Effect of Osteopontin Alleles on β -Glucan-Induced Granuloma Formation in the Mouse Liver

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The granuloma formation is a host defense response against persistent irritants. Osteopontin is centrally involved in the formation of granulomas. Three osteopontin alleles, designated a, b, and c, have been found in mice. Here we used a murine model of zymosan (β -glucan)-induced granuloma formation in the liver to determine possible functional differences between the osteopontin alleles in cell-mediated immunity. In contrast to mice with alleles a or c, mice with the allele b was defective in granuloma formation. As detected by mRNA expression, cytokines and chemokines known to be critically involved in granuloma formation were elicited in liver tissue, regardless of the osteopontin allele expressed. Alignment of the deduced amino acid sequences showed that unlike osteopontin c, b differs from a in 11 amino acids. All three osteopontin alleles had normal cell-binding properties. However, only the b allelic form was defective in the induction of cell migration as tested with dendritic cells. In conclusion, generation of a granulomatous response in mice depends critically on the presence of a functional osteopontin allele. Defective granuloma formation in mice with allele b is likely to be because of an impaired chemotactic function of the osteopontin b protein on immuno-competent cells. (*Am J Pathol* 2004, 164:567–575)

The granulomatous response is one form of chronic inflammation in which monocytes, macrophages, epithelioid cells, and multinucleated giant cells are involved and is the central mechanism to defend the host against persistent irritants.¹ Zymosan is the cell wall fraction of yeast containing an insoluble form of β -1,3-glucan and induces foreign body type hepatic granulomas after intravenous injection.^{2,3} Accumulation of monocytes and macrophages into the site of inflammation is affected by

at least two factors. The migration of monocytes from the circulation is promoted by chemotactic agents such as chemokines, complement components, and microbial products whereas the immobilization of macrophages within the lesion is mediated by cell adhesion factors.^{4,5}

Osteopontin (OPN) is a phosphorylated glycoprotein containing an arginine-glycine-aspartic acid (RGD) integrin-binding motif and is implicated in a variety of functions, including cell adhesion and migration.^{6–8} It is secreted by macrophages, T cells, and natural killer cells in a regulated manner.^{9–11} Recent studies have provided ample evidence that OPN has cytokine functions and plays a crucial role in various inflammatory and immune responses.^{12–17} The OPN protein is expressed during the formation of granulomatous lesions including tuberculosis and sarcoidosis and has been defined as an important factor during the formation of granulomas because OPN-deficient mice failed to develop granulomas after injection of polyvinylpyrrolidone.^{18–22} Resistance against various pathogens has been shown to be dependent on the presence of OPN.^{18,22–24} Nau and colleagues²⁵ demonstrated a reduced clearance of *Mycobacterium bovis* bacillus Calmette-Guérin in OPN-deficient mice. Three allelic forms of the murine OPN gene have been identified and the resistance to *Rickettsia tsutsugamushi* infection varies depending on the type of the OPN allele.²⁴ Thus, the presence or absence of OPN and the type of the OPN protein expressed decisively affects the outcome of host immune and inflammatory responses. This is further underlined by the finding that a number of single-nucleotide polymorphisms are present in the human OPN gene and interestingly an OPN polymorphism is associated with systemic lupus erythematosus.²⁶

Using the zymosan model we characterized the functional role of OPN alleles for the formation of liver foreign body granulomas. We found that in mice expressing OPN allele b granuloma formation was defective, compared to mice with alleles a or c. Zymosan injection induces the expression of inducible nitric oxide synthase (iNOS) mRNA only in the liver of mice with OPN a or c. The mRNA

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expression of the cytokines and chemokines involved in granuloma formation were similarly elicited in all groups. OPN c differs from a in only one, whereas OPN b differs from c in 10 amino acids. Functionally the allelic b form is defective in the induction of dendritic cell (DC) migration, but functions as a cell adhesive protein. These results suggest that granuloma formation in mice depends critically on the presence of a functional OPN allele. Defective granuloma formation in mice with OPN allele b seems to be associated with an impaired chemotactic function on immunocompetent cells. The functional impairment of OPN allele b may result from its various mutations, one of which locates close to the RGD sequence.

Materials and Methods

Animals

Male C57BL/6CrSlc (B6), BALB/cCrSlc (BALB/C), and C3H/HeJ (C3H) mice (specific pathogen-free) were purchased from SLC Japan (Shizuoka, Japan), and male CBA/JNCrj (CBA), SJL/JorlIcoCrj (SJL), and DBA/2NCrj (DBA) mice (specific pathogen-free) were purchased from Charles River Laboratories Japan.

Cell Lines

The highly metastatic B16-BL6 melanoma cell line²⁷ originally provided by Dr. I.J. Fidler, M.D. Anderson Cancer Center, Houston, TX, and the murine fibroblastic cell line L929²⁸ were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum.

Experimental Granuloma Formation

Zymosan (Sigma) was injected intravenously as a single dose of 750 μ g/head suspended in 200 μ l of sterile saline. Mice were sacrificed 7, 14, 21, and 28 days after zymosan injection and liver tissue was analyzed by histology and gene expression. Livers were longitudinally sectioned to obtain the possible largest area. Sections were stained with hematoxylin and eosin (H&E). The granuloma area (mm²) and liver area (mm²) per section were determined by NIH image 1.55 (public domain software). The percentage of granuloma area was calculated by the formula: granuloma area per section/liver area per section $\times$ 100.

RNase Protection Assays

Total RNA was prepared from murine liver using Trizol Reagent (Life Technologies). Twenty μ g of total RNA from each sample were hybridized to ³²P-labeled anti-sense RNA probes *in vitro*-transcribed from multi-probe template sets, mCK-2b, mCK-4, and mCK-5 (PharMingen) by the RiboQuant multi-probe RNase protection assay system as outlined by the supplier.

Quantitative Analysis on mRNA Expression

One μ g of total RNA was reverse-transcribed by using ReverTra Ace (Toyobo) in a total reaction volume of 20 μ l. The cDNAs were used as templates for polymerase chain reaction (PCR) using LightCycler-FastStart DNA Master SYBR Green I systems (Roche Diagnostics).

The primers used were: OPN (468-bp product): sense, 5'-ACGACCATGAGATTGGCAGTG-3'; anti-sense, 5'-TTACCTCAGTCCATAAGCCAA-3'; G3PDH (452-bp product): sense, 5'-ACCACAGTCCATGCCATCAC-3'; anti-sense, 5'-TCCACCACCCTGTTGCTGTA-3'; iNOS (213-bp product): sense, 5'-GCTTGCCCCCTGGAAGTTT-3'; anti-sense, 5'-CCTCACATACTGTGGACG-3'; β actin (349-bp product): sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; anti-sense, 5'-TAAACGCAGCTCAGTAACAGTCCG-3'.

Library Construction, Screening, and Sequencing

RNA was prepared from kidney of DBA mice by using Trizol Reagent. Polyadenylated RNA [poly(A)RNA] was isolated by mRNA purification kit (Amersham Pharmacia). RNA from indicated fractions was combined, concentrated by ethanol precipitation, and used as a template to synthesize a cDNA library by using SuperScript Choice System for cDNA Synthesis (Gibco BRL) and ligated into the phage vector, λ ZipLox (Gibco BRL). *Escherichia coli* strain, Y1090(ZL) (Gibco BRL) was used as the host for library plating. A total of 200,000 plaque-forming units (pfu) were screened with DNA probes labeled with [α -³²P]dCTP, using the Multiprime kit (Amersham Pharmacia). The cDNA library was plated at a density of 10,000 pfu per 95 $\times$ 135-mm plate. The GeneScreen Plus membranes (DuPont) were used for plaque hybridization screening. Plasmid pZL1-containing inserts were excised *in vivo* from independently selected six recombinant phages by introducing into the *E. coli* DH10B(ZIP) strain (Gibco BRL). The nucleotide sequences were obtained by electrophoresis on an automated DNA sequencer (model 373A, Applied Biosystems).

Construction of the GST-OPN Fusion Plasmid

PCR reactions were conducted using the cloned c allelic form of OPN (designated as OPN c hereafter) cDNA vector as a template, directed by primers: sense, 5'-GGATCCTCCCGGTGAAAGTG-3'; anti-sense, 5'-TTAGTTGACCTCAGAAGATGAAC-3'.

The PCR product was purified and cloned into pCRII vector as previously described.²⁹ The cloned cDNA was completely sequenced and inserted into the pGEX-3X vector in the same reading frame as a carrier gene (glutathione S-transferase, EC 2.5.1.18)³⁰ and transformed into *E. coli* DH5 α cells.

Protein Purification

The recombinant GST-OPN fusion proteins, namely OPN a, OPN b, and OPN c were prepared in *E. coli* as described previously.³¹ The GST fusion proteins were purified on glutathione-Sepharose columns as described.³⁰ The eluted proteins were subjected to gel filtration on a Sephadex G-25 column equilibrated with phosphate-buffered saline (PBS) and eluted with the same buffer. The purity of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Laemmli's system.

Cell Binding Assay

Plates (96 well) were precoated with various concentrations of GST and GST-OPN for 1 hour at 37°C, followed by treatment with 0.5% bovine serum albumin in RPMI 1640 medium for 10 minutes at 37°C to block nonspecific binding. Cells were suspended in Dulbecco's minimal essential medium containing 0.25% bovine serum albumin and 200 μ l of cell suspension (at a cell density of 5×10^4 cells/well) were applied to the 96-well plate and incubated for 1 hour at 37°C. Then the medium was removed from the plates and wells were washed with PBS twice. The adherent cells were fixed and stained with 0.5% crystal violet in 20% methanol for 30 minutes. All wells were rinsed three times with water and adherent cells were then lysed with 20% acetic acid. Absorbance at 595 nm was measured from the resulting supernatants from each well, using an immunoreader to determine the relative number of cells attached per well. All assays were performed in triplicate and at least three separate experiments were performed to obtain data.

In Vitro DC Migration Assay

In vitro DC migration was evaluated using a 48-well micro chemotaxis chamber (Costar) and cultured DCs as described previously.³² Murine DCs were generated as described previously.³² In brief, bone marrow cells from C57BL/6 mice with OPN a allele, suspended at a cell density of 10^6 /ml were cultured in the presence of 40 ng/ml of murine granulocyte macrophage colony-stimulating factor (PromoCell) and 10 ng/ml of interleukin (IL)-4 (PromoCell). On day 6, loosely adherent cells were recovered and fractionated by gradient centrifugation using 14.5% Metrizamide (Boehringer). The low-buoyant density cells were collected and washed. More than 80% of those cells expressed class II, CD11c, B7-1, and B7-2 and thus were designated as DCs.³³ Twenty-seven μ l of OPN at the indicated concentrations in Hanks' balanced salt solution, were added to the lower chamber. A polycarbonate filter (pore size 8 μ m, Costar) was layered onto the wells, covered by a silicon gasket, and followed by the top plate. For migration of DCs, 50 μ l of cell suspension (10^6 cells/ml) were added to the upper chamber. After the incubation (37°C, 5% CO₂ for 4 hours) filters were removed and stained with Diff-Quick (Baxter). Cells on the upper filter surface were removed, cells that had

Figure 1. Mice with OPN alleles a or c, but not b form zymosan-induced liver granulomas. Liver was obtained from mice with OPN allele a (B6 and BALB/c), OPN allele c (DBA), and OPN b allele (C3H, CBA, and SJL) at days 7, 14, and 21 after zymosan injection. The degree of granuloma per liver section was analyzed with NIH Image software and the percentage of granuloma area was calculated by the formula: granuloma (μm^2)/total liver area (μm^2) $\times$ 100. The results are shown as mean percentage of granuloma area $\pm$ SD of six different sections from three different mice (two sections per mice). ND, BALB/c liver tissues were not studied on day 14.

migrated to the filter bottom quantified counting four ocular grids.³⁴ Results are expressed as mean number of migrated cells/mm² $\pm$ SD of four chambers. Each experiment was performed in triplicate.

Results

OPN Allele b Mice Have an Impaired Formation of Zymosan-Induced Granulomas

Several reports indicated that allelic variations of OPN affect the susceptibility to infection.^{9,24,35} To determine whether the presence of a specific OPN allele affects the host immune responses *in vivo*, we studied the liver granuloma formation by intravenously injected zymosan in B6, BALB/c, C3H, CBA, SJL, and DBA mice. On day 7 marked liver granuloma formation was detected in B6 and BALB/c mice that express OPN allele a and DBA mice with OPN allele c. From days 14 to 21 the number of granulomas significantly decreased. By contrast in C3H, CBA, and SJL mice, which all express OPN allele b, a drastically reduced liver granuloma formation was detected on day 7 (Figure 1). In the following observation period from day 14 to day 21, formation of granuloma remained impaired, indicating that the reduced granulomatous response to zymosan in allele b mice was not because of a delay in granuloma formation. No significant granuloma formation was detected in C3H mice at day 28. Representative histology was shown in Figure 2. In addition, we found no significant histological difference in granuloma among mice of three OPN alleles at day 14 (Figure 2; J to L, with high-power views).

Induction of Chemokine Gene Expression Does Not Correlate with the Degree of Granuloma Formation

The regulated expression of a variety of cytokines and chemokines has been associated with granuloma formation. We set out to determine whether such cytokine expression patterns are affected by the presence of a certain OPN allele. mRNA expression of IL-1 β , macro-

Figure 2. Foreign body-type granuloma formation is reduced in mice with OPN allele b. H&E staining of liver sections obtained from mice with OPN a allele, B6 (**A, B, C, J**); OPN b allele, C3H (**D, E, F, K**); and OPN c allele, DBA (**G, H, I, L**) at 7 (**A, G**), 14 (**B, D, H, J, K, L**), 21 (**C, E, I**), and 28 (**F**) days after zymosan administration. Original magnifications: $\times 100$ (**A–I**); $\times 400$ (**J–L**).

phage colony stimulating factor (M-CSF), regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1 β (MIP-1 β), MIP-2, and monocyte chemoattractant protein-1 (MCP-1) were found to be similarly elicited in all animals strains after zymosan injection, independent of their OPN allelism (Figure 3). The only difference between OPN a allele mice and OPN b allele mice was an expression of IL-1 α that is not increased in the liver of C3H and CBA mice with OPN b allele, in which no significant granuloma formation was detected. However, significant induction of the IL-1 α gene was detected in SJL mice that also express the OPN b allele (Figure 3, A and C). Interestingly, mRNA of macrophage migration inhibitory factor (MIF) was constitutively expressed in all strains and did not

vary before and after zymosan injection. Our findings indicate that despite an inhibited granuloma formation in OPN allele b mice the expression of cytokine and chemokine genes did not differ from that of mice with normal granulomatous response.

iNOS mRNA Expression Is Defective in OPN Allele b Mice

iNOS is known to be up-regulated in macrophages on their activation in inflammatory responses. Previously it has been demonstrated that OPN is a negative feedback regulator of iNOS in murine macrophages *in vitro*.³⁶ We were interested in how iNOS expression is differentially

Figure 3. The OPN allelic form does not affect the cytokine or chemokine pattern expressed after zymosan accumulation in the liver. Total RNA was prepared from murine liver and 20 μ g of each sample were hybridized to 32 P-labeled anti-sense RNA probes that were *in vitro* transcribed from multi-probe template sets, mCK-2b (A, C) and mCK-4 and mCK-5 (B) (PharMingen) by using the RiboQuant Multi-Probe RNase Protection Assay Systems. The results are expressed as percentage of cytokine/chemokine expression, calculated by the formula: cytokine band intensity/GAPDH band intensity $\times$ 100. Mean data of two different mice at each time point (day 0, 1, 2, 3, and 7 after zymosan injection) are shown (A, B). Representative original data are shown in C.

regulated during granuloma formation and how its expression is affected by OPN alleles. On examination of iNOS mRNA expression in the liver we found an iNOS mRNA augmentation in mice with OPN a or c alleles after administration of zymosan. However, no significant induction of iNOS mRNA was detected in mice with OPN b allele (Figure 4A). In sharp contrast, OPN mRNA expression was augmented after zymosan injection in all allelic forms of mice examined (Figure 4B). Our findings indicate that iNOS is up-regulated in mice that show an effective granulomatous response to zymosan. However, iNOS level alterations are not exactly correlated with a modulation in the OPN mRNA levels of such tissues.

Figure 4. In contrast to mice with OPN alleles a or c, iNOS mRNA expression is not modulated in the liver mice with the OPN b allele. Liver tissue was obtained at the indicated time points from mouse strains B6 and BALB/c that have OPN allele a; DBA with allele c; or C3H, CBA, SJL, that all express the b allele. One μ g of total RNA was reverse-transcribed to cDNA in a total reaction volume of 20 μ l. One μ l of cDNA was used as template for PCR using LightCycler-FastStart DNA Master SYBR Green I systems (Roche Diagnostics). The mRNA expression of iNOS (A) and OPN (B) were detected by the LightCycler (Roche Diagnostics) PCR system and calculated by LightCycler Software version 3 (Roche Diagnostics). Data were standardized by β -actin (A) or G3PDH (B). The units represent the comparative amount of mRNA, calculated from the calibration curves of the standard plasmids. Data represent mean of two (A) or mean $\pm$ SD of three (B) independent experiments. *, Data are significantly different from that at day 0 ($P < 0.05$, one-way analysis of variance; Student's *t*-test).

Nucleotide and Deduced Amino Acid Sequence of Cloned OPN c cDNA

The sequences of OPN alleles a and b have previously been published and differ in 11 amino acid positions.^{9,37} However, the sequence of OPN c has not been deposited in the screened gene databases. To determine the sequence of OPN c allele, we obtained a cDNA library from kidney of DBA/2Ncrj mice. Approximately 10^5 colonies from that cDNA were screened and the nucleotide sequences of cDNA inserts from six independent clones were determined. All six clones derived from DBA mice (OPN c allele) exhibited the sequence shown in Figure 5A. Amino acid sequence comparison of OPN alleles a, b, and c (Figure 5B) showed that OPN a and c differ in only one amino acid position. Alleles b and c differed in 10 amino acids. The exchange of only one amino acid between OPN a and c make it conceivable that there are no, or little biological differences between these proteins. However the significantly diverging sequence of allele b compared to the other two, can explain functional differences described in our experiments.

Recombinant Proteins of OPN Alleles a, b, and c Support Cell Adhesion

OPN is abundantly secreted in granulomas.^{20,22,25} We tested the hypothesis whether a reduced granuloma formation in OPN mice with allele b could be explained by an impaired cell-binding capacity of inflammatory cells invading the site of granuloma formation. We therefore generated GST fusion proteins of the three OPN alleles and investigated their potential to support cell adhesion. We chose two cell lines that have previously been shown to have a high OPN-binding affinity: the highly metastatic

Figure 5. Comparison of the OPN alleles. **A:** The nucleotide sequence of the OPN c is shown. The signal sequence is **underlined**. The deduced amino acid sequence is shown using the single letter code. **B:** Alignment of the deduced amino acid sequences of OPN alleles. **Boxes** indicate the mutation sites. The potential phosphorylation sites are **underlined bold** and the Arg-Gly-Asp (RGD) motif is **double underlined**. *, Terminal codon.

melanoma cell line B16-BL6 and the nonmetastatic fibroblast cell line L929. For all recombinant GST-OPN alleles we found comparable binding of B16-BL6 and L929 cells (Figure 6).

OPN Allele b Protein Has a Significantly Reduced Ability to Induce DC Migration

The formation of granulomas depends on the migration of immunocompetent cells into the site of zymosan deposition. OPN is known to have a chemotactic function for macrophages as well as T cells that are recruited to the site of granuloma formation.^{20,22,25} Because the three OPN alleles were not different regarding their cell binding capacities, we speculated that the OPN b protein could

Figure 6. GST-fusion proteins of OPN alleles a, b, or c have similar cell-binding capacities. Cell binding of B16-BL6 (**A**) and L929 (**B**) cells to plates precoated with various concentrations of GST, GST-alleles a, b, and c OPN was performed as described in Materials and Methods.

Figure 7. Induction of DC migration is only detectable at high concentration of OPN allele b, whereas alleles a and c induce DC migration throughout a wide range of protein concentrations. DC migration assays were performed as described in Materials and Methods. Data are expressed as the mean number of migrated cells/mm² ± SD of four chambers. * and **, Data are significantly different from control at $P < 0.05$ and $P < 0.001$, respectively, one-way analysis of variance (analysis of variance, Tukey's multiple comparison test).

be altered in its chemotactic function. DCs are highly motile cells that are known to play a critical role in granuloma formation.^{32,38} Recently we demonstrated that OPN is an important chemoattractant for murine DC.³³ We therefore assessed the chemotactic ability of the three recombinant OPN alleles to attract DCs in modified Boyden chamber migration assays (Figure 7). Recombinant GST-OPN a and c induced DC migration throughout the whole range of tested protein concentrations in a dose-dependent manner, whereas a significant induction of DC migration by GST-OPN b protein was only detected at a concentration of 20.0 $\mu\text{g/ml}$. However, DC migration induced by this OPN b concentration was significantly lower than by the corresponding concentrations of recombinant OPN a and c. The promigratory effect of OPN b at 20.0 $\mu\text{g/ml}$ was approximately equivalent to that of OPN allele a at a 40-fold lower protein concentration.

Discussion

In the present study, we demonstrate that the zymosan-induced liver granuloma formation significantly varies among mouse strains. In the liver of mice with OPN a and c alleles, a marked granuloma formation was detected that peaked on day 7 after intravenous zymosan application. In sharp contrast, no significant granuloma formation was found in livers of mice with OPN b allele. This was not because of a delay in granuloma formation in these mice because no significant granulomatous response was detected within 28 days after zymosan was

deposited in the liver. Importantly, three OPN b homozygous mouse strains with otherwise different genetic background all had impaired granulomatous responses (Figures 1 and 2), indicating that the defective granuloma formation was independent of other genetic differences between these mice.

The formation of granulomas is a central mechanism to defend the host from persistent irritants.¹ It has been shown that the Kupffer cells play an important role in zymosan-induced liver granuloma formation. After they pick up the zymosan antigen they start to produce cytokines and chemokines, which lead to the recruitment of inflammatory cells into the liver.^{2,39} We speculated that the expression patterns of such inflammatory mediators could be differentially regulated in mice with different OPN alleles, thereby leading to modifications in granuloma formation. However, by RNase protection assay we found that after zymosan injection IL-1 β , M-CSF, RANTES, MIP-1 β , MIP-2, and MCP-1 were similarly induced in mice with OPN a, b, and c alleles. To our surprise, in our experiments the expression of these mediators was up-regulated both in granuloma-forming and nonforming animals. Our findings indicate that a fully functional form of OPN is essential for the formation of a granulomatous response. The unaltered expression of the above mediators could be explained either by an unaffected function of epitopes in the OPN b protein that modulated cytokine or chemokine function or a possible downstream location of OPN of the investigated inflammatory mediators.

iNOS expression is known to be up-regulated in granulomatous inflammation.^{40–42} Here we detected an augmented iNOS mRNA expression only in the liver of mice with OPN a and c alleles, that form normal granulomas, but not OPN b carrying unresponsive mice. By investigating monocytic cell lines it was previously reported that *in vitro* the expression of OPN is up-regulated by NO and at the same time, OPN is a negative feedback regulator that downmodulates the expression of iNOS.³⁶ Based on these findings, because of a functional defect of the OPN b protein an iNOS overexpression could be expected in OPN b animals. However, in our *in vivo* system, mice with OPN allele b did not up-regulate iNOS expression, in contrast to animals with OPN a or c. These unexpected differences may be explained by the complex cytokine milieu expressed during *in vivo* granuloma formation involved in NO synthesis. Other strong inflammatory mediators might overrule the *in vitro* described regulatory function of OPN on iNOS expression during the initiation of granulomas. Alternatively, the levels of iNOS in this study simply reflected the presence of macrophages. The reduction of iNOS in mice with OPN b allele leads to the amelioration of inflammatory responses and liver injury. It has been shown that iNOS regulates production of cytokines and chemokines that are important for recruitment of inflammatory cells.⁴³ In addition, iNOS negatively regulates function of hepatocyte tight junction, which is critical for the integrity of hepatobiliary system.⁴⁴

The difference in granuloma formation among mice with different OPN alleles can be explained by the amount of OPN protein produced after zymosan injection. In this regard, it has been shown that peritoneal cells

derived from mice with OPN a allele exhibited early and high OPN gene expression response on *Rickettsia tsutsugamushi* (RT) infection. Although those with OPN b and c alleles exhibited later and lower OPN gene expression.^{9,24} Thus, it is possible that gene polymorphism seen in OPN gene may affect the levels of transcription of OPN gene, and result in the alteration of protein expression. Our data demonstrated that OPN RNA expression was augmented in mice of all three alleles after zymosan injection (Figure 4). Although in allele b, augmentation of gene expression occurred at later time, the degree of gene expression was significant (Figure 4). Our findings were consistent with a previous report that unlike RT infection, intraperitoneal injection of concanavalin A resulted in the significant expression of OPN gene even in mice with OPN b allele.⁹ Thus, it is possible that the induction of OPN gene expression varies depending on the type of antigen used. In this regard, it is important to clarify the amount of OPN protein after zymosan injection in various mouse strains. Thus, we constructed an enzyme-linked immunosorbent assay with monoclonal and polyclonal antibodies specifically recognizing murine OPN to measure OPN protein levels in mouse plasma.³³ We found that this enzyme-linked immunosorbent assay could detect the a and c allelic forms of murine recombinant OPN, however failed to detect the b allelic protein, although the epitope detected by the used antibodies exists in the OPN b protein (data not shown). Although we cannot rule out the possibility that the amount of OPN protein after zymosan injection varied depending on the OPN allele mice carried (presumably high in OPN a and c alleles and lower in OPN b allele), it is possible that OPN gene polymorphism influences the function of OPN protein. We speculated that the failure to detect the allele b protein is because of a conformational alteration of OPN b. The complete nucleotide sequence of the a and b allelic forms of OPN have been described, we cloned the c allelic form to compare differences between all three alleles.^{9,37} The alignment of deduced amino acid sequences showed that although OPN a and c differed only in one position, OPN b showed 11 mutations compared to a and 10 compared to OPN c (Figure 5). Substitutions in OPN b are located close to the functionally important RGD-motif and within potential phosphorylation sites. Phosphorylation of OPN is known to be decisive for OPN functional binding in several systems.^{22,45} Thus, it is possible that the substitutions in the OPN b protein may change the protein conformation and/or phosphorylation and render OPN defective at least for some of its described functions as a chemokine, cytokine, or adhesive protein, thereby explaining the defective formation of granulomas in allele b-carrying mice. In this regard, the physicochemical nature of three OPN allele proteins should be determined in the future.

Based on these findings we went on to determine the differences of OPN alleles for two well-characterized OPN functions that are both decisively involved in granuloma formation; cell adhesion and cell migration. We selected two cell lines, that were previously shown to bind to OPN; L929 cells bind to OPN through the interaction of the RGD motif with $\alpha v \beta 3$ integrin, whereas B16-

BL6 cells express additional RGD-independent binding site(s) for OPN.⁴⁶ Both cell lines could bind to all allelic forms of OPN/GST fusion proteins, indicating that impaired adhesion mechanisms are unlikely to cause the reduced granuloma formation in mice expressing the OPN b allele.

In the liver, blood DCs interact with Kupffer cells and use their migrational capacities to undergo a blood lymph translocation via the hepatic sinusoids to access liver-draining lymph nodes, as an important mechanism for the initiation of cell-mediated immunity in this organ.^{47,48} Recently we reported that OPN is a potent chemotactic protein for DCs.³³ Here we investigated, whether all allelic forms of OPN could support DC migration. Interestingly we found that compared to alleles a and c only OPN allele b was defective in inducing DC migration. The defective chemotactic function of OPN allele b could explain the reduced formation of granulomas in several ways. We had previously described that OPN-induced DC migration is important in cell-mediated immunity.³³ Thus, an impaired migratory potential of DCs because of a defective OPN function could lead to the attenuation of granulomatous response. However, in addition to its effect on DC, OPN allele b is likely to be impaired in its chemotactic function for other immunocompetent cells, thereby leading to a reduced influx of inflammatory cells, such as T cells or macrophages during zymosan-induced liver inflammation.⁴⁹ OPN is known to interact with a variety of receptors such as $\alpha v\beta 3$, $\alpha v\beta 1$, $\alpha v\beta 5$, $\alpha 4\beta 1$, $\alpha 9\beta 1$, and certain CD44 isoforms. As shown the OPN b allele is not impaired in its cell-binding ability, while at the same time it has no chemotactic potential. We therefore speculate that the OPN b allele might be defective only in its binding to some but not all OPN receptors expressed on the cells involved in granuloma formation, thereby being restricted only in certain functions. Exactly which OPN receptors bind the different alleles has to be subject of further investigation.

In conclusion, we have demonstrated that OPN is critically involved in the formation of foreign body liver granulomas because mice that express OPN allele b but not a or c are impaired in their granuloma formation. The reduced granuloma response in OPN b mice most likely results from the defective function of the OPN b protein to induce chemotactic migration of immunocompetent cells including DCs, while normal cell-binding capacities are retained. Compared to OPN a or c alleles the OPN b protein contains mutations within functional domains relevant for OPN-receptor interactions. These mutations could be responsible for a functional impairment of some but not all OPN functions through defective interactions with selective OPN receptors.

References

1. Boros DL: Granulomatous inflammations. *Prog Allergy* 1978, 24:183-267
2. Bouwens L, Wisse E: Proliferation, kinetics, and fate of monocytes in rat liver during a zymosan-induced inflammation. *J Leukoc Biol* 1985, 37:531-543
3. Yamada M, Naito M, Takahashi K: Kupffer cell proliferation and glu-

- can-induced granuloma formation in mice depleted of blood monocytes by strontium-89. *J Leukoc Biol* 1990, 47:195-205
4. Allison AC: Macrophage activation and nonspecific immunity. *Int Rev Exp Pathol* 1978, 18:303-346
5. Carlos TM, Harlan JM: Leukocyte-endothelial adhesion molecules. *Blood* 1994, 84:2068-2101
6. Oldberg A, Franzen A, Heinegard D: Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci USA* 1986, 83:8819-8823
7. Singh RP, Patarca R, Schwartz J, Singh P, Cantor H: Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages in vitro and its effect upon macrophages in vivo. *J Exp Med* 1990, 171:1931-1942
8. Liaw L, Skinner MP, Raines EW, Ross R, Chersesh DA, Schwartz SM, Giachelli CM: The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of $\alpha v\beta 3$ in smooth muscle cell migration to osteopontin in vitro. *J Clin Invest* 1995, 95:713-724
9. Patarca R, Freeman GJ, Singh RP, Wei FY, Durfee T, Blattner F, Regnier DC, Kozak CA, Mock BA, Morse III HC, Jerrells TR, Cantor H: Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. *J Exp Med* 1989, 170:145-161
10. Pollack SB, Linnemeyer PA, Gill S: Induction of osteopontin mRNA expression during activation of murine NK cells. *J Leukoc Biol* 1994, 55:398-400
11. Kawashima R, Mochida S, Matsui A, YouLuTuZ Y, Ishikawa K, Toshima K, Yamanobe F, Inao M, Ikeda H, Ohno A, Nagoshi S, Uede T, Fujiwara K: Expression of osteopontin in Kupffer cells and hepatic macrophages and Stellate cells in rat liver after carbon tetrachloride intoxication: a possible factor for macrophage migration into hepatic necrotic areas. *Biochem Biophys Res Commun* 1999, 256:527-531
12. Patarca R, Wei FY, Singh P, Morasso MI, Cantor H: Dysregulated expression of the T cell cytokine Eta-1 in CD4-8-lymphocytes during the development of murine autoimmune disease. *J Exp Med* 1990, 172:1177-1183
13. Lampe MA, Patarca R, Iregui MV, Cantor H: Polyclonal B cell activation by the Eta-1 cytokine and the development of systemic autoimmune disease. *J Immunol* 1991, 147:2902-2906
14. Murry CE, Giachelli CM, Schwartz SM, Vracko R: Macrophages express osteopontin during repair of myocardial necrosis. *Am J Pathol* 1994, 145:1450-462
15. Tian JY, Sorensen ES, Butler WT, Lopez CA, Sy MS, Desai NK, Denhardt DT: Regulation of no synthesis induced by inflammatory mediators in RAW264.7 cells: collagen prevents inhibition by osteopontin. *Cytokine* 2000, 12:450-457
16. Yamamoto S, Nasu K, Ishida T, Setoguchi M, Higuchi Y, Hijiya N, Akizuki S: Effect of recombinant osteopontin on adhesion and migration of P388D1 cells. *Ann NY Acad Sci* 1995, 760:378-380
17. Giachelli CM, Lombardi D, Johnson RJ, Murry CE, Almeida M: Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. *Am J Pathol* 1998, 152:353-358
18. Nau GJ, Guilfoile P, Chupp GL, Berman JS, Kim SJ, Kornfeld H, Young RA: A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis. *Proc Natl Acad Sci USA* 1997, 94:6414-6419
19. Carlson I, Tognazzi K, Manseau EJ, Dvorak HF, Brown LF: Osteopontin is strongly expressed by histiocytes in granulomas of diverse etiology. *Lab Invest* 1997, 77:103-108
20. O'Regan AW, Chupp GL, Lowry JA, Goetschkes M, Mulligan N, Berman JS: Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties in vitro. *J Immunol* 1999, 162:1024-1031
21. Chiba S, Rashid MM, Okamoto H, Shiraiwa H, Kon S, Maeda M, Murakami M, Inobe M, Kitabatake A, Chambers AF, Uede T: The role of osteopontin in the development of granulomatous lesions in lung. *Microbiol Immunol* 2000, 44:319-332
22. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H: Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 2000, 287:860-864
23. Groves MG, Rosenstreich DL, Taylor BA, Osterman JV: Host de-

- fenses in experimental scrub typhus: mapping the gene that controls natural resistance in mice. *J Immunol* 1980, 125:1395–1399
24. Patarca R, Saavedra RA, Cantor H: Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. *Crit Rev Immunol* 1993, 13:225–246
25. Nau GJ, Liaw L, Chupp GL, Berman JS, Hogan BL, Young RA: Attenuated host resistance against *Mycobacterium bovis* BCG infection in mice lacking osteopontin. *Infect Immun* 1999, 67:4223–4230
26. Forton AC, Petri MA, Goldman D, Sullivan KE: An osteopontin (SPP1) polymorphism is associated with systemic lupus erythematosus. *Hum Mutat* 2002, 19:459
27. Matsumoto Y, Saiki I, Murata J, Okuyama H, Tamura M, Azuma I: Recombinant human granulocyte colony-stimulating factor inhibits the metastasis of hematogenous and non-hematogenous tumors in mice. *Int J Cancer* 1991, 49:444–449
28. Minamoto S, Mori H, Hatakeyama M, Kono T, Doi T, Uede T, Taniguchi T: Characterization of the heterodimeric complex of human IL-2 receptor alpha. Beta chains reconstituted in a mouse fibroblast cell line, L929. *J Immunol* 1990, 145:2177–2182
29. Starr L, Quaranta V: An efficient and reliable method for cloning PCR amplification products: a survey of point mutations in integrin cDNA. *Biotechniques* 1992, 13:612–618
30. Smith DB, Johnson KS: Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 1988, 67:31–40
31. Xuan JW, Hota C, Chambers AF: Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. *J Cell Biochem* 1994, 54:247–255
32. Sozzani S, Sallusto F, Luiniv W, Zhou D, Piemonti L, Allavena P, Van Damme J, Valitutti S, Lanzavecchia A, Mantovani A: Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. *J Immunol* 1995, 155:3292–3295
33. Weiss JM, Renkl AC, Maier CS, Kimmig M, Liaw L, Ahrens T, Kon S, Maeda M, Hotta H, Uede T, Simon JC: Osteopontin is involved in the initiation of cutaneous contact hypersensitivity by inducing Langerhans and dendritic cell migration to lymph nodes. *J Exp Med* 2001, 194:1219–1229
34. Lukas M, Stossel H, Hefel L, Imamura S, Fritsch P, Sepp NT, Schuler G, Romani N: Human cutaneous dendritic cells migrate through dermal lymphatic vessels in a skin organ culture model. *J Invest Dermatol* 1996, 106:1293–1299
35. Groves MG, Rosenstreich DL, Taylor BA, Osterman JV: Host defenses in experimental scrub typhus: mapping the gene that controls natural resistance in mice. *J Immunol* 1980, 125:1395–1399
36. Guo H, Cai CQ, Schroeder RA, Kuo PC: Osteopontin is a negative feedback regulator of nitric oxide synthesis in murine macrophages. *J Immunol* 2001, 166:1079–1086
37. Ono M, Yamamoto T, Nose M: Allelic difference in the nucleotide sequence of the Eta-1/Op gene transcript. *Mol Immunol* 1995, 32:447–448
38. Standiford TJ, Kunkel SL, Phan SH, Rollins BJ, Strieter RM: Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J Biol Chem* 1991, 266:9912–9918
39. Moriyama H, Yamamoto T, Takatsuka H, Umez H, Tokunaga K, Nagano T, Arakawa M, Naito M: Expression of macrophage colony-stimulating factor and its receptor in hepatic granulomas of Kupffer-cell-depleted mice. *Am J Pathol* 1997, 150:2047–2060
40. Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, Willoughby DA: Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci USA* 1994, 91:2046–2050
41. Hogaboam CM, Chensue SW, Steinhauser ML, Huffnagle GB, Lukacs NW, Strieter RM, Kunkel SL: Alteration of the cytokine phenotype in an experimental lung granuloma model by inhibiting nitric oxide. *J Immunol* 1997, 159:5585–5593
42. Hogaboam CM, Gallinat CS, Bone-Larson C, Chensue SW, Lukacs NW, Strieter RM, Kunkel SL: Collagen deposition in a non-fibrotic lung granuloma model after nitric oxide inhibition. *Am J Pathol* 1998, 153:1861–1872
43. Setoguchi K, Takeya M, Akaike T, Suga M, Hattori R, Maeda H, Ando M, Takahashi K: Expression of inducible nitric oxide synthase and its involvement in pulmonary granulomatous inflammation in rats. *Am J Pathol* 1996, 149:2005–2022
44. Mazzon E, Cuzzocrea S: Role of iNOS in hepatocyte tight junction alteration in mouse model of experimental colitis. *Cell Mol Biol* 2003, 49:45–57
45. Ek-Rylander B, Flores M, Wendel M, Heinegard D, Andersson G: Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. *J Biol Chem* 1994, 269: 14853–14856
46. Katagiri YU, Murakami M, Mori K, Iizuka J, Hara T, Tanaka K, Jia WY, Chambers AF, Uede T: Non-RGD domains of osteopontin promote cell adhesion without involving alpha v integrins. *J Cell Biochem* 1996, 62:123–131
47. Yoneyama H, Matsuno K, Zhang Y, Murai M, Itakura M, Ishikawa S, Hasegawa G, Naito M, Asakura H, Matsushima K: Regulation by chemokines of circulating dendritic cell precursors, and the formation of portal tract-associated lymphoid tissue, in a granulomatous liver disease. *J Exp Med* 2001, 193:35–49
48. Kudo S, Matsuno K, Ezaki T, Ogawa M: A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. *J Exp Med* 1997, 185:777–784
49. Weber GF, Zawadeh S, Hikita S, Kumar VA, Cantor H, Ashkar S: Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. *J Leukoc Biol* 2002, 72:752–761