Amorphous silica nanoparticles (nSP50) exacerbate hepatic damage through the activation of acquired cell-mediated immunity

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Abstract

Due to their innovative functions, the use of nanoparticles in various industries has been expanding. However, a key concern is whether nanoparticles induce unexpected biological effects. Although many studies have focused on innate immunity, information on whether nanoparticles induce biological responses through effects on acquired immunity is sparse. Here, to assess the effects of amorphous silica nanoparticles on acquired immunity, we analyzed changes in acute toxicities after pretreatment with amorphous silica nanoparticles (50 nm in diameter; nSP50). Pretreatment with nSP50 biochemically and pathologically exacerbated nSP50-induced hepatic damage in immunocompetent mice, while pretreatment with nSP50 did not exacerbate hepatic damage in immunodeficient mice. Consistent with this, the depletion of CD8+ cells with an anti-CD8 antibody in animals pretreated with nSP50 resulted in lower plasma levels of hepatic injury markers such as ALT and AST after an intravenous administration compared to treatment with an isotype-matched control antibody. Finally, stimulation of splenocytes promoted the release of IFN-γ in nSP50-pretreated mice regardless of the stimulator used. Moreover, the blockade of IFN-γ decreased plasma levels of ALT and AST levels in nSP50-pretreated mice. Collectively, these data show that nSP50-induced acquired immunity leads to exacerbation of hepatic damage through the activation of cytotoxic T lymphocytes.

Abbreviations

ALT Alanine Aminotransferase
AST Aspartate Aminotransferase
CD Cluster of Differentiation
CTL Cytotoxic T Lymphocytes
DAMP Danger-associated Molecular Pattern
FBS Fetal Bovine Serum
IFN Interferon
PBS Phosphate-buffered Saline
SCID Severe Combined Immunodeficiency Disease

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1. Introduction

The development of products containing nanoparticles (i.e., less than 100 nm in short diameter) has expanded significantly in various industries [1]. For example, amorphous silica nanoparticles are frequent components of cosmetics [2] and foods [3]. As the size of these particles decreases, their reactivity at the tissue interface and their tissue penetration increases and their in vivo kinetics change [4, 5]. Given this potential change in kinetics, concerns have emerged regarding the acquisition of unexpected biological effects as particle size is refined [6]. Therefore, we need to identify potential sources of unpredictable adverse biological effects due to increasingly smaller nanoparticles and to optimize the form of nanoparticles used in various manufactured products [7, 8].

From this viewpoint, biological evaluation of nanoparticles focusing on innate immunity and inflammatory response has been reported. For example, some nanoparticles activate innate immunity, including the NLRP3 inflammasome, and induce chronic inflammatory diseases, like pneumoconiosis due to asbestos [9–11]. Moreover, acquired immunity is activated subsequent to innate immunity and mediates immunological memory in settings including vaccination [12], allergic reaction [13, 14], and anaphylaxis [15]. In consideration with the production amount and the way to use of nanoparticles, we are thought to be exposed to them continuously. Hence, some biological effects would be occurring due to acquired immune responses which is activated after continuous exposure to nanoparticles. As previously shown, the pretreated mice with silver nanoparticles developed allergic inflammation in response to the silver [16], suggesting that metallic nanoparticles have the potential to induce biological effects through acquired immunity. However, it currently is unclear whether non-metallic nanoparticles pose adverse biological effects mediated through the induction of acquired immunity.

Amorphous silica nanoparticles are non-metallic nanoparticles that people are frequently and repeatedly exposed to because they are components of daily products [17]. By focusing on their potential to induce various acute toxicities, we have sought to define the effects of multiple exposure of silica nanoparticles on the acquired immune system. For instance, amorphous silica nanoparticles increasingly induced dysregulated coagulation [18] and hepatic damage [19] as their particle size became very small. Also, a drop in rectal temperature and inflammatory responses involving macrophages were induced once silica nanoparticles decreased to approximately 50 nm in diameter [20, 21]. Here, we used amorphous silica nanoparticles that were 50 nm in diameter (nSP50) to assess their effects on hepatic damage, coagulation dysregulation, and rectal temperature as mediated through acquired immunity and the mechanism through which these effects were achieved.

2. Materials and methods

2.1. Mice

Male BALB/cCrslc, BALB/cSlc-nu/nu, and C3H/HeNSlc mice were purchased from SLC Japan (Shizuoka, Japan), and male CB17/Icr–Prkdcscid/CrlCrj (C.B–17 SCID) mice were purchased from Charles River Japan (Kanagawa, Japan). All mice were used at 7 to 11 wk of age. Mice were housed in a ventilated animal room maintained at 25 ± 2°C with a 12-h light/12-h dark cycle. Mice had free access to water and a standard MF diet (Oriental Yeast Co., Ltd., Tokyo, Japan). To ensure ethical treatment of animals, all experiments were performed in accordance with the institutional guidelines of Osaka University.

2.2. Amorphous silica nanoparticles and reagents

Solutions of amorphous silica particles were purchased from Micromod Partikeltechnologie (Rostock, Warnemünde, Germany); solutions of amorphous silica particles were stored at room temperature. Before use, the solution was sonicated for 5 min at 400 W and mixed for 1 min by using a benchtop vortexer. The hydrodynamic diameters of nSP50 in PBS was tested by dynamic light scattering (Zetasizer Nano-S; Malvern Instruments., Malvern, UK) and were 41.97 ± 0.08 nm (figure S1 (available online at stacks.iop.org/NANOX/3/015002/media)). Based on the Stokes’ law, the supernatant was recovered by centrifugation at 20,600 g for 20 min at room temperature, which is a condition containing almost no nSP50. Anti-mouse CD8 antibody was purified from ascitic fluid collected from nude mice (BALB/cSlc-nu/nu) after the transplantation of 53–6.72 hybridoma cells (American Type Culture Collection, Manassas, VA, USA). Anti-mouse IFN-γ antibody (clone R4–6A2) and the isotype-matched rat IgG2a control antibody were purchased from BioLegend (San Diego, CA, USA). The isotype-matched rat IgG1 control antibody was purchased from Bio X Cell (Lebanon, NH, USA). Anti-mouse CD16/CD32 antibody, phycoerythrin–conjugated anti-mouse CD3 monoclonal antibody (clone OKT3), and fluorescein isothiocyanate–conjugated anti-mouse CD8 monoclonal antibody (clone 53–6.7) were purchased from eBiosciences (San Diego, CA, USA).
2.3. Sample preparation and administration to mice
For pretreatment of mice, nSP50 were diluted to 12.5 mg ml\(^{-1}\) in PBS solution by using 10 \(\times\) PBS solution and sterile water; mice were anesthetized by using isoflurane (Wako, Osaka, Japan), and a micro-injector (TERUMO, Tokyo, Japan) was used to inject each ear with 10 \(\mu\)l of PBS or 12.5 mg ml\(^{-1}\) nSP50 in PBS. For challenge administration, nSP50 was diluted in PBS to 50 or 80 mg nSP50 kg\(^{-1}\) body weight of mice; 1 wk after the last pretreatment, each mouse was injected intravenously with 180 to 220 \(\mu\)l of nSP50 (50 or 80 mg nSP50 kg\(^{-1}\) body weight of mice) through the tail vein. These solutions were prepared immediately before use.

2.4. Biochemical analysis
Plasma was harvested by centrifuging whole blood at 3,000 \(\times\) g. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by using a biochemical auto-analyzer (FUJI DRI-CHEM; Fujifilm, Tokyo, Japan).

2.5. Histologic analysis
After blood collection (see the Supplementary Methods), residual blood was removed by perfusing livers with PBS. Then each liver was removed and placed in fixative solution (10% neutral-buffered formaldehyde; Nacalai Tesque, Kyoto, Japan). Livers were embedded in paraffin blocks and sliced. Liver sections were stained by using hematoxylin and eosin and evaluated histologically at the Applied Medical Research Laboratory (Osaka, Japan). For each sample, several representative signs of hepatic damage (blood stasis, vacuolar degeneration, inflammatory cell infiltration, necrosis of hepatocytes) were scored as 0 (none), 1 (very mild), 2 (mild), 3 (moderate), or 4 (severe) by laboratory staff at the Applied Medical Research Laboratory, who were blinded to treatment group.

2.6. Serum transfer experiment
For serum transfer experiments, ears of donor mice (BALB/cCrSlc, male, 6 wk) were pretreated intradermally each week for 4 wk with PBS or 12.5 mg ml\(^{-1}\) nSP50. At 1 wk after the last pretreatment, blood was harvested from the donor mice through cardiac puncture. These blood samples were incubated for 1 h at 37 °C and then for 5 h at 4 °C. After incubation, samples were centrifuged at 5,000 \(\times\) g for 15 min at 4 °C to obtain serum. Immediately after collection, all serum samples were pooled, and 500 \(\mu\)l of the pooled serum were injected intraperitoneally into 11-wk-old recipient mice. At 24 h after serum transfer, recipient mice were challenged by receiving nSP50 through tail vein injection.

2.7. In vivo cell depletion–cytokine blockade experiment
For the cell-depletion experiment, nSP50-pretreated mice were treated intraperitoneally with 500 \(\mu\)g per mouse of anti-mouse CD8 antibody or IgG2a isotype-matched control antibody in PBS; 24 h later, mice received nSP50 through their tail veins. For comparison, additional mice were treated with anti-mouse IFN-γ antibody or IgG1 isotype-matched control antibody; immediately after antibody treatment, mice received nSP50 for the cytokine blockade experiment. Cell depletion was evaluated by flow cytometry (FACS Aria Cell Sorter; BD Bioscience, San Jose, CA, USA); by 6 h after challenge.

2.8. Isolation of spleen and primary culture of splenocytes
After blood collection, spleens were harvested aseptically, dissociated through a cell strainer and single-cell suspensions of splenocytes were created by resuspending cell pellets in culture medium, and cell counts were obtained (Coulter, TOMY Digital Biology, Tokyo, Japan). Cell suspensions were brought to a final concentration of 1.0 \(\times\) 10^7 cells ml\(^{-1}\) in culture medium. The splenocyte culture medium was Roswell Park Memorial Institute 1640 medium (RPMI-1640; Wako) containing 0.2 mM L-glutamine; 1 \(\times\) Minimum Essential Medium Non-Essential Amino Acids (Nacalai Tesque); 50 \(\mu\)M 2-mercaptoethanol (GIBCO, Kanagawa, Japan); 1% antibiotics (Antibiotic-antimycotic, Invitrogen, Carlsbad, CA, USA); and 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), which had been inactivated through incubation at 56 °C for 30 min.

2.9. Quantification of IFN-γ in the supernatant of restimulated splenocytes
Samples (100 \(\mu\)l each; 1.0 \(\times\) 10^6 cells well\(^{-1}\)) of single-cell suspensions of splenocytes were placed in each well of 96-well round-bottom plates (IWAKI). Immediately, 100 \(\mu\)l of nSP50 (final concentrations 5, 50, and 500 \(\mu\)g ml\(^{-1}\)) diluted in culture medium was added to individual wells. After incubation in 5% CO_2 at 37 °C for 72 h, supernatants were collected by centrifugation at 300 g for 5 min at 4 °C. Supernatants were collected and tested
for IFN-γ concentration by using a sandwich enzyme-linked immunosorbent assay kit (catalog #555138; BD Bioscience) as per the recommended protocol.

2.10. Statistical analysis
Statistical analyses were performed by using OriginPro 9J (OriginLab, Northampton, MA, USA). All data are presented as means ± S.E.M. Significant differences between control groups and experimental groups were determined by using Tukey’s multiple comparison test with a one-way ANOVA or two-way ANOVA, or non-parametric Steel-Dwass’s multiple comparison test; P-values less than 0.05 were considered significant.

3. Results

3.1. Identification of adverse biological effects due to acquired immunity
In this research, we focused on the effect of multiple exposure of nSP50 on their acute toxicities because acquired immune responses are different from responses of initial exposure. First, to evaluate whether nSP50-induced acute toxicities differed when mice were pretreated with nanoparticles, mice were intradermally pretreated with PBS (control) or nSP50 and then intravenously challenged with PBS or nSP50 (80 mg kg⁻¹) to induce acute toxicities (figure 1(a)). In the mice challenged with nSP50, neither the expected drop in rectal temperature (figure S2a) nor the number of platelets (figure S2b) differed significantly between PBS-pretreated and nSP50-pretreated groups; hematologic analysis (see the Supplementary Methods) did not reveal any changes between the nSP50-pretreated and PBS-pretreated groups (figure S3a and S3b).

In contrast, plasma levels of ALT (figure 1(b)) and AST (figure 1(c)) were significantly increased in the nSP50-pretreated/nSP50-challenged group compared with the PBS-pretreated/nSP50-challenged group. Staining with hematoxylin and eosin demonstrated that vacuolar degeneration and necrosis of hepatocytes occurred more frequently in liver sections from nSP50-pretreated mice than from PBS-pretreated mice (figure 1(d)). In addition, histologic scores were higher for nSP50-pretreated mice than the PBS-pretreated group (table 1), indicating that pretreatment with nSP50 exacerbated hepatic damage. Therefore, exacerbation of hepatic damage was identified as an adverse biological effect of multiple exposure of nSP50.

We then sought to ensure that the exacerbation of hepatic damage due to multiple exposure of nSP50 was universal across mouse strains. In C3H/HeNSlc mice, plasma levels of ALT (figure 1(e)) and AST (figure 1(f)) continued to be higher in the nSP50-pretreated/nSP50-challenged group compared with the PBS-pretreated/nSP50-challenged group, as they were in BALB/cCrSlc mice. These results suggest that exacerbation of hepatic damage is versatile effect of pretreating mice with nSP50.

Silica is biodegraded only slightly and therefore likely accumulates in the body [22], prompting concern regarding potential effects due to the presence of contaminants in preparations of nSP50. We therefore evaluated whether mice that received multiple pretreatments with nSP50 or the solution in which the particles are suspended (as a potential source of contaminants) influenced nSP50-induced hepatic damage. However, plasma levels of ALT (figure 1(g)) and AST (figure 1(h)) did not differ significantly between PBS-pretreated/PBS-challenged and nSP50-pretreated/PBS-challenged mice or between PBS-pretreated/nSP50-challenged and supernatant-pretreated/nSP50-challenged groups. These results suggest that the observed exacerbation of hepatic damage was not due to cumulative effects of pretreatment or to contaminants in the nSP50 formulation; that is, nSP50 itself exacerbated hepatic damage in challenged mice.

3.2. Clarification of mechanisms underlying on the exacerbated hepatic damage
Next, to clarify the underlying mechanisms, we focused on the acquired immunity–related response. To this end, we intradermally injected immunodeficient C.B-17 SCID mice with PBS or nSP50 and then challenged them with PBS or nSP50 to induce liver damage. The resulting plasma levels of ALT (figure 2(a)) and AST (figure 2(b)) did not differ significantly between the PBS-pretreated/nSP50-challenged and nSP50-pretreated/nSP50-challenged groups. Immunodeficient C.B-17 SCID mice lack T and B lymphocytes [23] but not innate immune cells such as NK cells and granulocytes. In this regard, this result suggests that antibodies and B cells driven by humoral immunity or cytotoxic T lymphocytes driven by the cell-mediated immunity could be involved in the nSP50-exacerbated hepatic damage.

Then, to clarify the types of acquired immunity involved in the nSP50-induced exacerbation of hepatic damage, serum transfer experiments and cell depletion analysis were performed. First, to assess the involvement of antibody-mediated immunity, non-treated (recipient) mice were injected with serum from nSP50-pretreated (donor) mice or PBS-pretreated mice (figure 2(c)). ALT (figure 2(d)) and AST (figure 2(e)) levels in mice did not differ significantly between those that received serum from nSP50-pretreated mice versus PBS-pretreated mice, suggesting that antibody-mediated immunity has a negligible effect on the exacerbation of nSP50-induced hepatic damage. We then depleted mice of CD8⁺ T cells and CD4⁺ cells (figure 2(f)); these markers are
respectively expressed on the cell surface of cytotoxic T lymphocytes (CTLs) and T helper (Th) cells [24]. Flow cytometry confirmed that the numbers of CD8+ (figure S4a) and CD4+ cells (figure S4b) in splenocytes were decreased by 6 h after challenge with nSP50. In CD4+ cells depleted mice, plasma levels of ALT (figure S5a) and...
AST (figure S5b) of pretreated mice with nSP50 were reduced compared with those of the group treated with the isotype-matched control antibody, however, they didn’t slightly change compared with nSP50-pretreated/nSP50-challenged and non-depleted group. On the other hand, plasma levels of ALT single dose of amorphous silica nanoparticles like 10 nm or 50 nm. The mechanism underlying nSP50-induced hepatic damage is independent of a coagulation disorder, unlike the hepatic damage due to silica nanoparticles that are 10 nm in diameter, which is achieved primarily through derangement of coagulation [20].

Moreover, flow cytometry showed that the proportions of B and T cells among total splenocytes (figure S6a) and the ratios of CTLs and T-helper cells to total T cells (figure S6b) did not differ significantly between PBS- and nSP50-pretreated groups. Subsequently, to confirm the ability of CD8+ T cells to be activated after nSP50 pretreatment, we placed splenocytes in medium with or without nSP50 (5, 50, or 500 μg ml–1) or in medium containing concanavalin A, a T cell receptor–independent activator [25]. We then measured the concentration of IFN–γ, a kind of cytokines which activates CD8+ T cells [26], in the culture supernatants at 72 h after stimulation. The concentrations of IFN–γ were increased in the splenocyte cultures derived from nSP50-pretreated/nSP50-challenged mice compared with those from PBS-pretreated mice (figure 3(a)). In this regard, the proportion of CTLs was not different in any groups (figure S7a), while killing activity of CTLs in the liver pretreated with nSP50 was higher than PBS-pretreated groups (figure S7b); CD107a (LAMP-1) is an indicator of killing activity, which is exposed to cell surface in association with the release of cytotoxic granules. These results suggest that the exacerbation of hepatic damage resulting from multiple exposure of nSP50 was due to differences in T cell activity rather than to changes in T cell number; these findings imply that IFN–γ may be a mediator in the mechanism of exacerbation.

We then inhibited IFN–γ activity (figure 3(b)). Plasma levels of ALT (figure 3(c)) and AST (figure 3(d)) of pretreated mice with nSP50 and then treated with a neutralizing antibody for blockade of IFN–γ activity were partially reduced compared with those of the group treated with the isotype-matched control antibody, whereas the non-blockade group showed exacerbation of hepatic damage. These results indicate that IFN–γ played an indirect and partial role in the exacerbation of the hepatic damage induced by multiple exposure of nSP50.

4. Discussion

The present study showed that multiple exposure of nSP50 exacerbated the nSP50-induced hepatic damage but not the drop in rectal temperature or decrease in platelet number that are associated with nSP50 given as a single dose [20]. These adverse effects are observed by single dose of amorphous silica nanoparticles like 10 nm or 50 nm. The mechanism underlying nSP50-induced hepatic damage is independent of a coagulation disorder, unlike the hepatic damage due to silica nanoparticles that are 10 nm in diameter, which is achieved primarily through derangement of coagulation [20]. Given that silica nanoparticles are recognized by the macrophage class A scavenger receptor [27] and that platelets are activated through a scavenger receptor–dependent mechanism [28], macrophages might recognize silica nanoparticles in a size-specific manner and mediate size-specific biological responses. In fact, as previously shown, the inflammatory responses of macrophages were most severe in response to silica nanoparticles that were approximately 50 nm in diameter [21]. Furthermore, it is possible that nanoparticles’ dynamics would be changed by acquired immunity and it affects emergence of adverse effects. From this viewpoint, we measured the abundance of silicon in the liver by Inductively Coupled Plasma Optical Emission Spectrometer. BALB/c CrSlc mice were intradermally pretreated with nSP50 (125 μg...
Figure 2. Changes in hepatic damage through acquired immunity-related response. C.B-17 SCID mice were pretreated intradermally once weekly for 4 wk with nSP50 (125 μg in 10 μl per ear) or PBS; 1 wk later, mice received nSP50 (80 mg kg⁻¹) or PBS by intravenous injection. Plasma levels of (a) ALT and (b) AST were measured at 6 h after challenge (n = 10). Data are expressed as mean ± S.E.M. (c) In the serum transfer experiment, BALB/cCrSlc (donor) mice were pretreated intradermally once weekly for 4 wk with nSP50 (125 μg in 10 μl per ear) or PBS; 1 wk later, mice were euthanized, and samples of donor serum were injected intraperitoneally into untreated (recipient) mice. At 24 h after serum injection, recipient mice were intravenously injected with nSP50 (80 mg kg⁻¹). Plasma levels of (d) ALT and (e) AST were measured at 6 h after treatment with nSP50 (n = 10–14). Data are expressed as mean ± S.E.M.; **, P < 0.01. (f) In the cell depletion experiment, BALB/cCrSlc mice were pretreated intradermally once weekly for 4 wk with nSP50 (125 μg in 10 μl per ear) or PBS; 1 wk later, mice were injected intraperitoneally with anti-mouse CD8 antibody or isotype control antibody (500 μg per mouse). At 24 h after antibody treatment, mice received nSP50 (50 mg kg⁻¹) or PBS by intravenous injection. Plasma levels of (g) ALT and (h) AST were measured at 6 h after treatment with nSP50 or PBS (n = 9–15). Data are expressed as mean ± S.E.M.; *, P < 0.05; **, P < 0.01 by the non-parametric test.
in 10 μl per ear) or PBS once weekly for 4 weeks; one week later, mice received nSP50 (80 mg kg⁻¹) or PBS by intravenous injection. Although the abundance of silicon in the liver was measured at 6 h after challenge, silicon elements was not detected in any groups with nSP50 injection due to their lower abundance compared to the minimum limit of detection (data not shown). Hence, to fully understand the biological effects of nanoparticle-induced acquired immunity, we need to explore the association among these adverse biologic effects, particle size, and the kinetics of nanoparticles, focusing particularly on the ways in which cells recognize nanoparticles.

CD8⁺ CTLs may play an important—albeit partial—role in the exacerbation of nSP50-induced hepatic damage. According to several studies that evaluated the activation of innate immunity by fine particles, silica nanoparticles are phagocytosed by macrophages or dendritic cells [29] and activate innate immunity through the following mechanisms: (i) activation of the NLRP3 inflammasome [30]; (ii) production of prostaglandin, a danger-associated molecular pattern (DAMP) [31]; and (iii) release of other DAMPs due to cell death [32].

Figure 3. Relationship of IFN-γ to the exacerbation of hepatic damage. BALB/c mice were pretreated intradermally once weekly for 4 wk with nSP50 (125 μg in 10 μl per ear) or PBS; 1 wk later, mice received nSP50 (80 mg kg⁻¹) or PBS by intravenous injection. At 6 h after treatment with nSP50 or PBS, mice were euthanized, and single-cell suspensions of splenocytes were prepared. (a) At 72 h after the addition of medium only, nSP50 (5, 50, or 500 μg ml⁻¹) to splenocyte cultures, the concentrations of IFN-γ in the culture supernatants were measured. Data are expressed as mean ± S.E.M (n = 5). **, P < 0.01. (b) For the IFN-γ blockade experiment, BALB/cCrSlc mice were pretreated intradermally once weekly for 4 wk with nSP50 (125 μg in 10 μl per ear) or PBS; 1 wk later, mice were injected intraperitoneally with anti-mouse IFN-γ antibody or isotype control antibody (500 μg per mouse) followed immediately by intravenous injection of nSP50 (50 mg kg⁻¹) or PBS. Plasma levels of (c) ALT and (d) AST were measured at 6 h after treatment with nSP50 or PBS (n = 9–14). Data are expressed as mean ± S.E.M; P-value was calculated by the non-parametric test.
Therefore, although further analysis is necessary, nSP50 might activate innate immunity through the same process as do other fine particles, with the subsequent activation of acquired immunity upon the delivery of additional exposure. Finally, IFN-γ is important in the targeting of damaged or infected hepatocytes after treatment with nSP50, resulting in the exacerbation of hepatic damage. IFN-γ is a type I cytokine that activates CTLs and is produced mainly by CD4+ T cells and only slightly by CD8+ T cells [33–35]. Therefore, we suppose that CD4+ T cells activate CD8+ T cells, which then directly kill or remove hepatocytes after challenge with nSP50. Given the results from CD8+ and CD4+ cell depletion experiment, IFN-γ experiment, and killing activity of CD8+ T cells in the liver, CD8+ T cells possibly attack hepatocytes directly; while CD4+ T cells and IFN-γ play an indirect role in exacerbated hepatic damage. Furthermore, how CD8+ cells become involved in a type I immune response, that is, activated by antigen presentation via MHC class I, must be elucidated. Typically, MHC class I presents cytosolic proteins (intracellular proteins), but a ‘cross-presentation pathway’ has been reported in which extracellular antigens are presented via MHC class I [36]. In this context, we need to clarify how nanoparticles as extracellular antigens drive the cross-presentation pathway, or how the exacerbation of hepatic damage we identified is not an antigen-specific response. Asbestos and crystalline silica, which cause pneumoconiosis and silicosis, are reported to activate T lymphocytes non-specifically and antigen-independently [37]. Therefore, antigen specificity in the context of the exacerbated hepatic damage must be scrutinized further.

To elucidate the detailed mechanism through which nSP50 promotes acquired immunity, learning how antigen processing, antigen presentation, and hepatocyte killing are accomplished is essential. In the re-stimulation experiment, IFN-γ production by splenocytes did not differ significantly between treatment with nSP50 and medium or between doses of nSP50. These findings suggest that the epitope involved in the exacerbation of hepatic damage is not nSP50 itself. In this context, nanoparticles reportedly can bind autologous protein and form complexes called ‘protein coronas’ [38, 39]. In these complexes, autologous protein is denatured [40, 41] and thus may acquire antigenicity. Hence, focusing on the denatured autologous proteins in the protein corona to identify cryptic epitopes will facilitate the design of nanoparticles that are not recognized by the immune system. From this viewpoint, the exacerbation of hepatic damage is not an nSP50-specific response. The composition of the protein corona is influenced by the surrounding biological fluids and the physical properties of the nanoparticles, such as their size and surface properties [42]. Moreover, these differences in the composition of the protein corona are believed to affect the cellular uptake of nanoparticles. In fact, the characteristics of nanoparticles influence their binding of human blood proteins and that the development of a protein corona inhibited the uptake of silver or silica nanoparticles by phagocytes [43, 44]. Therefore, understanding the relationship among the physicochemical properties of the particles, components of the protein corona, and immunogenicity might be key to elucidating the mechanisms behind the sensitization effects of nSP50 and their surface modification–dependent cross-reactivity. For these reasons, in addition to the relationship to particle size, the association between various physicochemical properties of nanoparticles and the biological effects related to acquired immunity should be explored.

5. Conclusion

We demonstrated that multiple exposure of nSP50 exacerbate the hepatic damage initiated by a single dose and that this exacerbation is mediated through acquired immunity. Mechanistically, CD8+ T lymphocytes and IFN-γ play critical roles in this process. Our current findings suggest that amorphous silica nanoparticles, a kind of non-metallic nanoparticles, can activate acquired immunity and generate adverse biological effects.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).
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Ethics approval and consent to participate

All the experiments involving mice were performed in accordance with the animal welfare guidelines of Osaka University.

Conflict of interests

The authors declare no conflict of interest.

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