F V K L BamHI hAPRIL aa 95-233 GGT GGA TCC AAG AAG CAG CAC TCT GTC CTG CAC CTG GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT GTG ACA GAG GTG ATG TGG CAA CCA GCT 1089 G Q H S v L Н L v Ρ Ν А т s K D D s D v т Е v W Q G S к к Т м Р CTT AGG CGT GGG AGA GGC CTA CAG GCC CAA GGA TAT GGT GTC CGA ATC CAG GAT GCT GGA GTT TAT CTG CTG TAT AGC CAG GTC CTG TTT CAA GAC GTG 1188 G 0 G G v R 0 D Α G v т. Y s R G D т 0 Α v т v т. 0 37 т. F 0 D 175 ACT TTC ACC ATG GGT CAG GTG GTG TCT CGA GAA GGC CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA AGT ATG CCC TCC CAC CCG GAC CGG GCC 1287 G 0 v 37 S R F G Q G R Q E T т. F R C т R S м P S н P р R 22 TAC AAC AGC TGC TAT AGC GCA GGT GTC TTC CAT TTA CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG GCA AGG GCG AAA CTT AAC CTC TCT CCA 1386 s V F I P G HLHQGDILS v RAR L N EcoRI IRES - GFP CAT GGA ACC TTC CTG GGG TTT GTG AAA CTG TGA TGA CCT GAA TTC CGC CCC TCT CCC TCC CCC CCT AAC GTT ACT GGC CGA AGC CGC TTG GAA TAA 1485 G T. G F v К L GGC CGG TGT GCG TTT GTC TAT ATG TTA TTT TCC ACC ATA TTG CCG TCT TTT GGC AAT GTG AGG GCC CGG AAA CCT GGC CCT GTC TTC TTG ACG AGC ATT 1584 CCT AGG GGT CTT TCC CCT CTC GCC AAA GGA ATG CAA GGT CTG TTG AAT GTC GTG AAG GAA GCA GTT CCT CTG GAA GCT TCT TGA AGA CAA ACG ACT 1683 GTA GCG ACC CTT TGC AGG CAG CGG AAC CCC CCA CCT GGC GAC AGG TGC CTC TGC GGC CAA AAG CCA CGT GTA TAA GAT ACA CCT GCA AAG GCG GCA CAA 1782 CCC CAG TGC CAC GTT GTG AGT TGG ATA GTT GTG GAA AGA GTC AAA TGG CTC TCC TCA AGC GTA TTC AAC AAG GGG CTG AAG GAT GCC CAG AAG GTA CCC 1881 CAT TGT ATG GGA TCT GAT CTG GGG CCT CGG TGC ACA TGC TTT ACA TGT GTT TAG TCG AGG TTA AAA AAC GTC TAG GCC CCC GA ACC ACG GGG ACG TGG 1980 TTT TCC TTT GAA AAA CAC GAT GAT AAT ATG GCC ACA ACC ATG GTG AGC AAG GGC GAG GGC GAG GGC GTG GTG GTG GTG GTG CCC ATC CTG GTC GAG CTG GAC 2079 GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCT GGC GAG GGC GAG GGC GAT GCC ACC TAC GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG 2178 CTG CCC GTG CCC TGG CCC ACC CTC GTG ACC CTG ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC CAC ATG AAG CAC GAC TTC TTC AAG 2277 TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC ACC ATC TTC TTC AAG GAC GGC GAC ACC TAC AAG ACC CGC GAC GTG AAG TTC GAG GGC GAC ACC 2376 CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC ATC CTG GGG CAC AAG CTG GAG TAC AAC TAC AAC AGC CAC AAC GTC TAT 2475 ATC ATG GCC GAC AAG CAG AAG AAC GGC ATC AAG GCG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC CTG CCG GAC CAC TAC CAG CAG 2574 AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT CAC 2673 ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG CTG TAC AAG TAA TGA ATT AAT TAA GAA TTA TCA AGC TTA TCG ATA 2772

Xbal Apal Sp6

CCG TCG AGT AGA TGA CTA GTC TAG AGG GCC CTA TTC TAT AGT GTC ACC TAA AT



(ps2883) Fc hAPRIL mono1 hBAFF mono2 hBAFF mono3 IRES GFP in PCR3

																													Sall	/			
			Τ7						Hir	ndIII				HA	sigr	nal													Xho	Ì	hlg	G1 a	a 245-470
-TA	ATA	CGA	CTC	ACT	ATA	GGG	AGA	CCC	AAG	CTT	AAT	CAA	AAC	ATG	GCT	ATC	ATC	TAC	CTC	ATC	CTC	CTG	TTC	ACC	GCT	GTG	CGG	GGC	CTC	GAC	AAA	ACT	99
CAC	ACA	TGC	CCA	CCG	TGC	CCA	GCA	ССТ	GAA	CTC	CTG	GGG	GGA	M	А	I GTC	I TTC	Y CTC	L	I	L	L	F	T	AGAC	ACC	R	G ATG	L ATC	D TCC	K	T> ACC	198
Н	Т	C	P	P	C	P	A	P	E	L	L	G	G	P	S	v	F	L	F	P	P	K	P	K	D	T	L	M	I	s	R	T>	190
CCT	GAG	GTC	ACA	TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	297
P	E	V	T	C	V	V	V	D	V	S	Н	E	D	P	E	V	K	F	N	W	Y	V	D	G	V	E	V	H	N	A	K	T>	206
K	P	R	GAG	GAG	CAG	Y	N	AGC	ACG T	Y	R	GTG V	GTC V	AGC	v	CTC L	ACC T	v	CTG T.	H	CAG	GAC	TGG	CTG T.	N	GGC	K	GAG	Y	K	TGC	AAG K>	396
GTC	TCC	AAC	AAA	GCC	CTC	CCA	GCC	ccc	ATC	GAG	AAA	ACC	ATC	TCC	AAA	GCC	AAA	GGG	CAG	ccc	CGA	GAA	CCA	CAG	GTG	TAC	ACC	CTG	ccc	CCA	TCC	CGG	495
v	S	N	К	А	L	Р	А	Р	I	Е	K	т	I	S	к	А	К	G	Q	Р	R	Е	Р	Q	v	Y	т	L	Ρ	Ρ	S	R>	
GAT	GAG	CTG	ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	GGC	TTC	TAT	CCC	AGC	GAC	ATC	GCC	GTG	GAG	TGG	GAG	AGC	AAT	GGG	CAG	CCG	GAG	594
AAC	AAC	TAC	AAG	ACC	ACG	CCT	ccc	GTG	TTG	GAC	TCC	GAC	GGC	TCC	TTC	TTC	CTC	TAC	AGC	AAG	CTC	ACC	GTG	GAC	AAG	AGC	AGG	TGG	CAG	CAG	GGG	AAC	693
N	N	Y	К	т	т	Ρ	Р	v	L	D	s	D	G	s	F	F	L	Y	s	к	L	т	v	D	к	S	R	W	Q	Q	G	N>	
GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CTG	TCT	CCG	GGT	AAA	AGA	TCT	CCG	CAG	CCG	CAG	CCG	792
v	г	5	C	5	v	Pi	п	Б	A	ц		1			1	<u>v</u>		5	ц	5	ц	5	P	G	к	ĸ	5	P	Q	r	Q	F>	
	000	a a c	000		000		000		<i>cc</i> ,	паа	PSU		hAP		aas) 5-2	33	CT C	ama	a.a.	ama	CMM	000	2 000		000	200	паа		C N m	6 20	шаа	0.0.1
K	P	CAG O	P	K	P	GAA E	P	GAA	GGA	S	L	CAG O	K	K	O	H	S	V	L	H	L	V	P	I	N	A	T	S	K	D	D	s>	091
GAT	GTG	ACA	GAG	GTG	ATG	TGG	CAA	CCA	GCT	CTT	AGG	CGT	GGG	AGA	GGC	CTA	CAG	GCC	CAA	GGA	TAT	GGT	GTC	CGA	ATC	CAG	GAT	GCT	GGA	GTT	TAT	CTG	990
D	v	т	Е	v	М	W	Q	Р	А	L	R	R	G	R	G	L	Q	Α	Q	G	Y	G	v	R	I	Q	D	А	G	V	Y	L>	
CTG T.	TAT	AGC	CAG	GTC	CTG T.	TTT	CAA	GAC	GTG	ACT	TTC	ACC	ATG M	GGT	CAG	GTG	GTG	TCT	CGA	GAA	GGC	CAA	GGA	AGG	CAG	GAG	ACT	CTA L	TTC	CGA	TGT	ATA T>	1089
AGA	AGT	ATG	ccc	TCC	CAC	CCG	GAC	CGG	GCC	TAC	AAC	AGC	TGC	TAT	AGC	GCA	GGT	GTC	TTC	CAT	TTA	CAC	CAA	GGG	GAT	ATT	CTG	AGT	GTC	ATA	ATT	CCC	1188
R	S	М	Р	s	Н	Р	D	R	А	Y	N	s	С	Y	s	А	G	v	F	Н	L	Н	0 Barr	G	D	I	L	s	v	I	I	P>	
																				link	er		Bg	<u>ííí</u> h	BAF	Fa	a 14	0-28	35				
CGG	GCA	AGG	GCG	ААА	CTT	AAC	CTC	TCT	CCA	CAT	GGA	ACC	TTC	CTG	GGG	TTT	GTG	ААА	CTG	GGA	GGA	GGT	GGA	TCT	GAA	ACA	GTC	ACT	CAA	GAC	TGC	TTG	1287
R	А	R	А	К	L	N	L	S	Ρ	Н	G	т	F	L	G	F	v	к	L	G	G	G	G	s	Е	т	v	т	Q	D	С	L>	
CAA	CTG	ATT	GCA	GAC	AGT	GAA	ACA	CCA	ACT	ATA	CAA	AAA	GGA	TCT	TAC	ACA	TTT	GTT	CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGG	GGA	AGT	GCC	CTA	GAA	GAA	1386
AAA	GAG	AAT	ААА	ATA	TTG	GTC	AAA	GAA	ACT	GGT	TAC	TTT	TTT	ATA	TAT	GGT	CAG	GTT	TTA	TAT	ACT	GAT	AAG	ACC	TAC	GCC	ATG	GGA	CAT	CTA	ATT	CAG	1485
К	Е	N	K	I	L	v	К	Е	т	G	Y	F	F	I	Y	G	Q	v	L	Y	т	D	К	т	Y	А	М	G	Н	L	I	Q>	
AGG	AAG	AAG	GTC	CAT	GTC	TTT	GGG	GAT	GAA	TTG	AGT	CTG	GTG	ACT	TTG	TTT	CGA	TGT	ATT	CAA	AAT	ATG	CCT	GAA	ACA	CTA	CCC	AAT	AAT	TCC	TGC	TAT	1584
TCA	GCT	GGC	ATT	GCA	AAA	CTG	GAA	GAA	GGA	GAT	GAA	CTC	CAA	CTT	GCA	r ATA	CCA	AGA	GAA	AAT	GCA	CAA	ATA	TCA	CTG	GAT	GGA	GAT	GTC	ACA	TTT	TTT	1683
s	А	G	I	А	к	L	Е	Е	G	D	Е	L	Q	L	А	I	Р	R	Е	N	А	Q	I	s	L	D	G	D	v	т	F	F>	
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GGT	GCA	TTG	AAA	CTG	CTG	GGA	GGA	GGA	GGA	TCC	GAA	ACA	GTC	ACT	CAA	GAC	TGC	TTG	CAA	CTG	ATT	GCA	GAC	AGT	GAA	ACA	CCA	ACT	ATA	CAA	ААА	GGA	1782
G	A	L	K	L	L	G	G	G	G	S	Е	Т	V	Т	Q	D	С	L	Q	L	I	A	D	S	E	Т	Р	Т	I	Q	K	G>	1001
S	Y	ACA T	F	GTT V	P	TGG	T.	CTC T.	AGC	F	K	AGG R	GGA	AGT	GCC	CTA T.	GAA	GAA	K	GAG	N	K	T	TTG T.	V	K	GAA	ACT T	GGT	Y	F	TTT F>	1881
ATA	TAT	GGT	CAG	GTT	TTA	TAT	ACT	GAT	AAG	ACC	TAC	GCC	ATG	GGA	CAT	CTA	ATT	CAG	AGG	AAG	AAG	GTC	CAT	GTC	TTT	GGG	GAT	GAA	TTG	AGT	CTG	GTG	1980
I	Y	G	Q	v	L	Y	т	D	к	т	Y	A	М	G	Н	L	I	Q	R	к	К	v	Н	v	F	G	D	Е	L	s	L	v>	
ACT	TTG L	TTT	CGA	TGT	ATT	CAA	AAT	ATG M	CCT	GAA	ACA T	CTA T.	CCC	AAT	AAT	TCC	TGC	TAT	TCA	GCT	GGC	ATT	GCA A	AAA	CTG T.	GAA	GAA	GGA	GAT	GAA	CTC L	CAA	2079
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CTT L	GCA	T	P	AGA R	GAA	N	GCA	CAA 0	T	S	CTG L	GAT	GGA	GAT	v	ACA T	F	F	GGT	GCA	TTG T.	K	CTG T.	CTG T.	TGA *	CTA	GTC	CTG	AAT	TCC	GCC	CCT	2178
CTC	ССТ	ccc	ccc	ccc	CTA	ACG	TTA	CTG	GCC	GAA	GCC	GCT	TGG	AAT	AAG	GCC	GGT	GTG	CGT	TTG	TCT	ATA	TGT	TAT	TTT	CCA	CCA	TAT	TGC	CGT	CTT	TTG	2277
GCA	ATG	TGA	GGG	CCC	GGA	AAC	CTG	GCC	CTG	TCT	TCT	TGA	CGA	GCA	TTC	CTA	GGG	GTC	TTT	ccc	CTC	TCG	CCA	AAG	GAA	TGC	AAG	GTC	TGT	TGA	ATG	TCG	2376
TGA	AGG	AAG	CAG	TTC	CTC	TGG	AAG	CTT	CTT	GAA	GAC	AAA	CAA	CGT	CTG	TAG	CGA	CCC	TTT	GCA	GGC	AGC	GGA	ACC	CCC	CAC	CTG	GCG	ACA	GGT	GCC	TCT	2475
CCT	CAA	GCG	TAT	TCA	ACA	AGG	GGC	TGA	AGG	ATG	CCC	AGA	AGG	TAC	CCC	ATT	GTA	TGG	GAT	CTG	ATC	TGG	GGC	CTC	GGT	GCA	CAT	GCT	TTA	CAT	GTG	TTT	2673
AGT	CGA	GGT	TAA	AAA	ACG	TCT	AGG	CCC	CCC	GAA	CCA	CGG	GGA	CGT	GGT	TTT	CCT	TTG	AAA	AAC	ACG	ATG	ATA	ATA	TGG	CCA	CAA	CCA	TGG	TGA	GCA	AGG	2772
GCG	AGG	AGC	TGT	TCA	CCG	GGG	TGG	TGC	CCA	TCC	TGG	TCG	AGC	TGG	ACG	GCG	ACG	TAA	ACG	GCC	ACA	AGT	TCA	GCG	TGT	CTG	GCG	AGG	GCG	AGG	GCG	ATG	2871
GCT GCT	TCA	GCC	GCA GCT	AGC	CCG	ACC	ACA	AGT TGA	AGC	AGC	ACG	ACT	тст	GCA TCA	AGC AGT	CCG	CCA	TGC	CCT	AAG	GCT	ACG	TCG	AGG	AGC	GCA	CCA	TCT	ACG TCT	TCA	AGG	AGT	2970 3069
ACG	GCA	ACT	ACA	AGA	CCC	GCG	CCG	AGG	TGA	AGT	TCG	AGG	GCG	ACA	ccc	TGG	TGA	ACC	GCA	TCG	AGC	TGA	AGG	GCA	TCG	ACT	TCA	AGG	AGG	ACG	GCA	ACA	3168
TCC	TGG	GGC	ACA	AGC	TGG	AGT	ACA	ACT	ACA	ACA	GCC	ACA	ACG	TCT	ATA	TCA	TGG	CCG	ACA	AGC	AGA	AGA	ACG	GCA	TCA	AGG	CGA	ACT	TCA	AGA	TCC	GCC	3267
ACA GCA	ACA	TCG AGT	AGG	ACG	GCA TGA	GCG	TGC	AGC	CCA	ACG	ACC AGA	AC'I' AGC	ACC GCG	AGC	AGA ACA	ACA TGG	TCC	TGC	TCG TGG	GCG AGT	ACG	TGA	CCG	TGC	CCG	GGA	TCA	ACA CTC	ACC TCG	ACT GCA	ACC	ACG	3465
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Xbal Apal Sp6

AGC TGT ACA AGT AAT GAA TTA ATT AAG AAT TAT CAA GCT TAT CGA TAC CGT CGA GTA GAT GAC TAG TCT AGA GGG CCC TAT TCT ATA GTG TCA CCT AAA 3564

т


T7

Flag hAPRIL mono1 hBAFF mono2 hBAFF mono3 IRES GFP in PCR3

HindIII

-TA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT AAT CAA AAC ATG GCT ATC ATC TAC CTC ATC CTC TC ACC GCT GTG CGG GGC GAT TAC AAA GAC 99 IYLILLF т v А R G D Y Κ Pstl hAPRIL aa 95-233 GAT GAC GAT AAA GGA CCC GGA CAG GTG CAG CTG CAG AAG AAG CAG CAC TCT GTC CTG CAC CTG GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT 198 G Ρ G Q v Q L Q К К Q Н s v L Н L А GTG ACA GAG GTG ATG TGG CAA CCA GCT CTT AGG CGT GGG AGA GGC CTA CAG GCC CAA GGA TAT GGT GTC CGA ATC CAG GAT GCT GGA GTT TAT CTG CTG 297 G v G v Е м W 0 Р Α T. R R G R T. Q А Q Y G R Т 0 D Α G Y T. T.2 TAT AGC CAG GTC CTG TTT CAA GAC GTG ACT TTC ACC ATG GGT CAG GTG GTG TCT CGA GAA GGC CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA 396 0 D т М G 0 v v s Е G 0 G R 0 Е R S 0 т. v F R т т. F C R> AGT ATG CCC TCC CAC CCG GAC CGG GCC TAC AAC AGC TGC TAT AGC GCA GGT GTC TTC CAT TTA CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG 495 Р D R А Y Ν s С Y s А G v F HLHQGDILSV Bamb М Р S н I I Р R> Bgill hBAFF aa 140-285 A R A K L N L S P H G T F L G F V K L G G G G S E T V T Q D C L Q> CTG ATT GCA GAC AGT GAA ACA CCA ACT ATA CAA AAA GGA TCT TAC ACA TTT GTT CCA TGG CTT CTC AGC TTT AAA AGG GGA AGT GCC CTA GAA GAA AAA 693

D s I Q К G s Y т F v Ρ W L L s F K R G s А GAG AAT AAA ATA TTG GTC AAA GAA ACT GGT TAC TTT TTT ATA TAT GGT CAG GTT TTA TAT ACT GAT AAG ACC TAC GCC ATG GGA CAT CTA ATT CAG AGG 792 G G т D м н 0 Τ. т Α T. AAG AAG GTC CAT GTC TTT GGG GAT GAA TTG AGT CTG GTG ACT TTG TTT CGA TGT ATT CAA AAT ATG CCT GAA ACA CTA CCC AAT AAT TCC TGC TAT TCA 891 н v F G D Е т. S L v т L F R С 0 Ν М Р Е т Τ. Р N N S C S> GCT GGC ATT GCA AAA CTG GAA GAA GGA GAT GAA CTC CAA CTT GCA ATA CCA AGA GAA AAT GCA CAA ATA TCA CTG GAT GGA GAT GTC ACA TTT TTT GGT 990 G I A K L E E G D E L Q L A I Р R Е N A 0 т S D G D v А T. т F G>

linker BamHI hBAFF aa 140-285

GCA TTG AAA CTG CTG GGA GGA GGA GGA GGA TCC GAA ACA GTC ACT CAA GAC TGC TTG CAA CTG ATT GCA GAC AGT GAA ACA CCA ACT ATA CAA AAA GGA TCT 1089 A L K L L G G G G S E T V T Q D C L Q L I A D S E T P T I Q K G S> TAC ACA TTT GTT CCA TGG CTT CTC AGC TTT AAA AGG GGA AGT GCC CTA GAA GAA AAA GAG AAT AAA ATA TTG GTC AAA GAA ACT GGT TAC TTT TTT ATA 1188 G s Е K R А L к Е Ν ĸ L TAT GGT CAG GTT TTA TAT ACT GAT AAG ACC TAC GCC ATG GGA CAT CTA ATT CAG AGG AAG AAG GTC CAT GTC TTT GGG GAT GAA TTG AGT CTG GTG ACT 1287 G Q Y т D K T Y A M G H L I Q R к v н v F G D Е S L L К L T> TTG TTT CGA TGT ATT CAA AAT ATG CCT GAA ACA CTA CCC AAT AAT TCC TGC TAT TCA GCT GGC ATT GCA AAA CTG GAA GAA GGA GAT GAA CTC CAA CTT 1386 s G R C 0 Ν М Р Е т L Р Ν Ν С Y S А Ι А Κ L E Е GDELO T.> т. т

EcoRI IRES - GFP

Sp6

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Xbal Apal

GTA CAA GTA ATG AAT TAA TTA AGA ATT ATC AAG CTT ATC GAT ACC GTC GAG TAG ATG ACT AGT CTA GAG GGC CCT ATT CTA TAG TGT CAC CTA AAT



T7

Flag hBAFF mono1 hAPRIL mono2 hAPRIL mono3 IRES GFP in PCR3

HindIII

-TA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT AAT CAA AAC ATG GCT ATC ATC TAC CTC ATC CTC TC ACC GCT GTG CGG GGC GAT TAC AAA GAC 99 Pstl hBAFF aa 140-285 т v А R G D Y Κ GAT GAC GAT AAA GGA CCC GGA CAG GTG CAG CTG CAG GAA ACA GTC ACT CAA GAC TGC TTG CAA CTG ATT GCA GAC AGT GAA ACA CCA ACT ATA CAA AAA 198 v G Ρ G Q v Q L Q E т т Q D С L Q L А D GGA TCT TAC ACA TTT GTT CCA TGG CTT CTC AGC TTT AAA AGG GGA AGT GCC CTA GAA GAA AAA GAG AAT ATA ATA TTG GTC AAA GAA ACT GGT TAC TTT 297 G S Y T F V P W L L S F K R G S A L E E K E N K I J. V K F T C V F~ TTT ATA TAT GGT CAG GTT TTA TAT ACT GAT AAG ACC TAC GCC ATG GGA CAT CTA ATT CAG AGG AAG AAG GTC CAT GTC TTT GGG GAT GAA TTG AGT CTG 396 G 0 D К М G Н 0 R v н v G D Е v т. т Y А L к к F т. S T.> GTG ACT TTG TTT CGA TGT ATT CAA AAT ATG CCT GAA ACA CTA CCC AAT AAT TCC TGC TAT TCA GCT GGC ATT GCA AAA CTG GAA GAA GAA GAA GAA CTC 495 G D E L> BamHH F С I O N M Р Е Т L P N N S С Y S A G IAKLEE т L R

Bgill hAPRIL aa 95-233

 \underline{linker} \underline{Mim} hAPRIL as 9 caa ctt gca ata cca aga gaa aat gca caa ata tca ctg gat gga gat gtc aca ttt ttt ggt gca ttg aaa ctg ctg gga gga gga gga tct aag aag 594 Q L A I P R E N A Q I S L D G D V T F F G A L K L L G G G G G S K K> CAG CAC TCT GTC CTG CAC CTG GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT GTG ACA GAG GTG ATG TGG CAA CCA GCT CTT AGG CGT GGG AGA 693 v L v Р N А т s K D D s D v т Е v М W Q Ρ А L R GGC CTA CAG GCC CAA GGA TAT GGT GTC CGA ATC CAG GAT GCT GGA GTT TAT CTG CTG TAT AGC CAG GTC CTG TTT CAA GAC GTG ACT TTC ACC ATG GGT 792 R D G s 0 0 Α T. Τ. 0 T. D т CAG GTG GTG TCT CGA GAA GGC CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA AGT ATG CCC TCC CAC CCG GAC CGG GCC TAC AAC AGC TGC TAT 891 S R Е G 0 G R 0 Е т L F R С т R S М Р s н Ρ D R А Y N S С Y> AGC GCA GGT GTC TTC CAT TTA CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG GCA AGG GCG AAA CTT AAC CTC TCT CCA CAT GGA ACC TTC CTG 990 К I P RARA T. N T. S н G s А Р т ғ T.>

linker BamHI hAPRIL aa 95-233

GGG TTT GTG AAA CTG GGA GGA GGA GGA TCC AAG AAG CAG CAC TCT GTC CTG CAC CTG GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT GTG ACA 1089 т. C G G G c Н s 37 Н р N D GAG GTG ATG TGG CAA CCA GCT CTT AGG CGT GGG AGA GGC CTA CAG GCC CAA GGA TAT GGT GTC CAG ATC CAG GAT GCT GGA GTT TAT CTG CTG TAT AGC 1188 R G R G Q Q G G v R D R L А Q А CAG GTC CTG TTT CAA GAC GTG ACT TTC ACC ATG GGT CAG GTG GTG TCT CGA GAA GGC CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA AGT ATG 1287 L Q D v т F T M G Q v v SREG QGRQETLF R С R s M> Q Ι CCC TCC CAC CCG GAC CGG GCC TAC AAC AGC TGC TAT AGC GCA GGT GTC TTC CAT TTA CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG GCA AGG 1386 v OGDIL S н Р D R А Y Ν s С Y S А G F H L Н S v Ι Ι Ρ R А R>

EcoRI IRES - GFP

Sp6

GCG AAA CTT AAC CTC TCT CCA CAT GGA ACC TTC CTG GGG TTT GTG AAA CTG TGA TGA CCT GAA TTC CGC CCC TCT CCC TCC CCC CCC AAC GTT ACT 1485 н G v т. N т. s Р G т F L F К A L N L S F H G T F L G F V K L C ACC ATA TTG CCG TCT TTT GCC AAT GTG AGG GCC CGG AAA CCT GGC 1584 CCT GTC TTC TTG ACG AGC ATT CCT AGG GGT CTT TCC CCT CTC GCC AAA GGA ATG CAA GGT CTG TTG AAT GTC GTG AAG GAA GCA GTT CCT CTG GAA GCT 1683 TCT TGA AGA ACA ACG TCT GTA GCG ACC CTT TGC AGG CAG CGG AAC CCC CCA CCT GGC GAC AGG TGC CTC TGC GGC CAA AAG CCA CGT GTA TAA GAT 1782 ACA CCT GCA AAG GCG GCA CAA CCC CAG TGC CAC GTT GTG AGT TGG ATA GTT GTG GAA AGA GTC AAA TGG CTC TCC TCA AGC GTA TTC AAC AAG GGG CTG 1881 AAG GAT GCC CAG AAG GTA CCC CAT TGT ATG GGA TCT GAT CTG GGG CCT CGG TGC ACA TGC TTT ACA TGT GTT TAG TCG AGG TTA AAA AAC GTC TAG GCC 1980 CCC CCG ACC ACC GGG ACG TGG TTT TCC TTT GAA AAA CAC GAT GAT AAT ATG GCC ACA ACC ATG GTG ASC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG GTG GTG 2079 CCC ATC CTG GTC GAG CTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCT GGC GAG GGC GAG GGC GAT GCC ACC TAC GGC AAG CTG ACC CTG AAG 2178 TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC TGG CCC ACC CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC CAC ATG 2277 AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC ACC ATC TTC TTC AAG GAC GAC GAC TAC AAG ACC CGC GCC GAC 2376 GTG AAG TTC GAG GGC GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC ATC CTG GGG CAC AAG CTG GAG TAC AAC 2475 TAC AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC AAG CAG AAG AAC GGC ATC AAG GCG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC GGC AGC GTG CAG 2574 CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC GAC AAC CAC TAC CTG AGC AAC CAC CAG TCC GCC CTG AGC AAA GAC 2673 CCC AAC GAG AAG CGC GAT CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG CTG TAC AAG TAA TGA ATT AAT TAA 2772

Xbal Apal

GAA TTA TCA AGC TTA TCG ATA CCG TCG AGT AGA TGA CTA GTC TAG AGG GCC CTA TTC TAT AGT GTC ACC TAA AT



T7

Flag hBAFF mono1 mono2 mono3 IRES GFP in PCR3

HindIII

-TA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT AAT CAA AAC ATG GCT ATC ATC TAC CTC ATC CTC CTG TTC ACC GCT GTG CGG GGC GAT TAC AAA GAC 99 Pstl hBAFF aa 140-285 v т А R G D Y Κ GAT GAC GAT AAA GGA CCC GGA CAG GTG CAG CTG CAG GAA ACA GTC ACT CAA GAC TGC TTG CAA CTG ATT GCA GAC AGT GAA ACA CCA ACT ATA CAA AAA 198 G Р G Q v Q L Q Е т v т Q D С L Q D L А GGA TCT TAC ACA TTT GTT CAC TGG CTT CTC AGC TAT AAA AGG GGA AGT GCC CTA GAA GAA AGA AAA GAG AAT AAA ATA TTG GTC AAA GAA ACT GGT TAC TTT 297 G S Y T F V P W L L S F K R G S A L E E K E N K I L V K E T G Y F> TTT ATA TAT GGT CAG GTT TTA TAT ACT GAT AAG ACC TAC GCC ATG GGA CAT CTA ATT CAG AGG AAG AAG GTC CAT GTC TTT GGG GAT GAA TTG AGT CTG 396 G D К М G Н R Н v G D Е 0 v т. т А L 0 к к v F т. S T.> GTG ACT TTG TTT CGA TGT ATT CAA AAT ATG CCT GAA ACA CTA CCC AAT AAT TCC TGC TAT TCA GCT GGC ATT GCA AAA CTG GAA GAA GAA GAA GAA CTC 495 G D E L> Bam∰ I O N M Р Е т L P N N S С Y S A G IAKLEE т L F R С

hBAFF aa 140-285

linker

CAA CTT GCA ATA CCA AGA GAA AAT GCA CAA ATA TCA CTG GAT GGA GAT GTC ACA TTT TTT GGT GCA TTG AAA CTG CTG GGA GGA GGA GGA TCT GAA ACA 594 G D 37 G G G G G TT CAL GAC TEC TEC CAL CTE ATT GCA GAC AGT GAL ACA CCA ACT ATA CAL ANA GGA TCT TAC ACA TTT GTT CCA TEG CTT CTC AGC TTT ANA AGG 693 D С Q А D s Е т Р т I Q К G s v Ρ W s GGA AGT GCC CTA GAA GAA AAA GAG AAT AAA ATA TTG GTC AAA GAA ACT GGT TAC TTT TTT ATA TAT GGT CAG GTT TTA TAT ACT GAT AAG ACC TAC GCC 792 к к G D Е G Т 0 T. К ATG GGA CAT CTA ATT CAG AGG AAG AAG GTC CAT GTC TTT GGG GAT GAA TTG AGT CTG GTG ACT TTG TTT CGA TGT ATT CAA AAT ATG CCT GAA ACA CTA 891 G н т. 0 R к v н v F G D Е т. S L v т т. F R C т 0 N м Р E CCC AAT AAT TCC TGC TAT TCA GCT GGC ATT GCA AAA CTG GAA GAA GGA GAT GAA CTC CAA CTT GCA ATA CCA AGA GAA AAT GCA CAA ATA TCA CTG GAT 990 Р N S C S A G I A K L E E G D E L Q L A I R E N A 0 Р N Y т S T. D>

linker BamHI hBAFF aa 140-285

GGA GAT GTC ACA TTT TTT GGT GCA TTG AAA CTG CTG GGA GGA GGA GGA GGA GGA ACC GAA ACA GTC ACT CAA GAC TGC TTG CAA CTG ATT GCA GAC AGT GAA ACA 1089 G G G G s Е m 37 т D CCA ACT ATA CAA AAA GGA TCT TAC ACA TTT GTT CCA TGG CTT TCC AGC TTT AAA AGG GGA AGT GCC CTA GAA GAA AAA GGA ATA AAA ATA TTG GTC AAA 1188 s R G s L А L Е K L GAA ACT GGT TAC TTT TTT ATA TAT GGT CAG GTT TTA TAT ACT GAT AAG ACC TAC GCC ATG GGA CAT CTA ATT CAG AGG AAG GTC CAT GTC TTT GGG 1287 G Q v L Y т D т Y G Н L Q R к Н G> G Y Κ А М Ι Κ Ι GAT GAA TTG AGT CTG GTG ACT TTG TTT CGA TGT ATT CAA AAT ATG CCT GAA ACA CTA CCC AAT AAT TCC TGC TAT TCA GCT GGC ATT GCA AAA CTG GAA 1386 E т. S т. т L F R С т 0 Ν М Ρ Е т L Р Ν Ν s С Y S А G А к т. E>v т GAA GGA GAT GAA CTC CAA CTT GCA ATA CCA AGA GAA AAT GCA CAA ATA TCA CTG GAT GGA GAT GTC ACA TTT TTT GGT GCA TTG AAA CTG CTG TGA CTA 1485 Е G D E L Q L A I P R E N A Q I S L D G D v т F F G А L к L Τ.

Enzyme cutters

EcoRI IRES - GFP

Xbal Apal

TCA CTC TCG GCA TGG ACG AGC TGT ACA AGT AAT GAA TTA ATT AAG AAT TAT CAA GCT TAT CGA TAC CGT CGA GTA GAT GAC TAG TCT AGA GGG CCC TAT 2871

____Sp6

TCT ATA GTG TCA CCT AAA T

(ps694) m BAFF full in PCR3

		T7	,					ŀ	lind	III				E	Bam	HI	
TAA	TAC	GAC	TCA	CTA	TAG	GGA	GAC	CCA	AGC	TTG	GTA	CCG	AGC	TCG	GAT	CCA	51
СТА	GTA	ACG	GCC	GCC	AGT	GTG	CTG	Eco GAA	DRI TTC	AGG	AAA	GCC	m acc	BAF ATG M	GAT D	-309 GAG E>	102
TCT	GCA	AAG	ACC	CTG	CCA	CCA	CCG	TGC	CTC	TGT	TTT	TGC	TCC	GAG	AAA	GGA	153
S	A	K	T	L	P	P	P	C	L	C	F	C	S	E	K	G>	
GAA	GAT	ATG	AAA	GTG	GGA	TAT	GAT	CCC	ATC	ACT	CCG	CAG	AAG	GAG	GAG	GGT	204
E	D	M	K	V	G	Y	D	P	I	T	P	Q	K	E	E	G>	
GCC	TGG	TTT	GGG	ATC	TGC	AGG	GAT	GGA	AGG	CTG	CTG	GCT	GCT	ACC	CTC	CTG	255
A	W	F	G	I	C	R	D	G	R	L	L	A	A	T	L	L>	
CTG	GCC	CTG	TTG	TCC	AGC	AGT	TTC	ACA	GCG	ATG	TCC	TTG	TAC	CAG	TTG	GCT	306
L	A	L	L	S	S	S	F	T	A	M	S	L	Y	Q	L	A>	
GCC	TTG	CAA	GCA	GAC	CTG	ATG	AAC	CTG	CGC	ATG	GAG	CTG	CAG	AGC	TAC	CGA	357
A	L	Q	A	D	L	M	N	L	R	M	E	L	Q	S	Y	R>	
GGT	TCA	GCA	ACA	CCA	GCC	GCC	GCG	GGT	GCT	CCA	GAG	TTG	ACC	GCT	GGA	GTC	408
G	S	A	T	P	A	A	A	G	A	P	E	L	T	A	G	V>	
AAA	CTC	CTG	ACA	CCG	GCA	GCT	CCT	CGA	CCC	CAC	AAC	TCC	AGC	CGC	GGC	CAC	459
K	L	L	T	P	A	A	P	R	P	H	N	S	S	R	G	H>	
AGG	AAC	AGA	CGC	GCT	TTC	CAG	GGA	CCA	GAG	GAA	ACA	GAA	CAA	GAT	GTA	GAC	510
R	N	R	R	A	F	Q	G	P	E	E	T	E	Q	D	V	D>	
CTC	TCA	GCT	CCT	ССТ	GCA	CCA	TGC	CTG	ССТ	GGA	TGC	CGC	CAT	TCT	CAA	CAT	561
L	S	A	P	Р	A	P	C	L	Р	G	C	R	H	S	Q	H>	
GAT	GAT	AAT	GGA	ATG	AAC	CTC	AGA	AAC	ATC	ATT	CAA	GAC	TGT	CTG	CAG	CTG	612
D	D	N	G	M	N	L	R	N	I	I	Q	D	C	L	Q	L>	
ATT	GCA	GAC	AGC	GAC	ACG	CCG	ACT	ATA	CGA	AAA	GGA	ACT	TAC	ACA	TTT	GTT	663
I	A	D	S	D	T	P	T	I	R	K	G	T	Y	T	F	V>	
CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGA	GGA	AAT	GCC	TTG	GAG	GAG	AAA	GAG	AAC	714
P	W	L	L	S	F	K	R	G	N	A	L	E	E	K	E	N>	
AAA	ATA	GTG	GTG	AGG	CAA	ACA	GGC	TAT	TTC	TTC	ATC	TAC	AGC	CAG	GTT	CTA	765
K	I	V	V	R	Q	T	G	Y	F	F	I	Y	S	Q	V	L>	
TAC	ACG	GAC	CCC	ATC	TTT	GCT	ATG	GGT	CAT	GTC	ATC	CAG	AGG	AAG	AAA	GTA	816
Y	T	D	P	I	F	A	M	G	H	V	I	Q	R	K	K	V>	
CAC	GTC	TTT	GGG	GAC	GAG	CTG	AGC	CTG	GTG	ACC	CTG	TTC	CGA	TGT	ATT	CAG	867
H	V	F	G	D	E	L	S	L	V	T	L	F	R	C	I	Q>	
AAT	ATG	CCC	AAA	ACA	CTG	CCC	AAC	AAT	TCC	TGC	TAC	TCG	GCT	GGC	ATC	GCG	918
N	M	P	K	T	L	P	N	N	S	C	Y	S	A	G	I	A>	
AGG	CTG	GAA	GAA	GGA	GAT	GAG	ATT	CAG	CTT	GCA	ATT	CCT	CGG	GAG	AAT	GCA	969
R	L	E	E	G	D	E	I	Q	L	A	I	P	R	E	N	A>	
CAG	ATT	TCA	CGC	AAC	GGA	GAC	GAC	ACC	TTC	TTT	GGT	GCC	CTA	AAA	CTG	CTG	1020
Q	I	S	R	N	G	D	D	T	F	F	G	A	L	K	L	L>	
ТАА *	ACT	AGT	ССТ	EC GAA	DRI TTC	Pstl TGC	AGA	TAT	CCA	TCA	CAC	No TGG	otl cgg	CCG	Xh CTC	OI GAG	1071
		٧Ŀ		۸					~								

(ps2355)

m BAFF H242A full in PCR3

		T7	,					ŀ	lind					E	Bam	HI	
TAA	TAC	GAC	TCA	CTA	TAG	GGA	GAC	CCA	AGC	TTG	GTA	CCG	AGC	TCG	GAT	CCA	51
								Fo	-BI							~~~	
CTA	GTA	ACG	GCC	GCC	AGT	GTG	CTG	GAA	TTC	AGG	AAA	GCC	M ACC	ATG M	GAT D	-309 gag E>	102
TCT	GCA	AAG	ACC	CTG	CCA	CCA	CCG	TGC	CTC	TGT	TTT	TGC	TCC	GAG	AAA	GGA	153
S	A	K	T	L	P	P	P	C	L	C	F	C	S	E	K	G>	
GAA	GAT	ATG	AAA	GTG	GGA	TAT	GAT	CCC	ATC	ACT	CCG	CAG	AAG	GAG	GAG	GGT	204
E	D	M	K	V	G	Y	D	P	I	T	P	Q	K	E	E	G>	
GCC	TGG	TTT	GGG	ATC	TGC	AGG	GAT	GGA	AGG	CTG	CTG	GCT	GCT	ACC	CTC	CTG	255
A	W	F	G	I	C	R	D	G	R	L	L	A	A	T	L	L>	
CTG	GCC	CTG	TTG	TCC	AGC	AGT	TTC	ACA	GCG	ATG	TCC	TTG	TAC	CAG	TTG	GCT	306
L	A	L	L	S	S	S	F	T	A	M	S	L	Y	Q	L	A>	
GCC	TTG	CAA	GCA	GAC	CTG	ATG	AAC	CTG	CGC	ATG	GAG	CTG	CAG	AGC	TAC	CGA	357
A	L	Q	A	D	L	M	N	L	R	M	E	L	Q	S	Y	R>	
GGT	TCA	GCA	ACA	CCA	GCC	GCC	GCG	GGT	GCT	CCA	GAG	TTG	ACC	GCT	GGA	GTC	408
G	S	A	T	P	A	A	A	G	A	P	E	L	T	A	G	V>	
AAA	CTC	CTG	ACA	CCG	GCA	GCT	CCT	CGA	CCC	CAC	AAC	TCC	AGC	CGC	GGC	CAC	459
K	L	L	T	P	A	A	P	R	P	H	N	S	S	R	G	H>	
AGG	AAC	AGA	CGC	GCT	TTC	CAG	GGA	CCA	GAG	GAA	ACA	GAA	CAA	GAT	GTA	GAC	510
R	N	R	R	A	F	Q	G	P	E	E	T	E	Q	D	V	D>	
CTC	TCA	GCT	CCT	CCT	GCA	CCA	TGC	CTG	CCT	GGA	TGC	CGC	CAT	TCT	CAA	CAT	561
L	S	A	P	P	A	P	C	L	P	G	C	R	H	S	Q	H>	
GAT	GAT	AAT	GGA	ATG	AAC	CTC	AGA	AAC	ATC	ATT	CAA	GAC	TGT	CTG	CAG	CTG	612
D	D	N	G	M	N	L	R	N	I	I	Q	D	C	L	Q	L>	
ATT	GCA	GAC	AGC	GAC	ACG	CCG	ACT	ATA	CGA	AAA	GGA	ACT	TAC	ACA	TTT	GTT	663
I	A	D	S	D	T	P	T	I	R	K	G	T	Y	T	F	V>	
CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGA	GGA	AAT	GCC	TTG	GAG	GAG	AAA	GAG	AAC	714
P	W	L	L	S	F	K	R	G	N	A	L	E	E	K	E	N>	
AAA	ATA	GTG	GTG	AGG	CAA	ACA	GGC	TAT	TTC	TTC	ATC	TAC	AGC	CAG	GTT	CTA	765
K	I	V	V	R	Q	T	G	Y	F	F	I	Y	S	Q	V	L>	
TAC	ACG	GAC	CCC	ATC	TTT	GCT	ATG	GGT	CAT	GTC	ATC	CAG	AGG	AAG	AAA	GTA	816
Y	T	D	P	I	F	A	M	G	H	V	I	Q	R	K	K	V>	
GCC	GTC	TTT	GGG	GAC	GAG	CTG	AGC	CTG	GTG	ACC	CTG	TTC	CGA	TGT	ATT	CAG	867
A	V	F	G	D	E	L	S	L	V	T	L	F	R	C	I	Q>	
AAT	ATG	CCC	AAA	ACA	CTG	CCC	AAC	AAT	TCC	TGC	TAC	TCG	GCT	GGC	ATC	GCG	918
N	M	P	K	T	L	P	N	N	S	C	Y	S	A	G	I	A>	
AGG	CTG	GAA	GAA	GGA	GAT	GAG	ATT	CAG	CTT	GCA	ATT	CCT	CGG	GAG	AAT	GCA	969
R	L	E	E	G	D	E	I	Q	L	A	I	P	R	E	N	A>	
CAG	ATT	TCA	CGC	AAC	GGA	GAC	GAC	ACC	TTC	TTT	GGT	GCC	CTA	AAA	CTG	CTG	1020
Q	I	S	R	N	G	D	D	T	F	F	G	A	L	K	L	L>	
				Eco	oRI	Pstl						N	otl		Xh	ol	
ТАА *	ACT	AGT	ССТ	GAA	TTC	TGC	AGA	TAT	CCA	TCA	CAC	TGG	CGG	CCG	CTC	GAG	1071
		Xt	bal	Ap	al				S	p6							

(ps2575)

m BAFF E278K full in PCR3

		T7	,					ŀ	lind					E	Bam	HI	
TAA	TAC	GAC	TCA	CTA	TAG	GGA	GAC	CCA	AGC	TTG	GTA	CCG	AGC	TCG	GAT	CCA	51
								Ec	oRI				m	RAF	E 1.	300	
CTA	GTA	ACG	GCC	GCC	AGT	GTG	CTG	GAA	TTC	AGG	AAA	GCC	ACC	ATG M	GAT D	GAG E>	102
TCT	GCA	AAG	ACC	CTG	CCA	CCA	CCG	TGC	CTC	TGT	TTT	TGC	TCC	GAG	AAA	GGA	153
S	A	K	T	L	P	P	P	C	L	C	F	C	S	E	K	G>	
GAA	GAT	ATG	AAA	GTG	GGA	TAT	GAT	CCC	ATC	ACT	CCG	CAG	AAG	GAG	GAG	GGT	204
E	D	M	K	V	G	Y	D	P	I	T	P	Q	K	E	E	G>	
GCC	TGG	TTT	GGG	ATC	TGC	AGG	GAT	GGA	AGG	CTG	CTG	GCT	GCT	ACC	CTC	CTG	255
A	W	F	G	I	C	R	D	G	R	L	L	A	A	T	L	L>	
CTG	GCC	CTG	TTG	TCC	AGC	AGT	TTC	ACA	GCG	ATG	TCC	TTG	TAC	CAG	TTG	GCT	306
L	A	L	L	S	S	S	F	T	A	M	S	L	Y	Q	L	A>	
GCC	TTG	CAA	GCA	GAC	CTG	ATG	AAC	CTG	CGC	ATG	GAG	CTG	CAG	AGC	TAC	CGA	357
A	L	Q	A	D	L	M	N	L	R	M	E	L	Q	S	Y	R>	
GGT	TCA	GCA	ACA	CCA	GCC	GCC	GCG	GGT	GCT	CCA	GAG	TTG	ACC	GCT	GGA	GTC	408
G	S	A	T	P	A	A	A	G	A	P	E	L	T	A	G	V>	
AAA	CTC	CTG	ACA	CCG	GCA	GCT	CCT	CGA	CCC	CAC	AAC	TCC	AGC	CGC	GGC	CAC	459
K	L	L	T	P	A	A	P	R	P	H	N	S	S	R	G	H>	
AGG	AAC	AGA	CGC	GCT	TTC	CAG	GGA	CCA	GAG	GAA	ACA	GAA	CAA	GAT	GTA	GAC	510
R	N	R	R	A	F	Q	G	P	E	E	T	E	Q	D	V	D>	
CTC	TCA	GCT	CCT	ССТ	GCA	CCA	TGC	CTG	CCT	GGA	TGC	CGC	CAT	TCT	CAA	CAT	561
L	S	A	P	Р	A	P	C	L	P	G	C	R	H	S	Q	H>	
GAT	GAT	AAT	GGA	ATG	AAC	CTC	AGA	AAC	ATC	ATT	CAA	GAC	TGT	CTG	CAG	CTG	612
D	D	N	G	M	N	L	R	N	I	I	Q	D	C	L	Q	L>	
ATT	GCA	GAC	AGC	GAC	ACG	CCG	ACT	ATA	CGA	AAA	GGA	ACT	TAC	ACA	TTT	GTT	663
I	A	D	S	D	T	P	T	I	R	K	G	T	Y	T	F	V>	
CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGA	GGA	AAT	GCC	TTG	GAG	GAG	AAA	GAG	AAC	714
P	W	L	L	S	F	K	R	G	N	A	L	E	E	K	E	N>	
AAA	ATA	GTG	GTG	AGG	CAA	ACA	GGC	TAT	TTC	TTC	ATC	TAC	AGC	CAG	GTT	CTA	765
K	I	V	V	R	Q	T	G	Y	F	F	I	Y	S	Q	V	L>	
TAC	ACG	GAC	CCC	ATC	TTT	GCT	ATG	GGT	CAT	GTC	ATC	CAG	AGG	AAG	AAA	GTA	816
Y	T	D	P	I	F	A	M	G	H	V	I	Q	R	K	K	V>	
CAC	GTC	TTT	GGG	GAC	GAG	CTG	AGC	CTG	GTG	ACC	CTG	TTC	CGA	TGT	ATT	CAG	867
H	V	F	G	D	E	L	S	L	V	T	L	F	R	C	I	Q>	
AAT N	ATG M	ссс Р - 247	ааа к 7 К	ACA T	CTG L	CCC P	AAC N	AAT N	TCC S	TGC C	TAC Y	TCG S	GCT A	GGC G	ATC I	GCG A>	918
AGG	CTG	AAA	GAA	GGA	GAT	GAG	ATT	CAG	CTT	GCA	ATT	CCT	CGG	GAG	AAT	GCA	969
R	L	K	E	G	D	E	I	Q	L	A	I	P	R	E	N	A>	
CAG	ATT	TCA	CGC	AAC	GGA	GAC	GAC	ACC	TTC	TTT	GGT	GCC	CTA	AAA	CTG	CTG	1020
Q	I	S	R	N	G	D	D	T	F	F	G	A	L	K	L	L>	
				ECO		Pstl	_					N	זנ		xn	01	
ТАА *	ACT	AGT	ССТ	GAA	TTC	TGC	AGA	TAT	CCA	TCA	CAC	TGG	CGG	CCG	CTC	GAG	1071
		Xb	bal	Ар	al				S	Sp6							

(ps2583) m BAFF E247K full in PCR3

	T7							ŀ	Hind				BamHI				
TAA	TAC	GAC	TCA	CTA	TAG	GGA	GAC	CCA	AGC	TTG	GTA	CCG	AGC	TCG	GAT	CCA	51
CTA	GTA	ACG	GCC	GCC	AGT	GTG	CTG	Eco GAA	DRI TTC	AGG	ААА	GCC	m acc	BAF ATG M	GAT	-309 GAG E>	102
TCT	GCA	AAG	ACC	CTG	CCA	CCA	CCG	TGC	CTC	TGT	TTT	TGC	TCC	GAG	AAA	GGA	153
S	A	K	T	L	P	P	P	C	L	C	F	C	S	E	K	G>	
GAA	GAT	ATG	AAA	GTG	GGA	TAT	GAT	CCC	ATC	ACT	CCG	CAG	AAG	GAG	GAG	GGT	204
E	D	M	K	V	G	Y	D	P	I	T	P	Q	K	E	E	G>	
GCC	TGG	TTT	GGG	ATC	TGC	AGG	GAT	GGA	AGG	CTG	CTG	GCT	GCT	ACC	CTC	CTG	255
A	W	F	G	I	C	R	D	G	R	L	L	A	A	T	L	L>	
CTG	GCC	CTG	TTG	TCC	AGC	AGT	TTC	ACA	GCG	ATG	TCC	TTG	TAC	CAG	TTG	GCT	306
L	A	L	L	S	S	S	F	T	A	M	S	L	Y	Q	L	A>	
GCC	TTG	CAA	GCA	GAC	CTG	ATG	AAC	CTG	CGC	ATG	GAG	CTG	CAG	AGC	TAC	CGA	357
A	L	Q	A	D	L	M	N	L	R	M	E	L	Q	S	Y	R>	
GGT	TCA	GCA	ACA	CCA	GCC	GCC	GCG	GGT	GCT	CCA	GAG	TTG	ACC	GCT	GGA	GTC	408
G	S	A	T	P	A	A	A	G	A	P	E	L	T	A	G	V>	
AAA	CTC	CTG	ACA	CCG	GCA	GCT	CCT	CGA	CCC	CAC	AAC	TCC	AGC	CGC	GGC	CAC	459
K	L	L	T	P	A	A	P	R	P	H	N	S	S	R	G	H>	
AGG	AAC	AGA	CGC	GCT	TTC	CAG	GGA	CCA	GAG	GAA	ACA	GAA	CAA	GAT	GTA	GAC	510
R	N	R	R	A	F	Q	G	P	E	E	T	E	Q	D	V	D>	
CTC	TCA	GCT	CCT	CCT	GCA	CCA	TGC	CTG	CCT	GGA	TGC	CGC	CAT	TCT	CAA	CAT	561
L	S	A	P	P	A	P	C	L	P	G	C	R	H	S	Q	H>	
GAT	GAT	AAT	GGA	ATG	AAC	CTC	AGA	AAC	ATC	ATT	CAA	GAC	TGT	CTG	CAG	CTG	612
D	D	N	G	M	N	L	R	N	I	I	Q	D	C	L	Q	L>	
ATT	GCA	GAC	AGC	GAC	ACG	CCG	ACT	ATA	CGA	AAA	GGA	ACT	TAC	ACA	TTT	GTT	663
I	A	D	S	D	T	P	T	I	R	K	G	T	Y	T	F	V>	
CCA	TGG	CTT	CTC	AGC	TTT	AAA	AGA	GGA	AAT	GCC	TTG	GAG	GAG	AAA	GAG	AAC	714
P	W	L	L	S	F	K	R	G	N	A	L	E	E	K	E	N>	
AAA	ATA	GTG	GTG	AGG	CAA	ACA	GGC	TAT	TTC	TTC	ATC	TAC	AGC	CAG	GTT	CTA	765
K	I	V	V	R	Q	T	G	Y	F	F	I	Y	S	Q	V	L>	
TAC Y	ACG T	GAC D	CCC P	ATC I F	TTT F 247	GCT A	ATG M	GGT G	CAT H	GTC V	ATC I	CAG Q	AGG R	AAG K	AAA K	GTA V>	816
CAC	GTC	TTT	GGG	GAC	AAG	CTG	AGC	CTG	GTG	ACC	CTG	TTC	CGA	TGT	ATT	CAG	867
H	V	F	G	D	K	L	S	L	V	T	L	F	R	C	I	Q>	
AAT	ATG	CCC	AAA	ACA	CTG	CCC	AAC	AAT	TCC	TGC	TAC	TCG	GCT	GGC	ATC	GCG	918
N	M	P	K	T	L	P	N	N	S	C	Y	S	A	G	I	A>	
AGG	CTG	GAA	GAA	GGA	GAT	GAG	ATT	CAG	CTT	GCA	ATT	CCT	CGG	GAG	AAT	GCA	969
R	L	E	E	G	D	E	I	Q	L	A	I	P	R	E	N	A>	
CAG	ATT	TCA	CGC	AAC	GGA	GAC	GAC	ACC	TTC	TTT	GGT	GCC	CTA	AAA	CTG	CTG	1020
Q	I	S	R	N	G	D	D	T	F	F	G	A	L	K	L	L>	
				EC		Pstl	_					N	oti				
ТАА *	ACT	AGT	ССТ	GAA	TTC	TGC	AGA	TAT	CCA	TCA	CAC	TGG	CGG	CCG	СТС	GAG	1071
		Xb	bal	Ap	al				s	p6							



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

		pBluescrip	t		
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	В	amHI pBlue	escript
GGGGGATGGT	TCCTGTTAAG	GGGATCCCTG	CAGCCCGGGG	GATCCACTAG	
TTCTAGAGCG	GCCGCCACCG	CGGTGGAGCT	CCAGCTTTTG	TTCCCTTTAG	700
TGAGGGTTAA	TT				



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

DBLuescript CARTAGGACT CACTATAGGG CGAATTGGGT ACCGGGCCCC CCCTCGAGGT Hindili mBAFF 3' sequence CGACGGTATC GATAGCTG CATCGCGAGG CTGGAAGAAG GAGATGAGAA 100 CGACGCTTGCA ATACCTGG AGAATGCACA GATTTCACCC AACGGAGAG ACCGCCTT TGGTGCCCTA AAACTGCTGT AACTCACTG CTGGAAGAGC (CCCTCGAACT TGGTGCACAC CAGAAATAT AACAGGAAGC CACAACGCAC GGTGTGCCATG TGAGTATGA GAAACGGAGC CGCGCCTCAG AAAGACCGGAC AGGGAAAATAT AACAGGAAGC CACAACCGAA GTGTGCCATG TGAGTTATGA GAAACGGAGC CGCGCCTCAG AAAGACCGGA 400 CGAGGAAGAC GCTTTTCTCC AGTCCTTGC CACACGCAC CGCAACCTGT TGCTTTGCCT TGGGTGACAC ATGTCCAGAA TGCAGGGAGA TTTCCTTGTT 500 TTGCGATTG CCATGAGAGA GAGGCCCCAC ACTGGCGGAGA TTTCCTTGTT 500 TCACGCATAG TCCAGGAAT TACCTGCCT TCCTAGCCA ACTGCAGGCC ACTGGAGACT BamHI TCACGCCTAG TCCTAGGAT TACTCACGCA ACTGCAGGCA A00 DBLuescript

 $\begin{array}{c} & \text{pBluescript} \\ \hline \text{tccactagtt ctagagegeg cgccaccgcg gtggagetcc agettttgtt} \\ \text{ccctttagtg agggttaatt} \end{array}$



(ps2846) Targeting vector for mBAFF E247K knock-in in pBS

							mu BAFF S	Spel genomic	fragment		
	Т3	pE	Bluescript			Spel	with exons	5 and 6			
AATTAACCCT	CACTAAAGGG	AACAAAAGCT	GGAGCTCCAC	CGCGGTGGCG	GCCGCTCTAG	AACTAGTTGC	ATCTATCCTG	CCAGTGATGG	ACTTATAGCC	100	
TGGAATTATA	AGCTAACATA	AACACTTCCT	CCCTTAAGTT	GCTTTTTTTT	TTTTCAGAGT	GGGACTCTGA	AGCGTGTAAA	TGTATACTTT	AGGACATGTG		
ACATCAGTGA	CCTAAAACCT	AGTTCACACC	CAGAGTGAGG	CATGTCATCT	TAGAGTGTCT	CTCAACAGGG	GGACATCAAT	AAAACCCAAA	AGGTAGTACT		
TTTTTTTATC	TGTAGTTTAA	CTAATATCCT	GAAGTTCTAA	GTACATTTTA	AGTGGGCCAT	GATATTTATA	CTTAAGTTTT	CAATTCCCGG	AGCTGTTAAC	500	
ATGTCACTTG	GTTTGCTCTC	TTAGAGGATG	TTCTGGTGGC	ATTTGAAAGC	ACAGTTAACT	TTAATCAATT	ATGATATGGG	AGGTTATATA	TAACCTATGA		
TTGTGTGTGC	CTATGTATTG	TATTAAGTGT	TGTGTATACC	TGTGTTGTGT	GCACCTGTGT	TGTGTGCACT	GTTATGTGTA	CTGTGTTGTA	TGAGCTGTGT		
TGTGTGCACC	TGTGTTGTGT	GCACCTGTGT	TGTATACACC	TGTGTTGTGT	GCACTGTGTT	GTATGAACTG	TGTTGTGTGC	ACCTGTGTTG	TATGAACTGT		
TGTTGTGTGTGC	ACCTGTGTGTTG	TGTTCAGTGT	TGTGTGTGCACC	TGTGTTGTGTGT	GTACTGTGCT	ATGTGTGTACCC	ATGCTGTGTG	CACCTGTGCT	CATGTACACA	1000	
ATAGAAGAAG	AACATGTAGA	GTGAATGAAA	ACAAACAAAA	CAAAAGAAGC	ATGAGCTCTA	CCTTCTTAAT	AAGAGTAGTT	AACAAAAAAG	TAGCTACAAT		
GTAAAGAGCC	AGTAAATAAA	TTTAAATTAA	TGCAGAACAA	AATTCAGTAA	GTAAATACTT	TGGCCTAGAA	TATTTAAAAA	GTACTTTTAG	AAATCTATGT		
TTTACTTCAC	TTTGGTATCT	CTGCCAACTC	TGTAATGGGT	GGTGTTTCAT	TTTCCAGAAT	TTCAATAGCA	TGTTTTGCCA	AATGTTTTAC	AAAGACAGGA		
AATTGCCTGT	GGCTGATTAC	CACAGCAATC	ATCAGTTTGA	ATTATGTTTT	ACTTAATGTA	GAACTTACTA	ATTCAGTGAT	TATTAGATGC	ATATCTGCTG	1500	
CTCTATGTTC	AAACGATCAA	СТСАТТТТА	ATAATGTCAGG	AGCATAATCA	AGCTGGCCTG	ACCCCAGAGG	TTAGAACC	ATAGCAATAC	CTTTTCCCC	1500	
ACTGTTTGCT	TTGTTTTGTT	TGAGACAAAC	AAAACATGTA	ACCCTGACTC	TATGTAACCC	TGACTGTTCT	GGAACTTGCT	ATGATCGACC	AGGCTGGCCT		
TGAACTCCCA	ACTTGGGGTC	TAGACTTGTA	ACAATCTTTA	TTTTACAATG	CCCGAGTTGT	TACAGTCTTT	ACGTTAATTC	TTTCTCAGGT	TGTTCAGTAC		
TCAATGATCT	AACACACCTT	GGGCACAAAA	TTCCATTTAA	ACAAAATAAG	CACTGGCTTG	TTGGAGACGT	CTTGCTAGGA	TCTTGTTGTA	CACAGAGAGT		
AAAGAGGGCT	TGCTTTTGAT	ATTCGTGTTT	GATGTGACCT	GCTTCTCTGA	GTCTGTTATC	CTGATGAGCA	ATGGGGGAAT	TTTTTATTTAA	GAAAATTCCT	2000	
TGACTTCCTA	CITIGAGAAGA	AAAGTGTGGT	GACCATCCCT	GTGGCACCTC	AGGGCCGCTC	CGCTCTCTGG	AACACAAGCA	ATGGAAGTAG	CTCCAGGAAG		
CCCCCCAGGA	TCTCCCCAAC	CCCCATCCAT	CTATGATAAT	GCTTCCTTGA	GGAGCCAGCC	TAACGATCCT	TACTATGACC	TGAATTCACG	GTGGTCTTTG		
CTTTGTTTTA	CTGTGGGCAT	TTTTTTTCTCA	TTATTTTTCT	TTTTGTTTGT	TTTTCGAGAC	AGGGTTTCTC	TGTGTTGCCC	TGGCTGCCCT	GGAATTCACT		
CTGTAGACCA	GGCTGGCTTA	TGAACTCAGA	AATCTGCCTG	CCTCTCCCTC	CCAAGTGCTG	GGATCAAAGG	CATGTGCCAC	CACGCTCGGC	TTGCTGAGGA	2500	
CATTTTTTTT	TTTTTTTTTTT	TTAAAGATTT	ATTTATTTAT	TACATGTAAG	TACACTGTAG	CTGTCTTCAG	ACACACCAGA	AGAGGGCGTC	AGATCTCGTT		
ACAGATGGTT	GTGAGCCACC	ATGTGGTTGC	TGGGATTTGA	ACTTTGGACC	TTCGGAAGAG	CAGTCGGGTG	CTCTTACCCA	CTGAGCCATC	TCACCAGCCC		
ACTACATT	ATTTTTTAACT	TACATTCAAATT	TTCAGAAAAC	ATGAGCTTCT	ACACCCAACA	TCTTCTGGTA	AGGACCTTAT	TTGAATCAGG	CANTRIAGATI		
TCTGAGTTTA	TAAAGAAAAC	ТАААТСААТА	ACAAATAGAG	ACACCTAGAC	TCCCAAGTTC	ACTGTAGCCA	AGATTACAAC	AGCCAAAAAA	ATCAACTTGG	3000	
TGTCCGTCGC	CAGAGGAACG	GGTAATAGTG	GTATGGGCAC	TGGGCACTGA	ATGAACCTGT	GAGACAGACG	CAGAAATATA	AATGTCACTA	CAGTATCGCT		
TTGGACTTGT	TTGACTGGTC	TTGGAACCCA	TACCCCCTTT	TTGCTTATTG	AGGTGACATT	TTTAGACTGG	CTTAGTGTTT	CCCATTGTAC	AAGAATGGTC		
					Apal						
TGGTGTATTT	AGACTTTCCA	CACCATTIGGT	TGCTATTCTG	TGCTCACTGT	GCAGGGGGCCC	CCCCACTACC	CCAGCTGCTT	CCCTTCATT	AGTGTGTGGC		
TTTTTTCTCTC	TTCCTGGGTG	CGAGCTCTAG	GAAGCGCCTT	GCTCCAGTCT	GGTCTGGTGG	CTTGATTCCC	TAGCCTGTTG	CCCTTTCTGC	TCTTCTTGTC	3500	
AACCCTGAGC	ACAGTGTCTT	CCTGTCACTC	AGCATAGAAT	TAAGGAAATG	TCACTGTACT	GAAAGCCCTT	TCAGCTTCTG	AACACTGTAG	TCTTAGCACA		
CAGGTTCTGA	GCAGTTTTCA	GTGCAGGGCA	TATCCTCGTT	GCTGTCTGTG	TCATTTCCTA	GTTGTGTTTT	ACGGCCAAGT	TGGTGGTGGC	CTCACTTCCT		
CCAGATACAT	TTTGTTAAGT	TCTTCACCGA	ATCACCCAGG	AGATGGAAGA	AGACAATCTC	CCAGTGTGTT	GTGCAGAGGA	ACAGGTACTC	TCTCTCCTAG		
ATCCTCCCCA	AAACCCGGCC	TTTATCAGGA	AATAGCCAGT	GTGCAGAACA	AGAAAAGAAA	ATATAAGCAA	GAGCTGTGGA	ATGGGGTCTC	TTAGCACATG	4000	
CAICCAGGII	ICCCAIGCIA	AAAACAIGII	IGGCAGAIAG	CCCAAIGGAG	GACAGICCII	CICCICCICC	Itereficer	cercerring	evon 5	4000	
TATACGATGT									CAULT J		
1111110011101	GCTCTTTTAA	ATGTAGTGGG	AAGAAAAAGT	GCTTTTTACG	ATTTACTGTC	TCATGAGTAA	TTCTCTCTCT	GCTGCAGGAA	CTTACACATT		
	GCTCTTTTAA	ATGTAGTGGG	AAGAAAAAGT	GCTTTTTACG	ATTTACTGTC	TCATGAGTAA	TTCTCTCTCT	GCTGCAGGAA	exon 5		
TGTTCCATGG	CTTCTCAGCT	TTAAAAGAGG	AAGAAAAAGT	GCTTTTTACG	ATTTACTGTC AGAACAAAAT	TCATGAGTAA AGTGGTGAGG	TTCTCTCTCT	GCTGCAGGAA ATTTCTTCAT	CTTACACATT exon 5 CTACAGCCAG		
TGTTCCATGG	CTTCTCAGCT ACTCTCCCCT	ATGTAGTGGG TTAAAAGAGG ACCCCTGGTC	AAGAAAAAGT AAATGCCTTG CGACGTTTTC	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC	ATTTACTGTC AGAACAAAAT ACGTCTGGTG	TCATGAGTAA AGTGGTGAGG ACATATCCCG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT	ATTTCTTCAT	CTTACACATT exon 5 CTACAGCCAG on 6		
TGTTCCATGG GTAGCGTCAA	CTTCTCAGCT ACTCTCCCCT	ATGTAGTGGG TTAAAAGAGG ACCCCTGGTC	AAGAAAAAGT AAATGCCTTG CGACGTTTTC	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hind	TCATGAGTAA AGTGGTGAGG ACATATCCCG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT	GCTGCAGGAA ATTTCTTCAT <u>ex</u> CAGGTTCTAT	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC		
TGTTCCATGG GTAGCGTCAA CATCTTTGCT	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG	ATGTAGTGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAAGCT	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC	GCTGCAGGAA ATTTCTTCAT EXC CAGGTTCTAT GATGTATTCA	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC		
TGTTCCATGG GTAGCGTCAA CATCTTTGCT	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG	ATGTAGTGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG exon	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAQCT E247K	TCATGAGTAA AGTGGTGAGG ACATATCCCG IIII TAGCCTGGTG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC	4500	
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC	CTTCTCAGCT ACTCTCCCCCT ATGGGTCATG CCAACAATTC	TTAAAAGAGG ACCCCTGGTC TCATCCAGAG <u>exon</u> CTGCTACTCG	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA <u>6</u> GCTGGTATGT	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA	ATTTACTGTC AGAACAAAAAT ACGTCTGGTG Hinc GGGACAACT E247K AGACCTGCTT	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGGAAT	CTACACAGT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECOBV/Noti	4500	
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTTGGGA	TTAAAAGAGG ACCCCTGGTC TCATCCAGAG <u>exon</u> CTGCTACTCG	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT	ATTTACTGTC AGAACAAAAAT ACGTCTGGTG <u>Hinc</u> GGGACAACT E247K AGACCTGCTT GTGCTGTTTA	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG TTTTACAATA	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTGT	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGAAAA	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI	4500	
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTTGGGA IOXI	TTAAAAGAGG ACCCCTGGTC TCATCCAGAG <u>exon</u> CTGCTACTCG	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hino GGGAQAAGCT E247K AGACCTGCTT GTGCTGTTTA	TCATGAGTAA AGTGGTGAGG ACATATCCCG IIII TAGCCTGGTG AGCAAGATGG TTTTACAATA	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGAAAA	CTTACACAT exon 5 CTACAGGCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGCCCG	4500	
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CATAACTTCG	CTTCTCAGCT ACTCTCCCCCT ATGGGTCATG CCAACAATTC TGCTTTGGGA IOXI TATAGCATAC	ATGTAGGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG exon CTGCTACTCG TCTTAATGGA ATTATACGAA	AAGAAAAAGT AAATGCCTTG CGACGTTTCC GAAGAAAAGTA <u>6</u> GCTGGTATGT AAGACCTTCT GTTATGAATT	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hing GGGAQAAG E247K AGACCTGCTT GTGCTGTTTA TGTGGTTTTC	TCATGAGTAA AGTGGTGAGG ACATATCCCG IIII TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGGAAT ACGGAGAAAA TCCACCCAGG	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT	4500	
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CATAACTTCG TTCCACCCAA	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTGGGA TATAGCATAC TGTCGAGCAG	ATGTAGTEGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG <u>exon</u> CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTTG	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCCCGGGCAG	ATTTACTGTC AGAACAAAAT ACGTCTGGTG <u>Hinc</u> GGGAQAAGCT E247K AGACCTGCTT GTGCTGTTTA GTGGCTGTTTC GTGGCACTTT	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCCCGGAA	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGAAAA TCCACCCAGG CCCCTATTTG	CTTACACATT exon 5 CTACAGCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT TTTATTTTC	4500	Lox
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CATAACTTCG TTCCACCCAA TAAATACATTCG	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTTGGGA TATAGCATAC TGTCGAGCAG CAATATGTA	ATGTAGTEGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG exon CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTTG TCCCGCTCATG	AAGAAAAAGT AAATGCCTTG GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG AGACAATAAC	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCTCGAGCAG CCTCGATAAAT	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAGCT E247K AGACCTGCTT GTGCTGTTTA TGTGGTTTTC GTGCCACTTT GCTCCAATAA	TCATGAGTAA AGTGGTGAGG ACATATCCCG IIII TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT TATTGAAAAA	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GCACGCGAA GGAAGACCCC	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGAAAA TCCACCAGG CCCCTATTG TGAGGCGGAA	CTTACACATT exon 5 CTACAGCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT TTTATTTTC AGAACCACCT	4500	Lox P
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CCATAACTTCG TTCCACCCAA TAAATACATT GTGGAATGTG	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTATGGGATAC TATAGCATAC CAAATATGTA TGTCCAGCAG CAAATATGTA	ATGTAGGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG exon CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTTG TCCGCTCATG GGTGTGGAAA	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG AGACAATAAC GTCCCAGGC	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCCCGGGCAG CCTGATAAAT TCCCCCCAGCAG	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAGCT E247K AGACCTGCTT GTGCTGTTTAT GCTGCAACTATA GCTCCAATAA GCACAACTAT	TCATGAGTAA AGTGGTGAGG ACATATCCCG IIII TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT TATTGAAAAA GCAAAGCTG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCCCGGAA GGAAGAGTCC CATCTCAATT	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGAAAA TCCACCCAGG CCCCTATTTG TGAGGCGGAA AGTCAGCABC	CTTACACATT exon 5 CTACAGCAG on 6 ACACGGACCC GAATATGCCC CCTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT TTTATTTTC AGAACCAGCT CAGGCGCCCC	4500	Lox P SV
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CCAAAAGTGC TTCCACCCAA TAAATACATT GTGGAATGTG AAGTCCCCAG CTCCGCCCCAG	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTGGGA IOXÍ TATAGCATAC TGTCGAGCAG GCTCCCCAGC TGTCGAGCAG TCCGCCCAT	ATGTAGGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG exon CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTTG TCCGCTCATG GGTGTGGAAA AGGCAGAAGT	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT CTAAGAGGAAT GTCACCAGGAG AGACAATAAC GTCCCCAGGC ATGCCAAAGCA	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCCGGGCAG CCTGATAAAT TCCCCAGCAG TGCATCTCAA	ATTTACTGTC AGAACAAAAT ACGTCTGGTG GGGAQAAGCT E247K AGACCTGCTT GTGCTGTTTAT GCTGCAGTATTT TTAGTCAGCA ATTTATCGAG	TCATGAGTAA AGTGGTGAGG ACATATCCCG IIII TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT TATTGAAAAA GCAAAGCTG ACCATAGTCC	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCGCGGAA GGAAGAGTCC CACTTCAATT CGCCCCTAAC	GCTGCAGGAA ATTTCTTCAT CAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGGAAAA TCCACCCAGG CCCCTATTG TGAGGCGGAA AGTCAGCAAC TCCGCCCATC	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT TTTATTTTC AGAACCAGCT CAGGAGTGGA CCGCCCCTAA	4500 5000	Lox P SV40
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CATAACTTCG TTCCACCCAA AGGCAATGTG AAGTCCCCAG CTCCGCCCAG CTCCGCCCAG	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTTGGGA IOXÍ TATAGCATAC TGTCAGCTAG GCTCCCCAGC TTCCCCCAC TTTTTTGGAG	ATGTACTEGG TTAAAAGAGG ACCCCTGGTC TCATCCAAGAG CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTG GGTGTGGAAA AGGCAGAAGT TCTCCGCCCC	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG GTCCCCAGGC ATGCCAAGCA ATGGCTGACT TTGCAAAGAT	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCCCGGGCAG CCCGGGCAG TGCATCTCAA AATTTTTTTT	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAGT E247K AGACCTGCTT GTGCTGTTTA GTGGCTGTTTA GTGCACTTT GTGCACTTT TTAGTCACAA ATTTATCACAA ACAGGATCAG	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT GCAAAGCATG ACCATAGTCC AGGCCGAGGC GATCGTTTCG	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCGCGGAA GGAACAGCTC CATCTCAATT CGCCCCTAAC CGCCTCGGCC CATCATTCAA	GCTGCAGGAA ATTTCTTCAT	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT TTTATTTTC CAGGACTGGA CCGCCCTAA TCCAGAAGTA TGCACGCAGG	4500 5000	Lox P SV40 pr
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CCAAAAGTGC CCACAACATTG TTCCACCCAA AGTCCCCAG CTCCGCCCAG CTCACGCAAGC TTCTCCGGCC	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTGGGA Ioxí TATAGCATAC GCTCGAGCAG TGTCAGCTAG GCTCCCCACC TTTTTTGGAG GCTTGGGTGG	ATGTAGTEGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTG TCCGCTCATG TCTCCGCCCC GCCTAGGCTT AGAGGCTATT	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG AGACAATAAC ATGGCCAAGCA ATGGCTGACT CGGCTATGAC	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCCGGGCAG GCCTGGATCAGAG AATTTTTTT CGATCAAGAG TGGGCACAAC	ATTTACTGTC AGAACAAAAT ACGTCTGGTG <u>Hinc</u> GGGA(AAGT E247K AGACCTGCTT GTGCTGTTTA GTGCGTGTTTA GTGCGCACTTT GCTCCAATAA ATTTATCCAGA ATTTATCCAGA AGCAATCGG	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT TATTGAAAA AGCAAGGCATG ACCATAGTCC AGGCCGAGGC CTGCTCTGAT	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCGCGGAA GGAAGAGCTC CATCTCAATT CGCCCCTAAC CGCCCTGGCC CATGATTGAA	GCTGCAGGAA ATTTCTTCAT GX GAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGAAAA TCCACCCAGG CCCTATTG TGAGGCGAA TCCACCCAGT TCGAGCGCAT TCTGAGCACT TCTGAGCACT	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGAATGT TTTATTTTC AGAACCAGCT AGAACCAGCA TCCAGAAGTA ACCCACCAGG AGCCCCAGGG	4500	Lox P SV40 prom
TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CATAACTTCG TTCCACCCAA TAAATACATTCG GTGAAATGTG AAGTCCCCAG GTGAGGAGGC CTCCGCCCAG GTGAGGAGGC CGCCCGGTTCT	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTGGGA IOXÍ TATAGCATAC TGTCAGCAG GCAATATGTA GCTCCCCAGC TTCCCCCCAT TTTTTTGGAG CCTTGGCTGG TTTTTTGCAA	ATGTAGGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG CTGCTACTCG TCTTAATGGA ATTATACGAA TGTGGTTTTG TCCGCCCCATG GGTGTGGAAA AGGCAGAAGT TCTCCGCCCC GCCTAGGCTT GACCGCCTG	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG AGACAATAAC ATGCCAAGCA ATGCCAAGCA ATGCCAAAGCA TTGCAAAGCA TTGCCAAAGCA CGGCTATGAC	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCTCGAGCAG CCTCGATAGACT TGCCCCAGCAG TGCATCTCAA AATTTTTTTT TCCCCAGCAG TGGCACACAC TGGATGACACA	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAGCT E247K AGACCTGCTTT GTGCTGTTTC GTGCGCTGTTTA GCTGCAGTAT CCTCAATAA ATTTAGCAGCA ACTTAGCAGCA ACAGGATGAG GCAAAACCGG GCAAAACCGG	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAAT TATTGAAAA GCAAAGCATG ACCATAGTCC AGCCCGAGCC GATCGTTCG GATCGTTCGA	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCGCGGAA GGAAGACTCC CATCTCAATT CGCCCTAAC CGCCCTCGCCC CATGATGAA CCCCCGTGT TATCGTGCCT	GCTGCAGGAA ATTTCTTCAT GXGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGGAAAA TCCACCCAGG CCCCTATTG TGAGGCGGAA AGTCAGCGAC TCCGGCCATC TCCGGCTATC CAAGATGGAT CCGGCTGTC GGCCACGACG	CTTACACATT exon 5 CTACAGCCAG on 6 ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/Noti CTGATGGCCG CCTGGAATGT TTTATTTTC AGAACCACCT CAGGTGTGGA CCGCCCCTAA TCCAGCAGGG GCGCTCCTT	4500 5000 5500	Lox P SV40 promote
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TGTTCCATGG GTAGCGTCAA CATCTTTGCT AAAACACTGC CCAAAAGTGC CCAAAAGTGC CCATAACTTCG TTCCACCCAA CTCCGCCCAG CTCCGCCCAG CTCCGCCCAG CGCCAGCCGATC CGACAGCAGAAGTA GCACGCCCAA CCGCCTGGGT TACGGTATCG GACGCCCAA CCACCCCGGGC CATCAAAAAG CCATTGGGGC CCTGCCCCCG TTTTTATAAA	CTTCTCAGCT ACTCTCCCCT ATGGGTCATG CCAACAATTC TGCTTTGGGA Ioxí TATAGCATAC TGTCAGCAGAG CAAATATGTA GCTCCCCACC TTCCGCCCAT TTCTTGGAG GGCTGGGGGG GGTTGGGGGG GGATGGAAGC CGGCCACCAC CCGCCCCCCA ACCGATCAG ACCGAATAAA CAAAATAAA CAAAATAAA	ATGTAGIGGG TTAAAAGAGG ACCCCTGGTC TCATCCAGAG exon CTGCTACTCG TCTTAATGGA TGTGGTTTG TCCGCTCATG GGTGTGGAA AGGCAGAAGT TCCCGCCCC GCCTAGGCAT GGCCTAGAG GTCATGCAAT CGGCTCGTGA GGCATCTCTC CCGGCTCGTGA AGGCACTGTG GGACTTCTCC GGGCCGCATA GAGATGGGGA	AAGAAAAAGT AAATGCCTTG CGACGTTTTC GAAGAAAGTA 6 GCTGGTATGT AAGACCTTCT GTTATGAATT CAAGAGGAAG AGACAATAAC GTCCCCAGG ATGCAAAGAT ATGCCTACGA CGGCGTATGAC CCGGGAAGGGA GCGCCGCGCTG GATCAGGATG CCCATGCCGA ATGCCCTTCT TTCCCCAC CCCCCCCA TTGGCAGAGGCA ACTTCGTATA TGGAGAGGCA	GCTTTTTACG GAGGAGAAAG ACTGTCCTTC CACGTCTTTG AGCTGTCCTA GCCATAGACT CCTCGAGCAG CCTGGAGCAG CCTGGATCAGAA TGCCTGGCGGGAG TGCATCAGAA CATACGCTTG ATCTGGACGAT ACTCTGGACGA ACTCTCTTCTTC GGGGGAGGCT TGTCCATAAA CCCACCCCC IOXP GCATACCATTA	ATTTACTGTC AGAACAAAAT ACGTCTGGTG Hinc GGGAQAAG E247K AGACCTGGTT GTGGTGTTTC GTGGTGTTTC GTGCGCACTTT GCTCCAATAA ATTTAGCAGCA ACAGAATGG GCAAAGTAGG GCAAAGTGG GCAGAAGTGG GCAGAAGTCAG AGACAATCGG CCGAATATCA ATATTGCCGA TGGCGGAGTTC AAAGTTCGGG AACTGAAACA CGCGGGGTTC CAAGTTCGGG TACCAAGTTA GAAGAACAC	TCATGAGTAA AGTGGTGAGG ACATATCCCG III TAGCCTGGTG AGCAAGATGG TTTTACAATA AAGAGGAAGC TCGGGGAAA ACCATAGTCC GGCAAGCATG ACCATAGTCC AGGCCTGAC TGCCCGGGCC TGCCGGGCCA TGCGGGGCA TGCGGGGCA CTTCCGGGGCC TTCTGAGCGG CTTCCGGAACA GGTCCCAGG GGTCCCAGG TGAAGGCCCA TGGAAGGCCCA	TTCTCTCTCT CAAACAGGCT GCTTGTTTCT ACCCTGTTCC TGTAGATTGT ATTTGTGTTG AAAAAGCCTC GTGCGCGGAA GGAAGAGTCC CATCTCAATT CGCCCTAAC CGCCCTCGGCC CATCGCCGTGT TATCGTGCTG GACCACCAAG CACCCCAAG CACCCCATGG GACCACCAAG GACCTCTGGGG GACTCTGGGG CAATACCGAA CTGGCCATCT GGCCTCCCAG GACTCTCCGGC CTGCCACTCT GGCCTCCCAG CACACCAAGAAACC	GCTGCAGGAA ATTTCTTCAT GX GAGGTTCTAT GATGTATTCA GCTGGGGAAT ACGGAGGAAA TCCACCCAGG CCCTATTTG TGAGGCGCAT TCGAGCTATT CAACATCAGCTAT TCCGCACACG GTTCGCCAGG GTTCGCCAGG GTTCGCCAGG GTTCGCCAGG GTTCGCCAGG GTTCGCCAGG GTTCGCCAGG GTCGACCGCC GCGAACCCG CCACACCGCCG CCACCCCG CCACCCCGC CCACCCCG CCACCCCGCT CCCACCCCG CCCACCCCG CCCACCCCG CCCACCCCG CCCACCCCG CCCACCCCG CCCACCCCG CCCCCCCC	CTTACACATT exon 5 CTACAGCCAG ACACGGACCC GAATATGCCC CTTATTCCCC ECORV/NotI CTGATGGCCG CCTGGATGGC CCTGGATGGC CCTGGACAGGA CCGCCCTAA TCCAGCGCGG GGCGTCCTT TTGCTCCTGC CATCGACCAGGG ACCCCAGGGA CTCAAGCGGA CTCAAGCGGA CCTCATGCTG CACCGACCAGC GGCGCCAGCC GGCGCCAGCC AGTCATACCA	4500 5000 5500 6000 6500	Lox P SV40 promoter NeoR TK polyA Lox P
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