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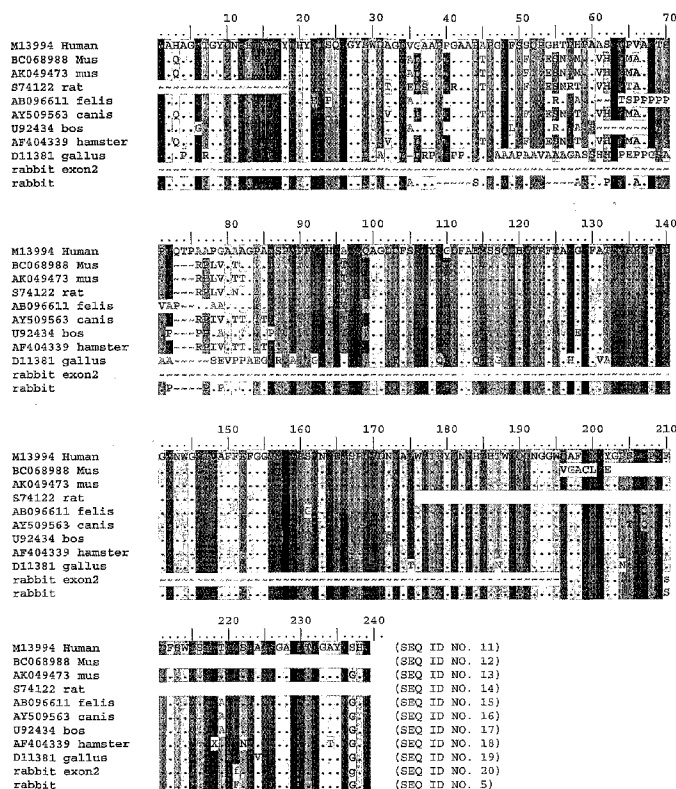
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(54) Title: SUPPRESSION OF B-CELL APOPTOSIS IN TRANSGENIC ANIMALS EXPRESSING HUMANIZED IMMUNOGLOBULIN



(57) Abstract: ABSTRACT The invention provides a novel approach to increase immunoglobulin expression in non-human transgenic animals. For instance, the invention provides a method to increase humanized immunoglobulin production in animals genetically engineered to express one or several human or humanized immunoglobulin transloci. This can be done by overexpressing the apoptosis inhibitor, i.e. a rabbit bcl-2, whose expression is driven by a B-cell specific promoter specifically in the B-cell of the animal, thereby enhancing the survival of B-cells. This invention further relates to a method for 'selectively enhancing the survival of exogenous B-cells, that is B-cells expressing any immunoglobulin transgene locus, over the survival of endogenous B-cells that do not express the transgene locus. Selectivity is achieved by expressing the apoptosis-inhibitor only within exogenous B-cells, that is, by coupling exogenous immunoglobulin expression with apoptosis inhibitor expression. This latter method allows for increased expression and production of the transgene encoded product(s) over the endogenously produced immunoglobulin of the transgenic animal. The invention also provides a novel apoptosis-inhibitor, rabbit bcl-2.



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SUPPRESSION OF B-CELL APOPTOSIS IN TRANSGENIC ANIMALS EXPRESSING HUMANIZED IMMUNOGLOBULIN

Field of the Invention

5 This invention relates to methods for enhancing the survival of B-cells in animals undergoing short-term lymphopoiesis. This invention further relates to methods for enhancing the survival of B-cells of transgenic animals expressing an exogenous immunoglobulin or immunoglobulin chain transgene locus for increasing the production of immunoglobulins. This invention further relates to a method for selectively enhancing the survival of exogenous B-cells
10 expressing any immunoglobulin transgene locus over endogenous B-cells that do not express the transgene locus by selectively expressing any apoptosis-inhibitor only within exogenous B-cells expressing the transgene-encoded immunoglobulin, but not within B-cells expressing endogenous immunoglobulin. This method allows for the increased expression and production of the transgene encoded product(s) over the endogenously produced immunoglobulin of the
15 transgenic animal. The invention also provides a novel apoptosis-inhibitor, rabbit bcl-2.

Background Art

The generation of mice expressing human-mouse chimeric antibodies has been described by Pluschke *et al.*, *Journal of Immunological Methods* 215: 27-37 (1998). The generation of
20 mice expressing human immunoglobulin polypeptides has been described by Neuberger *et al.*, *Nature* 338: 350-2 (1989); Lonberg *et al.*, *Int. Rev. Immunol.* 13(1):65-93 (1995); and Bruggemann *et al.*, *Curr. Opin. Biotechnol.*, 8(4): 455-8 (1997). Generation of transgenic mice using a BAC clone has been described by Yang *et al.*, *Nat. Biotechnol.* 15: 859-65 (1997). The generation of cows expressing human antibodies has been described by Kuroiwa *et al.*, *Nature*
25 *Biotech* 20(9): 889-894 (2002).

Transgenesis in animals has been described by Wall RJ, *Theriogenology* 57(1): 189-201 (2002). The generation of transgenic rabbits has been described by Fan, J. *et al.*, *Pathol Int.* 49: 583-94 (1999); and Brem *et al.*, *Mol. Reprod. Dev.* 44: 56-62 (1996). The production of transgenic chicken has been described by Etches *et al.*, *Methods in Molecular Biology* 62: 433-
30 450 (1997); and Pain *et al.*, *Cells Tissues Organs* 165(3-4): 212-9 (1999); and Sherman *et al.*, *Nature Biotech* 16:1050-1053 (1998).

Rabbits with impaired immunoglobulin expression have been described by Chen *et al.*, *J. Immunol.* 150:2783-2793 (1993); and Lamoyi E, and Mage RG., *J. Exp. Med.* 162:1149-1160 (1985). A gamma-globulinemic chicken has been described by Frommel *et al.*, *J. Immunol.* 105(1): 1-6 (1970); and Benedict *et al.*, *Adv. Exp. Med. Biol.* 88(2): 197-205 (1977).

5 The cloning of animals from cells has been described by T. Wakayama *et al.*, *Nature* 394:369-374 (1998); J. B. Cibelli *et al.*, *Science* 280:1256-1258 (1998); J.B. Cibelli *et al.*, *Nature Biotechnology* 16:642-646 (1998); A. E. Schnieke *et al.*, *Science* 278: 2130-2133 (1997); and K.H. Campbell *et al.*, *Nature* 380: 64-66 (1996). Nuclear transfer cloning of rabbits has been described by Stice *et al.*, *Biology of Reproduction* 39: 657-664 (1988); Challah-Jacques *et al.*,
10 *Cloning and Stem Cells* 8(4):295-299 (2003).

The production of non-human transgenic animals expressing human(ized) immunoglobulin transloci and the production of antibodies from such transgenic animals have been described in detail in PCT Publication Nos. WO 92/03918, WO 02/12437, and in U.S. Patent Nos. 5,545,807, 5,814,318; and 5,570,429. Homologous recombination for chimeric
15 mammalian hosts is exemplified in U.S. Patent No. 5,416,260. A method for introducing DNA into an embryo is described in U.S. Patent No. 5,567,607. Maintenance and expansion of embryonic stem cells is described in U.S. Patent No. 5,453,357.

The cleavage activities of viral proteins containing 2A peptide sequences have been described by Palmenberg *et al.*, *Virology* 190:754-762 (1992); Ryan *et al.*, *J Gen Virol* 72:2727-
20 2732 (1991); Donnelly *et al.*, *J Gen Virol* 82:1027-1041 (2001); Donnelly *et al.*, *J Gen Virol* 82:1013-1025 (2001); Szymaczak *et al.*, *Nature Biotech* 22(5):589-594 (2004).

So far, studies of the relative contribution of cell survival mechanisms regulated by the apoptosis inhibitor bcl-2, have been performed mainly in mice. The effect of bcl-2 expression on cell survival has been described by McDonnell *et al.*, *Cell*, 57:79-88, (1989); Strasser *et al.*,
25 *Current Topics in Microbiology and Immunology*, 166:175-181, (1990); Knott *et al.*, *Hybridoma*, 15 (5):365-371, (1996); Smith *et al.*, *J. Exp. Med.*, 191(3):475-784 (2000); Strasser *et al.*, *PNAS*, 88:8661-8665, (1991) and Kumar *et al.*, *Immunology Letters*, 65:153-159, (1999). The effect of the apoptosis inhibitor bcl-x_L expression on cell survival has been described by Takahashi *et al.*, *J. Exp. Med.*, 190(3): 399-409 (1999).

30 Mechanisms of B-cell development such as continuous and short-term B lymphopoiesis have been reviewed in Lanning D, Osborne BA, Knight, KL., Immunoglobulin genes and generation of antibody repertoires in higher vertebrates: a key role of GALT. *Molecular Biology*

of B-cells. Alt F.W., Honjo T, Nueberger, M. S., Eds. Elsevier London, p 443 (2004); and Flajnik M.F., Comparative analysis of immunoglobulin genes: surprises and portents. *Nat. Rev. Immunol.* 2:688, (2002).

Since production of antibodies in larger transgenic animals like rabbits, chickens, sheep
5 and cows is favored from the standpoint of antibody yield, creation of larger founder animals
with B-cell apoptosis inhibition expressing higher amounts of transgene-encoded products is
highly desirable. However, B-cell development differs significantly in species undergoing short-
term lymphopoiesis (like rabbits, chickens, sheep and cows) relative to animals characterized by
continuous B lymphopoiesis (like mice). Thus, it is unclear if apoptosis inhibitors can be used
10 with the same success in animals undergoing short-term lymphopoiesis as in the more
extensively studied animals with continuous B lymphopoiesis, or, what the impact of apoptosis
inhibitors on antibody production and/or antibody affinities will be.

Summary of the Invention

15 In one aspect, the invention provides a polypeptide comprising a novel apoptosis-
inhibitor polypeptide, namely, the rabbit bcl-2 polypeptide of SEQ ID NO: 5. In a particular
embodiment, the invention provides a chimeric molecule comprising the rabbit bcl-2 polypeptide
of SEQ ID NO: 5 fused to a heterologous amino acid sequence. In a further embodiment, the
heterologous amino acid sequence is an epitope sequence. In another embodiment, the
20 heterologous amino acid sequence is an immunoglobulin sequence. In yet another embodiment,
the immunoglobulin sequence is an Fc region of an immunoglobulin. The present invention also
provides a nucleotide sequences encoding the rabbit bcl-2 polypeptide of SEQ ID NO: 5. In one
aspect, the invention provides a vector, expression cassette or transgenic expression construct
comprising the nucleic acid molecule that encodes the rabbit bcl-2 polypeptide. In another
25 aspect, the invention provides an isolated host cell transformed with the nucleic acid sequences
encoding the rabbit bcl-2 polypeptide of SEQ ID NO: 5. In a further aspect, the invention
provides an isolated host cell transformed with the vector, expression cassette or transgenic
expression construct comprising the nucleic acid molecule that encodes the rabbit bcl-2
polypeptide.

30 In some aspects, any apoptosis inhibitor gene can be used, for example, an apoptosis
inhibitor selected from the group consisting of bcl-2, caspase-9-DN mutants, baculovirus p35,
caspase-9S, crmA, z-VAD-fmk, z-DEVD-fmk, B-D-fmk, z-YVAD-fmk, Bcl-x_L, Mcl-1, XIAP,

TIAP, KIAP, NAIP, cIAP1, cIAP2, API1, API2, API3, API4, HIAP1, HIAP2, MIHA, MIHB, MIHC, ILP, ILP-2, TLAP, survivin, livin, apollon, BRUCE, MLIAP, SODD and FLIP and variants thereof. In some specific embodiments, the apoptosis inhibitor gene may be a mammalian bcl-2 gene. In some preferred embodiments, the mammalian bcl-2 gene is selected from the group consisting of human bcl-2, mouse bcl-2 and rabbit bcl-2 of SEQ ID NO: 6. In a preferred embodiment, the bcl-2 is the rabbit bcl-2 of SEQ ID NO: 5.

In one aspect, the invention provides a transgenic expression construct comprising a nucleic acid molecule that encodes an apoptosis inhibitor driven by a B-cell specific promoter/enhancer and thus, is specifically expressed in B-cells.

In another aspect, the invention provides a transgenic expression construct comprising a transgene encoding a fusion-protein comprising polypeptide sequences in the following order: a) an immunoglobulin or immunoglobulin chain; b) a self-cleaving peptide; c) an apoptosis inhibitor; and optionally, d) a protease cleavage site between a) and b).

The present invention further provides a method for enhancing the expression of an immunoglobulin or immunoglobulin chain in a transgenic animal undergoing short-term lymphopoiesis, comprising introducing into the transgenic animal undergoing short-term lymphopoiesis at least one transgene construct comprising an apoptosis-inhibitor transgene driven by a B-cell specific promoter/enhancer whereby apoptosis of the B-cells carrying said transgene construct is inhibited and production of the immunoglobulin or immunoglobulin chain is enhanced.

In a further aspect, the present invention provides a method for enhancing the expression of an immunoglobulin or immunoglobulin chain in the short-term lymphopoietic transgenic animal that further comprises introducing into the transgenic animal at least one more transgene encoding for an exogenous immunoglobulin or immunoglobulin chain transgene locus. In this method, the two transgenes can both be present on the same or on different transgenic expression vectors. In the latter case, the different transgenic expression vectors can be introduced into the transgenic animal either at the same time or sequentially.

The present invention also provides a method for selectively enhancing the expression of an exogenous immunoglobulin or immunoglobulin chain within an exogenous B-cell of a non-human transgenic animal, comprising introducing into the animal, a transgene construct encoding a fusion-protein comprising polypeptide sequences in the following order: a) an immunoglobulin or immunoglobulin chain; b) a self-cleaving peptide; c) an apoptosis

inhibitor, and, optionally; d) a protease cleavage site between a) and b), whereby survival of the exogenous B cell and exogenous immunoglobulin production are enhanced.

In any aspect, the protease cleavage site used in any of the transgenic constructs or methods described above, is selected from the group consisting of sites for aspartic proteases, cysteine proteases, metalloproteases, serine proteases and threonine proteases. In preferred
5 embodiments, the furin cleavage site is used.

In all aspects of the invention, the B-cell specific promoter/enhancer may be selected from the group consisting of promoters/enhancers of CD19, CD20, CD21, CD22, CD23, CD24, CD40, CD72, Blimp-1, CD79b, mb-1, tyrosine kinase blk, VpreB, immunoglobulin heavy chain,
10 immunoglobulin kappa light chain, immunoglobulin lambda-light chain and immunoglobulin J-chain, or modifications thereof. In specific embodiments, the B-cell specific promoter/enhancer is the immunoglobulin kappa light chain gene promoter or a modification thereof.

In all aspects of the invention, the preferred exogenous immunoglobulin(s)/ immunoglobulin chain transgene locus is the human/ humanized immunoglobulin heavy and/or
15 light chain sequence.

In all aspects of the invention, the self-cleaving peptide of the invention can be obtained from viral 2A/2B or 2A-like/2B sequences. Thus, the virus may be selected from the group consisting of the picornaviridae virus family, the equine rhinitis A (ERAV) virus family, the picornavirus-like insect virus family and from the type C rotavirus family. The virus may also
20 be selected from the group consisting of the foot and mouth disease virus (FMDV), the equine rhinitis A (ERAV) virus, and the *Thosea asigna* virus (TaV).

In a further aspect of the invention, the invention relates to non-human transgenic animals comprising the transgenic constructs described above. For instance, the apoptosis inhibitor transgenes are preferably introduced into animals undergoing short-term lymphopoiesis. These
25 include, but are not limited to, rabbits, birds, chickens, sheep, goats, cows, swine, horses and donkeys. These short-term lymphopoietic animals may further comprise transgenes, for instance, encoding an immunoglobulin(s)/ immunoglobulin chain transgene. On the other hand, the fusion-protein encoding transgenes can be introduced into any non-human animal.

Thus, in most aspects of the invention, unless specified, non-human animals are selected
30 from the group consisting of rodents (e.g. mice, rats), rabbits, birds (e.g. chickens, turkeys, ducks, geese, etc.), cows, pigs, sheep, goats, horses, donkeys and other farm animals. In some aspects of the invention, the non-human transgenic animal can either substantially stops antibody

diversification by gene rearrangement early in life or substantially stops antibody diversification within the first month of its life. In a specific embodiment, the non-human transgenic animal is the rabbit.

Brief Description of Drawings

Figure 1 shows an amino acid alignment of the rabbit polypeptide sequence (SEQ ID NO:5) with other bcl-2 molecules derived from other species (SEQ ID NOS: 11-20).

Figure 2: SEQ ID NO: 1; A synthetic human bcl-2 apoptosis inhibition vector under the control of the kappa 1 B cell specific promoter.

Figure 3: SEQ ID NO: 2; DNA fragment encoding rabbit IgG M2-self cleaving peptide F2A-human bcl2 fusion protein FRT rpsL-neo FRT.

Figure 4: SEQ ID NO: 3; DNA fragment encoding rpsL-neo flanked with FRT and FRT2 sites.

Figure 5: SEQ ID NO: 4; DNA fragment encoding rabbit IgM-M2-self cleaving peptide F2A-codon optimized human bcl2 fusion protein flanked with FRT and FRT2 sites.

Figure 6: SEQ ID NO: 5; The rabbit bcl-2 polypeptide sequence.

Figure 7: SEQ ID NO: 6; DNA fragment encoding rabbit IgM-M2-self cleaving peptide F2A-codon optimized human bcl2 fusion protein.

Figure 8: SEQ ID NO: 7; DNA fragment encoding rabbit IgM-M2-self cleaving peptide F2A-human bcl2 fusion protein.

Figure 9: SEQ ID NO: 8; DNA fragment encoding an IgG-M2-self cleaving peptide F2A-codon optimized human bcl2 fusion protein.

Figure 10: SEQ ID NO: 9; DNA fragment encoding rabbit IgM-M2-furin cleavage site-self cleaving peptide F2A-human bcl2 fusion protein.

Figure 11: SEQ ID NO: 10; DNA fragment encoding rabbit IgG-M2-furin cleavage site-self cleaving peptide F2A-codon optimized human bcl2 fusion protein.

Detailed Description of the Invention

Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* 2nd ed., J. Wiley

& Sons (New York, NY 1994), and March, *Advanced Organic Chemistry Reactions, Mechanisms and Structure* 4th ed., John Wiley & Sons (New York, NY 1992), provide one skilled in the art with a general guide to many of the terms used in the present application.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

“B-cells” are defined as B-lineage cells that are capable of undergoing rearrangement of immunoglobulin gene segments and expressing immunoglobulin genes at some stage in their life cycle. These cells include, but are not limited to, early pro-B-cells, late pro-B-cells, large pre-B-cells, small pre-B-cells, immature B-cells, mature B-cells, memory B-cells, plasma cells, etc.

“Apoptosis-inhibitors” refer to a molecule or substance the presence or expression of which provides a reduction of apoptosis in target cells, regardless of the underlying mechanism. Preferably, the apoptosis-inhibitor reduces apoptosis of a target cell by at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% relative to apoptosis in the absence of the inhibitor.

The term “human Ig gene translocus or locus or segment” as used herein includes both naturally occurring sequences of a human Ig gene locus or a segment thereof, degenerate forms of naturally occurring sequences of a human Ig gene locus or segments thereof, as well as synthetic sequences that encode a polypeptide sequence substantially identical to a polypeptide encoded by a naturally occurring sequence of a human Ig gene locus or a segment thereof. In this context, by “substantially” is meant the degree of amino acid sequence identity is preferably at least about 85%-95%, or more preferably at least about 90%-95%, or even more preferably at least about 95%, or most preferably at least about 98%. In a particular embodiment, the human Ig gene segment renders the immunoglobulin molecule non-immunogenic in humans. Here, the terms “human or humanized immunoglobulin (Ig) heavy and/or light chain locus” or “human or human(ized) Ig locus” are used interchangeably.

The terms “human antibody” and “human immunoglobulin” are used herein to refer to antibodies and immunoglobulin molecules comprising fully human sequences.

The terms “humanized antibody” and “humanized immunoglobulin,” as used herein, mean an immunoglobulin molecule comprising at least a portion of a human immunoglobulin

polypeptide sequence (or a polypeptide sequence encoded by a human immunoglobulin gene segment). The humanized immunoglobulin molecules of the present invention can be isolated from a transgenic non-human animal engineered to produce humanized immunoglobulin molecules. Such humanized immunoglobulin molecules are less immunogenic to primates, especially humans, relative to non-humanized immunoglobulin molecules prepared from the animal or prepared from cells derived from the animal. Humanized immunoglobulins or antibodies include immunoglobulins (Igs) and antibodies that are further diversified through gene conversion and somatic hypermutations in gene converting animals. Such humanized Ig or antibodies are not "human" since they are not naturally made by humans (since humans do not diversify their antibody repertoire through gene conversion) and yet, the humanized Ig or antibodies are not immunogenic to humans since they have human Ig sequences in their structure.

"Transgenes or transgene constructs" are DNA fragments with sequences encoding naturally or synthetic proteins normally not found in the animal or cells of the animal. The term "transgene construct" is used herein to refer to a polynucleotide molecule, which contains a structural "gene of interest" and other sequences facilitating gene transfer. This invention refers to at least two transgene constructs: 1) the rabbit bcl-2 apoptosis inhibitor transgene driven by a B-cell specific promoter, and, 2) the human Ig locus-self-cleaving peptide-apoptosis-inhibitor transgene construct.

"A transgenic expression vector or expression construct" refers to DNA fragments which encode, besides one or several transgene constructs of the invention, other regulatory DNA sequences required either for temporal, cell specific, or enhanced expression of the transgene(s) of interest, within specific cells of the non-human transgenic animal.

The "human(ized) Ig locus - self-cleaving peptide - apoptosis-inhibitor transgene or transgene construct" refers to a transgene construct that is transcribed into a single mRNA, which is translated into two polypeptides, namely, the human(ized) immunoglobulin chain and an apoptosis-inhibitor, due to a self-cleaving mechanism discussed below.

The term "self-cleaving peptide" as used herein refers to a peptide sequence that is associated with a cleavage activity that occurs between two amino acid residues within the peptide sequence itself. For example, in the 2A/2B peptide or in the 2A/2B-like peptides, cleavage occurs between the glycine residue on the 2A peptide and a proline residue on the 2B peptide. This occurs through a 'ribosomal skip mechanism' during translation wherein, normal

peptide bond formation between the 2A glycine residue and the 2B proline residue of the 2A/2B peptide is impaired, without affecting the translation of the rest of the 2B peptide. Such ribosomal skip mechanisms are well known in the art and are known to be used by several viruses for the expression of several proteins encoded by a single messenger RNA.

5 The terms “endogenous Ig (immunoglobulin)-expressing B-cells” and “endogenous B-cells” are used interchangeably, and refer to those B-cells that express the animal’s endogenous immunoglobulin locus.

The terms “exogenous Ig (immunoglobulin)-expressing B-cells” and “exogenous B cells” refer to those B-cells of a non-human animal that undergo productive rearrangement of an
10 exogenous human(ized) Ig translocus introduced into such B-cells. The human(ized) Ig locus is introduced into such B-cells as a separate expression construct or as part of the same expression construct also encoding the apoptosis-inhibitor. Productive rearrangement of the human(ized) Ig locus results in the expression of the human(ized) Ig and the transgene encoded apoptosis-inhibitor. As a result, apoptosis in B-cells expressing exogenous immunoglobulin is inhibited
15 and cell survival is enhanced.

By “B-cell specific expression of the apoptosis inhibitor gene” is meant, expression of the apoptosis inhibitor gene product preferably within immune cells, more preferably within B-cells. Specific expression of the apoptosis inhibitor gene within immune cells or B-cells is achieved using immune-specific or preferably, using B-cell specific promoters to drive the expression of
20 the apoptosis inhibitor gene.

By “selective expression of the apoptosis-inhibitor” is meant, expression of the apoptosis-inhibitor gene product preferentially within exogenous B-cells rather than within endogenous B cells expressing the native immunoglobulins of the transgenic animal. Preferably, the expression level of the apoptosis-inhibitor is at least about 2-fold, more preferably at least about 5-fold, even
25 more preferably at least about 10-fold, most preferably at least about 50-fold more in exogenous B-cells as compared to expression in endogenous B-cells.

“Antibodies” (Abs) and “immunoglobulins” (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen
30 specificity. The term “antibody” is used herein in the broadest sense and specifically covers, without limitation, monoclonal antibodies (including full length monoclonal antibodies),

polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired specificity.

The term "Ig gene segment" as used herein refers to segments of DNA encoding various portions of an Ig molecule, which are present in the germline of animals and humans, and which are brought together in B-cells to form rearranged Ig genes. Thus, Ig gene segments as used herein include V gene segments, D gene segments, J gene segments and C region gene segments. Functional rearrangement of VDJ or VJ segments results in the expression of immunoglobulin heavy or light chain.

The terms "antibody diversity" and "antibody repertoire" are used interchangeably, and refer to the total of all antibody specificities that an organism is capable of expressing.

An Ig locus having the capacity to undergo gene rearrangement and gene conversion is also referred to herein as a "functional" Ig locus, and the antibodies with a diversity generated by a functional Ig locus are also referred to herein as "functional" antibodies or a "functional" repertoire of antibodies.

The term "monoclonal antibody" is used to refer to an antibody molecule synthesized by a single clone of B-cells.

The term "polyclonal antibody" is used to refer to a population of antibody molecules synthesized by a population of B-cells.

The terms "polynucleotide" and "nucleic acid" are used interchangeably, and, when used in singular or plural, generally refer to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules.

One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified

for stability or for other reasons are “polynucleotides” as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term “polynucleotides” as defined herein. In general, the term “polynucleotide” embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The term “non-human (transgenic) animal” as used herein includes, but is not limited to, mammals such as, for example, non-human primates, rodents (e.g. mice and rats), non-rodent mammals, such as, for example, rabbits, pigs, sheep, goats, cows, pigs, horses and donkeys, and birds (e.g., chickens, turkeys, ducks, geese and the like). The term “non-primate animal” as used herein includes, but is not limited to, mammals other than primates, including but not limited to the mammals specifically listed above.

The phrase “animals which create antibody diversity substantially by gene conversion and/or somatic hypermutation to create primary antibody repertoires” or “gene converting animals” and their grammatical equivalents, are used to refer to such animals in which the predominant mechanism of antibody diversification is gene conversion and/or hypermutation as opposed to gene rearrangement. Such animals include, but are not limited to, rabbits, birds (e.g., chickens, turkeys, ducks, geese and the like), cows and pigs. Particularly preferred non-human animals are rabbits and chickens.

By animals “stopping antibody gene rearrangement early in life” is meant those animals where the rearrangement of immunoglobulin genes stops typically within the first month of life. Examples of such animals are, without limitation, rabbits, birds (e.g. chickens), sheep, goats, cattle, swine and horses.

Detailed Description

This invention, at least in part, is based on the recognition that the production of immunoglobulin (including immunoglobulin chains) in a non-human transgenic animal undergoing short-term lymphopoiesis can be significantly increased by expressing an apoptosis inhibitor in the B cells of the animal. As a result, the survival of B cells is enhanced and the production of immunoglobulin is increased.

The invention is further based on the identification on a novel apoptosis inhibitor, rabbit bcl-2. Accordingly, in one embodiment, the invention relates to methods for increasing

immunoglobulin expression in non-human transgenic animals by overexpressing rabbit bcl-2 in the animals' B cells, using a B-cell specific promoter, thereby enhancing B-cell survival.

This invention further relates to a method for selectively enhancing the survival of exogenous B-cells, that is, B-cells expressing an immunoglobulin transgene locus, over the survival of endogenous B-cells that do not express such a transgene locus in non-human animals, undergoing short-term lymphopoiesis. Selectivity is achieved by coupling exogenous immunoglobulin expression with apoptosis inhibitor expression. In endogenous B-cells, the apoptosis inhibitor is not expressed and hence, apoptosis is not inhibited. Such selective expression results in the preferential production of the transgene expressed immunoglobulin over the endogenously produced immunoglobulin of the transgenic animal.

Overexpression of bcl-2 apoptosis inhibitors (other than the rabbit sequence first disclosed herein) has mainly been studied in mice which showed amplified and prolonged antibody responses to immunization due to a great excess of B lymphocytes, immunoglobulin-secreting cells, and serum immunoglobulins, attributable to increased longevity of B-lineage cells and antigen-specific memory B cells; McDonnell *et al.*, *Cell*, 57:79-88, (1989); Strasser *et al.*, *Current Topics in Microbiology and Immunology*, 166:175-181, (1990); Knott *et al.*, *Hybridoma*, 15 (5):365-371, (1996); Smith *et al.*, *J. Exp. Med.*, 191(3):475-784 (2000); Strasser *et al.*, *PNAS*, 88:8661-8665, (1991) and Kumar *et al.*, *Immunology Letters*, 65:153-159, (1999).

Apoptosis of targeted B-cell populations occurs routinely throughout B-cell development. Two major strategies for B-cell development have been identified through the study of different species: continuous B lymphopoiesis, as found in mice and humans, and short-term B lymphopoiesis followed by expansion in gut-associated lymphoid tissue (GALT), as found in chickens, rabbits, sheep and cows (reviewed in Lanning D, Osborne BA, Knight, KL., Immunoglobulin genes and generation of antibody repertoires in higher vertebrates: a key role of GALT. *Molecular Biology of B-cells*. Alt F.W., Honjo T, Nueberger, M. S., Eds. Elsevier London, p 443 (2004); and Flajnik M.F., Comparative analysis of immunoglobulin genes: surprises and portents. *Nat. Rev. Immunol.* 2:688, (2002)).

In species where continued B lymphopoiesis occurs, B-cells develop primarily in the bone marrow and fetal liver, and immunoglobulin genes diversify on-site through the process of combinatorial V(D)J joining. Most of the peripheral blood lymphocytes in such species are IgM⁺, IgD⁺, or naïve B-cells with undiversified VDJ and VJ genes, even in adults. Thus, there

may be lesser pressure to produce a B-cell compartment quickly in animals with continued B lymphopoiesis since new B-cells with novel antigenic specificities are produced continuously.

In contrast, in the GALT (gut-associated lymphoid tissue) species where B lymphopoiesis is brief, an initial pool of B-cells is formed early in life in tissues such as the yolk sac and spleen, and thereafter, immunoglobulin (Ig) genes diversify in the GALT. Because B lymphopoiesis arrest is rapid, this initial B-cell compartment must expand and diversify quickly to generate antibodies with biologically relevant specificities. For instance, somatic diversification of Ig genes begins even before birth in chickens, sheep and cows. As a consequence, nearly all of the VDJ genes within the peripheral blood lymphocytes of adult rabbits for example, are highly diversified and lack naïve B-cells. Yet, the adult rabbit is very capable of mounting primary antibody responses to previously unseen antigens. That is because B-cells migrating from the GALT to the periphery appear to be of the primary B-cell repertoire, and even though their VDJ genes are already diversified, these long-lived and/or self-renewing B-cells can maintain the functional antibody repertoire. It is also likely that, as in the case of rabbits, exogenous antigenic stimulation helps drive the diversification of the antibody repertoire in species with short B lymphopoiesis.

In normal mice, during primary T cell-dependent immune responses, somatic mutations of Ig V region genes occurs in germinal-center-B-cells thus generating variant B-cells that express immunoglobulins with altered affinities for the antigen. Variants with improved affinity are positively selected through the inhibition of apoptosis and eventually, such high affinity B-cells make up the majority of the antigen specific memory and antibody-forming B-cell populations. B-cells with a low affinity receptor fail to receive such antigen-dependent survival signals and undergo apoptosis. Such an increase in high-affinity B-cells, within memory and antibody-forming B-cell populations, is referred to as affinity maturation.

In bcl-2 transgenic mice, overexpression of bcl-2 results in the prevention of apoptosis not only of high affinity B-cells, but also of low affinity B-cells. Bcl-2 overexpressing mice have an excessive number of memory B-cells that are not affinity selected. In contrast, the stringent selection of high-affinity bone marrow antibody-forming cells in the bcl-2 mouse is not influenced by the bcl-2 transgene and their numbers remain unchanged compared to controls.

While the effects of overexpression of bcl-2 on B-cell survival and development in other animals undergoing continuous B lymphopoiesis may be similar to that in mice, its role in the

development of memory and/or antibody-forming B-cells of animals undergoing short-term B lymphopoiesis is unclear.

Therefore, the present invention is directed to methods for overexpressing apoptosis inhibitors, particularly in animals with short-term B lymphopoiesis like rabbits, birds, chickens, sheep, goats, cows, swine, horses and donkeys and enhancing B-cell survival in such transgenic animals. In addition, when these animals further express an Ig translocus, expression of the Ig translocus is enhanced or prolonged and since these are larger animals, their antibody yields should also be greater. Thus, this invention aims at creating larger founder animals producing higher amounts exogenous immunoglobulins through enhanced B-cell survival.

In one aspect, the present invention is directed to transgenic constructs useful for enhancing the survival of B-cells. Transgenes or transgene constructs are DNA fragments with sequences encoding for one, or several, natural or synthetic proteins not normally found in the animal or cells of the animal. The DNA fragment(s) may be introduced into the animal's genome by a variety of techniques including microinjection of pronuclei, transfection, nuclear transfer cloning, sperm-mediated gene transfer, testis-mediated gene transfer, and the like.

In one embodiment, the transgene construct comprises the nucleic acid molecule encoding the apoptosis inhibitor, rabbit bcl-2 polypeptide. By "nucleic acid molecule encoding the apoptosis inhibitor" is meant the native DNA sequence, as well as any codon optimized DNA sequence which encodes for the a polypeptide sequence identical to the native DNA sequence, but which has a different DNA sequence based on codon degeneracy. This concept is discussed in detail below. In another embodiment, the transgene construct comprises the nucleic acid molecule encoding any apoptosis inhibitor. The apoptosis-inhibitor gene, such as the rabbit bcl-2 gene or the human bcl-2 gene, is preferably expressed in B-cells of the transgenic animal by means of an immune-specific promoter, preferably a B-cell specific promoter. Therefore, apoptosis-inhibitor expression is enhanced preferably within B-cells alone leading to enhanced B-cell survival in the non-human transgenic animal. By "B-cell specific promoter" is meant the promoter/enhancers sequence of any B-cell specific genes, and/or variants or engineered portions thereof, that normally controls the expression of genes expressed in a B-cell, examples of which include, but are not limited to, promoters/enhancers of CD19, CD20, CD21, CD22, CD23, CD24, CD40, CD72, Blimp-1, CD79b (also known as B29 or Ig beta), mb-1 (also known as Ig alpha), tyrosine kinase blk, VpreB, immunoglobulin heavy chain, immunoglobulin kappa light chain, immunoglobulin lambda-light chain, immunoglobulin J-chain, etc. In a preferred

embodiment, the kappa light chain promoter/enhancer drives the B-cell specific expression of the rabbit bcl-2 apoptosis-inhibitor gene.

In yet another embodiment, the transgene construct comprising the nucleic acid molecule encoding the apoptosis inhibitor is coexpressed with a transgene construct comprising an
5 exogenous immunoglobulin or immunoglobulin (Ig) chain transgene locus. In this embodiment, both the Ig transgene locus and the apoptosis inhibitor transgene may be present on the same transgenic expression vector or on two different transgenic expression vectors. In the latter case, the two transgenic expression vectors may be introduced into the non-human transgenic animal either at the same time or sequentially.

10 The present invention also provides transgene constructs comprising a chimeric transgene that encodes for a fusion protein comprising a transgene encoding a fusion-protein comprising polypeptide sequences in the following order: a) an immunoglobulin or immunoglobulin chain; b) a self-cleaving peptide; c) an apoptosis inhibitor; and optionally, d) a protease cleavage site
15 between a) and b). Here, the expression of the apoptosis inhibitor is linked or coupled to the expression of the immunoglobulin heavy or light chain using mechanisms discussed below. This transgenic construct is also referred to as the Ig locus-protease cleavage site-self cleaving peptide-apoptosis inhibitor construct. In this construct, a protease cleavage site is optionally added to facilitate the removal of the F2A self-cleaving peptide sequence from the immunoglobulin; for instance, from the M2-exon of the Ig, to prevent any potential interference
20 of the F2A peptide sequence with signaling (and therefore B-cell development). The protease cleavage sites can be recognized by any constitutively expressed proteases. Protease cleavage sites useful herein include, but are not limited to, aspartic proteases, cysteine proteases, metalloproteases, serine proteases threonine proteases, etc. In a preferred embodiment, the protease cleavage site is the furin cleavage site.

25 The chimeric transgenes described above comprises DNA sequences encoding for a self cleaving peptide (for example, 2A peptide or 2A-like peptide). Insertion of a self-cleaving peptide-encoding sequence between the immunoglobulin-encoding sequence and an apoptosis-inhibitor sequence in the transgene results in production of one messenger RNA. Translation of this mRNA, however, results in two separate proteins, the immunoglobulin(s) and the apoptosis-inhibitor, due to the peptide's self-cleaving mechanism. Therefore, expression of the apoptosis-
30 inhibitor can be coupled to the functional rearrangement of VDJ or VJ segments.

In one such embodiment of the invention, the self-cleaving is mediated by 2A/2B peptides, or 2A-like/2B sequences of viruses that include the picornaviridae virus family, the equine rhinitis A (ERAV) virus family, the picornavirus-like insect virus family or from the type C rotavirus family. The picornaviridae virus family includes the entero-, rhino-, cardio- and aphtho- and foot-and-mouth disease (FMDV) viruses. The picornavirus-like insect virus family includes viruses such as the infectious flacherie virus (IFV), the *Drosophila C* virus (DCV), the acute bee paralysis virus (ABPV) and the cricket paralysis virus (CrPV) and the insect virus *Thosea asigna* virus (TaV). The type C rotavirus family includes the bovine, porcine and human type C rotaviruses. In further embodiments, the cleavage sequences may include 2A-like/2B sequences from either the poliovirus, rhinovirus, coxsackie virus, encephalomyocarditis virus (EMCV), mengovirus, the porcine teschovirus-1, or the Theiler's murine encephalitis virus (TMEV), etc. In a preferred embodiment, the self-cleaving protein sequence is either the 2A/2B peptide of the foot and mouth disease virus (FMDV), the equine rhinitis A (ERAV) virus, or the *Thosea asigna* virus (TaV); Palmenberg *et al.*, *Virology* 190:754-762 (1992); Ryan *et al.*, *J Gen Virol* 72:2727-2732 (1991); Donnelly *et al.*, *J Gen Virol* 82:1027-1041 (2001); Donnelly *et al.*, *J Gen Virol* 82:1013-1025 (2001); Szymaczak *et al.*, *Nature Biotech* 22(5):589-594 (2004). Thus, using the self-cleaving peptide, expression of the apoptosis inhibitor gene is linked or coupled to the expression of the Ig translocus within exogenous B-cells. Selective survival of exogenous B-cells over endogenous B-cells results in reduced endogenous immunoglobulin production but in a corresponding increase in production of the Ig translocus encoded polypeptide/protein.

While bcl-2 is discussed as a prototype of apoptosis-inhibitors, other apoptosis-inhibitors are also included for use in the chimeric transgene construct. These include, without limitation, caspase-9 dominant negative (caspase-9-DN) mutants, baculovirus p35, caspase-9S, crmA, z-VAD-fmk, z-DEVD-fmk, B-D-fmk, and z-YVAD-fmk, other bcl-2 family members like Bcl-x_L, Mcl-1, etc., inhibitors of proapoptotic molecules like Bax, Bak, Bad, inhibitors of "BH3 domain only" molecules like Bid, Bim, PUMA, Noxa, etc., other endogenous caspase inhibitors like IAP (inhibitor of apoptosis proteins) including, but not limited to XIAP, TIAP, KIAP, NAIP, cIAP1, cIAP2, API1, API2, API3, API4, HIAP1, HIAP2, MIHA, MIHB, MIHC, ILP, ILP-2, TLAP, survivin, livin, apollon, BRUCE, and MLIAP, etc., proteins like SODD and FLIP, etc. involved in the down-regulation of death receptors and variants thereof. In a specific embodiment, the apoptosis inhibitor gene may be a mammalian bcl-2 gene and in preferred embodiments, the mammalian bcl-2 gene is selected from the group consisting of human bcl-2, mouse bcl-2 and

rabbit bcl-2 of SEQ ID NO: 5. In a preferred embodiment, the rabbit bcl-2 gene of SEQ ID NO: 5 is used.

In yet another aspect of the invention, the transgene encodes immunoglobulin heavy chains and/or immunoglobulin light chains or parts thereof. The loci can be in germline configuration or in a rearranged form. The coding sequences or parts thereof may code for human immunoglobulins resulting in the expression of human(ized) antibodies.

The transgene(s) encoding human(ized) antibodies contain(s) an Ig locus or a large portion of an Ig locus, containing one or several human Ig segments (e.g., a human Ig V, D, J or C gene segment). Alternatively, the transgene is a human immunoglobulin locus or a large portion thereof. The transgene containing such a human Ig locus or such modified Ig locus or modified portion of an Ig locus, also referred to herein as "a human(ized) Ig translocus", is capable of undergoing gene rearrangement in the transgenic non-human animal thereby producing a diversified repertoire of antibodies having at least a portion of a human immunoglobulin polypeptide sequence.

Immunoglobulin heavy and light chain genes comprise several segments encoded by individual genes and separated by intron sequences. Thus genes for the human immunoglobulin heavy chain are found on chromosome 14. The variable region of the heavy chain (VH) comprises three gene segments: V, D and J segments, followed by multiple genes coding for the C region. The V region is separated from the C region by a large spacer, and the individual genes encoding the V, D and J segments are also separated by spacers.

There are two types of immunoglobulin light chains: κ and λ . Genes for the human κ light chain are found on chromosome 2 and genes for the human λ light chain are found on chromosome 22. The variable region of antibody light chains includes a V segment and a J segment, encoded by separate gene segments. In the germline configuration of the κ light chain gene, there are approximately 100-200 V region genes in linear arrangement, each gene having its own leader sequence, followed by approximately 5 J gene segments, and C region gene segment. All V regions are separated by introns, and there are introns separating the V, J and C region gene segments as well.

Additionally, the vectors containing either of the transgene constructs described above may further contain DNA sequences coding for antibiotic selection markers like gentamycin, neomycin or kanamycin etc. and/or other conventional components of expression vectors.

The present invention provides methods for enhancing the expression of immunoglobulins in a non-human transgenic animal undergoing short-term lymphopoiesis comprising introducing into the transgenic animal undergoing short-term lymphopoiesis, at least one transgene construct comprising an apoptosis-inhibitor transgene driven by a B-cell specific promoter/enhancer. Thus, apoptosis of such B-cells with the transgene construct is inhibited and production of the immunoglobulin or immunoglobulin chain is enhanced. In a further embodiment of this method, the non-human transgenic animal undergoing short-term lymphopoiesis may further comprise an exogenous immunoglobulin(s) or immunoglobulin chain transgene locus. This results in higher yields of the exogenous immunoglobulin which can greatly simplify antibody purification and production. In this instance, the apoptosis-inhibitor gene may be introduced, either, as part of a transgenic expression construct that also introduces the Ig translocus, or on different transgenic constructs.

The invention further provides another method for selectively enhancing the expression of an exogenous immunoglobulin(s)/immunoglobulin chain within an exogenous B-cell of a non-human transgenic animal, where expression of the exogenous immunoglobulin(s)/immunoglobulin chain and an apoptosis inhibitor transgene within the exogenous B-cell is coupled. Correspondingly, there is no expression of the apoptosis inhibitor in endogenous B-cells, or B-cells not expressing the Ig translocus. Due to productive rearrangement of the exogenous immunoglobulin translocus and an increase in exogenous B-cell survival, transgene-encoded immunoglobulin production is increased over endogenous immunoglobulin production. Thus, survival of the exogenous B cell is enhanced and exogenous immunoglobulin(s)/immunoglobulin chain production is also enhanced.

The present invention further provides nucleic acid sequences that encode for proteins, polypeptides or peptide sequences for rabbit bcl-2, which is an apoptosis-inhibitor. It is also contemplated that a given nucleic acid sequence for rabbit bcl-2 may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same protein. Furthermore, the term functionally equivalent codon is used herein to refer to codons that encode the same amino acid, for example, as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids, as discussed herein.

The rabbit bcl-2 DNA segments used in the present invention encompass biologically functional equivalent modified polypeptides and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally

within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by human may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein, to reduce toxicity effects of the protein in vivo to a subject given the protein, or to increase the efficacy of any treatment involving the protein.

Allowing for the degeneracy of the genetic code, the invention encompasses sequences that have at least about 50%, usually at least about 60%, more usually about 70%, most usually about 80%, preferably at least about 90% and most preferably about 95% sequence identity to the nucleotide sequence of the rabbit bcl-2 gene or the human bcl-2 gene, respectively. These are also referred to as codon optimized sequences and is discussed below under functionally equivalent codons.

The term biologically functional equivalent is well understood in the art and is further defined in detail herein. Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99% identical at the amino acid level are considered functionally equivalent to the rabbit bcl-2 polypeptide, provided the biological activity of the protein is maintained.

The term functionally equivalent codon is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA coding sequence, and nevertheless produce a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below.

In making such changes, the hydrophathic index of amino acids may also be considered. The importance of the hydrophathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the relative hydrophathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0. \pm .1); glutamate (+3.0. \pm .1); serine (+0.3); asparagine (+0.2) glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5. \pm .1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within \pm .2 is preferred, those that are within \pm .1 are particularly preferred, and those within \pm .05 are even more particularly preferred.

As outlined herein, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take into consideration the various foregoing characteristics are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure (Johnson 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

These principles may be used, in conjunction with the principles outlined above, to engineer second generation molecules having many of the natural properties of apoptosis-inhibitors with altered and improved characteristics.

Thus, variant nucleic acid sequences that encode for rabbit bcl-2 and functionally equivalent polypeptides of rabbit bcl-2 are useful as apoptosis-inhibitors in this invention.

The immune system's capacity to protect against infection rests in a genetic machinery specialized to create a diverse repertoire of antibodies. Antibody-coding genes in B-cells are assembled in a manner that allows to countless combinations of binding sites in the variable (V) region. It is estimated that more than 10^{12} possible binding structures arise from such mechanisms. In all animals, including humans, the antibody-making process begins by recombining variable (V), diversity (D) and joining (J) segments of the immunoglobulin (Ig) locus. Following this step, depending on the animal species, two general mechanisms are used to produce the diverse binding structures of antibodies.

In some animals, such as human and mouse, there are multiple copies of V, D and J gene segments on the immunoglobulin heavy chain locus, and multiple copies of V and J gene segments on the immunoglobulin light chain locus. Antibody diversity in these animals is generated primarily by gene rearrangement, i.e., different combinations of gene segments to form rearranged heavy chain variable region and light chain variable region. In other animals (e.g., rabbit, birds, e.g., chicken, goose, and duck, sheep, goat, and cow), however, gene rearrangement plays a smaller role in the generation of antibody diversity. For example, in rabbit, only a very limited number of the V gene segments, most often the V gene segments at the 3' end of the V-region, is used in gene rearrangement to form a contiguous VDJ segment. In chicken, only one V gene segment (the one adjacent to the D region, or "the 3' proximal V gene segment"), one D segment and one J segment are used in the heavy chain rearrangement; and only one V gene segment (the 3' proximal V segment) and one J segment are used in the light chain rearrangement. Thus, in these animals, there is little diversity among initially rearranged variable region sequences resulting from junctional diversification. Further diversification of the rearranged Ig genes is achieved by gene conversion a process in which short sequences derived from the upstream V gene segments replace short sequences within the V gene segment in the rearranged Ig gene. Additional diversification of antibody sequences may be generated by hypermutation.

Immunoglobulins (antibodies) belong into five classes (IgG, IgM, IgA, IgE, and IgD, each with different biological roles in immune defense. The most abundant in the blood and potent in response to infection is the IgG class. Within the human IgG class, there are four sub-classes (IgG1, IgG2, IgG3 and IgG4 isotypes) determined by the structure of the heavy chain constant regions that comprise the Fc domain. The F(ab) domains of antibodies bind to specific sequences (epitopes) on antigens, while the Fc domain of antibodies recruits and activates other components of the immune system in order to eliminate the antigens.

Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by covalent disulfide bond(s), while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains (Chothia *et al.*, *J. Mol. Biol.* 186:651 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. U.S.A.* 82:4592 (1985)).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, connected by three CDRs. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit

various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The creation of human-animal translocus allows for the creation of transgenic animals that express diversified, high-affinity human(ized) (polyclonal) antibodies in high yields. In general, the humanization of an immunoglobulin (Ig) locus in a non-human animal involves the integration of one or more human Ig gene segments into the animal's genome to create human(ized) immunoglobulin loci. Thus, creation of a human(ized) Ig heavy chain locus involves the integration of one or more V and/or D and/or J segments, and/or C region segments into the animal's genome. Similarly, the creation of a humanized Ig light chain locus involves the integration of one or more V and/or J segments, and/or C region segments into the animal's genome.

Regardless of the chromosomal location, the human(ized) Ig locus of the present invention has the capacity to undergo gene rearrangement and gene conversion and hypermutation in the non-human animal, thereby producing a diversified repertoire of human(ized) Ig molecules. An Ig locus having the capacity to undergo gene rearrangement and gene conversion is also referred to as a "functional" Ig locus and the antibodies with a diversity generated by a functional Ig locus are also referred to as "functional" antibodies or a "functional" repertoire of antibody molecules.

In one aspect, animals in which diversification of the antibody repertoire stops early in life are useful in the current invention. B-cells develop from hematopoietic stem cells. Prior to antigen exposure, B-cells undergo a series of maturation steps the end product of which is a mature B-cell, which expresses a unique membrane-associated IgM and often IgD on its cell surface along with other cell surface signaling molecules. While in humans, antibody diversification by gene rearrangement occurs throughout life, in other animals the diversification of antibody repertoire stops early in life, typically within the first month of life.

In one aspect of this invention, the animals to whom the DNA constructs of the invention can be administered include, but are not limited to, mammals (e.g. humans, non-human primates, rodents (e.g. mice and rats), non-rodents (e.g. rabbits, pigs, sheep, goats, cows, pigs, horses and donkeys), and birds (e.g., chickens, turkeys, ducks, geese and the like). The animals to whom the DNA constructs of the invention can be administered include 'gene converting animals', that is, animals that create antibody diversity substantially by gene conversion and/or somatic hypermutation (for e.g. rabbits, birds, cows, swine, etc.), and animals where antibody

rearrangement stops early in life, that is, typically, within the first month of life (for e.g. rabbits, birds, sheep, goats, cattle, swine, horses, etc.) .

Further, animals to whom the DNA constructs of the invention can be administered also include any of the non-human animal described above, further carrying a transgene encoding an exogenous immunoglobulin translocus, preferably, a human or humanized immunoglobulin heavy chain and/or immunoglobulin light chain sequence or parts thereof. The transgene locus can be either in the germline configuration or in a rearranged form. Since the transgenes encode for human or humanized immunoglobulins or parts thereof, it results in the generation of humanized antibodies. Thus, for example, using the methods described above, enhanced production of humanized antibodies, can be generated in target non-human animals using the rabbit bcl-2 apoptosis-inhibitor described in this invention.

According to the present invention, a transgenic animal capable of making human(ized) immunoglobulins is made by introducing into a recipient cell or cells of an animal, one or more of the transgenic vectors described herein above, one of which carries a human(ized) Ig locus, and deriving an animal from the genetically modified recipient cell or cells.

The recipient cells may, for example, be from non-human animals which generate antibody diversity by gene conversion and/or hypermutation, e.g., bird (such as chicken), rabbit, cows and the like. In such animals, the 3'proximal V gene segment is preferentially used for the production of immunoglobulins. Integration of a human V gene segment into the Ig locus on the transgene vector, either by replacing the 3'proximal V gene segment of the animal or by being placed in close proximity of the 3'proximal V gene segment, results in expression of human V region polypeptide sequences in the majority of immunoglobulins. Alternatively, a rearranged human V(D)J segment may be inserted into the J locus of the immunoglobulin locus on the transgene vector.

The transgenic vectors containing the genes of interest, namely, the human(ized) Ig locus and the apoptosis-inhibitor gene may be introduced into the recipient cell or cells and then integrated into the genome of the recipient cell or cells by random integration or by targeted integration.

For random integration, a transgenic vector containing a human(ized) Ig locus can be introduced into an animal recipient cell by standard transgenic technology. For example, a transgenic vector can be directly injected into the pronucleus of a fertilized oocyte. A transgenic vector can also be introduced by co-incubation of sperm with the transgenic vector before

fertilization of the oocyte. Transgenic animals can be developed from fertilized oocytes. Another way to introduce a transgenic vector is by transfecting embryonic stem cells and subsequently injecting the genetically modified embryonic stem cells into developing embryos. Alternatively, a transgenic vector (naked or in combination with facilitating reagents) can be directly injected into a developing embryo. Ultimately, chimeric transgenic animals are produced from the embryos which contain the human(ized) Ig transgene integrated in the genome of at least some somatic cells of the transgenic animal.

In a particular embodiment, a transgene containing a human(ized) Ig locus is randomly integrated into the genome of recipient cells (such as fertilized oocyte or developing embryos) derived from animal strains with an impaired expression of endogenous immunoglobulin genes. The use of such animal strains permits preferential expression of immunoglobulin molecules from the human(ized) transgenic Ig locus. Examples for such animals include the Alicia and Basilea rabbit strains, as well as agammaglobulinemic chicken strain, as well as immunoglobulin knock-out mice. Alternatively, transgenic animals with human(ized) immunoglobulin transgenes or loci can be mated with animal strains with impaired expression of endogenous immunoglobulins. Offspring homozygous for an impaired endogenous Ig locus and a human(ized) transgenic Ig locus can be obtained.

For targeted integration, a transgenic vector can be introduced into appropriate animal recipient cells such as embryonic stem cells or already differentiated somatic cells. Afterwards, cells in which the transgene has integrated into the animal genome and has replaced the corresponding endogenous Ig locus by homologous recombination can be selected by standard methods. See for example, Kuroiwa et al, Nature Genetics 2004, June 6. The selected cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. Enucleation of oocytes and nuclear transfer can also be performed by microsurgery using injection pipettes. (See, for example, Wakayama *et al.*, *Nature* (1998) 394:369). The resulting egg cells are then cultivated in an appropriate medium, and transferred into synchronized recipients for generating transgenic animals. Alternatively, the selected genetically modified cells can be injected into developing embryos which are subsequently developed into chimeric animals.

Further, according to the present invention, a transgenic animal capable of producing human(ized) immunoglobulins can also be made by introducing into a recipient cell or cells, one

or more of the recombination vectors described herein above, one of which carries a human Ig gene segment, linked to 5' and 3' flanking sequences that are homologous to the flanking sequences of the endogenous Ig gene segment, then selecting cells in which the endogenous Ig gene segment is replaced by the human Ig gene segment by homologous recombination, and
5 deriving an animal from the selected genetically modified recipient cell or cells.

Similar to the target insertion of a transgenic vector, cells appropriate for use as recipient cells in this approach include embryonic stem cells or already differentiated somatic cells. A recombination vector carrying a human Ig gene segment can be introduced into such recipient cells by any feasible means, e.g., transfection. Afterwards, cells in which the human Ig gene
10 segment has replaced the corresponding endogenous Ig gene segment by homologous recombination, can be selected by standard methods. These genetically modified cells can serve as nuclei donor cells in a nuclear transfer procedure for cloning a transgenic animal. Alternatively, the selected genetically modified embryonic stem cells can be injected into developing embryos which can be subsequently developed into chimeric animals.

15 In a specific embodiment, the transgene constructs of the invention may be introduced into the transgenic animals during embryonic life by directly injecting the transgenes into the embryo or indirectly by injecting them into the pregnant mother or into the egg-laying hen. As a consequence, due to the inhibition of apoptosis in exogenous B-cells, transgenic offspring will have increased production of human(ized) antibodies in response to immunization with antigens.

20 Transgenic animals produced by any of the foregoing methods form another embodiment of the present invention. The transgenic animals have at least one, i.e., one or more, human(ized) Ig loci in the genome, from which a functional repertoire of human(ized) antibodies is produced.

In a specific embodiment, the present invention provides transgenic rabbits expressing one or more human(ized) Ig loci and an apoptosis-inhibitor gene. The transgenic rabbits of the
25 present invention are capable of rearranging and gene converting the human(ized) Ig loci, and expressing a functional repertoire of human(ized) antibodies.

In another specific embodiment, the present invention provides transgenic chickens expressing one or more human(ized) Ig loci and a apoptosis-inhibitor gene. The transgenic chickens of the present invention are capable of rearranging and gene converting the
30 human(ized) Ig loci, and expressing a functional repertoire of human(ized) antibodies. In another specific embodiment, the present invention provides transgenic mice expressing one or more human(ized) V regions and the rabbit bcl-2 apoptosis-inhibitor gene. The human(ized) V

region comprises at least two human V gene segments flanked by non-human spacer sequences. The transgenic mice are capable of rearranging the human V elements and expressing a functional repertoire of antibodies.

Immunization with antigen leads to the production of human(ized) antibodies against the same antigen in said transgenic animals.

Although preferred embodiments of the present invention are directed to transgenic animals having human(ized) Ig loci, it is to be understood that transgenic animals having primatized Ig loci and primatized polyclonal antisera are also within the spirit of the present invention. Similar to human(ized) polyclonal antisera compositions, primatized polyclonal antisera compositions are likely to have a reduced immunogenicity in human individuals.

Once a transgenic non-human animal capable of producing diversified human(ized) immunoglobulin molecules is made (as further set forth below), human(ized) immunoglobulins and human(ized) antibody preparations against an antigen can be readily obtained by immunizing the animal with the antigen. A variety of antigens can be used to immunize a transgenic host animal. Such antigens include, microorganism, e.g. viruses and unicellular organisms (such as bacteria and fungi), alive, attenuated or dead, fragments of the microorganisms, or antigenic molecules isolated from the microorganisms.

Preferred bacterial antigens for use in immunizing an animal include purified antigens from *Staphylococcus aureus* such as capsular polysaccharides type 5 and 8, recombinant versions of virulence factors such as alpha-toxin, adhesin binding proteins, collagen binding proteins, and fibronectin binding proteins. Preferred bacterial antigens also include an attenuated version of *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*, or culture supernatant from these bacteria cells. Other bacterial antigens which can be used in immunization include purified lipopolysaccharide (LPS), capsular antigens, capsular polysaccharides and/or recombinant versions of the outer membrane proteins, fibronectin binding proteins, endotoxin, and exotoxin from *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*.

Preferred antigens for the generation of antibodies against fungi include attenuated version of fungi or outer membrane proteins thereof, which fungi include, but are not limited to, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Cryptococcus neoformans*.

Preferred antigens for use in immunization in order to generate antibodies against viruses include the envelop proteins and attenuated versions of viruses which include, but are not limited

to respiratory syncytial virus (RSV) (particularly the F-Protein), Hepatitis C virus (HCV), Hepatitis B virus (HBV), cytomegalovirus (CMV), EBV, and HSV.

Therapeutic antibodies can be generated for the treatment of cancer by immunizing transgenic animals with isolated tumor cells or tumor cell lines; tumor-associated antigens which include, but are not limited to, Her-2-neu antigen (antibodies against which are useful for the treatment of breast cancer); CD19, CD20, CD22 and CD53 antigens (antibodies against which are useful for the treatment of B-cell lymphomas), (3) prostate specific membrane antigen (PMSA) (antibodies against which are useful for the treatment of prostate cancer), and 17-1A molecule (antibodies against which are useful for the treatment of colon cancer).

The antigens can be administered to a transgenic host animal in any convenient manner, with or without an adjuvant, and can be administered in accordance with a predetermined schedule.

After immunization, serum or milk from the immunized transgenic animals can be fractionated for the purification of pharmaceutical grade polyclonal antibodies specific for the antigen. In the case of transgenic birds, antibodies can also be made by fractionating egg yolks. A concentrated, purified immunoglobulin fraction may be obtained by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

The fractionated human(ized) antibodies may be dissolved or diluted in non-toxic, non-pyrogenic media suitable for intravenous administration in humans, for instance, sterile buffered saline.

The antibody preparations used for administration are generally characterized by having immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody preparation may contain immunoglobulins of various isotypes. Alternatively, the antibody preparation may contain antibodies of only one isotype, or a number of selected isotypes.

For making a human(ized) monoclonal antibody, spleen cells are isolated from the immunized transgenic animal whose B-cells expressing the animal's endogenous immunoglobulin have been depleted. Isolated spleen cells are used either in cell fusion with transformed cell lines for the production of hybridomas, or cDNAs encoding antibodies are cloned by standard molecular biology techniques and expressed in transfected cells. The procedures for making monoclonal antibodies are well established in the art. See, e.g., European

Patent Application 0 583 980 A1 ("Method For Generating Monoclonal Antibodies From Rabbits"), U.S. Patent No. 4,977,081 ("Stable Rabbit-Mouse Hybridomas And Secretion Products Thereof"), WO 97/16537 ("Stable Chicken B-cell Line And Method of Use Thereof"), and EP 0 491 057 B1 ("Hybridoma Which Produces Avian Specific Immunoglobulin G"), the disclosures of which are incorporated herein by reference. In vitro production of monoclonal antibodies from cloned cDNA molecules has been described by Andris-Widhopf et al., "Methods for the generation of chicken monoclonal antibody fragments by phage display", *J Immunol Methods* 242:159 (2000), and by Burton, D. R., "Phage display", *Immunotechnology* 1:87 (1995), the disclosures of which are incorporated herein by reference.

In most instances the antibody preparation consists of unmodified immunoglobulins, i.e., human(ized) antibodies prepared from the animal without additional modification, e.g., by chemicals or enzymes. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

Embodiments of the invention are directed to transgenes comprising the rabbit bcl-2 apoptosis-inhibitor which is expressed specifically in B-cells using a B-cell specific promoter. Another embodiment is directed to transgenes comprising the Ig locus-self-cleaving peptide-apoptosis inhibitor transgene, where expression of the apoptosis inhibitor gene is coupled to the expression of the Ig locus. Various apoptosis-inhibitor genes described above and those known in the art, including the rabbit bcl-2 apoptosis inhibitor, can be used in this embodiment.

Further embodiments of the invention are directed to methods to enhance the survival of B-cells using the transgene constructs described above. When the rabbit bcl-2 transgenic construct is used, the transgene can be introduced into a transgenic animal further comprising a transgene encoding an immunoglobulin locus thereby specifically enhancing the survival of B-cells. When the Ig locus-self-cleaving peptide-apoptosis inhibitor transgene is used, exogenous B-cells selectively survive and productively produce the transgene encoded gene. Selectivity is achieved by coupling exogenous immunoglobulin expression with apoptosis inhibitor expression. In endogenous B-cells, the apoptosis inhibitor is not expressed and hence, apoptosis is not inhibited. Such selective expression results in the preferential production of the desired transgene expressed immunoglobulin over the endogenously produced immunoglobulin of the transgenic animal. Any variety of apoptosis-inhibitors, self-cleaving peptides or immunoglobulin genes described herein or well-known in the art can be used in this transgene

construct. In a preferred embodiment, the Ig locus of the transgene is a human(ized) immunoglobulin/ immunoglobulin chain translocus.

The invention also provides a novel apoptosis-inhibitor, rabbit bcl-2, which is useful for enhancing cell survival.

5 In one aspect of this invention, the non-human transgenic animal which expresses the rabbit bcl-2 apoptosis inhibitor is preferably an animal undergoing short-term lymphopoietic B-cell development discussed above, which includes, but is not limited to, animals like rabbits, chickens, sheep and cows, etc. Since these animals are larger, their antibody production and yields, using the methods described above, are also greater. In another aspect of the invention,
10 the non-human transgenic animal which expresses the Ig locus-self-cleaving peptide-apoptosis inhibitor, is any animal including rodents (e.g. mice, rats), rabbits, birds (e.g. chickens, turkeys, ducks, geese, etc.), cows, pigs, sheep, goats, horses, donkeys and other farm animals. In a further aspect, the transgenic animals used in the methods of the invention can either be gene converting animals or animals that can undergo antibody diversification by gene rearrangement
15 that stops early in life. In a preferred embodiment, the non-human transgenic animal is the rabbit.

Thus, the transgenic constructs, the vectors comprising the transgene constructs and the transgenic animals generated using the methods described above are all embodiments of the invention.

20 The invention is further illustrated, but by no means limited, by the following examples.

Example 1

Construction of a apoptosis-inhibitor expression vector with human bcl-2

Screening of rabbit genomic BAC libraries resulted in the identification of two BACs
25 (179L1 and 196O2; Gene Bank Accession Nos: AY495827, and AY495828, respectively) containing rabbit light chain K1 gene segments.

For the construction of a B-cell specific apoptosis-inhibitor expression vector, BAC
AY495827 was modified by homologous recombination in E.coli (ET cloning: E. Chiang Lee *et al.*, *Genomics* 73, 56-65 (2001); Daiguan Yu *et al.*, *PNAS* 97, 5978-5983 (2000); Muyrers *et al.*,
30 *Nucleic Acids Research* 27, 1555-1557 (1999); Zhang *et al.*, *Nature Biotechnology* 18, 1314-1317(2000)) and nucleotides 1 - 107795 and 142832 - 205141 were deleted. A synthetic human bcl-2 gene, under control of the kappa 1 promoter from AY495828 (pos. 114284-114570) further

connected to the rabbit beta globin polyA sequence, was synthesized. Downstream, a gentamycin selection cassette flanked by FRT-sites was introduced by overlap extension PCR. The bcl-2 – gentamycin cassette was amplified with primers having 50 bp homologies to the modified AY495827 BAC (SEQ ID NO:1). The sequence from nucleotide 134571 – 136019 on
5 BAC AY495827 was exchanged against the bcl-2 – gentamycin cassette (SEQ ID NO:1) by ET cloning. Positive clones were selected with gentamycin, analyzed by restriction enzyme digests and confirmed by sequencing. Subsequently, the gentamycin selection cassette was removed by FLP-recombination. The resulting construct was used to generate transgenic animals.

10 Example 2

Construction of a apoptosis-inhibitor expression vector with mouse bcl-2

Screening of a rabbit genomic BAC libraries resulted in the identification of two BACs (179L1 and 196O2; Gene Bank Accession Nos: AY495827, and AY495828, respectively) containing rabbit light chain K1 gene segments.

15 For the construction of a B-cell specific apoptosis-inhibitor expression vector, BAC AY495827 was modified by homologous recombination in E.coli (ET cloning: (E. Chiang Lee *et al.*, *Genomics* 73, 56-65 (2001); Daiguan Yu *et al.*, *PNAS* 97, 5978-5983 (2000); Muyrers *et al.*, *Nucleic Acids Research* 27, 1555-1557 (1999); Zhang *et al.*, *Nature Biotechnology* 18, 1314-1317(2000) and nucleotides 1 - 107795 and 142832 - 205141 were deleted. A synthetic mouse
20 bcl-2 gene under the control of the kappa 1 promoter from AY495828 (pos. 114284-114570), further connected to the rabbit beta globin polyA sequence, was synthesized. Downstream, a gentamycin selection cassette flanked by FRT-sites was introduced by overlap extension PCR. The bcl-2 – gentamycin cassette was amplified with primers having 50 bp homologies to the modified AY495827 BAC (SEQ ID NO:1). The sequence from nucleotide 134571 – 136019 on
25 BAC AY495827 was exchanged against the bcl-2 – gentamycin cassette by ET cloning. Positive clones were selected with gentamycin and analyzed by restriction enzyme digests and confirmed by sequencing. Subsequently, the gentamycin selection cassette was removed by FLP-recombination. The resulting construct was used to generate transgenic animals.

Example 3**Construction of a human(ized) heavy chain locus encoding a fusion protein consisting of the membrane forms of IgM and IgG, a 2A self-cleaving peptide, and apoptosis-inhibitor**

5 BAC and fosmid clones containing rabbit immunoglobulin heavy chain locus sequences were isolated from genomic DNA libraries using probes specific for the constant, variable, and joining gene segments or the 3' enhancer region. Isolated BACs and fosmid Fos15B were sequenced (Genebank acc. No. AY386695, AY386696, AY386697, AY386698). The J and C μ regions of AY386695 and the C δ region of AY386696 were exchanged with corresponding
10 human counterparts by homologous recombination in E.coli by ET cloning (E. Chiang Lee *et al.*, *Genomics* 73, 56-65 (2001); Daiguan Yu *et al.*, *PNAS* 97, 5978-5983 (2000); Muylers *et al.*, *Nucleic Acids Research* 27, 1555-1557 (1999); Zhang *et al.*, *Nature Biotechnology* 18, 1314-1317(2000)).

The four BACs were recombined by *in vitro* ligation and Cre-mediated recombination to
15 reconstitute a rabbit Ig locus with human J, C μ and C δ coding sequences.

To link the expression of bcl-2 to the expression of IgM and IgG, the coding sequence of bcl-2 was fused with the coding sequence of M2 membrane exons of IgM and IgG with a sequence coding for a F2A self-cleaving peptide.

For the insertion of a sequence encoding an IgG-M2-F2A-bcl-2 fusion protein the
20 following construct was generated. Sequences for homologous recombination were based on the sequence of BAC AY 386696. A DNA fragment (from 5' to 3') containing a KpnI site, a sequence identical to 50 nucleotides of C γ M2, a sequence encoding F2A, a sequence encoding a human bcl-2, an FRT5 site, and an EcoRI site was synthesized. The rpsL.Neo counter selection cassette was amplified using plasmid pSC101 *rpsL*-neo (Genebridges) as template. The
25 upstream primer contained a EcoRI and an FRT5 site, the downstream primer contained a sequence identical to 50 nucleotides downstream of C γ M2 and a XhoI site. The synthetic fragment and the PCR amplification product were ligated into the pcDNA3.1(+) vector, opened with KpnI and XhoI. The ligated cassette (SEQ ID NO: 2) was released with XhoI and KpnI and used for homologous recombination in E coli. Following transformation of the cassette into the
30 *E.coli* strain DH10B containing BAC AY 386696 and the plasmid pSC101-BAD-gba-tetra, expression of recombinases Red α/β was induced. Subsequently, kanamycin resistant clones

were selected and were analysed by restriction enzyme digestion and partial sequence analysis. Lastly, the RpsLNeo-resistance gene was deleted from the BAC by Flp-mediated recombination.

The resulting BAC clone was further modified through insertion of a sequence encoding an IgM-M2-F2A-bcl-2 fusion protein. Sequences for homologous recombination are based on the sequence of BAC AY 386696. The rpsL.Neo gene was amplified using plasmid pSC101 *rpsL*-neo (Genebridges) as the template. Primers contain sequences identical to IgG-M2 and flanking sequences, FRT- and FRT2-sites (SEQ ID NO: 3). The amplification product was inserted into BAC AY 386696 by ET cloning. Subsequently, the selection cassette was replaced with a DNA fragment encoding an IgM-M2-F2A-bcl-2 fusing protein (SEQ ID NO: 4). This DNA fragment was synthesized containing from 5' to 3'- an EcoRI site, an FRT site, a sequence encoding IgM-M2, a sequence encoding F2A and bcl-2, a FRT2 and an EcoRI site (SEQ ID NO: 4). The synthesized fragment was released with EcoRI and was used for the exchange of the rpsL.Neo gene with IgM-M2-F2A-bcl-2 by Flp-mediated recombination between FRT/FRT and FRT2/FRT2 sites. Positive clones are identified by restriction analysis and further analysed by partial sequencing.

The resulting BAC was combined with BACs containing different V-regions. BACs can be combined by ligation or recombination. The resulting constructs were used for the generation of transgenic animals.

Example 4

Generation of transgenic mice and rabbits expressing humanized heavy chain immunoglobulins

Transgenic rabbits and mice containing humanized heavy and light chain immunoglobulin loci and a apoptosis-inhibitor gene are generated by injection of DNA into the pronuclei of fertilized oocytes and subsequent transfer of embryos into foster mothers. Transgenic founder animals are identified by PCR. Expression of human(ized) immunoglobulin M and G is measured by ELISA. Expression of humanized IgG was 10-20 mg/ml.

Example 5

Generation of transgenic chicken expressing humanized heavy chain immunoglobulins

Transgenic chicken were generated by testis mediated gene transfer. DNA constructs (50ug) are mixed with 250ul lipofection reagent (superfect) in 500ul 0.9% NaCl and injected in

the testis of roosters. Three to four weeks later roosters with transgenic sperm are identified by PCR analysis and mated with hens. Transgenic offspring were identified by PCR. Expression of humanized IgG is 10-20 mg/ml.

All references cited throughout the disclosure along with references cited therein are
5 hereby expressly incorporated by reference.

While the invention is illustrated by reference to certain embodiments, it is not so limited. One skilled in the art will understand that various modifications are readily available and can be performed without substantial change in the way the invention works. All such modifications are specifically intended to be within the scope of the invention claimed herein.

WHAT IS CLAIMED IS:

- 1 1. A polypeptide comprising the rabbit bcl-2 polypeptide of SEQ ID NO: 5.
- 1 2. The polypeptide of claim 1 wherein said bcl-2 polypeptide of SEQ ID NO: 5 is fused to a
2 heterologous amino acid sequence.
- 1 3. The polypeptide of claim 2 wherein said heterologous amino acid sequence is an
2 epitope sequence.
- 1 4. The polypeptide of claim 2 wherein said heterologous amino acid sequence is an
2 immunoglobulin sequence.
- 1 5. The polypeptide of claim 4 wherein said immunoglobulin sequence is an Fc
2 region of an immunoglobulin.
- 1 6. An isolated nucleic acid molecule comprising a sequence encoding the rabbit bcl-
2 2 polypeptide of SEQ ID NO: 5.
- 1 7. A vector, expression cassette or transgenic expression construct comprising the
2 nucleic acid molecule of claim 6.
- 1 8. The transgenic expression construct of claim 7 wherein expression of said rabbit
2 bcl-2 apoptosis inhibitor is driven by a B-cell specific promoter/enhancer.
- 1 9. The transgenic expression construct of claim 8 wherein said B-cell specific
2 promoter/enhancer is selected from the group consisting of a promoter/enhancer of CD19, CD20,
3 CD21, CD22, CD23, CD24, CD40, CD72, Blimp-1, CD79b, mb-1, tyrosine kinase blk, VpreB,
4 immunoglobulin heavy chain, immunoglobulin kappa light chain, immunoglobulin lambda light
5 chain and immunoglobulin J-chain genes or modifications thereof.

1 10. An isolated host cell transformed with the vector, expression cassette or
2 transgenic expression construct of claim 7.

1 11. A method for enhancing the expression of an immunoglobulin or immunoglobulin
2 chain in a transgenic animal undergoing short-term lymphopoiesis, comprising introducing into
3 said transgenic animal undergoing short-term lymphopoiesis at least one transgene construct
4 comprising an apoptosis-inhibitor transgene driven by a B-cell specific promoter/enhancer to
5 produce said transgenic animal, whereby apoptosis of the B-cells carrying said transgene
6 construct is inhibited and production of the immunoglobulin or immunoglobulin chain is
7 enhanced.

1 12. The method of claim 11 wherein said transgenic animal is selected from the group
2 consisting of rabbits, birds, chickens, sheep, goats, cows, swine, horses and donkeys.

1 13. The method of claim 11 wherein said transgenic animal is a rabbit.

1 14. The method of claim 11 wherein said B-cell specific promoter/enhancer is
2 selected from the group consisting of CD19, CD20, CD21, CD22, CD23, CD24, CD40, CD72,
3 Blimp-1, CD79b, mb-1, tyrosine kinase blk, VpreB, immunoglobulin kappa light chain,
4 immunoglobulin lambda light chain and immunoglobulin J-chain promoters or modifications
5 thereof.

1 15. The method of claim 11 wherein said apoptosis-inhibitor is selected from the
2 group consisting of bcl-2, caspase-9-DN mutants, baculovirus p35, caspase-9S, crmA, z-VAD-
3 fmk, z-DEVD-fmk, B-D-fmk, z-YVAD-fmk, Bcl-x_L, Mcl-1, XIAP, TIAP, KIAP, NAIP, cIAP1,
4 cIAP2, API1, API2, API3, API4, HIAP1, HIAP2, MIHA, MIHB, MIHC, ILP, ILP-2, TLAP,
5 survivin, livin, apollon, BRUCE, MLIAP, SODD and FLIP and variants thereof.

1 16. The method of claim 11 further comprising introducing into said transgenic
2 animal undergoing short-term lymphopoiesis at least one more transgene encoding for an
3 exogenous immunoglobulin or immunoglobulin chain transgene locus.

1 17. The method of claim 16 wherein said exogenous immunoglobulin or
2 immunoglobulin chain is a human(ized) immunoglobulin heavy and/or light chain sequence.

1 18. The method of claim 17 wherein the exogenous immunoglobulin or
2 immunoglobulin chain transgene locus and the apoptosis-inhibitor transgene are both present on
3 the same transgenic expression vector.

1 19. The method of claim 17 wherein the exogenous immunoglobulin or
2 immunoglobulin chain transgene locus and the apoptosis-inhibitor transgene are present on
3 different transgenic expression vectors.

1 20. The method of claim 19 wherein each of said transgenic expression vectors are
2 introduced into the transgenic animal at the same time.

1 21. The method of claim 19 wherein each of said transgenic expression vectors are
2 introduced into the transgenic animal sequentially.

1 22. A non-human transgenic animal undergoing short-term lymphopoiesis comprising
2 at least one transgene construct comprising an apoptosis inhibitor transgene driven by a B-cell
3 specific promoter/enhancer.

1 23. A non-human transgenic animal undergoing short-term lymphopoiesis comprising
2 at least one transgene construct comprising an apoptosis inhibitor transgene driven by a B-cell
3 specific promoter/enhancer and at least one exogenous immunoglobulin or immunoglobulin
4 chain transgene locus.

1 24. The non-human transgenic animal of claim 23 wherein said exogenous
2 immunoglobulin or immunoglobulin chain is a human(ized) immunoglobulin heavy and/or light
3 chain sequence.

1 25. The non-human transgenic animal of claim 22 or 23 selected from the group
2 consisting of rabbits, birds, chickens, sheep, goats, cows, swine, horses and donkeys.

1 26. A transgenic expression construct comprising a transgene encoding a fusion-
2 protein comprising polypeptide sequences in the following order:

- 3 a) an immunoglobulin or immunoglobulin chain;
4 b) a self-cleaving peptide;
5 c) an apoptosis inhibitor; and optionally,
6 d) a protease cleavage site between a) and b).

1 27. The transgenic expression construct of claim 26 wherein said protease cleavage
2 site is selected from the group consisting of sites of aspartic proteases, cysteine proteases,
3 metalloproteases, serine proteases and threonine proteases.

1 28. The transgenic expression construct of claim 26 wherein said protease cleavage
2 site is the furin cleavage site.

1 29. The transgenic expression construct of claim 26 wherein said immunoglobulin
2 fragment is a fragment of a human(ized) immunoglobulin heavy and/or light chain.

1 30. The transgenic expression construct of claim 26 wherein said self-cleaving
2 peptide is derived from viral 2A/2B or 2A-like/2B peptides.

1 31. The transgenic expression construct of claim 30 wherein said virus is selected
2 from the group consisting of the picornaviridae virus family, the equine rhinitis A (ERAV) virus
3 family, the picornavirus-like insect virus family and from the type C rotavirus family.

1 32. The transgenic expression construct of claim 31 wherein said virus is selected
2 from the group consisting of the foot and mouth disease virus (FMDV), the equine rhinitis A
3 (ERAV) virus, and the *Thosea asigna* virus (TaV).

1 33. The transgenic expression construct of claim 26 wherein said apoptosis inhibitor
2 is selected from the group consisting of bcl-2, caspase-9-DN mutants, baculovirus p35, caspase-
3 9S, crmA, z-VAD-fmk, z-DEVD-fmk, B-D-fmk, z-YVAD-fmk, Bcl-x_L, Mcl-1, XIAP, TIAP,
4 KIAP, NAIP, cIAP1, cIAP2, API1, API2, API3, API4, HIAP1, HIAP2, MIHA, MIHB, MIHC,

5 ILP, ILP-2, TLAP, survivin, livin, apollon, BRUCE, MLIAP, SODD and FLIP and variants
6 thereof.

1 34. The transgenic expression construct of claim 26 wherein said apoptosis inhibitor
2 is mammalian bcl-2.

1 35. The transgenic expression construct of claim 34 wherein said mammalian bcl-2 is
2 selected from the group consisting of human bcl-2, mouse bcl-2 and rabbit bcl-2.

1 36. An isolated host cell transformed with the transgenic expression construct of
2 claim 26.

1 37. A method for selectively enhancing the expression of an exogenous
2 immunoglobulin or immunoglobulin chain within an exogenous B-cell of a non-human
3 transgenic animal, comprising introducing into said animal a transgene construct encoding a
4 fusion-protein comprising polypeptide sequences in the following order:

5 a) an immunoglobulin or immunoglobulin chain;

6 b) a self-cleaving peptide;

7 c) an apoptosis inhibitor, and, optionally;

8 d) a protease cleavage site between a) and b),

9 whereby survival of the exogenous B cell and exogenous immunoglobulin production
10 are enhanced.

1 38. The method of claim 37 wherein said exogenous immunoglobulin or
2 immunoglobulin chain is a human(ized) immunoglobulin heavy and/or light chain.

1 39. The method of claim 37 wherein said protease cleavage site is selected from the
2 group consisting of sites of aspartic proteases, cysteine proteases, metalloproteases, serine
3 proteases and threonine proteases.

1 40. The method of claim 37 wherein said protease cleavage site is the furin cleavage
2 site.

1 41. The method of claim 37 wherein said gene of a self-cleaving peptide is obtained
2 from viral 2A/2B or 2A-like/2B.

1 42. The method of claim 41 wherein said virus is selected from the group consisting
2 of the picornaviridae virus family, the equine rhinitis A (ERAV) virus family, the picornavirus-
3 like insect virus family and from the type C rotavirus family.

1 43. The method of claim 41 wherein said virus is selected from the group consisting
2 of the foot and mouth disease virus (FMDV), the equine rhinitis A (ERAV) virus, and the
3 *Thosea asigna* virus (TaV).

1 44. The method of claim 37 wherein said apoptosis-inhibitor is selected from the
2 group consisting of bcl-2, caspase-9-DN mutants, baculovirus p35, caspase-9S, crmA, z-VAD-
3 fmk, z-DEVD-fmk, B-D-fmk, z-YVAD-fmk, Bcl-x_L, Mcl-1, XIAP, TIAP, KLAP, NAIP, cIAP1,
4 cIAP2, API1, API2, API3, API4, HIAP1, HIAP2, MIHA, MIHB, MIHC, ILP, ILP-2, TLAP,
5 survivin, livin, apollon, BRUCE, MLIAP, SODD and FLIP and variants thereof.

1 45. The method of claim 37 wherein said non-human transgenic animal is selected
2 from the group consisting of rodents, primates, rabbits, birds, cows, pigs, sheep, goats, horses
3 and donkeys.

1 46. A non-human transgenic animal comprising at least one transgene construct
2 encoding a fusion-protein comprising polypeptide sequences in the following order:

- 3 a) an immunoglobulin or immunoglobulin chain;
4 b) a self-cleaving peptide;
5 c) an apoptosis inhibitor, and, optionally;
6 d) a protease cleavage site between a) and b),
7 whereby said fusion-protein is expressed.

2 47. The non-human transgenic animal of claim 46 wherein said animal is selected
3 from the group consisting of rodents, primates, rabbits, birds, cows, pigs, sheep, goats, horses
4 and donkeys.

FIG. 1

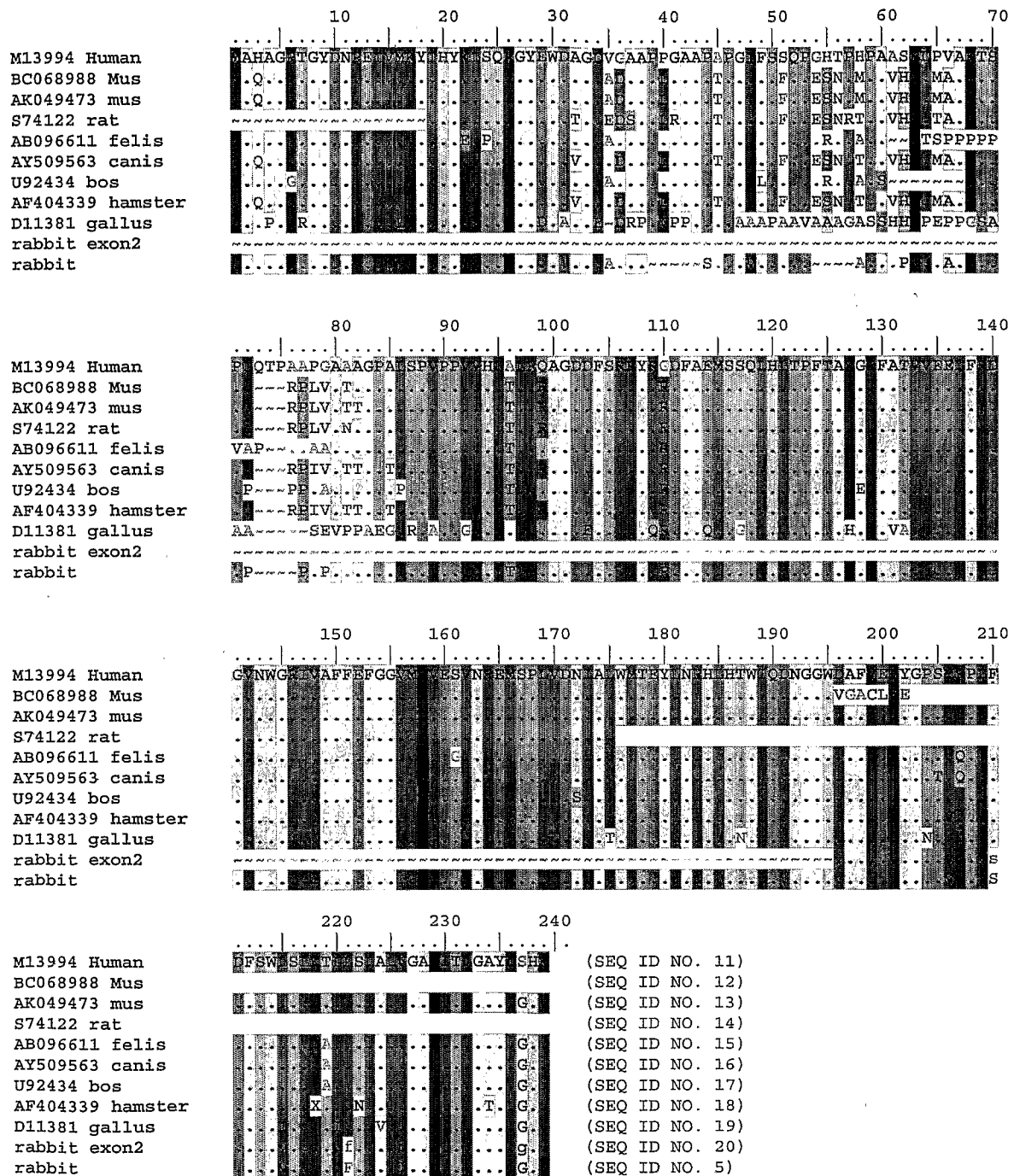
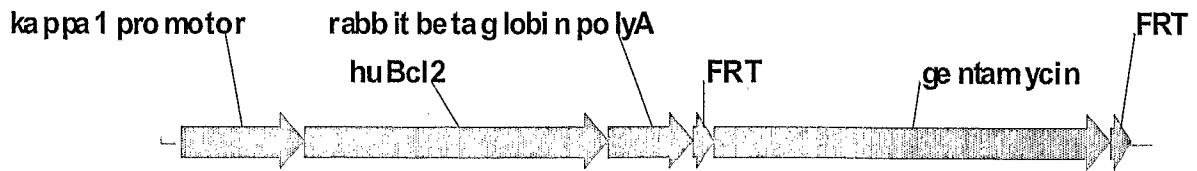


FIG. 2 (SEQ ID NO: 1)



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agtaggtt

FIG. 3 (SEQ ID NO: 2)

DNA fragment encoding rabbit IgG M2-selfcleaving peptide F2A-human bcl2 fusion protein
FRT rpsL-neo FRT

```

1  GGTACCTGAA ACACACCATC GCTCCCGACT ACAGGAACAT GATCGGGCAG GGGGCCGTGA
61 AACAGACTTT GAAATTTGAC CTTCTCAAGT TGGCGGGAGA CGTGGAGTCC AACCAGGGC
121 CCATGGCCCA CGCCGGGCGC ACTGGCTATG ATAATCGCGA AATTGTCATG AAGTATATTC
181 ACTACAAGCT CTCTCAAAGA GGATACGAGT GGGATGCGGG GGACGTGCGC GCTGCCCCAC
241 CTGGAGCTGC GCCGGCTCCA GGCATCTTTA GCAGCCAGCC GGGCCACACA CCTCACACCG
301 CTGCCTCCAG GGATCCGGTG GCACGGACCA GCCCTCTGCA AACTCCCGCC GCCCTGGGG
361 CTGCAGCGGG TCCCGCCTTG TCCCGGTGC CCCCTGTGGT GCACCTCACG CTGCGGCAGG
421 CGGGCGACGA CTTCAGCAGG CGCTACAGAA GAGACTTTGC CGAAATGTCC CGCCAGCTCC
481 ATCTGACCCC CTTACCCGCA CGAGGGAGGT TCGCCACCGT GGTGGAAGAA CTTTCCGCG
541 ACGTGTGAA CTGGGGCCGC ATCGTTGCCT TTTTGTAGTT CGGGGGGGTT ATGTGCGTGG
601 AATCAGTGAA CCGCGAAATG AGTCCCTTGG TCGACAACAT AGCTCTTTGG ATGACAGAGT
661 ACCTGAACCG GCATCTGCAT ACTTGGATAC AGGACAACGG AGGATGGGAT GCTTTTGTGT
721 AGCTGTACGG CCCATCAATG CGCCCCTTGT TCGACTTCAG CTGGTTGTCC CTGAAGACCG
781 TCCTGAGCCT CGCTCTTGTG GGCGCTGTFA TCACTTTGGG CGCTTATCTC GGACATAAAT
841 AAGAAGTTCC TATTCCGAAG TTCCTATTCT TCAAAAGGTA TAGGAACCTC GAATTCATTA
901 CACCAAGTGC AGTAAGCGGG CAAAGTCGGT TAATGTCAGT TTCAAAACGT CCACCCATCA
961 GGCCTGGTGA TGATGGCGGG ATCGTTGTAT ATTTCTTGAC ACCTTTTCGG CATCGCCCTA
1021 AAATTCGGCG TCCTCATATT GTGTGAGGAC GTTTTATTAC GTGTTACGA AGCAAAAGCT
1081 AAAACCAGGA GCTATTTAAT GGCAACAGTT AACCAGCTGG TACGCAAACC ACGTGCTCGC
1141 AAAGTTGCGA AAAGCAACGT GCCTGCGCTG GAAGCATGCC CGCAAAAACG TGGCGTATGT
1201 ACTCGTGTAT ATACTACCAC TCCTAAAAAA CCGAACTCCG CGCTGCGTAA AGTATGCCGT
1261 GTTCGTCTGA CTAACGGTTT CGAAGTGA CTCTACATCG GTGGTGAAGG TCACAACCTG
1321 CAGGAGCACT CCGTGATCCT GATCCGTGGC GGTGCTGTFA AAGACCTCCC GGGTGTTCGT
1381 TACCACACCG TACGTGGTGC GCTTGACTGC TCCGGCGTTA AAGACCGTAA GCAGGCTCGT
1441 TCCAAGTATG GCGTGAAGCG TCCTAAGGCT TAAGGAGGAC AATCATGATT GAACAAGATG
1501 GATTGCACGC AGGTTCCTCG GCCGCTTGGG TGGAGAGGCT ATTCCGGTAT GACTGGGCAC
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1621 TTCTTTTGT CAAGACCGAC CTGTCCGGTG CCCTGAATGA ACTGCAGGAC GAGGCAGCGC
1681 GGTATCGTG GCTGGCCACG ACGGGCGTTC CTTGCGCAGC TGTGCTCGAC GTTGTCACTG
1741 AAGCGGGAAG GGA CTGGCTG CTATTGGGCG AAGTGCCGGG GCAGGATCTC GTGTCATCTC
1801 ACCTTGCTCC TGCCGAGAAA GTATCCATCA TGGCTGATGC AATGCGGCGG CTGCATACGC
1861 TTGATCCGGC TACCTGCCCA TTCGACCACC AAGCGAAACA TCGCATCGAG CGAGCACGTA
1921 CTCGATGGA AGCCGGTCTT GTCGATCAGG ATGATCTGGA CGAAGAGCAT CAGGGGCTCG
1981 CGCCAGCCGA ACTGTTGCGC AGGCTCAAGG CGGCATGCC CGACGGCGAG GATCTCGTCG
2041 TGACCATAGG CGATGCCTGC TTGCCGAATA TCATGGTGA AAATGGCCGC TTTTCTGGAT
2101 TCATCGACTG TGGCCGGCTG GGTGTGGCGG ACCGCTATCA GGACATAGCG TTGGCTACCC
2161 GTGATATTGC TGAAGAGCTT GCGGCGCAAT GGGCTGACCG CTTCTCTGTG CTTTACGGTA
2221 TCGCCGCTCC CGATTGCGAG CGCATCGCCT TCTATCGCCT TCTTGACGAG TTCTTCTGAG
2281 AAGTTCCTAT TCCGAAGTTC CTATTCTTCA AAAGGTATAG GAACTTCGCC CTTCTCTCTC
2341 ACAGCTGCC TCCCTGGCCA GCAGGAGCCC CCGCTCCTC GAG

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FIG. 4: (SEQ ID NO: 3)

rpsL-neo flanked with FRT and FRT2 sites

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1  CGTCCATTCC CAACACATGA ACAGCATCTC ACGCCACCTC TGTTCCTGTC GAAGTTCCTA
61  TTCCGAAGTT CCTATTCTCT ACTTAGTATA GGAAGTTCAT TACACCAGTG TCAGTAAGCG
121 GGCAAAGTCG GTTAATGTCA GTTTCAAAAC GTCCACCCAT CAGGCCTGGT GATGATGGCG
181 GGATCGTTGT ATATTCTTGT ACACCTTTTC GGCATCGCCC TAAAATTCGG CGTCCTCATA
241 TTGTGTGAGG ACGTTTTTATT ACGTGTGTTAC GAAGCAAAAG CTAAAACCAAG GAGCTATTTA
301 ATGGCAACAG TTAACCAAGCT GGTACGCAAA CCACGTGCTC GCAAAGTTGC GAAAAGCAAC
361 GTGCCTGCGC TGGAAGCATG CCCGCAAAAA CGTGGCGTAT GTACTCGTGT ATATACTACC
421 ACTCCTAAAA AACCAGACTC CGCGCTGCGT AAAGTATGCC GTGTTCTGCT GACTAACGGT
481 TTCGAAGTGA CTTCTTACAT CGGTGGTGAA GGTCACAACC TGCAGGAGCA CTCCTGTATC
541 CTGATCCGTG GCGGTCTGTG TAAAGACCTC CCGGGTGTTC GTTACCACAC CGTACGTGGT
601 GCGCTTGACT GCTCCGCGCT TAAAGACCGT AAGCAGGCTC GTTCCAAGTA TGGCGTGAAG
661 CGTCCTAAGG CTTAAGGAGG ACAATCATGA TTGAACAAGA TGGATTGCAC GCAGGTTCTC
721 CGGCCGCTTG GGTGGAGAGG CTATTGCGGT ATGACTGGGC ACAACAGACA ATCCGCTGCT
781 CTGATGCCCG CGTGTTCGCG CTGTGAGCGC AGGGGCGCCC GGTTCCTTTT GTCAAGACCG
841 ACCTGTCCGG TGCCCTGAAT GAAGTGCAGG ACGAGGCAGC GCGGTATCGT TGGCTGGCCA
901 CGACGGGCGT TCCTTGCGCA GCTGTGCTCG ACGTTGTCAC TGAAGCGGGA AGGGACTGGC
961 TGCTATTGGG CGAAGTGCCG GGGCAGGATC TCCTGTCATC TCACCTTGCT CCTGCCGAGA
1021 AAGTATCCAT CATGGCTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC
1081 CATTGACCCA CCAAGCGAAA CATCGCATCG AGCGAGCACG TACTCGGATG GAAGCCGGTC
1141 TTGTCGATCA GGATGATCTG GACGAAGAGC ATCAGGGGCT CGCGCCAGCC GAACTGTTCTG
1201 CCAGGCTCAA GCGCGCATG CCCGACGGCG AGGATCTCGT CGTGACCCAT GGCATGCTT
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1381 TTGGCGGCGA ATGGGCTGAC CGCTTCTCTG TGCTTTACGG TATCGCCGCT CCCGATTGCG
1441 AGCGCATCGC CTTCTATCGC CTTCTTGACG AGTTCTTCTG AGAAGTTCCT ATTCGGAAGT
1501 TCCTATTCTC TAGAAAGTAT AGGAACTTCC CTGAGAAGGA TGTGCGAGGC CAAGAGACAA
1561 GCCCGCCGTG GCCCTGCTC

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FIG. 5: (SEQ ID NO: 4)

DNA fragment encoding rabbit IgM-M2-selfcleaving peptide F2A-codon optimized human bcl2 fusion protein flanked with FRT and FRT2 sites

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1  GAATTCGAAG TTCCTATTCC GAAGTTCCTA TTCTCTACTT AGTATAGGAA CTTCAGGTGA
61  AGCAGACTTT GAATTTTGAC CTTCTCAAGT TGGCGGGAGA CGTGGAGTCC AACCCAGGGC
121 CCATGGCCCA CGCCGGGCGC ACTGGCTATG ATAATCGCGA AATTGTCTAT AAGTATATTC
181 ACTACAAGCT CTCTCAAAGA GGATACGAGT GGGATGCGGG GGACGTCGGC GCAGCTCCAC
241 CTGGAGCTGC GCCGGCCCCC GGCATCTTTA GCAGCCAGCC GGGCCACACA CCTCACACCG
301 CTGCCTCCAG GGATCCGGTG GCACGGACCA GCCCTCTGCA AACTCCCGCC GCCCTGGGGG
361 CTGCAGCGGG TCCCGCCTTG TCCCGGGTGC CCCCTGTGGT GCACCTCAGC CTGCGGCAGG
421 CGGGCGACGA CTTACAGCAG CGCTACAGAA GAGACTTTGC CGAAATGTCC CGCCAGCTCC
481 ATCTGACCCC CTTACCGCA CGAGGGAGGT TCGCCACCGT GGTGGAAGAA CTTTCCGCG
541 ACGGTGTGAA CTGGGGCCGC ATCGTTGCCT TTTTGTAGTT CGGGGGGGTT ATGTGCGTGG
601 AATCAGTGAA CCGCGAAATG AGTCCCTTGG TCGACAACAT AGCTCTTTGG ATGACAGAGT
661 ACCTGAACCG GCATCTGCAT ACTTGGATAC AGGACAACGG AGGATGGGAT GCTTTTGTG
721 AGCTGTACGG CCCATCAATG CGCCCTTGT TCGACTTCAG CTGGTTGTCC CTGAAGACGC
781 TCCTGAGCCT CGCTCTTGTG GCGCCTGTGA TCACTTTGGG CGCCTATCTC GGACATAAAT
841 AAGAAGTTCC TATTCCGAAG TTCCTATTCT CTAGAAAGTA TAGGAAGTTC CTCGAGGAAT
901  TC

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FIG. 6: (SEQ ID NO: 5)

MAHAGRTGYDNREIVMKYIHYKLSQRGYEWDAAGDAGAASAPGVFSSQPAPAAPRDPAARTSP
PPPPAAAGPALSPVPPVHLLTLRQAGDDFSRRYRRDFAEMSSQLHLTPFTARGRFATVVEEL
FRDGVNWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEYLNRLHTWIQDNGGWDAFV
ELYGPSVRPLSDFSWVSLKTLFSLALIGACITLGAYLGHK*

FIG. 7 (SEQ ID NO: 6)

DNA fragment encoding rabbit IgM-M2-selfcleaving peptide F2A-codon optimized human
bcl2 fusion protein

aggtgaagcagactttgaattttgaccttctcaagttggcgggagacgtggagtgccaaccca
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FIG. 8 (SEQ ID NO: 7)

DNA fragment encoding rabbit IgM-M2-selfcleaving peptide F2A-human bcl2 fusion protein

aggatgaagcagactttgaattttgaccttctcaagttggcgggagacgtggagtgccaaccca
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FIG. 9 (SEQ ID NO: 8)

DNA fragment encoding an IgG-M2-selfcleaving peptide F2A-codon optimized human bcl2 fusion protein

Aggtgaagtggatcttctcgctccgtgggtggagctgaaacacaccatcgctcccgactacagg
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a

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FIG. 10 (SEQ ID NO: 9)

DNA fragment encoding rabbit IgM-M2-furin cleavage site-self cleaving peptide F2A-human bcl2 fusion protein

aggtgaagcgagcaaagcgaccggtgaaacagactttgaattttgaccttctcaagttggcg
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tga

FIG. 11 (SEQ ID NO: 10)

DNA fragment encoding rabbit IgG-M2-furin cleavage site-self cleaving peptide F2A-codon optimized human bcl2 fusion protein

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 <211> 1579
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Synthesized sequence

<400> 3
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 ggcaaaagtc gttaatgtca gtttcaaac gtccacccat caggcctggg gatgatggcg 180
 ggatcgttgt atatttcttg acaccttttc ggcacgccc taaaattcgg cgtcctcata 240
 ttgtgtgagg acgttttatt acgtgtttac gaagcaaaag ctaaaaccag gagctattta 300
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gtgcctgcgc  tggagcatg  cccgcaaaaa  cgtggcgtat  gtactcgtgt  atatactacc  420
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ttcgaagtga  cttcctacat  cgggtggtgaa  ggtcacaaac  tgcaggagca  ctccgtgatc  540
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<210> 4

<211> 902

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthesized sequence

<400> 4

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ccatggccca  cgccggggcg  actggctatg  ataatcgca  aattgtcatg  aagtatattc  180
actacaagct  ctctcaaaga  ggatacgagt  gggatgcggg  ggacgtcggc  gcagctccac  240
ctggagctgc  gccggcccc  ggcattctta  gcagccagcc  gggccacaca  cctcacaccg  300
ctgcctccag  ggatccgggt  gcacggacca  gccctctgca  aactcccgc  gccctgggg  360
ctgcagcggg  tcccgccttg  tcccgggtgc  cccctgtggg  gcacctcacg  ctgcggcagg  420
cgggcgacga  cttcagcagg  cgctacagaa  gagactttgc  cgaaatgtcc  cgccagctcc  480
atctgacccc  cttcacgcga  cgagggaggt  tcgccaccgt  ggtcgaagaa  cttttccgcg  540
acgggtgtgaa  ctggggccgc  atcgttcgct  tttttgagtt  cggggggggt  atgtgcgtgg  600
aatcagtga  ccgcgaaatg  agtcccttgg  tcgacaacat  agctctttgg  atgacagagt  660
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agctgtacgg  cccatcaatg  cgcccttctg  tcgacttcag  ctggttggtc  ctgaagacgc  780
tcctgagcct  cgctcttggt  ggccgctgta  tcactttggg  cgcctatctc  ggacataaat  840
aagaagttcc  tattccgaag  ttctatttct  ctgaaagta  taggaacttc  ctcgaggaat  900
tc  902

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<210> 5

<211> 226

<212> PRT

<213> Oryctolagus

<400> 5

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Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
 20          25          30
Gly Asp Ala Gly Ala Ala Ser Ala Pro Gly Val Phe Ser Ser Gln Pro
 35          40          45
Ala Pro Ala Ala Pro Arg Asp Pro Ala Ala Arg Thr Ser Pro Pro Pro
 50          55          60
Pro Pro Ala Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val
 65          70          75          80
His Leu Thr Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg
 85          90          95
Arg Asp Phe Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr
100          105          110

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Ala Arg Gly Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly
 115 120 125
 Val Asn Trp Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met
 130 135 140
 Cys Val Glu Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile
 145 150 155 160
 Ala Leu Trp Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile
 165 170 175
 Gln Asp Asn Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser
 180 185 190
 Val Arg Pro Leu Ser Asp Phe Ser Trp Val Ser Leu Lys Thr Leu Phe
 195 200 205
 Ser Leu Ala Leu Ile Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Gly
 210 215 220
 His Lys
 225

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 <211> 788
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Synthesized sequence

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 ctccacctgg agctgcgcgg gccctgggca tctttagcag ccagccgggc cacacacctc 240
 acaccgtgc ctccagggat ccggtggcac ggaccagccc tctgcaaact cccgccgccc 300
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 ggcaggcggg cgacgacttc agcaggcgct acagaagaga ctttgccgaa atgtcccgcc 420
 agctccatct gacccccttc accgcacgag ggaggttcgc caccgtggtc gaagaacttt 480
 tccgcgacgg tgtgaactgg ggccgcatcg ttgccttttt tgagttcggg ggggttatgt 540
 gcgtggaatc agtgaaccgc gaaatgagtc ccttggtcga caacatagct ctttggatga 600
 cagagtacct gaaccggcat ctgcataact ggatacagga caacggagga tgggatgctt 660
 ttgttgagct gtacggccca tcaatgcgcc ccttggtcga cttcagctgg ttgtccctga 720
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 ataaataa 788

<210> 7
 <211> 788
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Synthesized sequence

<400> 7
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 acatccatta taagctgtcg cagaggggct acgagtggga tgcgggagat gtgggcgccc 180
 cgccccggg ggccgcccc gcgcccggca tcttctctc gcagcccggg cacacgccc 240
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 ctgagtacct gaaccggcac ctgcacacct ggatccagga taacggaggc tgggatgcct 660
 ttgtggaact gtacggcccc agcatgcggc ctctgtttga tttctcctgg ctgtctctga 720
 agactctgct cagtttggcc ctggtgggag cttgcatcac cctgggtgcc tatctgggac 780
 acaagtga 788

<210> 8
 <211> 869

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<212> DNA

<213> Artificial Sequence

<220>

<223> Synthesized sequence

<400> 8

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cgggagacgt ggagtcacaac ccagggccca tggcccacgc cgggcgcact ggctatgata 180
atcgcgaaat tgtcatgaag tatattcact acaagctctc tcaaagagga tacgagtggg 240
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ccaccgtggt cgaagaactt ttccgcgacg gtgtgaactg gggccgcatac gttgcctttt 600
ttgagttcgg gggggttatg tgcgtggaat cagtgaaccg cgaaatgagt cccttggtcg 660
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acaacggagg atgggatgct tttgttgagc tgtacggccc atcaatgcgc cccttggtcg 780
acttcagctg gttgtccctg aagacgctcc tgagcctcgc tcttgtgggc gcctgtatca 840
ctttgggcgc ctatctcgga cataaataa 869

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<210> 9

<211> 809

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthesized sequence

<400> 9

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accgggagat agtgatgaag tacatccatt ataagctgtc gcagaggggc tacgagtggg 180
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acttcgccga gatgtccagg cagctgcacc tgacgccctt caccgcgcgg ggacgctttg 480
ccacggtggt ggaggagctc ttcagggaac ggggtgaactg ggggaggatt gtggccttct 540
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atcttctcct gctgtctctg aagactctgc tcagtttggc cctggtggga gcttgcataca 780
ccctgggtgc ctatctgggc cacaagtga 809

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<210> 10

<211> 884

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthesized sequence

<400> 10

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accttctcaa gttggcggga gacgtggagt ccaaccaggg gcccatggcc cacgccgggc 180
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atacttggat acaggacaac ggaggatggg atgcttttgt tgagctgtac ggcccatcaa 780
 tgcgcccctt gttcgacttc agctggttgt ccctgaagac gctcctgagc ctcgctcttg 840
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<210> 11
 <211> 239
 <212> PRT
 <213> Homo

<400> 11
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 1 5 10 15
 Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
 20 25 30
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 35 40 45
 Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
 50 55 60
 Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
 65 70 75 80
 Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Val Val His Leu Ala
 85 90 95
 Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
 100 105 110
 Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
 115 120 125
 Arg Phe Ala Thr Val Val Glu Leu Phe Arg Asp Gly Val Asn Trp
 130 135 140
 Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
 145 150 155 160
 Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
 165 170 175
 Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
 180 185 190
 Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
 195 200 205
 Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
 210 215 220
 Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
 225 230 235

<210> 12
 <211> 199
 <212> PRT
 <213> Mus

<400> 12
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 1 5 10 15
 Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
 20 25 30
 Gly Asp Ala Asp Ala Ala Pro Leu Gly Ala Ala Pro Thr Pro Gly Ile
 35 40 45
 Phe Ser Phe Gln Pro Glu Ser Asn Pro Met Pro Ala Val His Arg Asp
 50 55 60
 Met Ala Ala Arg Thr Ser Pro Leu Arg Pro Leu Val Ala Thr Ala Gly
 65 70 75 80
 Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu Arg Arg
 85 90 95
 Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met
 100 105 110
 Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala
 115 120 125
 Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile
 130 135 140
 Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn
 145 150 155 160

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Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu
 165 170 175
 Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
 180 185 190
 Val Gly Ala Cys Leu Val Glu
 195

<210> 13
 <211> 236
 <212> PRT
 <213> Mus

<400> 13
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 1 5 10 15
 Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
 20 25 30
 Gly Asp Ala Asp Ala Ala Pro Leu Gly Ala Ala Pro Thr Pro Gly Ile
 35 40 45
 Phe Ser Phe Gln Pro Glu Ser Asn Pro Met Pro Ala Val His Arg Asp
 50 55 60
 Met Ala Ala Arg Thr Ser Pro Leu Arg Pro Leu Val Ala Thr Thr Gly
 65 70 75 80
 Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu Arg Arg
 85 90 95
 Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met
 100 105 110
 Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala
 115 120 125
 Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile
 130 135 140
 Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn
 145 150 155 160
 Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu
 165 170 175
 Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
 180 185 190
 Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro Leu Phe Asp
 195 200 205
 Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala Leu Val Gly
 210 215 220
 Ala Cys Ile Thr Leu Gly Ala Tyr Leu Gly His Lys
 225 230 235

<210> 14
 <211> 154
 <212> PRT
 <213> Rattus

<400> 14
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 Glu Asp Ser Ala Pro Leu Arg Ala Ala Pro Thr Pro Gly Ile Phe Ser
 20 25 30
 Phe Gln Pro Glu Ser Asn Arg Thr Pro Ala Val His Arg Asp Thr Ala
 35 40 45
 Ala Arg Thr Ser Pro Leu Arg Pro Leu Val Ala Asn Ala Gly Pro Ala
 50 55 60
 Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu Arg Arg Ala Gly
 65 70 75 80
 Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met Ser Ser
 85 90 95
 Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala Thr Val
 100 105 110
 Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala
 115 120 125

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Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn Arg Glu
 130 135 140
 Met Ser Pro Leu Val Asp Asn Ile Ala Leu
 145 150

<210> 15
 <211> 235
 <212> PRT
 <213> Felis

<400> 15
 Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
 1 5 10 15
 Lys Tyr Ile His Tyr Glu Leu Pro Gln Arg Gly Tyr Glu Trp Asp Ala
 20 25 30
 Gly Asp Ala Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
 35 40 45
 Phe Ser Ser Gln Pro Gly Arg Thr Pro Ala Pro Ala Arg Thr Ser Pro
 50 55 60
 Pro Pro Pro Pro Val Ala Pro Ala Ala Ala Ala Ala Gly Pro
 65 70 75 80
 Ala Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu Arg Gln Ala
 85 90 95
 Gly Asp Asp Phe Ser Arg Arg Tyr Arg Asp Phe Ala Glu Met Ser
 100 105 110
 Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala Thr
 115 120 125
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 130 135 140
 Ala Phe Phe Glu Phe Gly Val Met Cys Val Glu Gly Val Asn Arg
 145 150 155 160
 Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu Tyr
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 35 40 45
 Phe Ser Phe Gln Pro Glu Ser Asn Pro Thr Pro Ala Val His Arg Asp
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 Pro Thr Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu Arg Arg
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 Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala
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 Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile
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Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn
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Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
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Asp Ala Phe Val Glu Leu Tyr Gly Pro Thr Met Gln Pro Leu Phe Asp
      195      200      205
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Gly Asp Ala Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
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Pro Pro Pro Pro Ala Ala Ala Ala Gly Pro Ala Pro Ser Pro Val Pro
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Pro Val Val His Leu Thr Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg
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Arg Tyr Arg Arg Asp Phe Ala Glu Met Ser Ser Gln Leu His Leu Thr
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Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
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Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Val Arg Pro Leu Phe Asp
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Arg Pro Ala Pro Gly Val His Leu Ala Leu Arg Gln Ala Gly Asp
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165      170      175
Arg His Leu His Asn Trp Ile Gln Asp Asn Gly Gly Trp Asp Ala Phe
180      185      190
Val Glu Leu Tyr Gly Asn Ser Met Arg Pro Leu Phe Asp Phe Ser Trp
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			20				25					30			
Ala	Cys	Ile	Thr	Leu	Gly	Ala	Tyr	Leu	Gly	His	Lys				
		35					40								

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2006/030250

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/82 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUROIWA Y ET AL: "Cloned transchromosomal calves producing human immunoglobulin" NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 20, no. 9, September 2002 (2002-09), pages 889-894, XP002976595 ISSN: 1087-0156 the whole document ----- -/--	1-47



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

17 November 2006

Date of mailing of the international search report

12/01/2007

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Authorized officer

Friedrich, Christof

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/030250

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JASPER P J ET AL: "B lymphocyte development in rabbit: progenitor B cells and waning of B lymphopoiesis" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, vol. 171, no. 12, 15 December 2003 (2003-12-15), pages 6372-6380, XP002368177 ISSN: 0022-1767 page 6372, column 1, paragraph 1 -----	1-47
A	JANANI RAMESH ET AL: "Effect of a bcl-2 transgene on production and localization of precursor B cells in mouse bone marrow" EXPERIMENTAL HEMATOLOGY (CHARLOTTESVILLE), vol. 26, no. 10, September 1998 (1998-09), pages 982-990, XP009074925 ISSN: 0301-472X the whole document -----	1-47
A	GRILLOT D A M ET AL: "bcl-x exhibits regulated expression during B cell development and activation and modulates lymphocyte survival in transgenic mice" JOURNAL OF EXPERIMENTAL MEDICINE, TOKYO, JP, vol. 183, February 1996 (1996-02), pages 381-391, XP002977276 ISSN: 0022-1007 figure 4 -----	1-47
A	WO 2004/037849 A2 (UNIV MINNESOTA [US]; VAN NESS BRIAN G [US]; LINDEN MICHAEL A [US]) 6 May 2004 (2004-05-06) the whole document -----	1-47
A	SONG ET AL: "Adenovirus-mediated Bcl-2 gene transfer inhibits apoptosis and promotes survival of allogeneic transplanted hepatocytes" SURGERY, C.V. MOSBY CO., ST. LOUIS,, US, vol. 130, no. 3, September 2001 (2001-09), pages 502-512, 530, XP005156362 ISSN: 0039-6060 the whole document ----- -/--	1-47

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2006/030250

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HUANG J ET AL: "Bcl-xL gene transfer protects the heart against ischemia/reperfusion injury" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 311, no. 1, 7 November 2003 (2003-11-07), pages 64-70, XP004465104 ISSN: 0006-291X the whole document</p>	1-47
A	<p>GUTHRIE H D ET AL: "Follicular expression of a human beta-cell leukaemia/lymphoma-2 (Bcl-2) transgene does not decrease atresia or increase ovulation rate in swine" REPRODUCTION FERTILITY AND DEVELOPMENT, vol. 17, no. 4, 12 April 2005 (2005-04-12), pages 457-466, XP002407867 ISSN: 1031-3613 the whole document</p>	1-47
A	<p>SHIN DONG HOON ET AL: "Localization of bcl-2 mRNA in the rabbit central nervous system" NEUROSCIENCE LETTERS, LIMERICK, IE, vol. 278, no. 1-2, 7 January 2000 (2000-01-07), pages 73-76, XP002390586 ISSN: 0304-3940 the whole document</p>	1-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2006/030250

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. type of material
- ☒ a sequence listing
- ☐ table(s) related to the sequence listing
- b. format of material
- ☒ on paper
- ☒ in electronic form
- c. time of filing/furnishing
- ☒ contained in the international application as filed
- ☒ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2006/030250

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004037849 A2	06-05-2004	AU 2003286515 A1	13-05-2004