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Research Article **Immunology**

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VSIG4, a B7 family–related protein, is a negative regulator of T cell activation

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T cell activation by APCs is positively and negatively regulated by members of the B7 family. We have identified a previously unknown function for B7 family–related protein V-set and Ig domain–containing 4 (VSIG4). In vitro experiments using VSIG4-Ig fusion molecules showed that VSIG4 is a strong negative regulator of murine and human T cell proliferation and IL-2 production. Administration to mice of soluble VSIG4-Ig fusion molecules reduced the induction of T cell responses in vivo and inhibited the production of Th cell–dependent IgG responses. Unlike that of B7 family members, surface expression of VSIG4 was restricted to resting tissue macrophages and absent upon activation by LPS or in autoimmune inflammatory foci. The specific expression of VSIG4 on resting macrophages in tissue suggests that this inhibitory ligand may be important for the maintenance of T cell unresponsiveness in healthy tissues.

Introduction

T cell responses are regulated by a complex network of activating and inhibitory signals. Recognition of peptides presented by MHC molecules is usually not sufficient for full T cell activation, but additional signals from costimulatory molecules are required (1–4). The most prominent costimulatory molecule expressed on T cells is CD28, interacting with the B7 family members CD80 and CD86 (5, 6). Engagement of CD28 facilitates T cell activation by enhancing TCR-mediated signaling and reducing the number of TCRs that need to be engaged for activation (7, 8). CTLA-4, a close homolog of CD28, also engages CD80 and CD86 (5, 6). Yet it serves a completely different function, since it reduces rather than enhances T cell responses.

Novel members of the CD28/B7 families have been identified recently. ICOS, engaging ICOSL (9, 10), has a function homologous to that of CD28 and generally enhances T cell responses; under some conditions, ICOS stimulation appears to selectively favor induction of Th2 cells (11, 12). Moreover, ICOS has been shown to mediate CD28-independent antiviral responses (13, 14) and to enhance antibody responses and germinal center formation (15, 16). Another new member of the family is the inhibitory receptor programmed death 1 (PD-1), which interacts with PD-ligand 1 (PD-L1) (B7-H1) and PD-L2 (B7-DC) (17–21). PD-1 has a function similar to that of CTLA-4 and downmodulates T cell responses (18, 19). The same is true for BTLA, a CD28 homolog interacting with herpesvirus entry mediator on APCs (22, 23). There are 2 more B7 homologs with unknown receptors on T cells, called B7-H3 (24) and B7-H4 (B7x, B7S1) (22, 25, 26). Their function is less well established. B7-H3 is upregulated upon inflammation and has been suggested to function as both a positive and negative regulator of T cell responses (27, 28). B7-H4 is also expressed on DCs upon activation and is thought to function as a negative regulator (25).

Here we report the identification of a novel function of V-set and Ig domain–containing 4 (VSIG4). In vitro experiments showed that VSIG4 is at least as potent at inhibiting T cell responses as PD-L1. Furthermore, VSIG4 inhibited proliferation of mouse as well as human T cells. In vivo, administration of VSIG4-Ig fusion molecules was able to inhibit the induction of CTL responses as well as the development of Th cell–dependent IgG responses. Hence, VSIG4 is a potent negative regulator of T cell responses.

Results

VSIG4, a B7 family–related protein. Considering the importance of B7 family members as regulators of immune responses, we set out to screen for members of this protein family. To do so, a search using HMMR software was performed in silico on a translated expressed sequence tag (EST) database using a hidden Markov model (HMM) profile of the ectodomain of all known B7 family members. Obtained hits were further narrowed using different filters as outlined in Methods. Two of the remaining hits turned out to be the mouse clones BC025105 and NM_177789, which were almost identical and obviously derived from the same mRNA encoding the protein VSIG4. The protein sequence encoded by these cDNAs displayed about 20% identity and shared conserved amino acids with known B7 family members (Figure 1). Based on this homology, we concluded that VSIG4 was a B7 family–related protein 3. In contrast to the B7 family members, which contain 2 IgG domains, VSIG4 contains 1 complete IgV-type domain and a truncated IgC-type domain.

Further screening with the mouse sequence led to the identification of the putative human ortholog named Ig superfamily protein 39 (Z39Ig; GeneBank accession number NM_007268). The amino acid sequence of Z39Ig shows 44% identity with mouse VSIG4 (Figure 1A). Although identities in a similar range are observed between human and mouse sequences for B7 family members (Figure 1B), this degree of identity is relatively low. However, the mod-

Nonstandard abbreviations used: AFC, antibody-forming cell; HMM, hidden Markov model; PD-1, inhibitory receptor programmed death 1; PD-L1, PD-ligand 1; VLP, virus-like particle; VSIG4, V-set and Ig domain–containing 4; Z39Ig, Ig superfamily protein 39.

Conflict of interest: L. Vogt, N. Schmitz, M. Bauer, H.I. Hilton, D. Gatto, P. Sebbel, R.R. Beerli, P. Saudan, and M.F. Bachmann are employed by Cytos Biotechnology AG and own stock or stock options in the company.

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A

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mVSIG4    1 meissgllflghlivltyghptlktpesvtgtwkgdvkiq
Z39Ig     1 .g.ll...l....t.d...r.i.ev......p.....nlp
mVSIG4   41 ciydplrgyrqvlvkwlvrhgsdsvtiflrdstgdhiqqa
Z39Ig    41 .t....q..t........qr...p........s.......
mVSIG4   81 kyrgrlkvshkvpgdvslqintlqmddrnhytcevtwqtp
Z39Ig    81 ..q...h............ls..e....s...........
mVSIG4  121 dgnqvirdkiielrvrkyn----------------------
Z39Ig   121 .....v....t....q.lsvskptvttgsgygftvpqgmr
mVSIG4  140 ----------pp-----------------------------
Z39Ig   161 islqcqargs..isyiwykqqtnnqepikvatlstllfkp
mVSIG4  142 -----------------------------------rin
Z39Ig   201 aviadsgsyfctakgqvgseqhsdivkfvvkdsskllktk
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mVSIG4  225 rvfarktsnseettrvttiat----depdsqalisdysdd
Z39Ig   321 .ah..eand.g..m..aif.sgcss...t..n.gnn...e
mVSIG4  261 pclsqeyqitir-------------------stmsipac
Z39Ig   361 ..ig.....iaqingnyarlldtvpldyefla.egksv.
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B

	mCD80	hCD86	mCD86	hB7-H1	mB7-H1	hB7-H2	mB7-H2	hPD-L2	mPD-L2	hB7-H3	mB7-H3	hB7-H4	mB7-H4	Z39Ig	mB7RP
hCD80	**48**	26	23	20	20	22	25	19	20	25	26	20	22	19	15
mCD80		29	26	23	21	22	24	22	20	25	25	22	22	17	16
hCD86			**56**	18	23	20	26	21	18	23	24	21	21	18	16
mCD86				20	20	22	23	17	20	24	26	19	21	18	14
hB7-H1					**70**	21	22	36	35	29	29	22	24	16	15
mB7-H1						22	22	38	34	29	29	25	25	16	18
hB7-H2							**48**	22	22	30	29	22	20	18	17
mB7-H2								20	21	28	27	18	18	19	19
hPD-L2									**72**	25	26	22	22	17	13
mPD-L2										24	24	21	20	16	17
hB7-H3											**92**	26	28	19	19
mB7-H3												25	28	20	20
hB7-H4													**90**	17	16
mB7-H4														18	18
Z39Ig															**44**

Figure 1
Sequence and homology of VSIG4. (**A**) Amino acid sequence alignment of murine VSIG4 with the putative human ortholog Z39Ig. The N-terminal signal sequences determined according to von Heijne (53) are underlined. The 2 Ig domains are in italics, and identical amino acids are indicated with dots. The gaps are indicated with dashes. Bold letters correspond to the predicted transmembrane domain. Overall, the 2 proteins show 44% identity, and within the common extracellular domain (aa 1–139), 78% identity was found between VSIG4 and Z39Ig. (**B**) Percentage of identity between the extracellular domains of known B7 superfamily members.

erate homology may be explained by the different length of the mouse (280 aa) and the human (399 aa) open reading frames. The putative human ortholog contains 2 complete extracellular IgG domains, like the members of the B7 family. Detailed analysis of the common sequence within the extracellular domain (aa 1–139) reveals 78% identity between the mouse and the human protein (Figure 1A). Moreover, *Vsig4* and *Z39Ig* are found in corresponding gene clusters on the mouse and human X chromosome, respectively (data not shown). Although Z39Ig has been described previously by others, little about its biological function is currently known (29–31). Recently, monocytes isolated from PBMCs and mature DCs derived from umbilical cord hematopoietic progenitor cells were shown to express Z39Ig, and Z39Ig has been proposed to be involved in the regulation of immune responses mediated by phagocytosis and/or antigen presentation (32).

In summary, VSIG4 contains typical features of B7 family members, suggesting that it is a B7 family–related protein. The degree of sequence identity and the chromosomal location identify Z39Ig as the human ortholog of VSIG4.

VSIG4 is specifically expressed on resting tissue macrophages. To characterize the expression pattern of *Vsig4*, the expression of *Vsig4* in various tissues and cell types was evaluated by quantitative PCR. *Vsig4* was found to be expressed at high levels in the liver, DCs, neutrophils, and macrophages. Low expression was found in various other tissues, including lung, heart, spleen, and lymph nodes, while no *Vsig4* expression was found in T and B cells (Figure 2A). Hence, *Vsig4* expression appears to be present in macrophages, neutrophils, and DCs and in a variety of peripheral tissues.

To determine cell-surface expression of VSIG4, we generated a polyclonal rabbit serum against the extracellular domain of VSIG4. We first tested the specificity of the rabbit serum in cell lines ectopically expressing VSIG4. To this end, EL4 cells transduced with a recombinant retrovirus expressing VSIG4 were generated (EL4-VSIG4). As shown in Figure 2B, VSIG4 surface expression was detected with anti-VSIG4 rabbit serum on EL4-VSIG4 cells, whereas no staining was observed on EL4 cells transduced with empty expression vector. No staining was observed with preimmune serum on either cell population (data not shown). To test whether the serum also recognizes VSIG4 in cell lysates, 293 cells were transiently transfected with a VSIG4 expression vector, and cells were lysed and analyzed by Western blot. A distinct band of 46 kDa was detected in cell lysates of 293 cells transfected with the VSIG4 expression vector, and no signal was observed in nontransfected 293 cells (Figure 2B). Taken together, these data demonstrate that the anti-VSIG4 rabbit serum specifically recognizes surface VSIG4 as well as VSIG4 protein in cell lysates.

Having established that anti-VSIG4 serum was specific, we set out to analyze VSIG4 surface expression in various cell types. Several bone marrow–derived cell types were analyzed for VSIG4 cell-surface expression. In accordance with the expression data shown in Figure 2A, naive and activated T and B cells did not show VSIG4 surface expression (data not shown). Moreover, naive and activated DCs, blood and splenic neutrophils, and granulocytes did not show VSIG4 surface expression (Figure 2C), although some of these cell types were positive for *Vsig4* mRNA expression. In contrast, a subpopulation of resting peritoneal macrophages expressed high

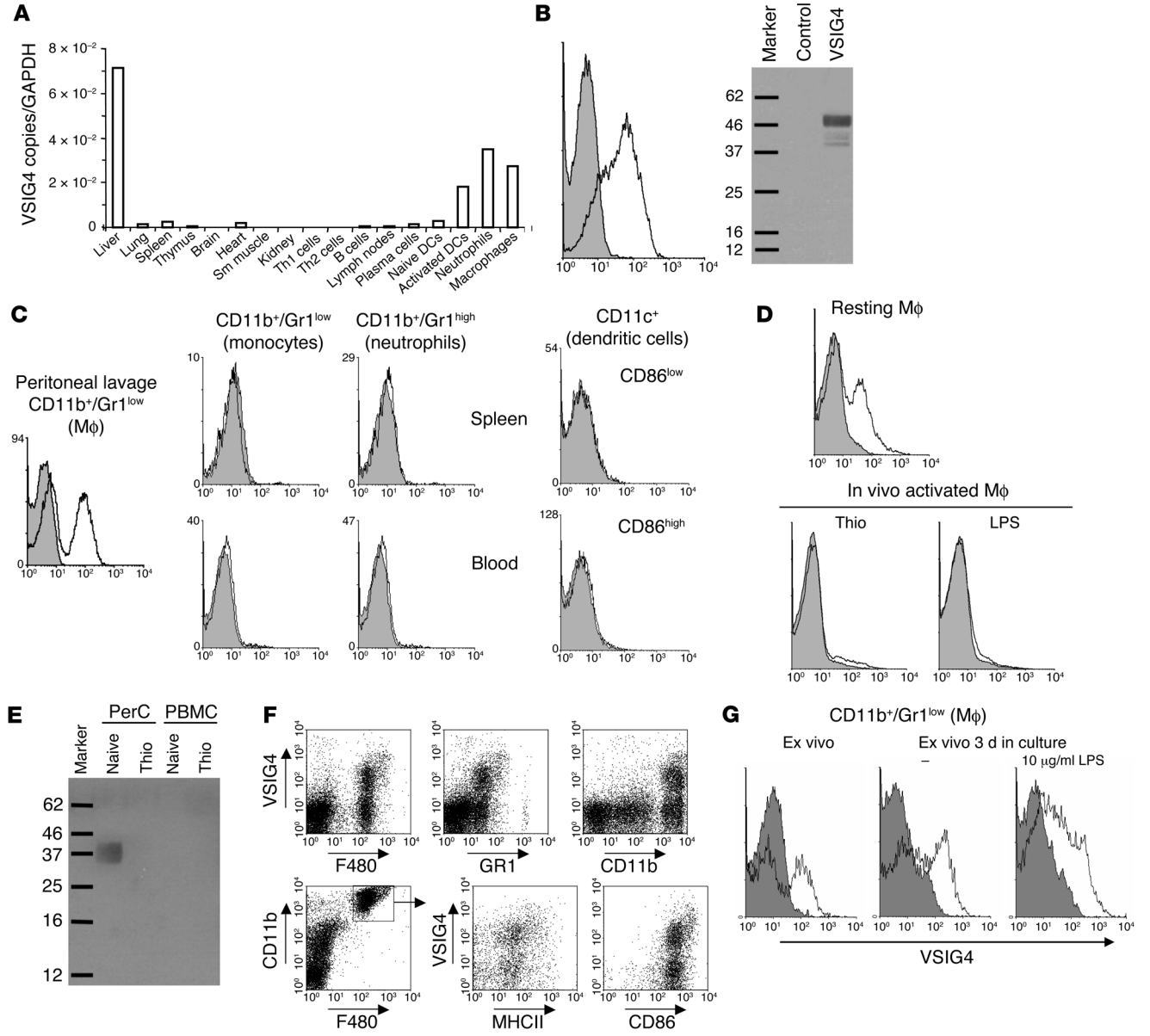

Figure 2

VSIG4 is expressed on resting peritoneal macrophages and downregulated upon activation. (**A**) VSIG4 copies normalized to GAPDH in the respective tissues or cell types are shown. Sm muscle, smooth muscle. (**B**) Characterization of anti-VSIG4 rabbit serum. Left: EL4 (filled histogram) or EL4-VSIG4 cells (black lines) were stained with anti-VSIG4. Right: Western blot analysis of lysates from 293 and 293-VSIG4 cells with anti-VSIG4. (**C**) Peritoneal macrophages (Mϕ), monocytes, and neutrophils from blood and spleen and activated and naive DCs were stained with anti-VSIG4 (black lines) and preimmune serum (filled histograms). Gating markers are indicated above the histograms. Half of the CD11b$^+$/Gr1$^-$ cells from the peritoneum (resident macrophages) showed VSIG4 expression. (**D**) Resting peritoneal macrophages and macrophages activated in vivo by thioglycolate (Thio) or LPS for 3 days were isolated, stained for the expression of VSIG4, and analyzed by FACS as described for **C**. Histograms of the gated CD11b$^+$/Gr1$^-$ cell population are shown. (**E**) Resting peritoneal macrophages and macrophages activated in vivo by thioglycolate and PBMCs from the same animals were isolated and analyzed for VSIG4 expression by Western blotting using polyclonal anti-VSIG4 antibodies. PerC, peritoneal cells. (**F**) Dot plots of cells isolated from the peritoneum and stained with the indicated cell-surface marker and VSIG4 rabbit serum. (**G**) Freshly isolated peritoneal macrophages were either stained directly or cultivated in the presence or absence of 10 μg/ml LPS for 3 days and stained with anti-VSIG4 (black lines) or preimmune serum (filled histogram). Histograms of the gated CD11b$^+$/Gr1^{low} cell population are shown. Representative stainings of at least 2 independent experiments are shown.

levels of VSIG4 (Figure 2C). In the next step, we analyzed VSIG4 expression on resting and activated macrophage populations. To this end, VSIG4 expression in resting peritoneal macrophages and peritoneal macrophages obtained after in vivo activation by LPS or thioglycolate for 3 days was assessed. In contrast to resting macrophages, inflammatory macrophages activated in vivo did not show VSIG4 expression (Figure 2D). These results were confirmed by Western blotting, since VSIG4 protein could only be

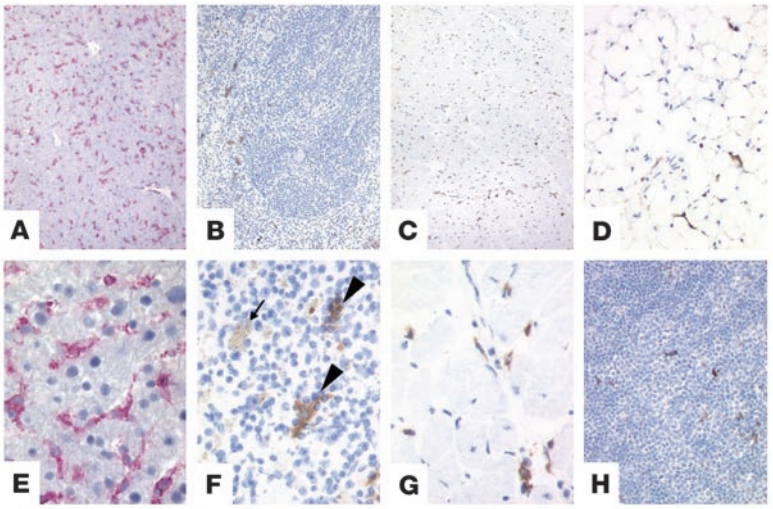

Figure 3
VSIG4 is expressed on resting tissue macrophages. Organs of untreated mice were assessed for VSIG4 expression by histology. (**A** and **E**) Kupffer cells lining the sinusoids of the liver were evenly positive for VSIG4. (**B** and **F**) Occasional macrophages of the red pulp of the spleen were positive, while macrophages of the white pulp were negative for VSIG4 (**B**). Within the red pulp (**F**), iron-laden macrophages (a weak granular signal was derived from the iron) were negative (small arrow), while other macrophages were weakly positive for VSIG4 (large arrowheads). (**C** and **G**) The myocardium showed an uneven distribution of VSIG4-positive macrophages. VSIG4 was also detected in tissue-resident macrophages of adipose tissue (**D**). VSIG4 was absent in thymic cortex and detected in rare macrophages of the thymic medulla (**H**). Representative stainings of at least 2 independent experiments are shown. Original magnification, ×60 (**A**–**D**); ×150 (**E**–**H**).

detected in cell lysates of naive peritoneal macrophages, whereas no VSIG4 could be detected in cell lysates of thioglycolate-treated macrophages. PBMCs from control and thioglycolate-injected mice also did not express VSIG4 (Figure 2E). The slightly faster migration of VSIG4 observed in lysates of peritoneal macrophages compared with the one observed in 293 cells (Figure 2B) could be explained by different glycosylation of the protein in the different cell types. Taken together, these results suggest that VSIG4 expression is restricted to resting macrophages and that the expression is lost upon activation. Since VSIG4 was only observed in a subpopulation of resting peritoneal macrophages, we wondered whether the VSIG4-positive population differed from the VSIG4-negative cells in the expression of different surface markers. To this end, cells from the peritoneum were isolated and stained with anti-VSIG4 and anti-CD11b, -GR1, -F480, and –MHC class II antibodies and analyzed by FACS. As shown in Figure 2F, the VSIG4-positive population was $CD11b^{high}$, $GR1^{low}$, and $F480^{high}$, demonstrating that only resident macrophages of the peritoneal lavage are positive for VSIG4 expression. However the VSIG4-positive population did not differ in the expression of either CD86 or MHC class II from the VSIG4-negative population of resident peritoneal macrophages (Figure 2F).

Taken together, these results suggest that VSIG4 expression is restricted to resting macrophages and is lost upon activation. To test this further, resting peritoneal macrophages were isolated from naive mice and either stained directly or cultivated for 3 days in the presence or absence 10 μg/ml LPS. As shown in Figure 2G, VSIG4 expression was reduced upon activation with LPS. Hence, VSIG4 is downregulated in vitro upon activation of the cells. The slightly reduced expression of VSIG4 observed in the cells that had not been activated compared with freshly isolated peritoneal macrophages may be explained by partial activation during cultivation of the cells in tissue culture plates. In summary, these results demonstrate that VSIG4 is expressed on naive resting macrophages and that the expression is lost upon activation of these cells. Moreover, infiltrating activated macrophages did not display detectable VSIG4 expression. The more restricted expression of VSIG4 assessed by antibody staining compared with that assessed by RT-PCR is not without precedent, since mRNA for B7-H1 is found in many tissues, while protein expression is largely restricted to professional APCs (17–21). In addition, most tissues, in particular the liver, contain resting macrophages as a potential source of VSIG4 expression. Indeed, histological analysis showed expression of VSIG4 macrophages in many tissues, including liver,

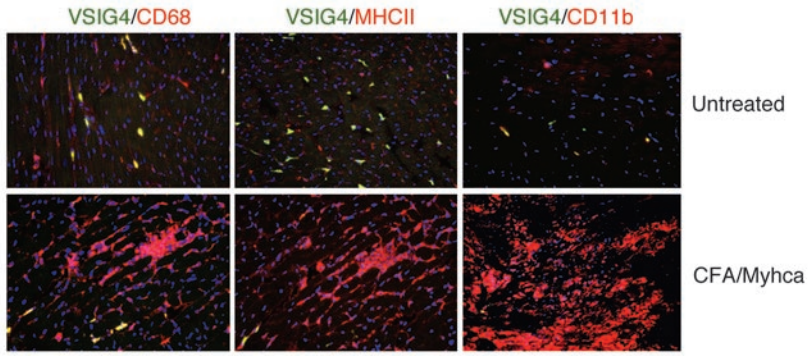

Figure 4
Macrophages in autoimmune infiltrates of the heart do not express VSIG4. Myocarditis was induced by immunization with α–myosin heavy chain–derived peptide in CFA (CFA/Myhca), and as a control, untreated mice were used. Heart sections from inflamed hearts and control hearts were stained with VSIG4 (green) together with CD68, MHC class II (MHC II), or CD11b (red) as indicated. In untreated mice, a fraction of CD68- or CD11b-positive tissue-resident macrophages coexpressed VSIG4. (Note that all VSIG4-positive cells coexpressed Cd11b; in some cases, Cd11b expression was low, precluding visualization of the expression in an overlay image). In contrast, MHC class II–positive DCs showed no coexpression of VSIG4. In CFA/Myhca-immunized mice, inflammatory foci included numerous CD68-positive macrophages that showed no coexpression of VSIG4. Occasional VSIG4-positive macrophages were detected in the myocardium surrounding the inflammatory focus. On a consecutive histological section, abundant MHC class II expression was detected within the inflammatory infiltrate, probably mostly on activated macrophages. Representative stainings of at least 2 independent experiments are shown. Original magnification, ×150.

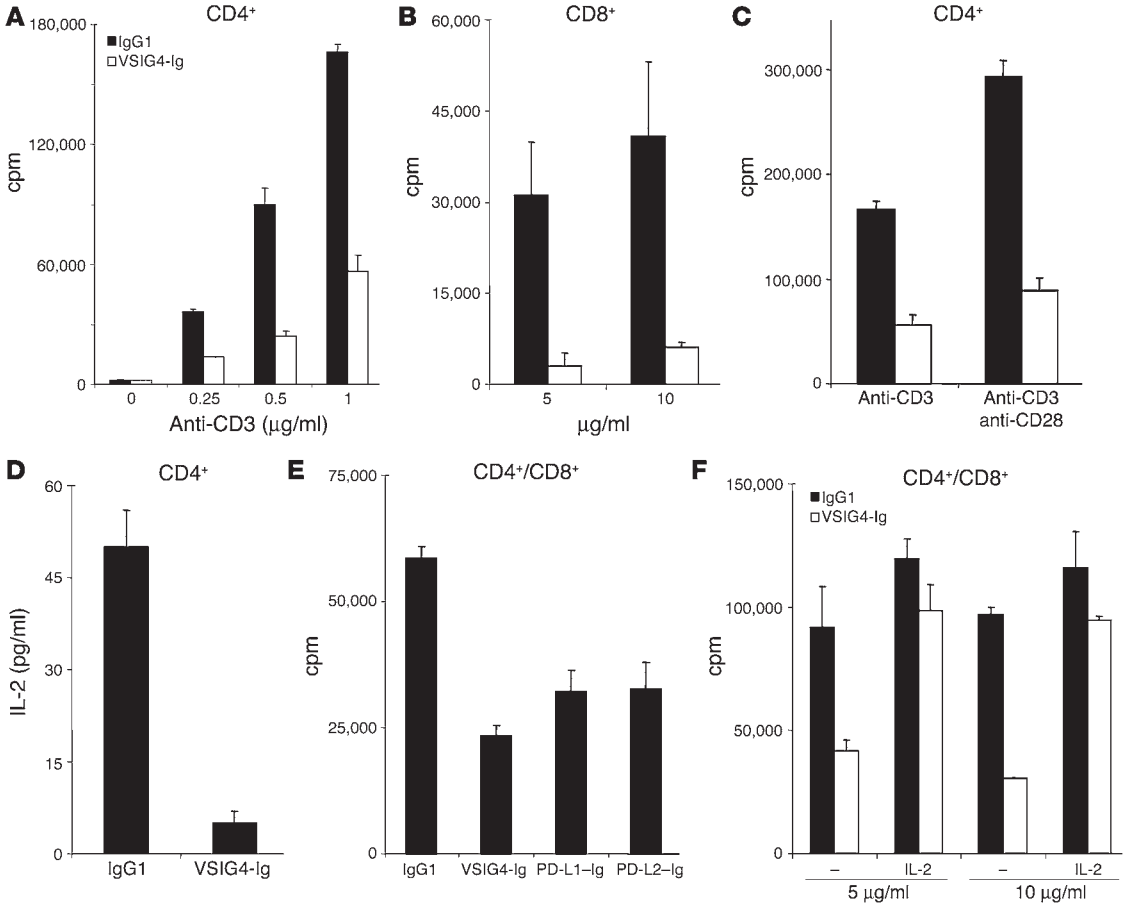

Figure 5

VSIG4 inhibits T cell proliferation and IL-2 production in vitro. (**A**) Plates were coated with anti-CD3 at the indicated concentrations in the presence of 5 μg/ml VSIG4-Ig or control IgG1. Proliferation of purified CD4+ T cells was monitored after 2 days by [3H]thymidine incorporation. (**B**) Plates were coated with anti-CD3 at 0.5 μg/ml in the presence of 5 or 10 μg/ml VSIG4-Ig or IgG1 at the indicated concentrations. CD8+ T cell proliferation was monitored after 2 days by [3H]thymidine incorporation. (**C**) Costimulation assays were performed with purified CD4+ T cells with a fixed concentration of anti-CD3 (0.5 μg/ml) in the presence or absence of anti-CD28 (2 μg/ml) with either 5 μg/ml VSIG4-Ig or control IgG1. (**D**) IL-2 production of CD4+ T cells stimulated with 0.5 μg/ml anti-CD3 in the presence of 5 μg/ml VSIG4-Ig or control IgG1. (**E**) Proliferation assays were performed in the presence of 0.5 μg/ml anti-CD3 and VSIG4-Ig, PD-L1–Ig, PD-L2–Ig, or control IgG1 at a concentration of 10 μg/ml. (**F**) Proliferation assays were performed in the presence of 0.5 μg/ml anti-CD3 and VSIG4-Ig or control IgG1 at a concentration of 5 or 10 μg/ml in the absence or presence of 10 ng/ml IL-2. Error bars represent standard deviations for data from experiments performed in triplicate. Representative data from least 2 independent experiments are shown. The difference between VSIG4-Ig– and IgG1-treated cells was significant in all panels shown ($P < 0.05$). In **F**, the difference between VSIG4-Ig– and IgG1-treated cells in the presence of IL-2 was not significant ($P > 0.05$).

thymus medulla, adipose tissue, and the heart (Figure 3; see also Figure 4 for costaining with CD68 and CD11b). In myocardium, a slightly uneven, at times patchy distribution of VSIG4-positive macrophages was seen. VSIG4 was not detected in intestines, kidney, skeletal muscle, salivary gland, lymph node, splenic white pulp, lung, and brain (data not shown).

VSIG4 is downregulated in autoimmune tissue. Since VSIG4 was downregulated on macrophages activated by LPS or thioglycolate, we wanted to assess whether VSIG4 may also be absent on macrophages in autoimmune tissue. Thus, female BALB/c mice were immunized and boosted with myosin-derived peptides, leading to autoimmune inflammation of the heart (33). Three weeks later, VSIG4 expression was assessed on control heart and diseased heart. In the healthy tissue, VSIG4 expression was found on a fraction of $CD68^+$ and $CD11b^+$ macrophages but not on MHC class II^{high} DCs (Figure 4). In contrast, VSIG4 expression was not found in the confluent inflammatory infiltrates of hearts with myocarditis (Figure 4). Very rarely, a single VSIG4-positive macrophage could be detected within an inflammatory focus, and macrophages outside inflammatory foci expressed VSIG4 in normal numbers and unremarkable distribution (data not shown). This indicates that absence of VSIG4 expression was restricted to actual sites of inflammation. These histological data further support the notion that VSIG4 is downregulated upon the activation of the cells.

VSIG4 is a negative regulator of T cell proliferation. To study the function of the putative member of the B7 family, the extracellular domain of VSIG4 (aa 1–186) was fused to the human IgG1 constant region and expressed in 293-EBNA cells. The soluble fusion molecule was named VSIG4-Ig. Bearing in mind the importance of costimulatory molecules in positive and negative regulation of T cell activation, we first tested the role of VSIG4 in vitro in T cell proliferation assays. Briefly, increasing concentrations of anti-CD3

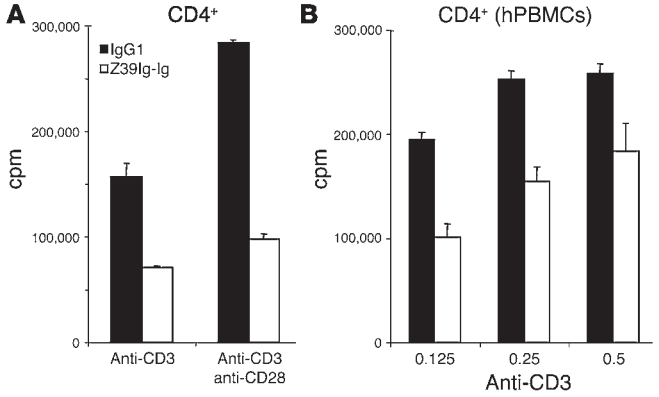

Figure 6

Z39Ig, the human homolog of VSIG4, inhibits T cell proliferation. (**A**) Proliferation assays were performed with purified mouse CD4+ T cells with 0.5 μg/ml anti-CD3 in the presence or absence of 2 μg/ml anti-CD28 with Z39Ig-Ig (human VSIG4) or control IgG1 at a concentration of 5 μg/ml. (**B**) Proliferation assays were performed with CD4+ T cells purified from human PBMCs in the presence of the indicated amounts of anti-CD3 together with Z39Ig-Ig (human VSIG4) or control IgG1 at a concentration of 20 μg/ml. Error bars represent standard deviations for data from experiments performed in triplicate. Representative data from at least 2 independent similar experiments are shown. The differences between Z39Ig-Ig– treated cells compared with control IgG1–treated cells were significant ($P < 0.05$) in both **A** and **B**.

antibody (0, 0.25, 5, and 1 μg/ml) were coated in the presence of 5 μg/ml VSIG4-Ig or control IgG1. Purified CD4+ T cells were added, and proliferation was assessed after 48 hours by [3H]thymidine incorporation. As shown in Figure 5A, dose-dependent proliferation of T cells was observed with increasing concentrations of anti-CD3 antibody. Inclusion of immobilized VSIG4-Ig led to a strong reduction in T cell proliferation as compared with IgG1 at all 3 anti-CD3 concentrations used. IL-2 levels were also strongly reduced in culture supernatants of CD4+ T cells incubated in the presence of VSIG4 (Figure 5B). The inhibitory activity of VSIG4 on CD8+ T cells was analyzed next (Figure 5D). Proliferation assays as described above were performed with a fixed concentration of anti-CD3 (0.5 μg/ml) in the presence of 5 or 10 μg/ml VSIG4-Ig or control IgG1. As shown in Figure 5B, the inclusion of immobilized VSIG4-Ig fusion protein strongly inhibited proliferation of CD8+ T cells at both concentrations of VSIG4-Ig.

Having established that VSIG4-Ig is a strong inhibitor of T cell proliferation induced by anti-CD3, we wondered whether VSIG4-Ig could also inhibit T cell proliferation induced by anti-CD3 and anti-CD28. For this purpose, costimulation assays were performed with a fixed concentration of anti-CD3 (0.5 μg/ml) in the presence or absence of anti-CD28 (2 μg/ml) with either 5 μg/ml VSIG4-Ig or control IgG1. Purified CD4+ T cells were added, and proliferation was assessed by [3H]thymidine incorporation. VSIG4-Ig strongly inhibited proliferation of anti-CD3 as well as anti-CD3/anti-CD28–stimulated T cells (Figure 5C).

To benchmark the potency of VSIG4 as a negative regulator of T cell activation, we compared its inhibitory properties with those of PD-L1 and PD-L2, two well-known inhibitors of T cell activation. To this end, costimulation assays were performed with a fixed concentration of anti-CD3 (1.0 μg/ml) in the presence of 10 μg/ml VSIG4-Ig, PD-L1–Ig, or PD-L2–Ig. VSIG4 inhibited T cell proliferation at least as potently as PD-L1 and PD-L2 (Figure 5E).

Since incubation of T cells with VSIG4-Ig led to the inhibition of T cell proliferation with a concomitant reduction in IL-2 secretion, we wondered whether T cell proliferation could be rescued by the addition of IL-2. To this end, costimulation assays were performed with a fixed concentration of anti-CD3 with either 5 or 10 μg/ml VSIG4-Ig or, as a control, human IgG1 in the presence or absence of 10 ng/ml IL-2. Purified T cells were added, and proliferation was assessed by [3H]thymidine incorporation. The inhibitory activity of VSIG4-Ig could be abolished by the addition of IL-2 (Figure 5F). These results confirm the specific inhibitory activity of VSIG4-Ig and rule out unspecific toxic effects of the protein preparation.

As outlined earlier, based on the high degree of conservation as well as the genomic locus, we postulated that Z39Ig is the human ortholog of VSIG4. To test whether Z39Ig also negatively regulates T cell proliferation, the extracellular domain of Z39Ig (aa 1–280) was fused to the human IgG1 constant region and expressed in 293-EBNA cells. Costimulation assays as described above were performed with a fixed concentration of anti-CD3 (0.5 μg/ml) in the presence or absence of anti-CD28 (2 μg/ml) with either 5 μg/ml Z39Ig-Ig or control IgG1. Purified murine CD4+ T cells were added, and proliferation was assessed by [3H]thymidine incorporation. As shown in Figure 6A, Z39Ig-Ig strongly inhibited proliferation of anti-CD3– as well as anti-CD3/anti-CD28–stimulated murine T cells, documenting that the inhibitory function of VSIG4 is conserved across the species barrier. Furthermore, simulation of human CD4+ T cells with anti-CD3 resulted in reduced proliferation in the presence of plastic-bound Z39Ig-Ig (Figure 6B). These results demonstrate that VSIG4 functions as a negative regulator of mouse as well as human T cells.

In conclusion, these data demonstrate that VSIG4 is a strong negative regulator of T cell activation, providing further evidence that VSIG4 is indeed a B7 family–related protein.

VSIG4 negatively regulates CD8+ T cell responses and Th cell–dependent IgG responses in vivo. Having established the function of VSIG4 in vitro, we further explored the function of VSIG4 in vivo. We have previously shown that immunization of mice with peptide p33 derived from lymphocytic CMV glycoprotein coupled to virus-like particles (VLPs) derived from the bacteriophage Qβ induce strong primary CTL responses in mice (34, 35). To evaluate the in vivo effect of VSIG4 on the induction of CTLs, mice were treated with soluble VSIG4 (VSIG4-Ig) or a control protein (human IgG1 antibody) during vaccination with VLPs (Figure 7A). Accordingly, mice were injected i.p. on days –1, 1, and 3 with either mVSIG4-Ig or control IgG1. On day 0 mice were immunized with 50 μg of p33-Qβ mixed with CpGs as an adjuvant. To analyze specific cellular immune responses, p33-specific CD8+ T cells were measured in the 2 experimental groups by p33-specific tetramer staining 7 days after immunization. As shown in Figure 7B, immunized mice treated with VSIG4-Ig had a roughly 2.3-fold reduction in the number of p33-specific T cells in the blood. To further assess cellular T cell responses, intracellular IFN-γ production by splenic CD8+ T cells was measured by intracellular staining followed by FACS. As shown in Figure 7C, a 3.5-fold reduction in frequencies of IFN-γ–producing cells was observed in mice treated with VSIG4-Ig. Hence, similar to what was observed in vitro, VSIG4 inhibits CD8+ T cell activation in vivo.

Humoral immune responses in the same mice were studied next. We have previously shown that Qβ VLPs induce an early, Th cell–independent IgM response followed by a Th cell–dependent IgG

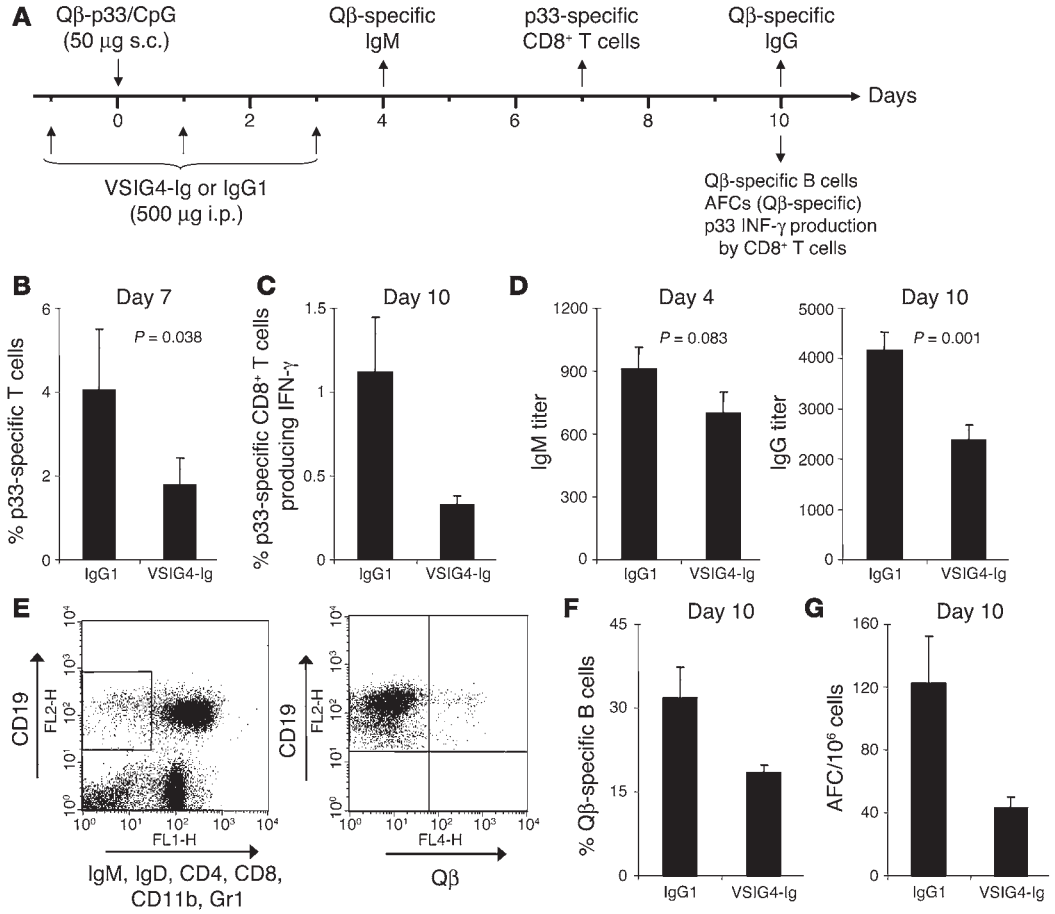

Figure 7

Soluble VSIG4 inhibits induction of CTL responses and Th cell–dependent humoral immune responses in vivo. (**A**) Experimental diagram. Mice were injected with VSIG4-Ig or control IgG i.p. on days –1, 1, and 3 and immunized with Qβ-p33 VLPs with CpGs on day 0. Qβ-specific IgM and IgG responses were measured after 4 and 10 days. p33-specific CD8+ T cells were determined in the blood by tetramer staining on day 7. Mice were sacrificed on day 10, and levels of Qβ-specific B cells, AFCs, and p33-specific IFN-γ–expressing CD8+ T cells were determined from spleens. (**B**–**G**) Averages of 2 independent experiments ± SEM are shown ($n = 8$). Differences between VSIG4-Ig– and IgG1-treated mice were significant in **B**, **C**, and the right panel of **D**, as well as **F** and **G**. *P* values obtained by Student's *t* test are shown. (**B**) Percentage p33-specific CD8+ T cells. (**C**) Percentage p33-specific CD8+ T cells after in vitro stimulation with p33-pulsed DCs 10 days after immunization. (**D**) ELISA titers of the Qβ-specific IgM and IgG responses. (**E**) Staining and gating strategy for the detection of isotype-switched B cells by FACS. Activated, isotype-switched B cells were found in the indicated gate. A representative example of Qβ-binding B cells in the indicated gate is shown in the right panel. (**F**) Percentage of Qβ-specific B cells within the indicated gate in **E** in mice 10 days after immunization. (**G**) Number of Qβ-specific AFCs per 10^6 splenocytes as determined by ELISPOT are shown.

response (34, 36). To evaluate the in vivo effect of VSIG4 on the induction of humoral responses, IgG antibody titers, the number of antibody-forming cells (AFCs), and the number of Qβ-specific B cells were determined 10 days after immunization. Moreover, Qβ-specific IgM antibody titers were measured on day 4. The Th cell–independent IgM response was only marginally affected by treatment with VSIG4-Ig. In contrast, the Th cell–dependent IgG response against Qβ was reduced about 2-fold in mice treated with VSIG4-Ig (Figure 7D).

To further characterize B cell responses, Qβ-specific B cells were enumerated by FACS (36). Activated and isotype-switched B cells downregulate surface expression of IgM and IgD. Thus, in order to specifically stain Qβ-specific, isotype-switched B cells, a population of CD19+ B cells expressing low levels of IgM and IgD was assessed for its ability to bind Qβ (Figure 7E). As shown in Figure 7F, the percentage of Qβ-specific, isotype-switched B cells was reduced by a factor of about 2 in the VSIG4-treated animals. Numbers of Qβ-specific AFCs were assessed by ELISPOT and found to be decreased by a factor of about 3 (Figure 7G).

In summary, treatment of immunized mice with VSIG4-Ig inhibited Th cell–dependent IgG responses, as documented by reduced specific IgG titers and reduced frequencies of Qβ-specific B cells and AFCs. In contrast, the Th cell–independent Qβ-specific IgM response was only marginally affected by VSIG4. Thus, VSIG4-Ig appears to inhibit B cell responses by blocking T help.

Discussion

The induction of specific T cell responses is a tightly regulated process. While research initially focused on costimulatory molecules enhancing T cell activation, the list of inhibiting receptor-ligand pairs is growing (23, 37, 38).The present study describes the identification of VSIG4 as potent inhibitor of T cell activation.

All B7 homologs described so far share a characteristic expression pattern: they are expressed at relatively low levels on resting cells and are upregulated in response to inflammatory signals. Some of the B7 family members, such as B7-1 and B7-2 as well as B7-H4, are largely restricted to professional APCs, such as DCs and macrophages, while others, e.g., B7-H1, are expressed rather in nonimmunological tissues, especially upon stimulation with inflammatory cytokines. It is thought that most of the B7 family members have the ability to engage both activating and inhibitory receptors on T cells; whether or not T cells become activated appears to depend on the timing of the interaction, since inhibitory receptors are preferentially expressed on activated cells (6). Interestingly, VSIG4 has a different expression pattern; it is highly expressed on resting, peripheral tissue macrophages and is downregulated or lost upon activation. In addition, little expression is found on macrophages in lymphoid organs. Hence, the main function of VSIG4 may not be the downregulation of an ongoing T cell response but rather the prevention of T cell activation in the intact tissue. Only upon inflammatory insult is VSIG4 downregulated or superseded by de novo infiltrating VSIG4-negative macrophages, which subsequently allow for proper T cell responses in peripheral tissues. It is tempting to speculate that VSIG4 may therefore be involved in the maintenance of peripheral T cell tolerance and/or nonresponsiveness. Indeed, VSIG4 was not present in the inflammatory infiltrates in tissue affected by autoimmune disease. This concept is supported by the finding that resting DCs induce T cell tolerance rather than T cell activation (39, 40). Additionally, and not mutually exclusively, it is possible that VSIG4 induces the differentiation of regulatory T cells. However, in preliminary in vitro experiments, we did not observe an increased production of anti-inflammatory cytokines by T cells upon stimulation with VSIG4 (data not shown).

The mechanism of VSIG4-mediated inhibition of T cell activation remains to be established. Although our data show that VSIG4 is able to directly inhibit T cell proliferation in vitro, it remains possible that VSIG4 may influence T cell activation indirectly in a manner similar to that described for CTLA-4. CTLA-4 is able to inhibit T cell activation through recruitment of the phosphatase shp-2, which reduces TCR-dependent signal transduction by dephosphorylation of signaling molecules (41, 42). Yet T cell responses mounted by CTLA-4–deficient, TCR-transgenic T cells are remarkably normal (43, 44). The more important function of CTLA-4 may therefore be to maintain peripheral tolerance. Indirect and direct mechanism(s) may account for this function, since CTLA-4 has been shown to be involved in the generation of regulatory T cells as well as inducing T cell anergy (45–49).

While we were revising the current manuscript, a study was published describing VSIG4 as a novel complement-binding protein, CRIg (binding C3b and iC3b), specifically expressed by tissue macrophages, in particular Kupffer cells. This is an intriguing finding demonstrating that the complement system has not only receptors for fine-tuning of B cell responses (CD21) but also links the innate immune system with the regulation of T cell activation. Interestingly, binding of C3b or iC3b to VSIG4/CRIg triggers internalization of the receptor (50), suggesting that activation of the complement cascade removes the inhibitory properties of tissue macrophages, promoting inflammation. Hence, VSIG4/CRIg may be a new pathway of adaptive immune regulation by the innate immune system.

In conclusion, VSIG4 was shown to be a strong inhibitor of T cell proliferation in vitro and in vivo. The restricted expression of VSIG4 on resting tissue macrophages suggests that VSIG4 plays an important role in the maintenance of T cell unresponsiveness in healthy tissue.

Methods

Cloning of full-length VSIG4, VSIG4 protein expression, and production of polyclonal antibodies. Protein sequences of both human and mouse B7 family members including CD80, CD86, B7-H1, B7-H2, and B7-H3 were used to generate an HMM. Translated EST sequences were subjected to an HMM search using HMMR software (51). Known proteins such as B7 family members, butyrophilins, and myelin oligodendrocyte glycoproteins were eliminated, and redundant sequences were grouped. Remaining sequences were further analyzed for the occurrence of a catalog of different features such as particular domains and specified intrinsic features (N-terminal signal sequence, transmembrane domain, and at least 1 IgG domain), which are included in the SMART (Simple Modular Architecture Research Tool) program (52).

The full-length mouse VSIG4 cDNA was generated by RT-PCR from mouse macrophage total RNA. The PCR was performed on mouse macrophage–derived cDNA as template using the High Fidelity PCR System (Roche Diagnostics) composed of a unique enzyme mix containing thermostable Taq DNA polymerase, a proofreading polymerase (catalog 1732650; Roche Diagnostics), and the primers LV80-mC18f (5′-GTAGCTTCAAATAGGATGGAG-3′) and LV81-mC18b (5′-AAACTGTGTTCAGCAGGCAG-3′). The resulting PCR product (867 bp) of mVSIG4 was cloned into the pGEM-T plasmid. The nucleic acid sequence of VSIG4 was then verified by DNA sequencing of several independent clones. Z39Ig was cloned as described above from spleen cDNA using the following primers: 5′-AGGAGGCTGGAAGAAAGGAC-3′ and 5′-CCCCCGGCAGAGATACTA-3′. VSIG4-Ig was prepared by fusing the coding region of the extracellular domain (aa 1–186) of VSIG4 to the constant region of human IgG1. Similarly, the extracellular domain of Z39Ig (aa 1–280) was fused to the constant region of human IgG1. The expression construct were transfected into EBNA cells (Invitrogen) using Lipofectamine Plus (Invitrogen) and the protein produced under serum-free conditions. VSIG4-Ig and Z39Ig fusion proteins were purified using a Protein A Sepharose column according to the manufacturer's recommendations (Pharmacia).

For the generation of polyclonal anti-VSIG4 serum, rabbits were immunized with VSIG4-Ig fusion protein. Ig-specific antibodies were quantitatively removed from VSIG4 rabbit serum on a Sepharose column coupled with IgG1.

Macrophage preparation and stainings. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing the peritoneum with 5 ml of PBS/1% BSA. To generate activated macrophages, BALB/c mice were either injected i.p. with thioglycolate (500 μl, 4% solution) or LPS (50 μg in 100 μl), and inflammatory macrophages were obtained from the peritoneum after 3 days, as described above. For the in vitro stimulation experiment, resident peritoneal macrophages were cultured for 3 days in 6-well plates in Iscove's modified Dulbecco's medium and completed with glutamine, antibiotics, and 10% FCS and the indicated amount of LPS (Sigma-Aldrich). Surface stainings were performed in the presence of Fc block (anti-CD16/CD32) on ice in PBS/0.1% FCS. All antibodies were from BD Biosciences — Pharmingen, with the exception of F480 (AbD Serotec) and CD68 (Acris Antibodies).

T cell proliferation assay. Purified T cells were stimulated by immobilized anti-CD3 antibody in the presence of immobilized mVSIG4-Ig fusion protein. The splenic T cells were purified from naive mice using CD4- and CD8-specific MACS beads (Miltenyi Biotec). For costimulation and inhibition assays, purified T cells (2×10^5 cells/well) were cultured in 96-well flat-bottom plates that were precoated at 4°C overnight with 75 μl/well of a solution containing the indicated concentrations of mouse anti–CD3ε

chain antibody NA/LE (145-2C11; BD Biosciences — Pharmingen), as well as the indicated concentrations of mVSIG4-Ig fusion protein or control proteins, such as antibody against mouse CD28 NA/LE (37.51; BD Biosciences – Pharmingen), recombinant mouse B7-H1–Ig chimera (catalog 1019-B7; R&D Systems), recombinant mouse PD-L2–Ig chimera (catalog 1022-PL; R&D Systems), and mouse gamma globulin (catalog 015-000-002; Jackson ImmunoResearch Laboratories Inc.). For measurement of T cell proliferation, the plates were cultured for 48–60 hours, and [^{3}H]thymidine (1 μCi/well) was added 10–16 hours prior to harvesting of the cultures. [^{3}H]thymidine incorporation was measured with a Wallac MicroBeta TriLux Liquid Scintillation counter (PerkinElmer).

In vivo assessment of T and B cell responses. Animal experiments were conducted according to guidelines set by the Swiss Federal Veterinary Office (BVET) and were approved by the Kantonale Veterinäramt of Zürich. For the animal studies, 6- to 18-week-old female C57BL/6 mice were used and kept in a specific pathogen–free facility at Cytos Biotechnology AG. Mice were injected i.p. with 500 μg of mVSIG4-Ig fusion protein or, alternatively, human IgG1κ (catalog I-5154; Sigma-Aldrich) on days –1, 1, and 3. Mice were immunized s.c. with 50 μg VLPs derived from the bacteriophage Qβ coupled with peptide p33 derived from lymphocytic choriomeningitis virus (Qβp33) in the presence of the CpG 1585 (5′-GGGGTCAACGTT-GAGGGGGG-3′ stabilized by phosphothioester bonds. Qβ-specific antibody responses were measured at the indicated time points as described previously (36). Peptide p33–specific T cell responses were measured by tetramer staining at the indicated time points, and intracellular cytokine staining was performed as described previously using peptide p33–loaded DCs for stimulation (35). Qβ-specific B cells were enumerated by flow cytometry as described previously (36). Qβ-specific AFCs were measured by ELISPOT as described previously (36).

Induction of myocarditis and histological analysis. Wild-type BALB/c female mice at age 8–10 weeks were immunized on days 0 and 7 with 100 μg of a murine-specific α–myosin heavy chain–derived peptide (myhc-α 614–629 [Ac-SLKLMATLFSTYASAD-OH]) in a 1:1 emulsion with CFA (1 mg/ml; H37Ra; Difco; BD Diagnostics). All experiments were in accordance with Swiss federal legislation were approved by the Kantonale Veterinäramt of Zürich. Twenty-one days after the immunization, hearts were removed, OTC embedded, and frozen. Seven-micrometer cryostat sections were fixed in acetone and then processed for antibody staining according to standard protocols. The following antibodies were used: anti–MHC class II (MCA46B; Serotec), anti-CD68 (MCA1957; Serotec), anti-CD11b (BD Biosciences — Pharmingen), and polyclonal anti-VSIG4 antibodies. For secondary incubation, HRP- or alkaline-phosphatase–labeled goat anti-rat Ig antisera were used. For 2-color immunofluorescence studies, Cy3-labeled anti-rat Ig and Alexa 488–labeled anti-rabbit Ig sera were used. DAPI-containing cover medium was used for counterstaining.

Statistics. Two-sided Student's *t* tests were performed using Excel Software. $P \leq 0.05$ was considered significant.

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