Anti-tumor peptide combining patterns of insect and mammalian immunologically relevant proteins.

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Abstract

Poor recognition of tumor cells is a true impediment in cancer immunology and immunotherapy. In this paper we describe the structure and the anti-tumor activity of a hybrid peptide combining patterns present in five classes of immunologically relevant molecules: the peptide-binding site of the immunoglobulin CDR3 region, the cystein-rich domain of macrophage scavenger receptors, the substrate binding site of transmembrane serine proteases, the octapeptide repeat region of prion proteins and alloferon, the peptide isolated from insects and capable of upregulating mammalian natural killer cell cytotoxicity towards tumor cells. The hybrid peptide referred to here as allostatin-1 was chemically synthesized and its anti-tumor activity was tested in DBA/2 mice grafted with syngenic P388D1 mouse lymphoma cells. Preventative vaccination with x-ray inactivated P388D1 cells caused tumoristatic effect in 27% of recipients however all the animals eventually developed tumors. Vaccination followed by allostatin-1 administration was tumoristatic in 65% and completely cured cancerous growth in 30% so that the total number of recipients benefitting from the treatment grew to 95%. Allostatin-1 demonstrated superior anti-tumor activity both in nonvaccinated and vaccinated animals as compared to the naturally occurring analog, alloferon. Sequence similarity analysis revealed that allostatin-like patterns are evolutionary conserved and widespread in the peptide-binding regions of mammalian proteins involved in non self recognition and cell-to-cell interaction. Thus, allostatin-1 represents a novel kind of adjuvant assisting the immune system in recognition and elimination of tumor cells and potentially applicable in cancer immunotherapy.

Keywords: anti-tumor immunity, tumor cell recognition, immunomodulatory peptides, adjuvants, alloferons, immunoglobulins, scavenger receptors, serine proteases, prion proteins

Introduction

A great number of immunologicals such as anti-tumor vaccines, monoclonal antibodies and immune response modifiers have been developed for the purpose of active and passive cancer immunotherapy. However, poor immunogenicity, great variability and immunosuppressive abilities of tumor cells considerably limit the immunotherapy efficacy (1, 2). Hunting for an immunological "magic bullet" assisting the immune system to recognize and attack cancerous growth remains one of most relevant directions in the field. Following this direction, we formerly isolated from an insect species, maggots of dipteran *Calliphora vicina,* a novel family of antiviral and anti-tumor peptides named alloferons (3). Natural killer (NK) lymphocytes were identified as the peptides pharmacological target in the mammalian immune system responding to alloferon-1 with immediate growth of cytotoxic activity (3-5). Other kinds of pharmacological activity like induction of interferon synthesis (3), suppression of virus proliferation (6-8), deblocking of NF-kB mediated signaling pathway (9), modification of cytokine production (10), and adjuvant activity in combination with cancer chemotherapy (11) have been published. Alloferon-1 antiviral efficacy correlated with enhancement of NK cell cytotoxicity was clinically proven in the treatment of herpes simplex (4) and human papilloma virus (12) infections.

Alloferons' physiological role in the host organism is not yet known however it is noticeable that cytotoxic hemocytes functionally similar to mammalian NK cells represent a significant portion of the maggot blood cells (13). From that standpoint alloferons look like an evolutionary conservative family of NK cell regulatory peptides active both in insects and mammals.

In this paper we have used alloferon-1 primary structure as a platform for design of a novel molecule demonstrating better anti-tumor activity and potentially applicable in the field of cancer immunotherapy. In the beginning we tried to identify alloferon-1 structural analogs among mammalian immunologically relevant peptides and proteins. No close analogs were found while substitution of two amino acids in the alloferon-1 sequence revealed similar patterns incorporated into human and mouse immunoglobulins, macrophage scavenger receptors, serine proteases and prion proteins playing key roles in innate and acquired anti-tumor immunity. The "humanized" peptide referred here to as allostatin-1 was chemically synthesized and tested in a mouse tumor transplantation model in order to characterize its anti-tumor activity in the regimen of monotherapy and in combination with a tumor antigen vaccination.

Results

Allostatin-1 structural analogs. Sequences of allostatin-1 analogs pertaining to human and mouse immune systems are listed in Table 1. Unlike alloferon-1, hydrophilic positively charged histidine in position 6 is substituted in allosatin-1 with hydrophobic tryptophan and hydrophobic valine in

position 11 with polar uncharged threonine. These two replacements revealed a series of analogous sequences present in different classes of mammalian immunologically relevant proteins as follows:

- Immunoglobulins; the heavy chain variable region contains a uniform 6 (mice) to 7 (human) amino acid motif homologous to the corresponding allostatin-1 fragment except histidine in position 9 is omitted in immunoglobulins. The motif belongs to the hypervariable region CDR3 determining antibody specificity. Nevertheless, it remains highly conserved throughout human and mouse immunoglobulins.
- Scavenger receptors; extracellular cystein-rich domains of human and mouse class B scavenger receptors contain an invariable sequence identical to the fragment 5-9 of allostatin-1 including histidine "missed" in the immunoglobulin CDR3.
- Transmembrane serine proteases; the substrate binding site of human and mouse transmembrane serine proteases holds an amino acid motif identical to the fragment 3-7 of allostatin-1.
- Extracellular serine proteases; some specialized serine proteases like coagulation factor VII and haptoglobin contain a sequence identical to the fragment 3-8 of allostatin-1.

Anti-tumor activity. Allostatin-1 and alloferon-1 anti-tumor activity was analyzed in naïve (non-vaccinated) animals (Fig. 1a) and animals preventively vaccinated with x-ray killed tumor cells (Fig. 1b). All control animals developed detectable carcinoma-like tumors *in situ* during the 20 days after inoculation with tumor cells. Allostatin-1 and alloferon-1 caused delay of tumor appearance in naïve animals as compared to the control group (Fig. 1a). The delay was more evident in the case of allostatin-1 treatment where the number of tumor-free animals significantly exceeded the control level at early stage of tumor appearance (days 15-20). However, the majority of treated animals finally developed tumors. Consideration must be given to the fact that the cancer proliferation rate in the model we have used was very fast and acquired immune response in these circumstances may not have enough time to contribute essentially in anti-tumor defense.

Results were different in preventively vaccinated mice (Fig. 1b). Vaccination-only treatment had a short-term tumoristatic effect on some of treated individuals. Allostatin-1 administration significantly suppressed tumor growth in most vaccinated animals compared to vaccination-only. One third of this group did not develop tumor over the whole checking period and seems to be completely cured. Alloferon-1 also increased vaccination efficacy, though the effect was significantly weaker compared to allostatin-1.

Detailed characteristics of cancerous growth in tumor bearing animals are summarized in Table 2. All the treatments significantly extended tumor latency period (interval between tumor cell inoculation and detectable tumor appearance) and reduced the volume of the first detected tumor as compared to the control group. The longest latency period was found in vaccinated animals treated with allostatin-

1 versus vaccination-only ($P<0.001$), allostatin-1 alone ($P<0.01$), alloferon-1 alone ($P<0.001$) and vaccination coupled with alloferon-1 treatment ($P<0.05$). Allostatin-1 treatment of naïve animals significantly extended the latency period over alloferon-1 as well ($P = 0.015$). Tumor growth rates demonstrate similar trends in the early stages of tumor development. All the treatments caused significant growth suppression in comparison with untreated control groups. Vaccination coupled with allostatin-1 treatment prevailed over vaccination-only ($P= 0.001$), allostatin-1 ($P< 0.05$) and alloferon-1 ($P< 0.001$) monotherapies. Allostatin-1 advantage over alloferon-1 in nonvaccinated individuals was also significant ($P< 0.01$). None of the treatments were effective in the advanced stages of cancerous growth.

Figure 2 illustrates the comparative anti-tumor efficacy of the treatments. The total number of positive responders benefiting from tumoricidal (complete tumor elimination) or tumoristatic (delay in detectable tumor appearance) effects significantly exceeded the control level in all groups except alloferon-1 treated naïve animals. The maximum overall efficacy was established in vaccinated individuals treated with allostatin-1. Total anti-tumor activity here significantly exceeded vaccination-only, allostatin-1 and alloferon-1 monotherapies as well as the vaccination combined with alloferon-1 treatment.

Discussion

Experiments with the mouse syngenic tumor transplantation model demonstrated potent allostatin-1 anti-tumor activity exceeding that of parental naturally occurring molecule, alloferon-1. The activity growth is correlated with two amino acid replacements in the alloferon-1 primary structure that make the resultant peptide similar to functionally relevant patterns of mammalian immunoglobulins, scavenger receptors and serine proteases referenced in Table 1. The allostatin-like pattern of the immunoglobulins belongs to CDR3, the region determining antibody (and B-cell receptor) complementarity to a variety of antigens. Despite the fact that CDR3 is the most variable part of immunoglobulins, the pattern is immutable within and between human and mouse genomes and therefore seems to show an important physiological function different from specific antigen complementarity and conserved throughout immunoglobulin evolution in mammals. This function may include binding of various peptidic structures. The fact that the allostatin-like pattern of all referenced in Table 1 immunoglobulins contains tryptophan-glycine site known to have peptide-binding activity supports such a possibility. Furthermore, the allostatin-like pattern of macrophage scavenger receptors belongs to the receptors' cystein-rich domain binding a variety of proteins located on the surface of microbial and apoptotic host cells (14). Another allostatin-like pattern is incorporated into the substrate binding site of serine proteases directly involved in protein-protein interaction. It is noteworthy that serine proteases, especially the transmembrane forms, implement

multiple functions in immune system regulation based on the binding of various signaling and effector proteins (15).

Prions are one more group of mammalian proteins containing allostatin-like patterns. Thus, human PrP gbAAB50777.1 comprises in its octapeptide repeat region ten copies of GWG triplet omnipresent in mammalian allostatin-1 analogs. A growing number of facts shows what an important place the cellular PrP isoform (PrP^c) holds in immune response regulation (16, 17). PrP^c has been detected on the surface of human T and B lymphocytes, NK cells, platelets, monocytes and dendritic cells. Although PrP^c immunoregulatory functions are not well understood, it seems to play a part in the formation of immune synapses (16), a process where reversible peptide binding capacity is indispensable.

Allostatin-1 intramolecular structure may be sketched out as a series of overlapping patterns pertaining to the mammalian immunologically relevant proteins and incorporated into an alloferon-like sequence (Table 3). Each pattern invariably contains GWG triplet and amino acid sequences specific to a particular class of the proteins: SQGT in human immunoglobulins, QH in scavenger receptors or VS in serine proteases. The patterns as a whole are evolutionary conservative and widespread in all proteins of the class. For example, any human and mouse immunoglobulin bears the GWGQGT pattern in its antigen binding CDR3 domain.

Overlapping of the patterns notably distinguishes allostatin-1 from known synthetic hybrid peptides constructed of sequentially linked parental sequences and having a, correspondingly, larger size and less predictable shape of resultant molecule. From the pharmaceutical standpoint, a smaller size molecule carrying the same or greater amount of biologically sensible information prerequisites potential advantages like accessibility of the pattern to its target, better physical stability and pharmacokinetic profile, reduced production cost etc.

Allostatin-1, taking into consideration its structural and functional peculiarities described in this paper, represents a novel chemical and biological entity simulating evolutionary conserved patterns of animal immune system and potentially applicable for cancer immunotherapy. Particularly, it may be used in combination with anti-tumor vaccines or independently as an adjuvant advising the immune cells to recognize poorly immunogenic tumor antigens. Currently available adjuvants were mainly developed for the purpose of anti-infective vaccination and are often not sufficiently effective as additives to cancer vaccines (18).

Materials and methods

1. Animals

Two-month old DBA/2 female mice with a body mass of 20-22 g were obtained from Rappolovo laboratory animal breeding nursery, St.Petersburg.

2. Tumor cells

Mouse lymphoma P388D1 cell line syngenic to DBA/2 mice was obtained from the Institute of Cytology cell culture center, St.Petersburg and used for transplantation or tumor antigen preparation.

3. Vaccination

P388D1 cells were inactivated by x-ray irradiation in a 15 000 rad dose and used as corpuscular tumor antigen. The antigen in a dose of 3 000 cells was inoculated twice into groin lymphatic nodes on days 1 (primary vaccination) and 13 (boosting vaccination) of the experiment.

4. Tumors cells transplantation

P388D1 tumor cells suspended in 200 µl of Hepes solution were inoculated subcutaneously in the mouse's spinal region 10 days after the boosting vaccination.

5. Anti-tumor activity assay

Allostatin-1 and alloferon-1 anti-tumor activity was assayed using DBA/2 mice grafted with 3000 P388D1 tumor cells per animal according to the protocol described formerly (11). The cells, unless rejected, form carcinoma-like solid tumor at the site of inoculation. Tumor *in situ* appearance and linear size measured as average of shortest and longest tumor diameter was monitored twice a week for 60 days after transplantation. Tumors in the untreated control animals became palpable within 20 days after transplantation and reached up to 3 cm in size in the next month. The 60 day checking period allows a conclusion to be drawn about transplanted tumor survival or elimination. In the latter case the anti-tumor effect was determined as tumoricidal. The effect was characterized as tumoristatic if a tumor appeared later than 20 days after transplantation when 100% of control animals developed palpable tumors. All animal experiments were approved by the author's institutional review board at the Institute of Cytology.

6. Peptides

Allostatin-1 is a linear peptide consisting of a 13 amino acid sequence His-Gly-Val-Ser-Gly-Trp-Gly-Gln-His-Gly-Thr-His-Gly with empiric formula $C_{56}H_{77}N_{21}O_{17}$ and molecular mass 1316 Da. The peptide in the form of acetic acid salt was synthesized by Diapharm Co, St.Petersburg by solid phase synthesis using Fmoc/But strategy and purified by reverse phase HPLC. Final purity of the peptide measured by HPLC was over 98%.

Alloferon-1 in the form of acetic acid salt was synthesized by Peptide synthesis ltd, Moscow as described (19). The peptide purity measured by HPLC was over 98%. Anti-tumor activity of the same product was previously tested using the DBA/2 mice P388D1 tumor transplantation model (11).

7. Peptides administration

Allostatin-1 and alloferon-1 in a single 25 µg dose diluted in 200 µl of Hepes solution were injected intraperitoneally three times: days 1, 13 and 22 of the experiment. The injections were synchronized

with primary vaccination, boosting vaccination and tumor transplantation, correspondingly. Control animals received an equal volume of the solvent.

8. Statistics and computation

Experimental data were summarized by descriptive statistics (mean and standard error of the mean for continued variables; frequency and percentage for categorical variables). Statistical analyses were performed using analysis of variances (F test) for continued variables and z test for categorical variables. A peptide sequence similarity study was performed using the SIB BLAST network service, NCBI BLAST software resources and UniProtKB protein knowledgebase.

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Figure legends

Figure 1. Progression of transplanted P388D1 tumor *in situ* appearance in naïve (A) and vaccinated (B) DBA2 mice treated with allostatin-1 or alloferon-1.

(A) Naïve animals. Allostatin-1 and alloferon-1 in a single 25 μg dose diluted in 200 μl of Hepes solution were injected intraperitoneally into DBA2 mice three times: days 1, 13 and 22 of the experiment. Control animals received an equal volume of the solvent. Syngenic P388D1 murine lymphoma cells suspended in 200 μl of Hepes solution were inoculated subcutaneously in the mouse's spinal region in a dose 3000 cells per animal at day 22 of the experiment. Tumor *in situ* appearance and linear size measured as an average of shortest and longest tumor diameter was monitored twice a week for 60 days after transplantation. Allostatin-1 and alloferon-1 caused more evident (z test) delay of tumor appearance in the case of allostatin-1 treatment where the number of tumor-free animals significantly exceeded the control level at days 15 ($P< 0.001$) and 20 ($P< 0.01$).

(B) Vaccinated animals. DBA2 mice were injected with the peptides or a solvent in the same way as the naïve animals. Simultaneously with the preparations' injections they were subcutaneously vaccinated with irradiated P388D1 cells at days 1 and 13 of the experiment and grafted at day 22 with viable P388D1 tumor cells identically to the naïve animals. Allostatin-1 administration significantly (z test) suppressed tumor growth in most vaccinated animals compared to vaccination-only ($P< 0.001$ days 15-25, < 0.01 days 30-35, <0.05 days 40-60). Alloferon-1 effect was significantly weaker compared to allostatin-1 ($P<0.01$ day 20, <0.05 day 35).

Figure 2. Comparative anti-tumor activity of P388D1 tumor vaccine, allostatin-1, alloferon-1 and their combination.

Tumoricidal activity is determined according to a number of tumor-free animals 2 months after tumor cells transplantation; tumoristatic activity corresponds to a number of animals developed detectable tumor later than in 20 days post transplantation. Total anti-tumor efficacy characterizing portion of animals benefited from the treatment is a sum of tumoricidal and tumoristatic effects. The total number of positive responders benefiting from tumoricidal (complete tumor elimination) or tumoristatic (delay in detectable tumor appearance) effects significantly exceeded the control level in all groups except alloferon-1 treated naïve animals. Total anti-tumor activity in vaccinated individuals treated with allostatin-1 significantly exceeded (z test) vaccination-only ($P< 0.001$), allostatin-1 ($P< 0.001$) and alloferon-1 ($P< 0.001$) monotherapies as well as vaccination combined with alloferon-1 treatment ($P= 0.002$).

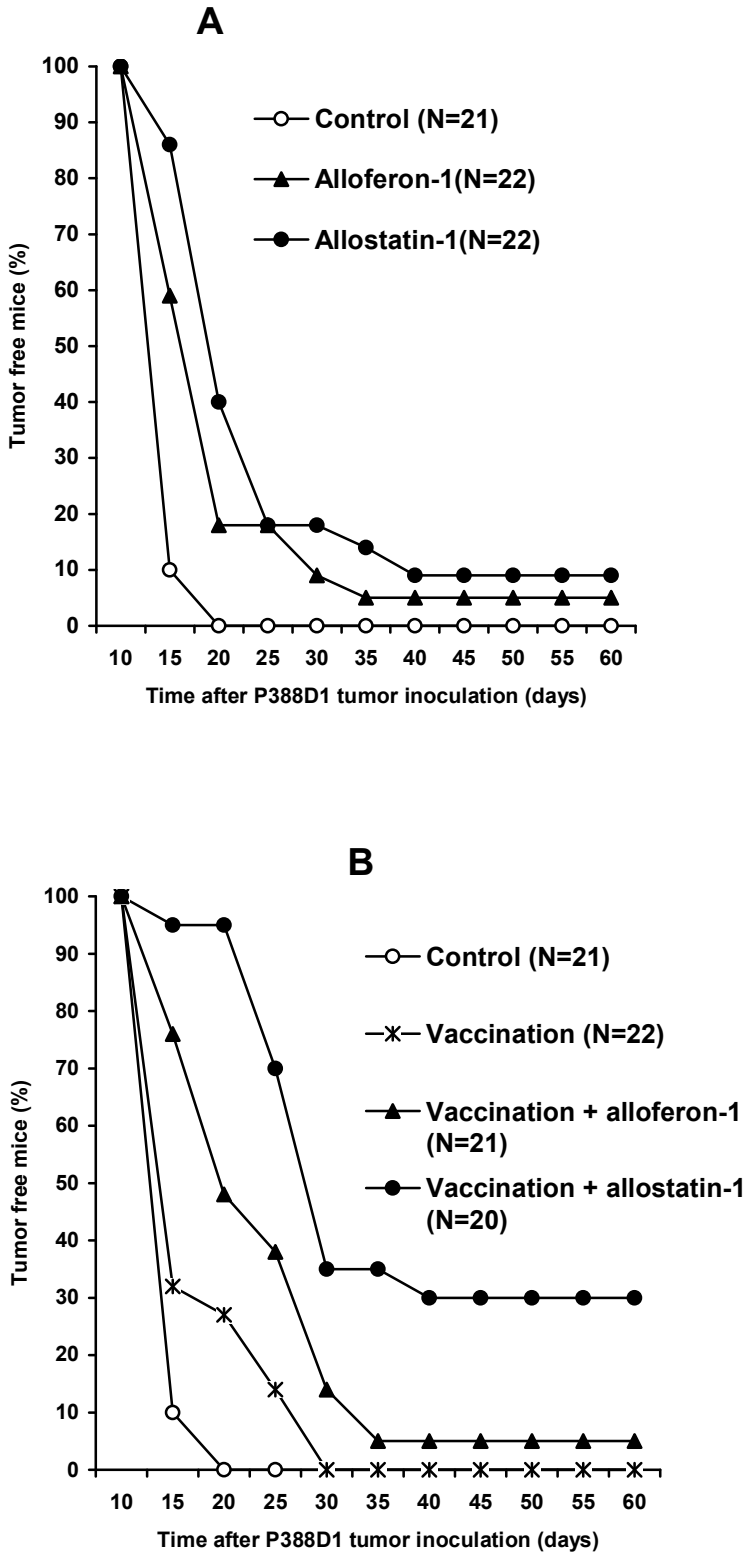
A
Control (N=21)
Alloferon-1(N=22)
Allostatin-1(N=22)
Tumor free mice (%)
Time after P388D1 tumor inoculation (days)
B
Control (N=21)
Vaccination (N=22)
Vaccination + alloferon-1 (N=21)
Vaccination + allostatin-1 (N=20)
Tumor free mice (%)
Time after P388D1 tumor inoculation (days)

Fig 1.

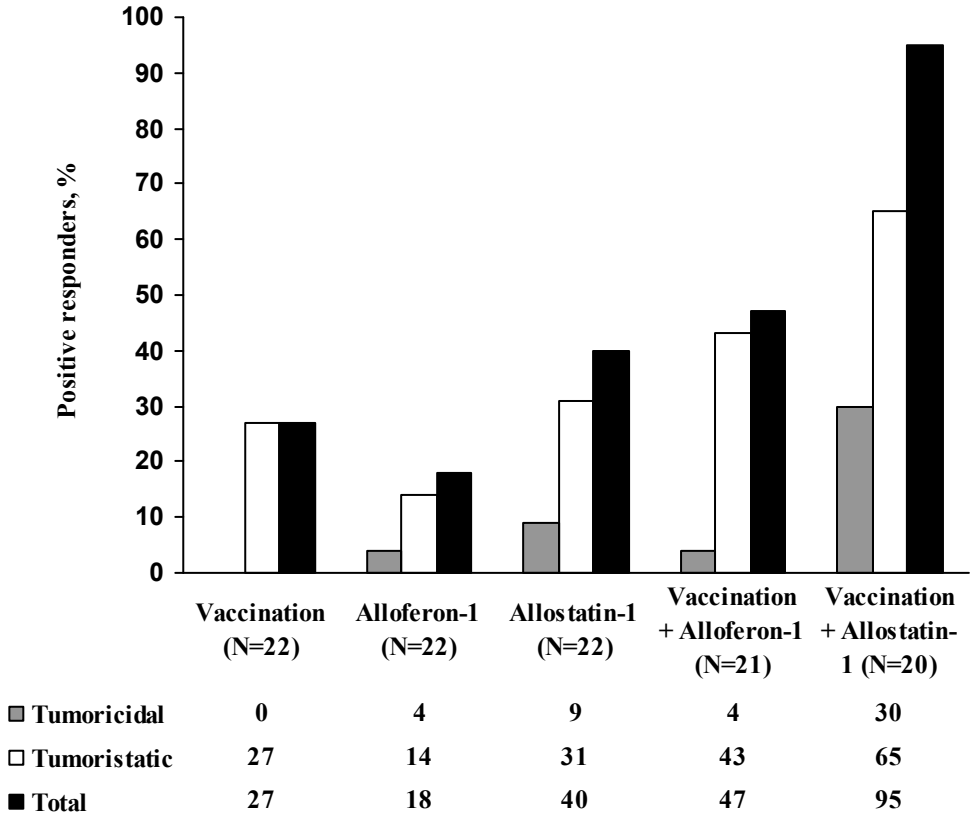
Positive responders, %
100
90
80
70
60
50
40
30
20
10
0
Vaccination (N=22)
Alloferon-1 (N=22)
Allostatin-1 (N=22)
Vaccination + Alloferon-1 (N=21)
Vaccination + Allostatin-1 (N=20)
Tumoricidal 0 4 9 4 30
Tumoristatic 27 14 31 43 65
Total 27 18 40 47 95

Fig 2.

Table 1. Allostatin-1 analogs from insect and mammalian immune systems

Peptide/Accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	Identity, %
Allostatin-1	H	G	V	S	**G**	**W**	**G**	Q	H	G	T	H	G	
Insect alloferons														
Calliphora vicina P83412.1 (Alloferon-1)	H	G	V	S	G	H	G	Q	H	G	V	H	G	85
Immunoglobulin heavy chain variable region														
Mouse gb|ABV01604.1| f 10-15; gb|AAX90105.1| f 13=18; gb|ABB46044.1| f 15-20; gb|AAA38086.1| f 76-81; gb|AEQ62373.1| f 94-99; emb|CAB64123.1| f 98-103					**G**	**W**	**G**	Q	-	G	T			86
Human gb|AAT02043.1| f 94-100; gb|AAZ08856.1| f 107-113; gb|AEX29562.1| f 109-115; dbj|BAI52147.1| f 119-125; gb|AAC18216.1| f 131-137; gb|AAB41737.1| f 134-140; gb|AAF15590.1| f 104-110				S	**G**	**W**	**G**	Q	-	G	T			88
Scavenger receptor extracellular cystein-rich domain														
Mouse |NP_001153838.1| f438-442 **Human** gb|AAH15651.1| f 36-40; emb|CAC84764.1| f 427-431; sp|Q9NR16.2| f 1119-1123					**G**	**W**	**G**	Q	H					100
Transmembrane serine proteases														
Mouse |NP_109634.2| f 334-346; |NP_032581.2| f 591-595 **Human** gb|AAI21803.1| f 300-305; gb|EAW67226.1| f 85-89			V	S	**G**	**W**	**G**							100
Extracellular serine proteases														
Haptoglobin **Mouse** |NP_059066.1| f 222-226 **Human** emb|CAA25927.1| f 223-228			V	S	**G**	**W**	**G**							100
Coagulation factor VII **Mouse** p70375 f 322-327 **Human** gb|ABN79862.1| f 148-153			V	S	**G**	**W**	**G**	**Q**						100

Table 2.Tumor growth characteristics in DBA/2 mice inoculated with P388D1 mouse lymphoma cells

Treatment	N_0	Tumor bearing animals		Tumor latency period, days	Size of first detected tumor		Tumor growth rate, mm/day	
		N	%		Diameter, mm	Volume, mm^3	Early stage[1]	Advanced stage[2]
Control	21	21	100	15.57 ± 0.39	12.76 ± 0.60	815	0.83 ± 0.046	1.30 ± 0.104
Vaccination	22	21	100	18.55 ± 1.18***	9.36 ± 0.61***	322	0.54 ± 0.046***	1.48 ± 0.088
Alloferon-1	22	21	95	17.38 ± 0.73***	9.33 ± 0.54***	319	0.56 ± 0.030***	1.35 ± 0.064
Allostatin-1	22	20	91	21.45 ± 1.44***	8.67 ± 0.48***	256	0.43 ± 0.034***	1.34 ± 0.069
Vaccination + Alloferon-1	21	20	95	22.65 ± 1.44***	8.50 ± 0.43***	241	0.38 ± 0.016***	1.50 ± 0.097
Vaccination + Allostatin-1	20	14	70*	27.36 ± 1.40***	8.79 ± 0.64***	267	0.33 ± 0.026***	1.60 ± 0.193

Significance compared to the control: *P<0.05 **P<0.01, ***P<0.001

[1] From tumor cell transplantation to detectable tumor appearance

[2] From detectable tumor appearance to the endpoint

Table 3. Allostatin-1 sequence embraces overlapping fragments present in mammalian immunologically relevant proteins

Allostatin 1	1 **H**	2 **G**	3 **V**	4 **S**	5 **G**	6 **W**	7 **G**	8 **Q**	9 **H**	10 **G**	11 **T**	12 **H**	13 **G**	Identity %
F_{Ig} - immunoglobulin type fragment				←	–	–	–	–	–	–	→			88
F_{sr} – scavenger receptor type fragment					←	–	–	–	→					100
F_{sp} – serine protease type fragment			←	–	–	–	→							100