Single domain intrabodies against WASP inhibit TCR-induced immune responses in transgenic mice T cells

Mitsuru Sato, Ryoko Sawahata, Chisato Sakuma, Takato Takenouchi & Hiroshi Kitani

Animal Immune and Cell Biology Research Unit, National Institute of Agrobiological Sciences, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan.

Intrabody technology provides a novel approach to decipher the molecular mechanisms of protein function in cells. Single domains composed of only the variable regions (VH or VL) of antibodies are the smallest recombinant antibody fragments to be constructed thus far. In this study, we developed transgenic (Tg) mice expressing the VH or VL single domains derived from a monoclonal antibody raised against the N-terminal domain of Wiskott–Aldrich syndrome protein (WASP), which is an adaptor molecule in immune cells. In T cells from anti-WASP VH and VL single domain Tg mice, interleukin-2 production induced by T cell receptor (TCR) stimulation were impaired, and specific interaction between the WASP N-terminal domain and the Fyn SH3 domain was strongly inhibited by masking the binding sites in WASP. These results strongly suggest that the VH/VL single domain intrabodies are sufficient to knockdown the domain function of target proteins in the cytosol.
expression of anti-WASP scFv intrabodies inhibited TCR-stimulation-induced IL-2 production without affecting TCR capping in T cells from anti-WASP scFv Tg mice39. These results strongly suggested that the WASP N-terminal domain plays a pivotal role in IL-2 production, but not in antigen receptor capping in the TCR signaling pathway.

To extend our earlier work in intrabody technologies, we previously constructed four types of single domain intrabodies derived from the anti-WASP N-terminus monoclonal antibody. These single domains were composed of the VH and VL regions with or without their leader sequences. These single domains were expressed at similar levels and showed the specific binding activity to the WASP N-terminal domain in gene-transfected NIH3T3 cells20. In this study, to assess the ability to inhibit IL-2 production upon TCR stimulation through the expression of anti-WASP single domain intrabodies in T cells, we developed Tg mice that expressed anti-WASP single domains. Anti-WASP single domains efficiently bound to WASP in these Tg mouse T cells, and their inhibitory effects on IL-2 production upon TCR stimulation were similar to those of anti-WASP scFv.

**Results**

Expression of anti-WASP scFv and single domains in gene-transfected T cells. Previously, we constructed two types of scFv39 and four types of single domains20 derived from the anti-WASP N-terminus monoclonal antibody with or without the leader sequences of the VH and VL region (Fig. 1a). To compare the expression levels of these scFv and single domains, the DNA constructs were transiently transfected into DO11-10 murine T cell hybridomas. In gene-transfected murine T cells high expression of the scFv and single domain constructs with leader sequences, SHL, SVH, and SVL, was observed, whereas the constructs without leader sequences, HL and VL, were showed lower expression levels and expression of VL was hardly detected in the western blot analysis with anti-Myc tag antibody (Fig. 1b, upper panel). Quite similar to DO11-10 murine T cells, expression levels of SHL, SVH, and SVL were higher than those of HL, VH, and VL in gene-transfected human Jurkat T cells (Fig. 1c, upper panel), suggesting that the expression efficiency of scFv and single domain constructs is dependent on the presence of leader sequences in T lymphocytes. The amounts of protein loaded in each lane were confirmed by Western blotting with anti-β-actin antibody (Fig. 1b and 1c, lower panel). We previously demonstrated that all of the single domains, SVH, VH, VL, and VL were expressed at comparable levels in gene-transfected NIH3T3 cells20. So, the expression efficiency of scFvs and single domains is variable between T lymphocytes and NIH3T3 fibroblasts, highly dependent on the presence of leader sequences in the former cell type.

Expression of SVH/SVL single domain and specific binding to WASP in T cells from Tg mice. As SVH and SVL DNA constructs containing their leader sequences were strongly expressed in gene-transfected T cells, we used these constructs for the development of transgenic mice to knockdown WASP N-terminal domain function (Fig. 1a). Tg mice expressing the WASP N-terminal domain (WASP15) and anti-WASP scFv with the leader signal sequence of the VH region (SHL) had been established previously (Fig. 1a)20.39. Two independent SVH (a and b) Tg and three independent SVL (c, d, and e) Tg founders were established, and T cells from C57BL/6, anti-WASP SHL Tg, SVH (a and b) Tg, and SVL (c, d, and e) Tg mice were lysed and immunoprecipitated with anti-Myc tag antibody. The expression pattern of anti-WASP SHL and SVL was similar to that of their transient expression in DO11-10 or Jurkat T cells (Fig. 1d, lower panel). Whereas SVH were expressed at the size of 18 kDa, and the additional two bands were detected as upper size of 20 kDa and 23 kDa. These bands may indicate the products from the transgenes of SVH DNA construct or some unrelated proteins, which are cross-reacted with anti-Myc tag antibody (Fig. 1d, lower panel). In this immunoprecipitation analysis, all single domains exhibited their efficient binding activity to WASP (Fig. 1d, upper panel). As a result, SVH-b and SVL-d Tg lines were used for the following experiments.

N-WASP, a ubiquitously expressed homologue of WASP, shares 50% sequence similarity with WASP and it contains the EVH1 domain. However, the binding of N-WASP and anti-WASP SHL, SVH, and SVL intrabodies was not observed in these Tg mice T cells (Fig. S1), suggesting that these anti-WASP intrabodies specifically binds to the WASP N-terminal domain in Tg mice T cells.

Subcellular localization of anti-WASP SVH/SVL single domains in T cells from Tg mice. To examine the subcellular localization of anti-WASP SHL/SVL single domains, T cells from anti-WASP SHL-Tg, SVH-Tg, and SVL-Tg mice were fractionated into their subcellular compartments, and each fractions were analyzed by Western blotting with anti-Myc tag antibody. In Tg T cells, anti-WASP SHL, SVH, and SVL were detected in both the cytosolic and membrane fractions, although the levels were higher in the latter fraction (Fig. 1e, upper panel). In general, antibodies with the leader signal sequences cross the rough endoplasmic reticulum (ER) membrane and enter the trans-Golgi network. However, some of anti-WASP scFv and single domains with leader sequences were detected in the cytoplasm. Although the reason and mechanism remained unknown, these scFv and single domains reside in the cytoplasm and efficiently bind to the target proteins as intrabodies. To validate each subcellular fraction, WASP (Fig. 1e, center panel) and Ribophorin I (Fig. 1e, lower panel) were detected by specific antibodies as cytosolic and membrane protein markers, respectively. These results demonstrated that neither fraction was cross-contaminated by the other fraction.

T cell development in WASP15-Tg, anti-WASP SHL-Tg, SVH-Tg, and SVL-Tg mice was similar to wild-type mice (Fig. S2), suggesting that overexpressed WASP15, anti-WASP SHL, SVH, and SVL do not have any adverse effect on lymphocyte development.

Impairment of IL-2 production in anti-WASP SVH/SVL single domain Tg T cells upon TCR stimulation. To assess the effects of anti-WASP SVH/SVL single-domain expression on IL-2 transcription, quantitative real-time PCR was performed using RNA isolated from the T cells of wild-type, WASP15-Tg, anti-WASP SHL-Tg, SVH-Tg, and SVL-Tg mice after TCR stimulation. In contrast to the apparent upregulation of IL-2 gene transcription upon TCR stimulation in wild-type T cells, T cells from WASP15-Tg mice exhibited less than one-third and T cells from anti-WASP SHL-Tg, SVH-Tg, and SVL-Tg mice exhibited approximately one-half of the levels of IL-2 transcription (Fig. 2a). The basal gene transcription of IL-2 was undetectable level in all mice T cells (data not shown). Impairment of IL-2 secretion from these Tg T cells was confirmed by ELISA (Fig. 2b), indicating that the function of the WASP N-terminal region (including the EVH1 domain) for IL-2 production upon TCR stimulation is inhibited by SVH/SVL single domain intrabodies in T cells from Tg mice. T cells from another SVH transgenic line and two additional SVL transgenic lines exhibited similarly impaired IL-2 production upon TCR stimulation (data not shown), suggesting that copy number and integration site had no adverse effects on the transgene.

Impaired antigen receptor-induced proliferation in anti-WASP SVH/SVL single domain Tg T cells. To assess the effects of anti-WASP SVH/SVL single-domain expression on T cell function, the proliferative response to stimulation with anti-CD3e antibody was examined. In parallel to IL-2 production upon TCR stimulation, T cells from each Tg mouse line exhibited reduced proliferative responses compared with wild-type T cells; respectively, WASP15-Tg, anti-WASP SHL-Tg, SVH-Tg, and SVL-Tg T cells exhibited one-third, two-thirds, one-half, and one-half of the proliferative response...
Figure 1 | Expression and binding activity of anti-WASP SHL, SVH, and SVL intrabodies in T cells from Tg mice. (a) Schematic representation of WASP, truncated WASP (WASP15), anti-WASP SHL, HL, SVH, VH, SVL, and VL constructs. The major functional domains of WASP [the EVH1 domain (EVH1), GTPase-binding domain (GBD), proline rich region (PRR), and verproline/cofiline/acidic domain (VCA)], the leader signal sequence (S), the VH and VL regions of anti-WASP N-terminus mAb, the flexible peptide liker (lin), and the T7- and Myc-tagged sequences are shown. (b) DO11-10 T cells and (c) Jurkat T cells were transfected with DNA encoding anti-WASP scFv and single domain derivatives. Western blot analysis showing the expression levels of each scFvs and single domains in transfected T cells. The immunoblots were probed with anti-Myc-tag pAb or anti-β-actin pAb. (d) T cells from wild-type, anti-WASP SHL Tg, SVH, VH (a and b) Tg, and SVL (c, d, and e) Tg mice were lysed and immunoprecipitated (IP) with anti-Myc-tag mAb. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb or anti-Myc-tag pAb. (e) T cell extracts from anti-WASP SHL, SVH, and SVL Tg mice were separated into cytosolic (C) and membrane (M) fractions. The fractionated cell extracts were analyzed by Western blotting with anti-Myc-tag pAb, anti-WASP pAb, or anti-Ribophorin 1 pAb. Full-length gels/blots are presented in Supplemental Fig. 1. Immunoblots are representative of three independent experiments.
exhibited by wild-type T cells (Fig. 3a). The addition of exogenous IL-2 restored the normal proliferative response to anti-CD3ε antibody stimulation in all Tg T cells (Fig. 3b). The basal cell proliferation was undetectable level in all mice T cells (data not shown). These findings indicate that the WASP N-terminal domain has a critical role in signaling through the TCR, but not the IL-2 receptor.

In contrast to the impairment of IL-2 production and antigen receptor-induced proliferation, T cells from each Tg mouse line exhibited normal actin polymerization induced by TCR stimulation (Fig. S3). Furthermore, immunofluorescent microscopy demonstrated that the extent of antigen receptor capping induced by TCR stimulation in all Tg mice T cells was similar to that in the wild-type mice (Fig. S4). These results strongly suggest that anti-WASP SHL, SVH, and SVL intrabodies specifically inhibit the WASP N-terminal domain function for IL-2 production, but do not affect the actin cytoskeletal rearrangement induced by TCR stimulation.

Lymphoid cells from anti-WASP SVH/SVL Tg mice immunized with ovalbumin exhibited impaired proliferative response and IL-2 production induced by secondary ovalbumin stimulation. To assess the effect of anti-WASP SVH/SVL single domain expression on lymphoid cells, a proliferation assay was performed on lymphoid cells derived from wild-type, WASP15-Tg, anti-WASP SHL-Tg, SVH-Tg, and SVL-Tg mice were cultured in anti-CD3ε antibody-coated dishes (see Experimental procedures). Each stimulation was performed in the absence (a) or presence (b) of exogenous IL-2 as indicated. After incubation for 48 h, 10 μM BrdU was added to the T cell cultures. The cells were incubated for an additional 16 h, and BrdU incorporation was quantified by ELISA. Values are expressed as mean ± SEM from three independent experiments. Statistical significance is indicated by P < 0.05, **P < 0.01, and ***P < 0.001.
proliferative response to secondary OVA stimulation were similar to those of WASP15 and anti-WASP SHL (Fig. 4a). As a negative control, lymphoid cells derived from OVA-immunized wild-type and all Tg mice did not proliferate in response to nonspecific antigen stimulation with BSA (Fig. 4a).

To evaluate IL-2 production induced by the specific antigen, single cell suspensions from lymph nodes of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice immunized with OVA were cultured with OVA for 24 h. In contrast to marked upregulation of IL-2 production in response to secondary OVA stimulation in wild-type lymphoid cells, lymphoid cells from WASP15 Tg and anti-WASP SHL Tg mice exhibited only one-fifth and lymphoid cells from anti-WASP SHL/SV single domain Tg mice exhibited one-half of wild-type IL-2 production levels (Fig. 4b). These findings indicate that expression of anti-WASP SHL/SV single domains induces impairment of the T cell immune response to the specific antigen in Tg mice.

Inhibition of the specific interaction between Fyn and WASP by expressing anti-WASP SHL/SV single domain intrabodies. Recently, we demonstrated that the SH3 domain of Fyn is a binding partner of the WASP N-terminal domain in mouse T cells, and that the inhibition of this interaction by the overexpression of WASP15 or anti-WASP scFv resulted in impaired IL-2 synthesis upon TCR stimulation22. To assess the inhibitory effect of anti-WASP SHL/SV single domain expression on the specific interaction between Fyn and WASP, T cell lysates from wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were incubated with glutathione S-transferase (GST) or GST–Fyn-SH3 fusion proteins, and pulled down with glutathione sepharose beads. In contrast to the strong binding of WASP to the GST–Fyn-SH3 in wild-type T cells, their interactions were markedly inhibited in T cells from WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice (Fig. 5a, upper panel). The protein levels of GST and Fyn-SH3 were comparable in the assay (Fig. 5a, lower panel). The expression levels of WASP in T cells from wild-type and each Tg mice were confirmed by Western blotting with anti-WASP and anti-β-actin antibodies (Fig. 5b). To confirm the inhibitory effect of the anti-WASP SHL/SV single domain on the specific binding between endogenous Fyn and WASP in T cells, T cell lysates from wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were immunoprecipitated with a monoclonal antibody (mAb) raised against Fyn, and immunocomplexes were immunoblotted with anti-WASP antibody. In the immunoprecipitation analysis, specific binding between endogenous Fyn and WASP was clearly detected in wild-type T cells (Fig. 5c, upper panel). In contrast, the interaction between Fyn and WASP was severely inhibited in WASP15 Tg T cells (Fig. 5c, upper panel). In addition, competitive binding of the truncated WASP to Fyn was demonstrated by immunoblotting with anti-T7-tag antibody (Fig. 5c, center panel). The production of anti-WASP SHL, SVH, or SVL intrabodies also inhibited the binding of Fyn and WASP (Fig. 5c, upper panel). Fyn was equivalently immunoprecipitated in T cells from all mouse lines (Fig. 5c, lower panel). These observations demonstrate that anti-WASP SHL/SV single domains inhibit the Fyn–WASP interaction by masking the WASP N-terminal domain, and their abilities are comparable to scFv intrabody in T cells from Tg mice.

Inhibition of the interaction between WASP N-terminal domain and WIP by expressing anti-WASP SHL/SV single domain intrabodies. WASP-interacting protein (WIP) is known to bind to the WASP N-terminal EVH1 domain23. Previously, we demonstrated that the overexpression of WASP15 or anti-WASP scFv inhibits the interaction between WASP N-terminal domain and WIP in each Tg mice T cells22. To assess the inhibitory effect of anti-WASP SHL/SV single domain expression on WIP–WASP interaction, T cell lysates from wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were immunoprecipitated with anti-WIP antibody, and immunocomplexes were immunoblotted with anti-WASP antibody. In wild-type T cells, a strong binding between WIP and WASP was observed (Fig. 5d, upper panel). In contrast, the WIP–WASP interaction was greatly inhibited in WASP15 Tg and anti-WASP SHL Tg T cells, and anti-WASP SHL/SV expression was also effectively inhibited the WIP–WASP interaction.

**Figure 4 | Proliferative response and IL-2 production of lymphoid cells from wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice stimulated with OVA.** Mice were immunized in both footpads with 100 μg of OVA in TiterMax gold (water-in oil adjuvant) (day 0). Inguinal, popliteal, and periaortic lymph nodes were removed, and single cell suspensions were prepared in RPMI 1640 medium (day 7). (a) Cells (1 × 10^6) were seeded in each well of 96-well tissue culture plates and cultured with the indicated amount of OVA or control BSA at 37°C for 48 h, then 10 μM BrdU was added to the lymphoid cell cultures. The cells were incubated for an additional 16 h, and then BrdU incorporation was quantitated by ELISA. Stimulation indices (see Experimental procedures) were calculated using the arithmetic mean values of triplicate cultures. (b) Lymphoid cells (1 × 10^6) were cultured with the indicated amount of OVA in 48-well tissue culture plates for 24 h. The IL-2 levels in culture supernatants were quantified by ELISA. Values are expressed as mean ± SEM from triplicate cultures, and are representative of three independent experiments. Statistical significance is indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.
interaction (Fig. 5d, upper panel). These results suggest that the WASP N-terminal domain has an affinity to WIP in T cells. Furthermore, to demonstrate the Fyn-WIP complex in TCR signaling, immunoprecipitates with anti-WIP antibody were immunoblotted with anti-Fyn pAb. The protein levels of WASP and β-actin in each Tg T cells were examined by immunoblotting. (c) T cell lysates of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were immunoprecipitated (IP) with anti-Fyn mAb. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb, anti-T7 pAb, or anti-Fyn pAb. (d) T cell lysates of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were immunoprecipitated (IP) with anti-WIP antibody. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb, anti-Fyn pAb or anti-WIP pAb. (e) T cells from wild-type, anti-WASP SHL, SVH, and SVL Tg mice were stimulated with anti-CD3ε antibody for the period indicated, lysed, and analyzed by Western blotting with anti-WASP pAb or anti-β-actin pAb. (f) Purified T cells from the spleens of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice with TCR stimulation were lysed and immunoprecipitated (IP) with anti-phosphotyrosine (p-Tyr) mAb. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb or anti-Fyn pAb. Full-length gels/blots are presented in Supplemental Fig. 5. Immunoblots are representative of three independent experiments.

Figure 5 | Inhibition of the WASP-Fyn and WASP-WIP interactions by anti-WASP SHL, SVH, and SVL intrabodies. (a) T cells from the spleens of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were lysed and incubated with GST or GST–Fyn-SH3 fusion protein non-covalently bound to glutathione sepharose beads. Bound proteins were analyzed by Western blotting with anti-WASP pAb or anti-GST pAb. (b) The protein levels of WASP and β-actin in each Tg T cells were examined by immunoblotting. (c) T cell lysates of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were immunoprecipitated (IP) with anti-Fyn mAb. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb, anti-T7 pAb, or anti-Fyn pAb. (d) T cell lysates of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were immunoprecipitated (IP) with anti-WIP antibody. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb, anti-Fyn pAb or anti-WIP pAb. (e) T cells from wild-type, anti-WASP SHL, SVH, and SVL Tg mice were stimulated with anti-CD3ε antibody for the period indicated, lysed, and analyzed by Western blotting with anti-WASP pAb or anti-β-actin pAb. (f) Purified T cells from the spleens of wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice with TCR stimulation were lysed and immunoprecipitated (IP) with anti-phosphotyrosine (p-Tyr) mAb. Immunocomplexes were analyzed by Western blotting with anti-WASP pAb or anti-Fyn pAb. Full-length gels/blots are presented in Supplemental Fig. 5. Immunoblots are representative of three independent experiments.
demonstrated that overexpression of the WASP N-terminal domain and anti-WASP scFv affects TCR-stimulation-induced tyrosine phosphorylation of WASP22. To assess whether the expression of anti-WASP SVH/SVL single domains affects TCR-stimulation-induced tyrosine phosphorylation of WASP was compared among wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg T cells by immunoprecipitation analysis. Tyrosine phosphorylation of WASP upon TCR stimulation was clearly detected in wild-type T cells, but was effectively reduced in anti-WASP SVH/SVL Tg T cells (Fig. 5f, upper panel). In contrast, Fyn was equivalently tyrosine phosphorylated upon TCR stimulation among all T cells from all mouse lines (Fig. 5f, lower panel). These results suggest that overexpression of the WASP N-terminal domain, anti-WASP N-terminus SHL, SVH, or SVL inhibits tyrosine phosphorylation of WASP by interrupting the binding of Fyn-SH3 to the WASP N-terminus, but does not affect the activation of Fyn upon TCR stimulation.

### Discussion

In the present study we demonstrated that intracellularly expressed SVH and SVL single domain intrabodies, the simplest intrabody structure derived from the variable region of the original mAb, retain their specific binding activity to the target signaling molecule and efficiently inhibit the TCR-induced immune response in a Tg mouse model.

Single domain intrabodies have several benefits in comparison to standard intrabodies to study the molecular mechanisms of protein function in the cells. Single domain intrabodies are the simplest in structure, composed of either VH or VL alone, but still work as intrabodies without intradomain disulfide bonds in murine NIH3T3 cells25, and T cells from Tg mice, as demonstrated in the present study. As Tanaka et al.26 suggested regarding anti-Ras VH/VL fragments, intradomain disulfide bonds may not be required for single variable domains to adapt a proper structure that allows them to interact specifically with target molecules in the cytoplasm. Utilization of properly designed single domain intrabodies could circumvent technical problem associated with ordinary intrabodies, such as cleavage of the intradomain disulfide bonds or improper folding of the variable region, mainly due to highly reducing cytoplasmic conditions8.

The leader signal peptide promotes the secretion of the newly synthesized antibodies. However, localization of anti-WASP SHL, SVH, and SVL with their leader sequences was detected not only in the membrane fraction but also in the cytoplasmic fraction in T cells from each Tg mice (Fig. 1e). In addition, anti-WASP SHL, SVH, and SVL were not detected in the T-cell culture supernatant (data not shown). Immunoglobulin heavy and light chains are cotranslocated and assembled with disulfide bonds in the ER lumen. An ER resident chaperon, Bip, binds to the constant region of immunoglobulin and maintains the integrity of the immunoglobulin form during translocation from ER to the secretory pathway27,28. Anti-WASP SHL, SVH, and SVL compose of only the variable regions, but not the constant regions of immunoglobulin, suggesting that the SHL, SVH, and SVL cannot be led to the inherent processing of the newly synthesized immunoglobulin.

Previously, we demonstrated that four types of anti-WASP single domains (SVH, VH, SVL, and VL) were expressed at similar levels regardless of the presence of the leader sequences in NIH3T3 cells29. However, in both murine and human T cells, the expression levels of SVH and SVL constructs with the leader sequences were greatly higher than VH and VL constructs which lack the leader sequences, as similar to anti-WASP scFv constructs (Fig. 1, b and c). These results suggest that the expression pattern of scFv and single domain constructs with or without their leader sequences may be different among cell types. Furthermore, the expression efficiency of the

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**Figure 6 | Impaired nuclear translocation of NFAT after TCR stimulation in T cells from WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice.** TCR-stimulated T cell extracts from wild-type, WASP15 Tg, anti-WASP SHL Tg, SVH Tg, and SVL Tg mice were separated into (a) cytosolic and (b) nuclear protein fractions. The fractionated cell extracts were analyzed by Western blotting with anti-NFATc2 pAb, anti-β-actin pAb, or anti-HDAC1 pAb. Full-length gels/blots are presented in Supplemental Fig. 6. Immunoblots are representative of three independent experiments.
recombinant antibody fragments with or without signal sequences may depend on the character of parental monoclonal antibody clones. T cells from anti-WASP SVH/SVL Tg mice exhibited impaired in IL-2 production upon TCR stimulation, at levels similar to those observed in T cells from anti-WASP SHL Tg mice (Fig. 2). Furthermore, the proliferative response and IL-2 production induced by secondary OVA stimulation were impaired in lymphoid cells from anti-WASP SVH/SVL Tg mice immunized with OVA (Fig. 4, a and b). These results indicate that anti-WASP SVH/SVL single domains inhibit the function of N-terminal domain of WASP in the immune response in T cells as efficiently as their parental anti-WASP scFv. In contrast, actin polymerization and antigen receptor capping in anti-WASP SHL, SVH, and SVL Tg T cells induced by TCR stimulation were unaffected (Fig. S3 and S4). Previously, we demonstrated that dominant negatively expression of WASP N-terminal domain (WASP15) also affected IL-2 production, but not affected TCR capping upon TCR stimulation22. Therefore, the WASP N-terminal domain has a critical role in IL-2 production, but not in actin cytoskeletal rearrangement after TCR signaling. Target-specific inhibition by anti-WASP N-terminal scFv, SVH, and SVL intrabodies strongly implicates the functional N-terminal domain of WASP in the T cell immune response. So, scFv and single domain intrabodies to WASP N-terminal domain would provide valuable tools to understand WASP downstream signaling pathways and function. Furthermore, the use of single domain intrabodies could be considered for potential therapeutic strategy against immunological disorders.

On the other hand, T lymphocytes from WAS patients with mutations in the N-terminal domain exhibit defects of both IL-2 production and cytoskeletal rearrangement upon TCR stimulation, which are similar to that of WASP knockout mice14–16. In addition, expression of WASP was null or barely detectable level in T cells from these WAS patients23. In contrast, the expression level of WASP was comparable between wild-type and Tg mice T cells (Fig. 5e). As de la Fuente et al24. demonstrated, the binding of WIP and WASP is essential for the stability of WASP in T cells, however WIP-WASP interaction was strongly interfered by the anti-WASP intrabodies in Tg mice (Fig. 5d). So, WASP may be somehow stabilized in our transgenic mice. The binding of anti-WASP intrabodies to the N-terminal domain of WASP might stabilize WASP as WIP does in wild-type mice, but this speculation needs to be verified.

Recently, we demonstrated that the WASP N-terminal domain specifically binds to the SH3 domain of Fyn tyrosine kinase in T cells. The interaction between WASP and Fyn was strongly inhibited by overexpression of the WASP N-terminal domain (WASP15) and anti-WASP scFv in T cells from each Tg mouse line25. In T cells from anti-WASP SVH/SVL single domain Tg mice, both SVH and SVL single domains were efficiently expressed and specifically bound to the WASP N-terminal domain (Fig. 1d), and inhibited specific interaction between WASP and Fyn by masking this domain (Fig. 5, a and b). These results indicate that anti-WASP SVH/SVL single domains inhibit the function of N-terminal domain of WASP upon TCR stimulation by the association of activated Fyn with the WASP N-terminal domain in T cells. In addition, T cells from WASP15 Tg mice exhibited impaired nuclear translocation of NFATc2 after TCR stimulation (Fig. 6). Badour et al26. demonstrated that TCR-induced phosphorylation of the WASP tyrosine residue at position 291 in human, and at position 293 in mouse, is required to induce NFAT translocation. Furthermore, by analyzing a series of WASP deletion mutants, Silvin et al27. demonstrated that the WASP homology 1 (WH1)/EVH1 domain, located in the WASP N-terminus, was responsible for NFAT transcriptional activity. Therefore, the specific interaction between Fyn and WASP through the N-terminal domain is necessary for the nuclear translocation of activated NFAT in T cells. The identification of molecules downstream of the Fyn–WASP–WIP complex in the TCR signaling cascade will provide insight into the molecular mechanism underlying IL-2 production in T cells.

As shown in Fig. 2 and 3, the inhibitory effects differ among the intervention strategies exploited: dominant-negative WASP15 exhibited the strongest level of inhibition, while anti-WASP scFv, SVH, and SVL exhibited moderate inhibition of IL-2 synthesis and T cell proliferation upon TCR stimulation. Over-expressed WASP15 specifically binds to the Fyn SH3 domain and strongly inhibits the interaction between Fyn and endogenous WASP. In addition, the Fyn SH3 domain may interact not only with the WASP N-terminus, but also with other proline-rich regions (PRRs) containing signaling molecules that play important roles in TCR signaling. As a result, WASP15 overexpression may broadly interfere the function of Fyn during TCR signaling. Unlike the overexpression of WASP15, anti-WASP scFv, SVH, and SVL intrabodies specifically bind to the WASP N-terminal domain, and masking this domain does not affect the function of the Fyn SH3 domain. Therefore, the difference in the magnitude of inhibition of IL-2 production may be explained by the difference in action mechanisms of the two interventions between WASP15 and anti-WASP N-terminus intrabodies.

Intrabody technology can be used both to elucidate disease mechanisms and to provide novel therapies. Particularly, in the pharmaceutical area, intrabodies have the potential to be a powerful tool for target discovery and validation. Intrabodies offer the possibility of selectively blocking the functions of a target molecule on a domain-specific or epitope-specific basis. Because each single domain intrabody contains only one variable region against the target epitope, the structural analysis of the docking site of the single domain may predict small compounds, which may have biological activity and specificity similar to that of the single domain28. Alternatively, the single domain intrabody itself would work as a therapeutic device after the transfection of intrabody DNA or protein. Regarding the latter case, recent technologies to deliver antibody or enzyme to the inside of target cells have been reported, such as peptide-mediated delivery using protein-transducing peptides (PTDs)29,30, or protein transfection using polyethyleneimine (PEI) as a transmembrane carrier31. Furthermore, the delivery of antibody-conjugated nanoparticles to the cytoplasm interrupted specific cell signaling22,32. Because of its minute size, the single domain fragment itself could easily be delivered into the cytoplasm, and might also be suitable for combining other application tools for target cell delivery. Finally, screening for or designing small-molecule drugs with the biological features of these anti-WASP single domain intrabodies may provide us with new, beneficial immunosuppressive agents.

Methods

Cells and electroporation. Expression vectors pCAG/SHL, HL, SVH, SVL, VH, or VL containing Myc-tagged anti-WASP scFv, VH, or VL constructs with or without the native leader signal sequences have been described previously33,34. The murine T-cell hybridoma DO-11.1038 and Jurkat cell line were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, 50 μM 2-mercaptoethanol (2-ME), 10 mM Hepes (all obtained from Life Technologies, Carlsbad, CA, USA), and 10% fetal calf serum (FCS). DO-11.10 cells and Jurkat cells, adjusted to a concentration of 5 × 106 cells/400 μl culture medium containing 1.25% dimethyl sulfoxide per cuvette, were electroporated using a Gene Pulser (Bio-Rad, Hercules, CA, USA) with 40 μg plasmid DNA at 290V and 960 F.

Generation of transgenic mice. The transgenes were excised from these expression vectors with SalI/NheI restriction enzymes, purified using agarose gel electrophoresis and a QiAquick Gel Extraction kit (Qiagen, Hilden, Germany), adjusted to a final concentration of 3 μg/ml, and microinjected into the fertilized egg pronuclei of inbred C57BL/6J mice. Next the injected eggs were then transferred into the oviducts of pseudopregnant female ICR mice. WASP15 Tg mice and anti-WASP SHL Tg mice were described previously35,36. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee at the National Institute of Agrobiological Sciences (approval ID: H19-001-1).

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Preparation of T cells. T cells were isolated from the spleens of WASP15 Tg, anti-WASP SHL Tg, SV1 Tg, SV1 Tg, and age-matched C57BL/6 mice, and purified by negative selection using microbeads conjugated to mouse CD45R antibody (BioLegend, San Diego, CA, USA).

Quantitative real-time PCR. Purified T cells (1 × 10⁶ cells) were seeded in 100-mm plastic dishes pre-coated with anti-CD3ε antibody (145-2C11, BioLegend) at 20 µg/ml and cultured at 37ºC in RPMI 1640 medium containing 10% fetal calf serum (FCS) for 14 h. RNA from the stimulated T cells was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA). cDNA was obtained using the SuperScript III First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The purity of the resulting cDNA was checked for a minimum of 260 nm absorbance. cDNA (1 µl) was added to 2 µl of Stratagene’s Brilliant SYBR Green PCR Master Mix and 0.2 µl of a primer mixture. PCR for β-actin was included in triplicate in each sample. The PCR conditions were 95ºC for 15 s followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s, and 72ºC for 1 min. The primer sequences are as follows:

- Forward primer: 5’-TTGATGGACCTACAGGATGC-3’
- Reverse primer: 5’-ATCCTGGGATGTTAGCTTA-3’

Statistical analysis. Statistical significance was assessed using Student’s t-test (p-values < 0.05).

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Acknowledgments

This work was supported in part by the NIAS Strategic Research Fund and the Animal Genome project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

Author contributions

M.S. and H.K. conceived and designed the experiments. M.S. and R.S. performed the experiments. C.S. and T.T. assisted with experiments and commented on the manuscript. M.S. and H.K. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sato, M., Sawahata, R., Sakuma, C., Takenouchi, T. & Kitani, H. Single domain intrabodies against WASP inhibit TCR-induced immune responses in transgenic mice T cells. *Sci. Rep.* **3**, 3003; DOI:10.1038/srep03003 (2013).

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