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(71) Applicant and

(72) Inventor: VASSILEV, Tchavdar [BG/BG]; 51 Simeon
Radev Street, 1618 Sofia (BG).

(72) Inventor; and

(75) Inventor/Applicant (for US only): TCHORBANOV, An-
drei [BG/BG]; 112 Vasil Levski Blvd, 1000 Sofia (BG).

(74) Agent: STEFANOVA, Stanislava; Po Box 70, 1797 Sofia
(BG).

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(54) Title: SUPPRESSOR OF DISEASE-ASSOCIATED AUTOREACTIVE B LYMPHOCYTES

(57) Abstract: The invention relates to a suppressor of disease-associated autoreactive B lymphocytes with affinity to three different B-lymphocyte receptors. The suppressor is build of an immunoglobulin G backbone and of two synthetic components and is designed for use treating autoimmune disorders.



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Suppressor of disease-associated autoreactive B lymphocytes

Field of the invention

The invention relates to a suppressor of disease-associated autoreactive B lymphocytes in autoimmune disorders with three different ligands incorporated into one single molecule and manifesting affinity to one activating and to two inhibiting B cell receptors.

Background of the invention

It is known that autoimmune diseases develop in approximately two percent of the human population. In these pathological states, the patients' own tissues and organs are attacked by the immune system by autoantibodies or by autoreactive T lymphocytes. Serious damage or destruction of the targeted tissue results from the autoimmune attack. Depending on the number of organs targeted, autoimmune diseases are divided into two groups – systemic and organ-specific. The anti-inflammatory and immunosuppressive drugs used presently to treat them in most cases diminish or delay the injury to the targeted tissue, but do not affect the primary disease itself. Even more, such medicines have serious and even life-threatening side-effects.

Autoreactive B-lymphocytes are logical targets for a selective therapeutic intervention in antibody-mediated autoimmune diseases and the efforts for their selective suppression and/or elimination are well founded. It has been shown that the disease-associated antibodies in lupus-prone (NZBx NZW)F1 mice are produced by B-1 cells. The continuous elimination of these cells from the very earliest age prevents the development of glomerulonephritis and increases significantly the animal's life-span [Murakami, M. et al. (1995) *Intern.Immunol.* 7,877]. This therapeutic approach is, however, not effective in MRL/lpr mice and in lupus patients as their DNA-specific B-lymphocytes belong to the B-2 type.

The studies of *R. Furie et al.* show that the selective induction of single signal anergy results in selective inactivation of disease-associated B cells. Their surface immunoglobulin receptor is engaged by an artificial molecule, containing oligovalent B cell epitopes, linked to a non-immunogenic carrier (LJP394). It suppresses *in vivo* DNA-specific B cells in BXSB mice developing spontaneously a lupus-like disease. The administration of this immunomodulator results in diminished kidney damage and in increased time of survival. LJP394 has already shown encouraging results in phase II of clinical trials [Furie, R.A. et al. (2001). *J Rheumatol* 28,257].

It is known that the effect of the binding of the immunoglobulin receptor on B cells largely depends on the signals from stimulatory and inhibitory co-receptors on the surface of the same cell [Ravech, J. et al. (2000), *Science* 290, 84]. The suppressive effect of the B lymphocyte receptor CD22 on the functions of these cells is well documented. Its physiological ligand remains unknown, but it is established that it binds to oligosaccharides with a terminal 2,6 sialic acid. It has been shown that the cross-linking of CD22 and of the IgM immunoglobulin receptors results in suppression of the cell's activity [Jin, L. et al. (2002) *J. Exp. Med.* 195,1199, Wakabayashi, C. et al. (2002), *Science* 298,2392].

The synthetic DWEYSVWLSN peptide is shown to mimic antigenically native (double stranded) DNA and to be bound by disease-associated anti-DNA antibodies [Putterman, C. et al. (2000), *J Immunol* 164,2542].

It is also well known that when immune complexes, composed of an IgG antibody and an antigen, cross-link the Fc γ R for IgG, type IIb (Fc γ RIIb) and the surface immunoglobulin receptor of B lymphocytes, the activity of the latter cells is specifically suppressed [Ravech, J. et al. (2000), *Science* 290, 84, Nimmerjahn, F. et al. (2005) *Science*, 310,1510]. Patent application PCT/BG04/00010A1 describes an agent for selective suppression of disease-associated B-lymphocytes, the agent being a chimeric antibody molecule that cross-links the B cell immunoglobulin receptors with pre-selected anti-self specificity with the inhibitory Fc γ RIIb receptors. This agent is active only on IgG receptor-expressing B lymphocytes.

WO03/030835A3 relates to a method for designing bispecific antibodies for regulating immune responses, wherein said bispecific antibodies bind to one activating and to one inhibiting receptor. As one of the possible inhibiting receptors FcγRIIB is chosen. CD22 is not mentioned. The bispecific antibodies, according to the above cited document, are only active if administered with an agent that stimulates the expression of an inhibiting receptor or one activating receptor or with additional therapeutics.

WO9707218 describes a fusion protein that cross-links activating antigen-specific receptors with inhibiting receptors on targeted B cells. However, this fusion protein binds only to the human FcγRIIB (CD32) and targets only B cells with IgG, but not these with immunoglobulin M antigen receptors [Wakabayashi, C. et al. (2002), *Science* 298,2392].

Description of the invention

The invention relates to a suppressor of disease-associated autoreactive B lymphocytes with affinity to inhibitory B-lymphocyte receptors possessing three different ligands, incorporated into one single molecule. The suppressor possesses affinity to one activating receptor and to two inhibiting B cell receptors and is build of an immunoglobulin G backbone and of two synthetic components. The first of the three ligands is a part of the immunoglobulin G backbone and the second and third ligands are the two synthetic components (Figure 1).

The first synthetic component, coupled to the immunoglobulin G backbone, is a peptide epitope with affinity to the activating immunoglobulin receptors on targeted autoreactive B cells. It is a DNA-mimotope peptide made of ten aminoacids - DWEYSVWLSN (SEQ ID NO.1), where a hexamethylene linker is added to the C-end of the peptide.

The second synthetic component coupled to the immunoglobulin G backbone, is a synthetic peptide GGPGG (SEQ ID NO. 2) with a hexamethylene group at its C-end. A STN epitope with a free terminal 2,6 sialic acid is coupled to the N-end of the peptide (Figure 2).

As an immunoglobulin G backbone a mouse monoclonal IgG antibody is used. It is obtained from IP 2-11-1 hybridoma cells grown in protein-free CHO medium. The immunoglobulin fraction of the synthetic medium is isolated after the end of the cultivation by ammonium sulfate precipitation and subsequent dialysis against phosphate-buffered saline pH 7.2.

After the purification stage the monoclonal IgG antibody is coupled to the modified DWEYSVWLSN and to the STN-containing peptides using 1-ethyl-3(3'-dimethylaminopropyl) carboimidine.HCl (EDC) [Bauminger, S. et al. (1980) *Methods in Enzymology*. 70,151].

The components for the construction of the trispecific immunoglobulin molecule are prepared as follows. A 1.5 mg/ml solution of the monoclonal IgG in phosphate buffer pH 6.0, 0.3 mg/ml solution of EDC in PBS pH 6.0 and 0.2 mg/ml solutions of both peptides in dimethylphormamide/ phosphate (ratio of 1:9) buffer are made. 7.5 ml of the mixed peptide solution is added to 3.75 ml EDC and to 10 ml of the IgG solution, the volume is brought to 150 ml with phosphate buffered saline pH 6.0. The mixture is incubated for 16 hours at +4 °C with constant stirring. After that it is dialyzed at +4°C against 200 volumes of PBS pH 7.0 for 16 hours and concentrated to a final volume of 20 ml using ultrafiltration through a 5 kD-pore membrane (*Amicon, Millipore Corp.*). This preparation contains the trispecific immunoglobulin molecules, composed of the monoclonal IgG backbone, the modified CD22-binding STN epitope and the DNA-mimicking DWEYSVWLSN peptide.

The protein content of chimera solution is determined spectrophotometrically at 280 nm. The purity of the constructed chimera is analyzed by SDS-PAGE under non-reducing conditions.

The ability of the trispecific immunoglobulin molecule according to the invention to engage the CD22 and FcγRIIb inhibitory receptors and to suppress selectively pathological DNA-specific B lymphocytes with IgM and with IgG antigen receptors is analyzed *in vivo* in animals that develop spontaneously an autoimmune disease, namely systemic lupus erythemathosus. The study showed that the trispecific immunoglobulin molecule bound the inhibitory receptors on targeted disease-associated

DNA-specific B cells and suppressed the activity of the latter. The healthy animals do not excrete proteins with the urine. Proteinuria levels correlate with the severity of renal involvement in lupus. The administration of the trispecific immunoglobulin molecule according to the invention resulted in the suppression of the IgM and IgG anti-DNA antibody levels, of proteinuria and in the increased survival time of the animals (Figures 3-5).

Brief description of the Figures

Figure 1 shows a schematic drawing of the trispecific immunoglobulin molecule, according to the invention. The DNA-mimicking and the STN epitopes are coupled to an IgG backbone.

Figure 2 represents a scheme of the constructed modified STN epitope.

Figure 3 shows the effect of the trispecific immunoglobulin molecule according to the invention on the levels of anti-DNA antibodies in treated lupus-prone MRL/lpr mice.

Figure 4 shows the effect of trispecific immunoglobulin molecule, according to the invention on albuminuria levels in treated MRL/lpr mice (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ relative to controls, unpaired t -test).

Figure 5 proves that the administration of the trispecific immunoglobulin molecule, according to the invention increases survival in female lupus-prone MLR/lpr mice (* $p < 0.05$ relative to controls, unpaired t -test).

The following **Examples** are set out without limiting the scope and the spirit of the invention.

Example 1. Construction of the modified peptides.

An oligosaccharide STN epitope with a free terminal sialic acid (purchased from Calbiochem, Darmstadt, Germany) was coupled to the N-end of a synthetic GGPGG peptide (SEQ ID No. 2). A hexamethylene group was added to the C-end of the same peptide. Synthesis was performed using the

Fmoc method [Chan, W. et al. (2000) *Fmoc Solid Phase Peptide Synthesis A Practical Approach*, Oxford University Press].

A decapeptide DWEYSVWLSN (SEQ ID NO. 1), that mimics antigenically DNA, was also coupled to the IgG backbone. A hexamethylene group was also added to the C-end of this peptide.

Example 2. Construction of the chimeric immunoglobulin molecule.

Both modified peptides are purified by the HPLC technology [Mehod, A.R. et al. (2002) *J. Chrom.A* 972,87] and then coupled to the mouse monoclonal IgG using 1-ethyl-3(3'-dimethylaminoprpyl) carboimidine.HCl (ECL) [Bauminger, S. et al. (1980) *Methods in Enzymology*. 70,151]. The components for the construction of the trispecific immunoglobulin molecule are prepared as follows. A 1.5 mg/ml solution of the monoclonal IgG in phosphate buffer pH 6.0, 0.3 mg/ml solution of EDC in PBS pH 6.0 and 0.2 mg/ml solutions of both peptides in dimethylphormamide/ phosphate (ratio of 1:9) buffer are made. 7.5 ml of the mixed peptide solution is added to 3.75 ml EDC and to 10 ml of the IgG solution, the volume is brought to 150 ml with phosphate buffered saline pH 6.0. The mixture is incubated for 16 hours at +4 °C with constant stirring. After that it is dialyzed at +4°C against 200 volumes of PBS pH 7.0 for 16 hours and concentrated to a final volume of 20 ml using ultrafiltration through a 5 kD-pore membrane (*Amicon, Millipore Corp.*). This preparation contains the trispecific immunoglobulin molecules, composed of the monoclonal IgG backbone, the modified CD22-binding STN epitope and the DNA-mimicking DWEYSVWLSN peptide. (Figure 1).

The protein content of the solution is determined spectrometrically at 280 nm. The purity of the constructed chimeric antibody is determined by SDS-PAGE under non-reducing conditions.

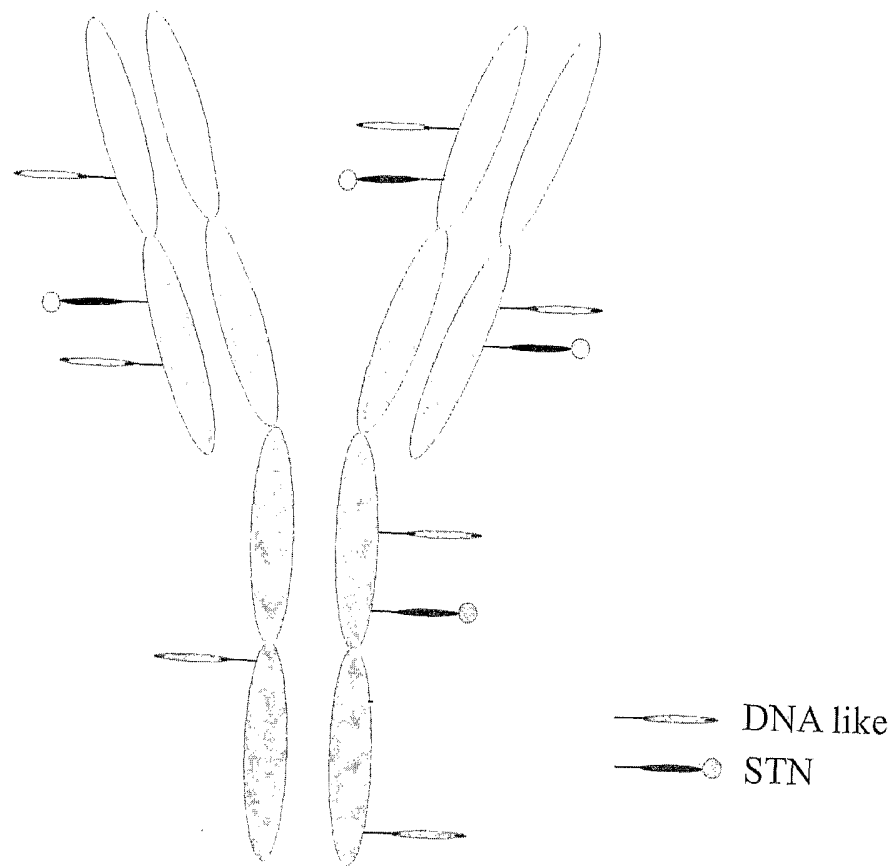
Example 3. Determination of the effect of the administration of the chimeric antibody in autoimmune animals, in particular in lupus-prone mice.

The constructed trispecific immunoglobulin molecule has been administered intravenously twice weekly (20 ug/dose) to groups of ten 7- and of 18-weeks old female MRL/lpr mice. Control group have been injected with the same amount of a similar modified antibody molecule that lacked the STN epitope and another control groups have been treated with PBS alone. It is known that female mice from this strain spontaneously develop at the age of 7-9 weeks a lupus-like disease accompanied by the appearance of high levels of disease-associated anti-double stranded DNA antibodies. These levels have been significantly reduced in the group treated with the trispecific immunoglobulin molecule, according to the invention (Figure 3).

The lupus glomerulonephritis results in kidney failure that is a major cause for the death of the animals. The quantity of proteins in the urine, a measure of the severity of kidney disease, has been determined by using a standard dry strip test (from Bayer, UK). The administration of the trispecific immunoglobulin molecule according to the invention, delays the increase of albuminuria that is seen in the control animals (Figure 4). The treated mice survive significantly longer than the control ones (Figure 5).

CLAIMS

1. Suppressor of disease- associated autoreactive B lymphocytes with affinity to inhibitory B-lymphocyte receptors, characterized in that three different ligands are incorporated into one single molecule manifesting affinity to one activating and to two inhibiting B cell receptors.
2. Suppressor according to Claim 1 characterized in that it is constructed of an immunoglobulin G backbone and two synthetic components coupled to it.
3. Suppressor according to Claims 1 and 2 characterized in that the first of the three ligands is a part of the immunoglobulin G backbone and the second and third ligands are the two synthetic components.
4. Suppressor according to Claims 1, 2 and 2 characterized in that the first of the synthetic components is a peptide epitope with affinity to the activating immunoglobulin receptors on targeted autoreactive B cells.
5. Suppressor according to Claims 1 and 2 characterized in that the second of the synthetic components has affinity to the inhibiting B cell receptor CD22.
6. Suppressor according to Claims 1, 2 and 3 characterized in that the immunoglobulin G backbone has affinity to the inhibiting B cell receptor Fc gamma RIIB.
7. Suppressor according to Claims 1, 2, 3 and 5 characterized in that the second synthetic component is composed of the peptide GGPGG (SEQ ID NO. 2) with a hexamethylene group at its C-end and a STN epitope with a free terminal 2,6 sialic acid at its N-end.
8. Use of the suppressor according to Claims 1-7 in the manufacture of a medicament for the treatment of disorders caused by disease-associated autoreactive B lymphocytes.

**Figure 1**

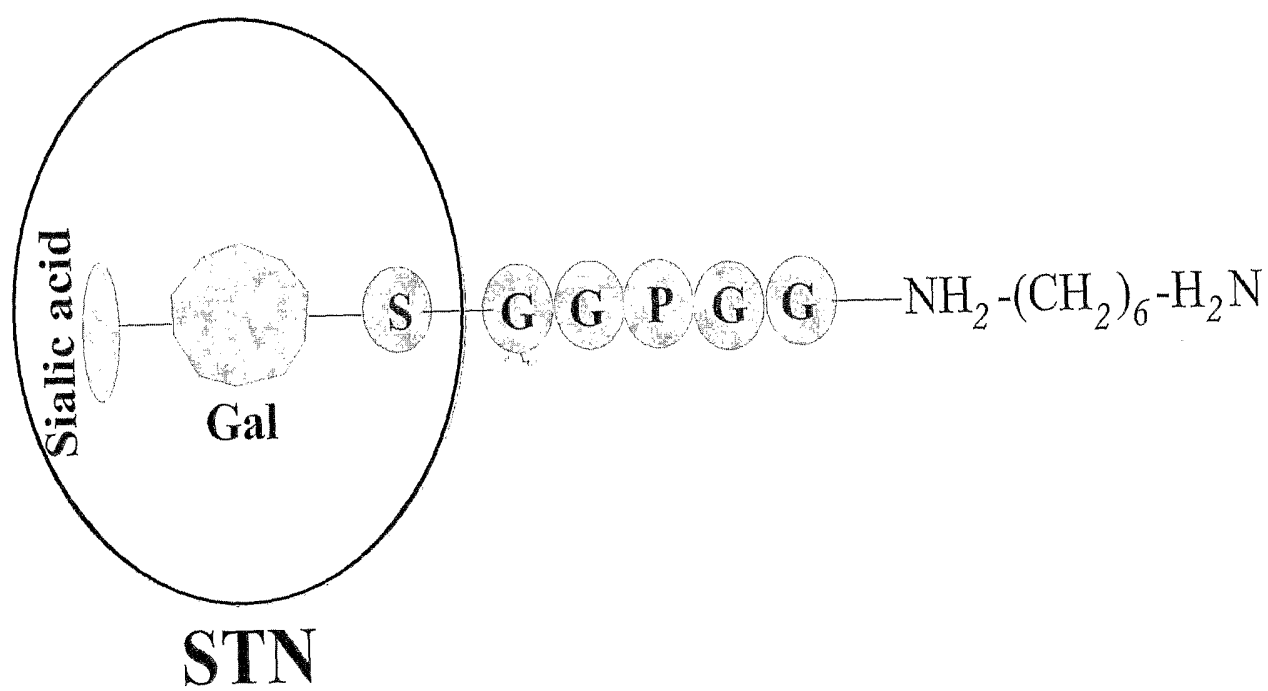


Figure 2

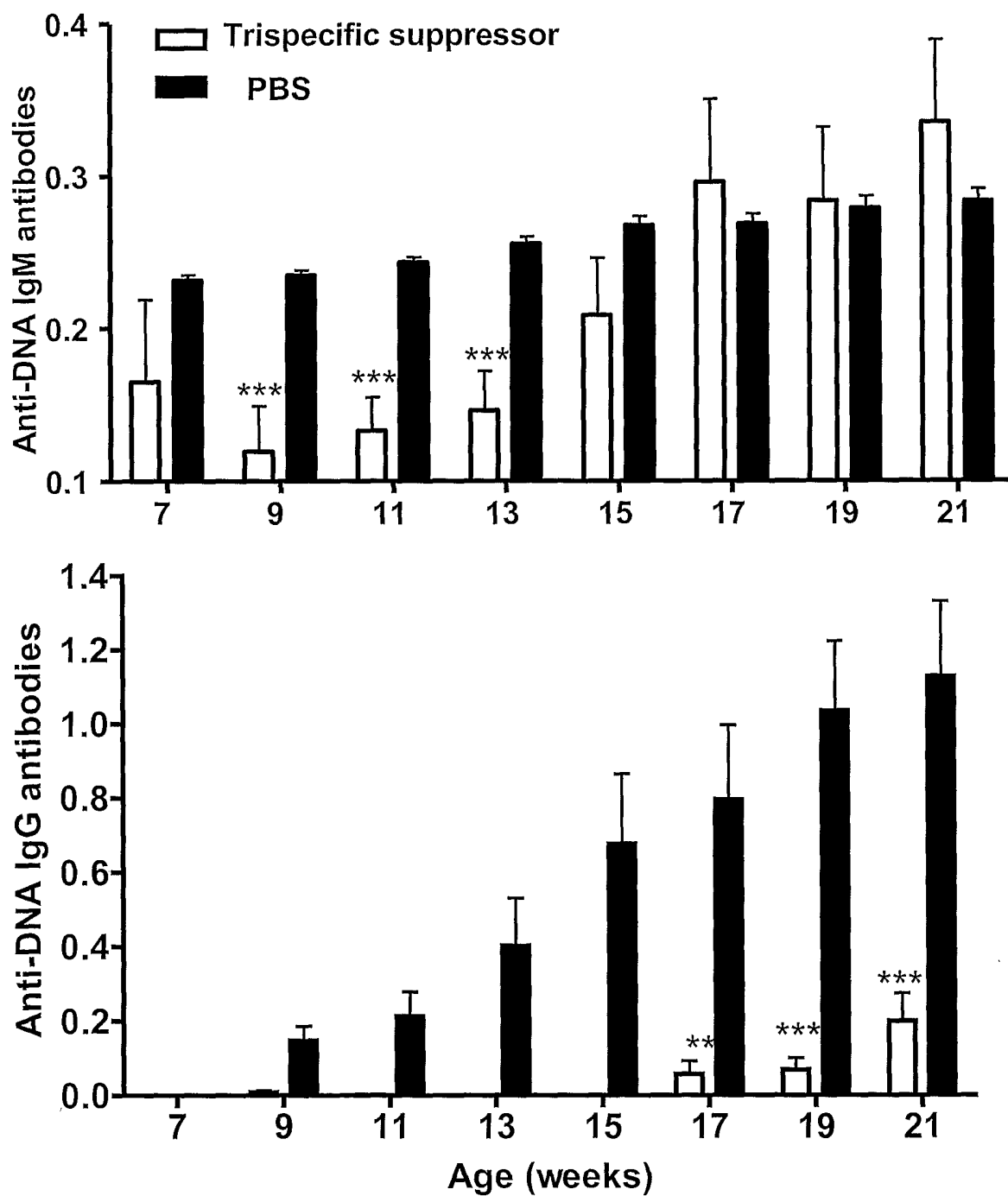


Figure 3

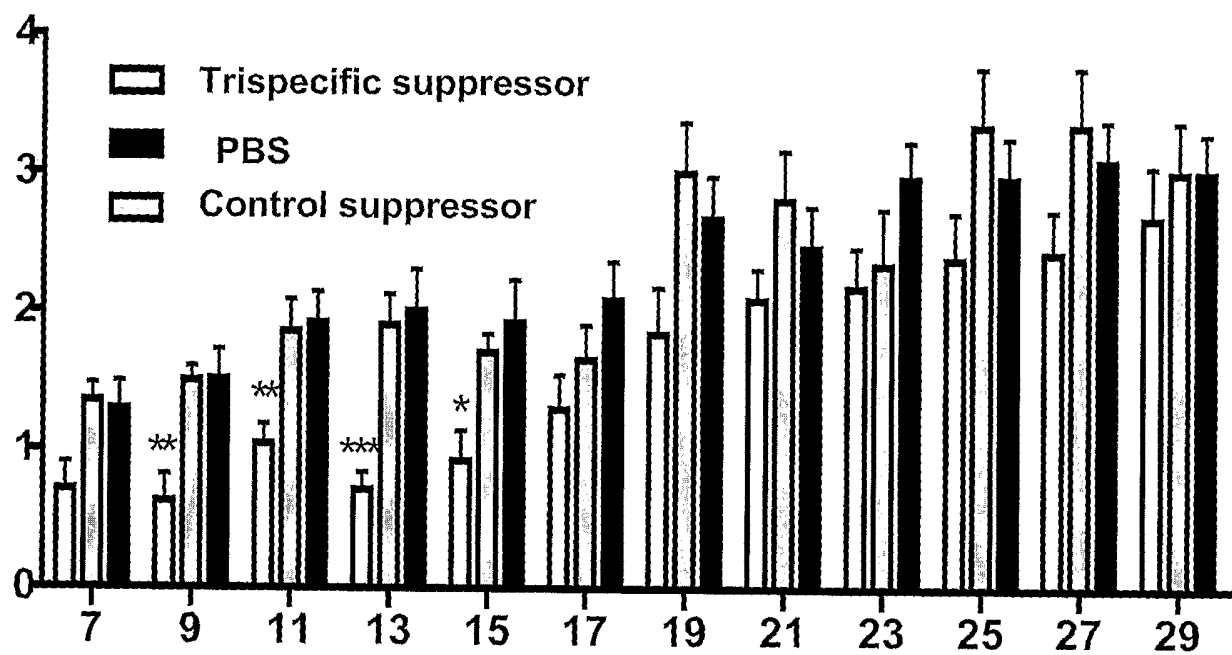


Figure 4

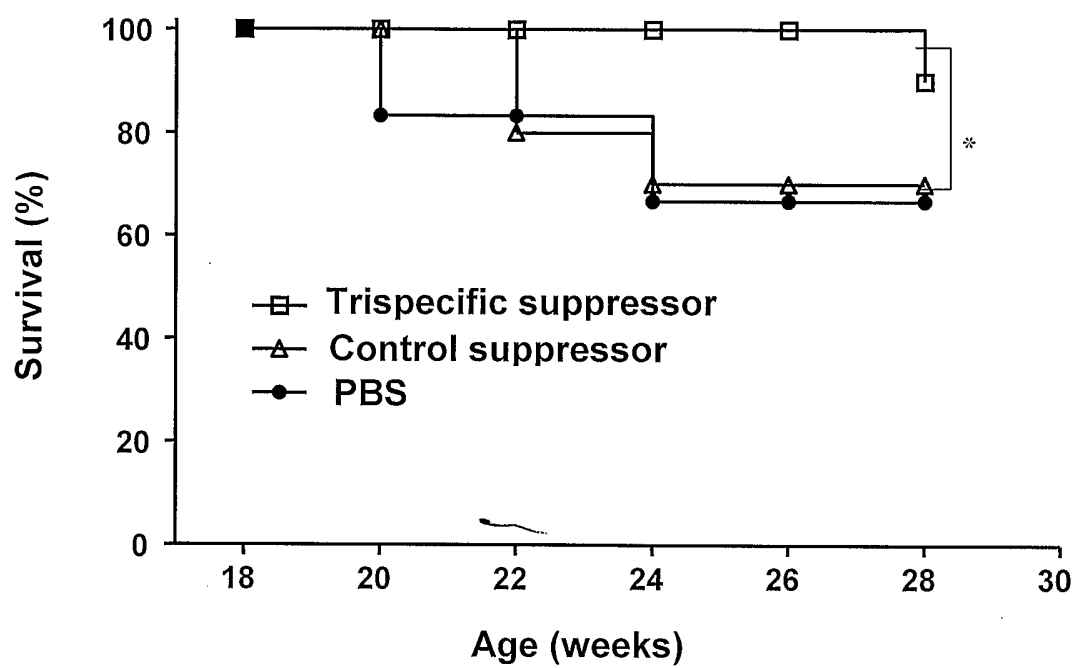


Figure 5

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