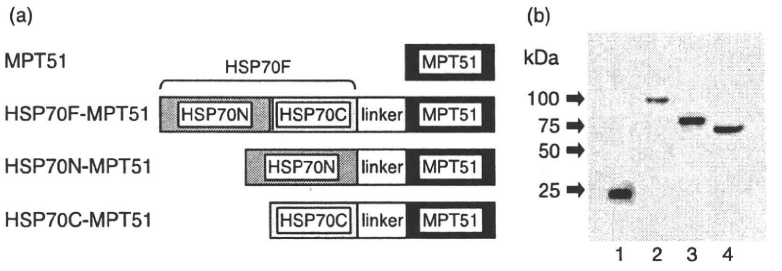

Fig. 2. Chimeric HSP70-MPT51 molecules. (a) Schematic diagram of chimeric molecules. HSP70F, HSP70N, and HSP70C mean full-length, the N-terminal domain, and the C-terminal domain of HSP70, respectively. (b) Expression of chimeric proteins in HEK293T cells. Cells were transfected with plasmids that encoded DNA for various MPT51 molecules, and the protein expression was detected by Western blotting using anti-MPT51 mAb (derived from a clone 2B11F5). 1, MPT51 (expected molecular weight: 27 979.17); 2, HSP70F-MPT51 (95 570.65); 3, HSP70N-MPT51 (67 382.45); and 4, HSP70C-MPT51 (57 774.05). Expected molecular sizes were calculated by an algorithm termed 'COMPUTE PI/MW TOOL' in EXPASY (http://expasy.org/tools/pi_tool.html).

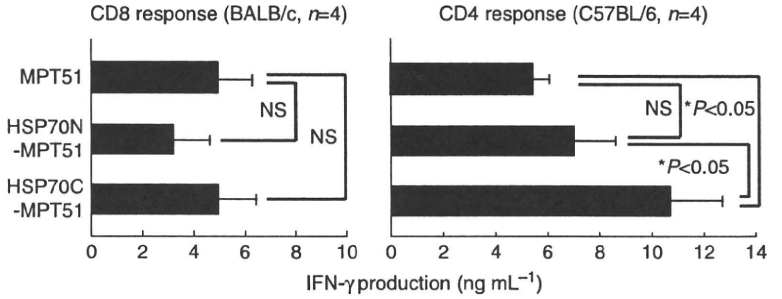

Fig. 3. Comparison of the enhancing effects of the N- and C-domains of HSP70 in DNA vaccination. BALB/c and C57BL/6 mice were immunized with plasmids that encoded *HSP70N-MPT51*, *HSP70C-MPT51*, or *MPT51* alone, and the effects were determined by an IFN-γ production assay using MPT51 peptides for stimulation. The results of four mice per group are presented as mean ± SD. NS, not significant; $*P < 0.05$. IFN-γ productions from unstimulated spleen cells were nearly undetectable in all the groups.

(Casares *et al.*, 1997; Barouch *et al.*, 2004). Therefore, intercellular antigen spreading seems particularly important to increase the number of APCs. In the present study, we attempted to establish a new fusion DNA vaccine against MPT51 using *M. tuberculosis* HSP70 as a partner to facilitate the receptor-mediated uptake of shed antigens from transfected somatic cells that underwent apoptosis and/or necrosis by professional APCs. With this new vaccine construct, we demonstrated that HSP70, especially its C-terminal domain, had an enhancing effect on the induction of MPT51-specific CD4+ (but not CD8+) T-cell responses.

Microbial and mammalian HSP70s effectively induce antigen-specific immune responses in various ways (Asea *et al.*, 2000; Kuppner *et al.*, 2001; Somersan *et al.*, 2001), and several receptors have been identified for them, that is, CD91 (Basu *et al.*, 2001), CD40 (Wang *et al.*, 2001; Becker *et al.*, 2002), CCR5 (Floto *et al.*, 2006), TLR-2, and TLR-4 (Asea *et al.*, 2002). Tobian *et al.* (2004a) have reported that exogenous *M. tuberculosis* HSP70 can CD91-dependently enhance the presentation capacity for ovalbumin (OVA)-derived major histocompatibility complex (MHC) class I peptides in macrophages and DCs. This has established CD91 as a receptor for prokaryotic as well as mammalian HSPs. On the other hand, they have also reported that the enhancing effect of *M. tuberculosis* HSP70 on the presentation of MHC class II peptides is CD91-independent (Tobian *et al.*, 2004b). HSP70 has also been reported to bind to a chemokine receptor CCR5 (Whittall *et al.*, 2006) and transduce various signals into DCs to enhance immune responses (Floto *et al.*, 2006). We reported previously that a fusion protein of MIP-1α and MPT51 is internalized preferentially into DCs via CCR5, and consequently, induces stronger MPT51-specific CD8+ and CD4+ T-cell responses than MPT51 alone (Uchijima *et al.*, 2008 and unpublished data). It is possible that *M. tuberculosis* HSP70 exerts its enhancing effect in the same way, although further analysis is necessary. Alternatively, additional signals via other HSP70 receptors, such as CD40 and/or TLRs, may contribute to the enhancing effects. Taken together, these observations suggest that activation signals transduced via HSP70 receptors other than CD91 play pivotal roles in the enhancement of immune responses, although CD91 may contribute to the internalization of MPT51 fusion proteins conjugated with HSP70.

The domain of HSP70 that is responsible for its enhancing effect is still a controversial subject. Some groups have reported that the C-terminal peptide-binding domain might act as a microbial adjuvant (Wang *et al.*, 2002; Qazi *et al.*, 2005). Wang and colleagues showed that the C-, but not the N-terminal domain upregulates the expression of cytokines and their receptors (IL-12, tumor necrosis factor-α, NO, C–C chemokines, and CCR7), costimulatory molecules (CD83, CD86, and CD80), and HLA class II molecules in human hematopoietic cells. Of these upregulated molecules, IL-12 is known to be a very potent cytokine for Th1 polarization (Trinchieri, 1994), but they showed Th1 skewing of immune responses only by an isotype analysis of immunoglobulins specific for HSP70 itself. Qazi and colleagues also described the effectiveness of the C-terminal domain, but they did not compare its effect with that of the N-terminal domain. Together with these observations, our current findings showing the enhancing effect on targeted-antigen-specific IFN-γ production by CD4+ T cells strengthen the rationale for the use of the C-terminal domain of HSP70 as a Th1-polarizing adjuvant. The enhancing effect of C-terminal domain of HSP70 is more prominent when used in protein immunization. As shown in Fig. 5b, the addition of HSP70C (and HSP70F) to MPT51 effectively induced antigen-specific immune responses, even if the antigen alone could not induce the response. In contrast, Udono *et al.* (2001) have identified amino acid residues 280–385 in the N-terminal ATPase domain of HSP70 as the most important region for cytotoxic T lymphocyte (CTL) induction. Huang *et al.* (2000) have also shown that the N-terminal domain of HSP70 (residues 160–370) is sufficient to stimulate the substantial generation of anti-OVA CTL in the absence of an adjuvant. At present, we have no evidence to explain why HSP70N did not show an enhancing effect on CD8+ T-cell responses in our system. Our construct contains the entire structure of the N-terminal domain while other effective constructs lack the extreme N-terminus, and this portion might have an inhibitory effect. Alternatively, the difference(s) in the immunogenicity of antigens (OVA vs. MPT51) and/or strain (C57BL/6 vs. BALB/c) may be accountable for the ineffectiveness. Besides the enhancing effect of the N-terminal domain on immune responses, however, its immunosuppressive role by the production of IL-10 and transforming growth factor-β has also been demonstrated in rats (Kimura *et al.*, 1998; Wendling *et al.*, 2000). In general, the C-terminal peptide-binding domain of HSP70 tends to facilitate CD4+ T-cell responses more effectively than CD8+ T-cell responses, which skews the cytokine milieu toward Th1.

IFN-γ-secreting CD4+ T cells known as Th1 cells are important mediators of tuberculosis protection (Cooper *et al.*, 1993; Flynn *et al.*, 1993), and attempts to induce tuberculosis antigen-specific Th1 cells have been the

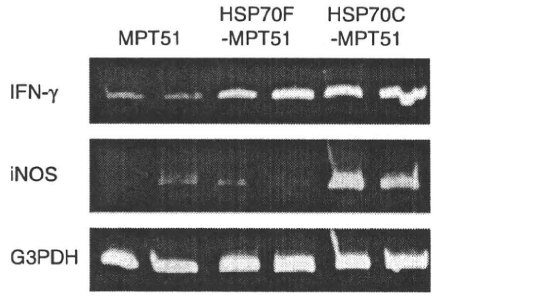

Fig. 4. Semi-quantitative RT-PCR for iNOS and IFN-γ. Spleen cells from immunized C57BL/6 mice were stimulated with MPT51 peptide (P171-190), and the expression of iNOS and IFN-γ was tested by semi-quantitative RT-PCR. G3PDH was used as a control.

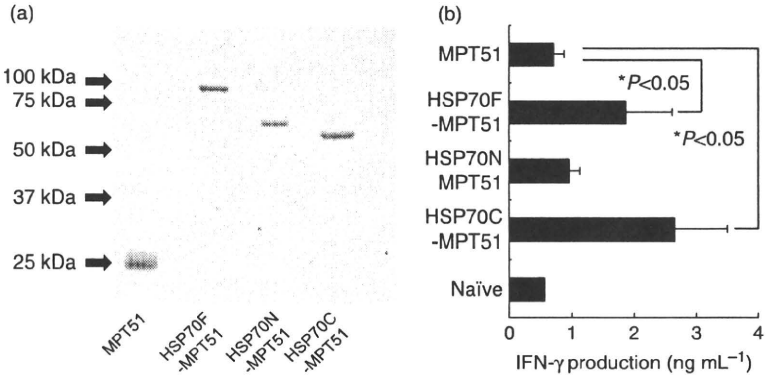

Fig. 5. Comparison of the enhancing effects of the N- and C-domains of HSP70 in protein vaccination. (a) SDS-PAGE analysis of recombinant proteins. Proteins were purified with Ni-NTA agarose, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. For the expected molecular sizes, see the legend for Fig. 2b. (b) MPT51-specific IFN-γ production. C57BL/6 mice were immunized with recombinant proteins, and their spleen cells were used for an IFN-γ production assay in response to the MPT51 peptide (P171–190). The results of four mice per group are presented as mean ± SD. $*P < 0.05$. IFN-γ productions from unstimulated spleen cells were nearly undetectable in all the groups.

dominant theme of most tuberculosis-vaccine development (Skeiky & Sadoff, 2006). Although CD8+ CTLs have also been reported to contribute to disease resistance (Flynn & Chan, 2001; Kaufmann, 2003), our current findings may pave the way for the establishment of a novel vaccine against *M. tuberculosis*.

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