HSP90α deficiency does not affect immunoglobulin gene hypermutation and class switch but causes enhanced MHC class II antigen presentation

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Abstract

Heat shock protein 90 (HSP90) is a molecular chaperone required for efficient antigen presentation and cross-presentation. In addition, HSP90 was recently reported to interact with and stabilize the activation-induced cytidine deaminase (AID) and plays a critical role in immunoglobulin gene hypermutation and class switch recombination. In mice and humans, there are two HSP90 isoforms, HSP90α and HSP90β, but the in vivo role of each isoform remains largely unknown. Here we have analyzed humoral immune responses in HSP90α-deficient mice. We found that HSP90α deficiency did not affect AID protein expression. B cell development and maturation, as well as immunoglobulin gene hypermutation and class switch, occurred normally in HSP90α-deficient mice. However, antibody production to a T-dependent antigen was elevated in the mutant mice and this was associated with enhanced MHC class II antigen presentation to T helper cells by dendritic cells. Our results reveal a previously unidentified inhibitory role for HSP90α in MHC class II antigen presentation and the humoral immune response. Along with our recent finding that HSP90α is required for antigen cross-presentation, these results suggest that HSP90α controls the balance of humoral and cellular immunity by dictating the fate of presentation of exogenous antigen.

Keywords: antigen presentation, class switch recombination, heat shock protein 90, humoral immune response, Ig gene hypermutation

Introduction

Heat shock protein 90 (HSP90) is a highly conserved molecular chaperone that functions in the folding, stabilization and degradation of a wide range of cellular proteins (1) and has been shown to play important roles in facilitating MHC class I and class II antigen presentation and cross-presentation (2, 3). More recently, HSP90 was reported to associate with the activation-induced cytidine deaminase (AID) (4), an enzyme essential for immunoglobulin gene hypermutation and class switch recombination (CSR) (5). Inhibition of HSP90 chaperone activity by geldanamycin or 17-demethoxygeldanamycin caused the ubiquitination and degradation of AID and reduced immunoglobulin gene hypermutation and CSR in B cells (4). In mice and humans, there are two HSP90 isoforms, HSP90α and HSP90β, encoded by the Hsp90aa1 and Hsp90ab1 genes, respectively (6). HSP90α and HSP90β are highly homologous, with 86% identity and 94% similarity at the amino acid level. Although the function of HSP90 has been extensively studied by using HSP90 inhibitors (7–13), these inhibitors inactivate both HSP90α and HSP90β, and the role of each HSP90 isoform remains largely unknown. HSP90α-deficient mice have recently been generated (14,15). The mutant mice developed normally with no apparent gross abnormalities, but homozygotes were sterile due to a block in meiotic progression during spermatogenesis.
cloned into the pCR2.1 vector for sequencing. Only unique sequences were analyzed in each mouse.

**Immunoblot analysis**

Splenocytes (1 x 10^6/ml) were cultured for 2 days in the presence of 50 μg/ml of LPS. The cells were then lysed on ice for 30 min in PBS containing 0.5% NP-40 and a protease inhibitor cocktail (1xComplete, Roche Diagnostics, Germany). The lysate was resolved in a 12.5% SDS PAGE and transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with Block Ace (DS Pharma Biomedical) at 4°C overnight, incubated with rat antibodies against AID (MAID-2, ebiosciences) followed by HRP-conjugated anti-rat IgG antibodies (#712-03S-15S, Jackson Laboratories) and developed with ECL Plus (GE Health).

**Antigen presentation assay**

Splenic B cells and DCs isolated from 15–19-week-old WT and Hsp90aa1−/− mice were analyzed for their ability to present ovalbumin (OVA) to Ob4, a T cell hybridoma specific for the OVApeptide present by H2-Aβ (19). B cells were purified using negative sorting with the IMag B Lymphocyte Enrichment Set except that biotinylated anti-CD11c Ab was included in the antibody cocktail to eliminate possible contamination by DCs. DCs were isolated using negative sorting with the IMag Dendritic Cell Enrichment Set (BD Biosciences). Ob4 T cells (5 x 10^4, 100 μl) were mixed with spleen B cells (5 x 10^4, 100 μl) or DCs (1 x 10^4, 100 μl) in 96-well U-bottom plates and OVA was then added at the indicated concentrations. The OVApeptide peptide was used as a positive control. The cells were cultured for 16 h and the amount of IL-2 in the supernatant was analyzed by ELISA.

**Results**

Normal B and T cell development and maturation in Hsp90aa1−/− mice

Total cell numbers in the bone marrow (BM), thymus and spleen were not significantly different between WT and Hsp90aa1−/− mice (Supplementary Table I is available at International Immunology Online). FACS analysis of BM cells from WT and Hsp90aa1−/− mice revealed a similar proportion of Pro-B (B220⁺CD43⁺IgM⁻), Pre-B (B220⁺CD43⁻IgM⁻), immature B (B220⁺CD19⁺IgM⁺) and re-circulating B (B220⁺CD19⁺IgM⁺) cells (Fig. 1A, upper and middle panels). The proportion of follicular (CD23⁺CD21⁺) and marginal zone (CD23⁺CD21⁺) B cells in the spleen was also similar between WT and Hsp90aa1−/− mice (Fig. 1A, lower panels). T cell development and differentiation appeared normal in Hsp90aa1−/− mice as

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*Role of HSP90α in the humoral immune response*

We found that HSP90α was required for efficient antigen cross-presentation in dendritic cells (DCs) by facilitating the cytosolic translocation of extracellular antigen (15). However, the effect of HSP90α ablation in MHC class II antigen presentation as well as immunoglobulin gene hypermutation and CSR is completely unknown.

In this study, we have analyzed the humoral immune response in HSP90α-deficient mice. We show that the absence of HSP90α did not affect B-cell development and maturation, or immunoglobulin gene hypermutation and CSR. However, antibody (Ab) production to a T-dependent (TD) antigen was elevated. Further analysis revealed that HSP90α-deficient DCs, but not B cells, showed enhanced MHC class II antigen presentation to T helper cells. Our results thus reveal a unique inhibitory role for the HSP90α isoform in MHC class II antigen presentation and in the humoral immune response.

**Methods**

**HSP90α-deficient mice**

The generation of HSP90α-deficient (Hsp90aa1−/+ ) mice in the C57BL/6 background has been described (15). The mice were maintained under specific pathogen-free conditions and all experiments were approved by the Animal Facility Committee of RIKEN Yokohama Institute (Permission number 20-025).

**FACS analysis, B and T cell purification, proliferation and class switch assays**

Antibodies used for flow cytometry analysis and cell culture have been described (16). Spleen B or T cells were purified using negative sorting with the IMag B or T Lymphocyte Enrichment Set (BD Biosciences, Mountain View, CA, USA), respectively. For proliferation assays, purified spleen B cells (5 x 10^5/ml, 100 μl) were seeded in 96-well flat-bottom plates and cultured in the presence of various stimuli. The cells were cultured for 2 days and pulsed for the last 6 h with ³H-thymidine (1 μCi/well). The mean ³H-thymidine incorporation and standard deviation were calculated for quadruplicate cultures. For CSR assays, purified spleen B cells were cultured in duplicate in 12-well plates (5 x 10^5/ml, 1 ml) in the presence either of LPS (10 μg/ml) plus IL-4 (20 ng/ml) or of CD40L (1:3 dilution) plus IL-4 (20 ng/ml) for 3 days and analyzed for IgG expression by FACS. T cell proliferation was performed as described previously (17).

**Somatic hypermutation of immunoglobulin genes**

Three Hsp90aa1−/+ and two wild type (WT) littermates (12 week old) were injected i.p. with 100 μg of 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken γ globulin (NP-CGG) precipitated with alum. Two weeks later, B220⁺PNA⁺ germinal center B cells were sorted from the spleen of each mouse and genomic DNA was extracted. The J₅₄ intronic region was amplified with forward primer J558Fr3 (5'-CAGCTTGACATCTGAGGACTTCGC-3') and reverse primer JHCHint (5'-TCCACCGACTCTCTGAGCACG-3') as described previously (18). PCR was carried out with KOD plus polymerase (Toyobo Ltd., Japan) under the following conditions: 94 °C for 5 min and then 94 °C for 20 s, 65 °C for 30 s and 68 °C for 70 s for 30 cycles. The PCR products were

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judged by CD4 and CD8 profiles in the thymus and spleen (Fig. 1B). Hsp90aa1<sup>−/−</sup> B cells exhibited reduced proliferative responses to anti-IgM stimulation but normal responses to CD40 ligation or LPS stimulation (Fig. 1C). Hsp90aa1<sup>−/−</sup> T cells responded normally to different doses of anti-CD3 or anti-CD3 + anti-CD28 stimulation (Supplementary Fig. 1 is available at International Immunology Online). These results collectively suggest that the absence of HSP90α had minimal effect on B- and T-cell development and maturation.

Normal immunoglobulin gene CSR and hypermutation in Hsp90aa1<sup>−/−</sup> mice

HSP90 was recently reported to associate with and stabilize AID and to be required for efficient immunoglobulin gene CSR and hypermutation (4). We found that WT and Hsp90aa1<sup>−/−</sup> spleen cells stimulated with LPS expressed similar levels of AID protein (Fig. 2A). Consistently, Hsp90aa1<sup>−/−</sup> spleen B cells switched normally from IgM to IgG<sub>1</sub> upon stimulation with either CD40L + IL-4 (Fig. 2B) or LPS + IL-4 (Fig. 2C). In addition, serum levels of each antibody isotype were similar between WT and HSP90aa1<sup>−/−</sup> mice (Supplementary Fig. 2, available at International Immunology Online). These results suggest that HSP90α is not essential for immunoglobulin gene CSR.

To address the role of HSP90α in immunoglobulin gene hypermutation, we analyzed the mutations in the J<sub>H4</sub> intronic region of the GC B cells isolated from spleens of immunized mice. We chose the J<sub>H4</sub> intronic region because mutations in this region were not selected by antigen and therefore

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**Fig. 1.** Normal B- and T-cell development and maturation in Hsp90aa1<sup>−/−</sup> mice. (A) FACS profiles of gated B220<sup>+</sup> cells in the BM (upper and middle panels) and spleen (lower panels). (B) CD4 and CD8 profiles of thymocytes (upper panels) and splenocytes (lower panels). (C) Proliferative responses of purified spleen B cells to various stimuli. Representative results of three pairs of mice (11–12 weeks old) are shown. *P < 0.05 (unpaired t-test).
represent unbiased mutations. We have analyzed two WT and three Hsp90aa1−/− mice and the results are summarized in Table 1 and Fig. 2D. No obvious differences were observed between WT and Hsp90aa1−/− mice in terms of the percentage of mutated sequences, total mutation frequency or the frequency at C/G and A/T pairs (Table 1). There was also no significant difference in the relative proportion of each type of base substitution (Fig. 2D). These results demonstrate that absence of HSP90α did not affect the stability and function of AID.

Fig. 2. Normal immunoglobulin gene CSR and hypermutation in Hsp90aa1−/− mice. (A) WT and Hsp90aa1−/− spleen cells expressed similar levels of AID protein upon LPS stimulation. Splenocytes cultured for two days in the presence of 50 μg/ml of LPS were analyzed for AID expression by immunoblot as described in Methods. Fresh splenocytes (Day 0) were used as a control. AID band intensities relative to β-actin are indicated. Similar results were obtained in two separate experiments. (B) and (C) Normal Ig gene CSR. Spleen B cells purified from 12-week-old mice were cultured for 3 days with CD40L + IL-4 (B) or LPS + IL-4 (C) as described in Methods. Left panels, FACS profiles after 3-day culture; right panel, kinetics of Ig gene CSR in WT and Hsp90aa1−/− B cells. The averages ± SD of two independent experiments (each in duplicate cultures) are shown. (D) Normal mutation patterns in the JH4 intronic region of the germinal center B cells in Hsp90aa1−/− mice. Relative representation of each type of base substitution is shown. The data were corrected for base composition of the 509-bp JH4 intronic region (A, 26.92%; T, 31.04%; C, 14.14%; G, 27.90%).
Elevated antibody production to a TD antigen in Hsp90aa1−/− mice

Consistent with the decreased responses to anti-IgM stimulation in Hsp90aa1−/− B cells (Fig. 1C), Hsp90aa1−/− mice produced a slightly reduced amount of NP-specific IgM and IgG3 Ab in response to the TI Ag NP-Ficoll (Fig. 3A), although the differences did not reach statistical significance. In contrast, when mice were immunized with the TD Ag NP-CGG, the production of the NP-specific IgG3 antibodies was significantly increased in Hsp90aa1−/− mice as compared with WT controls (Fig. 3B).

Hsp90aa1−/− DCs show enhanced antigen presentation to T helper cells

Successful humoral immune responses against TD antigen require the intimate interactions and the coordinated functions of T cells, B cells and DCs (20–22). Antigen presentation to T helper cells by B and DCs is a critical process for antibody production to TD antigen. We therefore analyzed MHC class II antigen presentation by freshly isolated splenic B cells or DCs. We used the H2-A�-restricted Ob4 T cell hybridoma specific for OVA266–277 peptide as responder cells. While Hsp90aa1−/− spleen B cells gave a similar dose-dependent response as did WT spleen B cells (Fig. 4A), Hsp90aa1−/− DCs exhibited enhanced presentation of OVA compared with WT DCs, especially at low concentrations of OVA (Fig. 4B). As a control, WT and Hsp90aa1−/− DCs pulsed with two different doses of OVA266–277 peptide induced comparable

### Table 1. Mutation frequency in WT and Hsp90aa1−/− mice

<table>
<thead>
<tr>
<th>JH4 intron (509bp)</th>
<th>WT (2 mice)</th>
<th>Hsp90aa1−/− (3 mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences</td>
<td>188</td>
<td>251</td>
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<tr>
<td>Mutated sequences (%)</td>
<td>110 (58.5%)</td>
<td>166 (66.1%)</td>
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<td>Total length of mutated sequences</td>
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<td>84,494</td>
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<td>Total number of mutations</td>
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<td>Overall mutation frequencya (×10−2/bp)</td>
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<td>Mutation frequency at C:G (×10−2/bp)</td>
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<td>0.31</td>
</tr>
<tr>
<td>Mutation frequency at A:T (%)</td>
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<tr>
<td>% mutation at C/G/A/T</td>
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<td>41.3:58.7</td>
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<tr>
<td>Total mutation frequencyb (×10−2/bp)</td>
<td>0.49</td>
<td>0.49</td>
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aTotal number of mutations divided by the length of mutated sequences.

bNumber of mutations divided by the length of total sequences.

**Fig. 3.** Elevated Ab production against TD Ag in Hsp90aa1−/− mice. (A) Responses to the TI Ag NP-Ficoll. The levels of NP-specific IgM (upper panel) and IgG3 (lower panel) in the serum are shown. (B) Responses to the TD Ag NP-CGG. The titers of NP-specific IgG1 are shown. Open and solid circles represent individual WT and Hsp90aa1−/− mice and a bar indicates the average. *P < 0.05 (unpaired t-test).
IL-2 production by Ob4 T cells (Fig. 4B), indicating that direct loading of the OVA peptide on MHC class II molecules was not affected by HSP90α-deficiency. It should be noted that DCs isolated from WT and Hsp90aa1−/− mice contained a similar proportion of CD11c high cells (Supplementary Fig. 3, available at International Immunology Online) and expressed similar levels of MHC class II, CD40, CD80 and CD86 (Fig. 4C). These results suggest that the enhanced presentation of OVA by Hsp90aa1−/− DCs was not due to elevated levels of MHC class II or co-stimulatory molecules but likely due to enhanced processing of OVA.

Discussion

HSP90 was recently shown to associate with and stabilize AID. Inhibition of HSP90 chaperone activity resulted in decreased AID levels and reduced immunoglobulin gene hypermutation and CSR in B cells (4). As HSP90 inhibitors inactivated both HSP90α and HSP90β, it was unclear whether HSP90α and HSP90β are both required for stabilizing AID. This study demonstrates that HSP90α is dispensable for immunoglobulin gene CSR and hypermutation, thereby suggesting that HSP90β alone is sufficient for maintaining the stability and function of AID. This conclusion is consistent with the previous finding that HSP90α and HSP90β can each independently associate with AID (4). Therefore, HSP90α and HSP90β appear to be functionally redundant in the regulation of AID stability. Immunoglobulin gene hypermutation is initiated by AID, which is thought to convert cytidine to uracil and generate a U/G mismatch on DNA. Mutations are introduced during replication and repair of the AID-triggered U/G lesions (23–26). In particular, the generation of mutations at A/T pairs is predominantly mediated by DNA polymerase η (POLH) (27–32) while the induction of C to G and G to C transversions is largely dependent on the deoxycytidyl transferase REV1 (17, 33, 34). The mechanism by which POLH and REV1 are recruited to the immunoglobulin gene loci to participate in the hypermutation processes remains unclear. Recently, HSP90 was shown to physically interact with and regulate the stability of POLH and REV1 and to be required for the recruitment of these molecules to the replication stalling sites in UV-damaged cells (35, 36). The normal frequency and patterns of A/T mutations as well as normal C to G and G to C transversion mutations in HSP90α-deficient mice, as revealed in this study, suggest that HSP90α is not required for the recruitment of POLH and REV1 during immunoglobulin gene hypermutation.

HSP90α-deficient DCs showed enhanced MHC class II antigen presentation compared with WT DCs. Notably, this enhancement was more prominent at low doses of OVA. Previous studies have shown that HSP90 inhibitors reduced MHC class II antigen presentation in DCs and macrophages suggesting that HSP90 positively regulates antigen presentation (12,13). In these experiments,
however, the activities of both HSP90α and HSP90β were affected and in some cases the expression of MHC class II and co-stimulatory molecules was also down-modulated. In a more recent study, siRNA-mediated knockdown of either HSP90α or HSP90β in human B cells was shown to inhibit MHC class II presentation of the diabetes autoantigen glutamic acid decarboxylase but did not affect the presentation of human serum albumin or the endogenous immunoglobulin κ chain (37). These results again suggest that both HSP90α and HSP90β are required for efficient MHC class II presentation in an antigen-specific manner. It should be noted that substantial residual HSP90α and HSP90β were expressed in the knockdown cells in these experiments (37). In contrast to these previous studies using HSP90 inhibitors or siRNA-mediated knockdown, the HSP90α-deficient DCs completely lack HSP90α isoform expression while having normal levels of HSP90β. MHC class II and co-stimulatory molecules (this study and reference 15). Our results clearly demonstrate that the HSP90α isoform does not facilitate, but inhibits, MHC class II presentation of OVA in DCs. It remains to be determined whether HSP90α also inhibits the presentation of other antigens or has different effect on MHC class II antigen presentation depending on the type of the antigen, for example, self versus non-self and extracellular versus intracellular antigen, and the route of antigen internalization.

We have recently found that HSP90α-deficient DCs show reduced cross-presentation of extracellular antigen (15). Detailed biochemical analysis revealed that HSP90α promoted the cytosolic translocation of extracellular antigen out of the endosomal compartment. The elevated MHC class II antigen presentation and decreased cross-presentation in HSP90α-deficient DCs suggest that HSP90α facilitates cross-presentation at the cost of MHC class II presentation. HSP90α thus controls the balance of humoral and cellular immunity by dictating the fate of presentation of exogenous antigen. The skewing of antigen presentation in HSP90α-deficient DCs in favor of the MHC class II pathway may suggest that HSP90α and HSP90β have distinct roles such that the lack of HSP90α cannot be fully compensated by HSP90β. Alternatively, HSP90α and HSP90β may be functionally equivalent, but sufficiently high levels of the total cytosolic HSP90 (HSP90α + HSP90β) are required for efficient antigen cross-presentation and for limiting MHC class II antigen presentation. In the latter case, lack of either HSP90α or HSP90β should result in a similar enhancement of MHC class II antigen presentation. It would be informative to analyze antigen presentation as well as immunoglobulin gene hypermutation and CSR in mice with conditional inactivation of HSP90β with or without HSP90α deficiency. Further studies are required to completely reveal the diverse functions of HSP90 in cells of the innate and adaptive immune systems. In conclusion, this study identified a unique inhibitory role for the HSP90α isoform in MHC class II antigen presentation and the humoral immune response.

Supplementary data
Supplementary data are available at International Immunology Online.

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