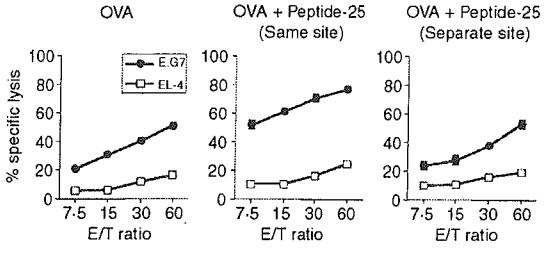

Lytic unit (/10^6)	OVA	OVA + Peptide-25 (Same site)	OVA + Peptide-25 (Separate site)
E.G7	1·8	15·1	1·7
EL-4	<0·01	<0·01	<0·01

Figure 3. Enhanced OVA-specific CD8$^+$ T-cell response induced by coimmunization with OVA and Peptide-25 at the same site. A group of mice was immunized with OVA (10 μg) and Peptide-25 (10 μg) in IFA at the same site subcutaneously. Another group of mice was immunized with OVA (10 μg) in IFA and Peptide-25 (10 μg) in IFA at two distant sites (separate sites). As a control, we also immunized a group of mice with OVA (10 μg) in IFA. Spleen cells from each group of mice 10 days after the immunization were subjected to OVA-specific CTL assay. CTL assay was conducted by incubating sensitized cells with ^{51}Cr-labelled E.G7 or ^{51}Cr-labelled EL-4 cells at various effector to target ratios at 37° for 4 hr. The values represent the geometric means and standard deviation of percentage specific lysis in triplicate cultures. Background lysis of the target cells was less than 10% of maximum ^{51}Cr-release. The number of LU was calculated from the dose–response curve obtained with each group. A representative result of a series of three experiments is shown.

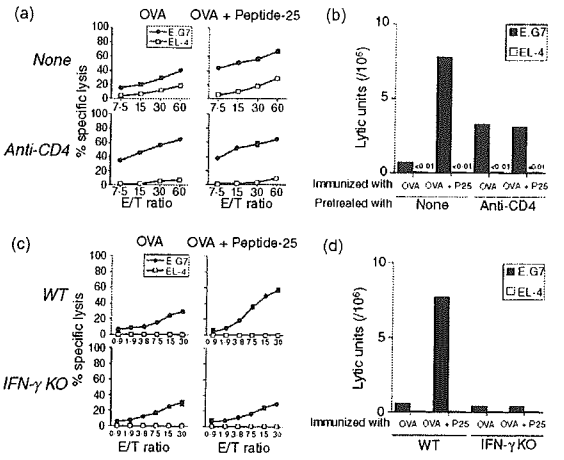

Figure 4. Role of CD4$^+$ T cells and IFN-γ in the enhancing effect of Peptide-25 on CTL response. (a,b) Two groups of wild-type mice were injected with anti-CD4 mAb (GK1.5) on days −13, −12, −11, −6, −5, −4, +1, +2 and +3 relative to immunization. Each group of mice was immunized with OVA (10 μg) in IFA or a mixture of OVA (10 μg) and Peptide-25 (10 μg) in IFA on Day 0. As controls, two other groups of mice had been treated with normal rat IgG in place of GK1.5 and immunized with OVA in IFA or a mixture of OVA and Peptide-25 in IFA. Spleen cells from each group of mice were subjected to *in vitro* OVA-specific CTL assay 10 days after the immunization. (c,d). Either wild-type or IFN-γ$^{-/-}$ mice with C57BL/6 background were immunized with OVA (10 μg) in IFA or a mixture of OVA (10 μg) and Peptide-25 (P25) (10 μg) in IFA. Spleen cells from each group of mice were subjected to OVA-specific CTL assay 10 days after the immunization. CTL assay (a,c) and LU calculations (b,d) were conducted as described in Figure 1.

described in the Materials and methods. As a control, rat IgG was administered in place of anti-CD4 to another two groups of mice. All groups of mice were immunized with OVA in IFA or with a mixture of OVA and Peptide-25 in IFA. As shown in Fig. 4(a,b), an enhanced CTL response specific for OVA, mounted in a culture of spleen cells taken from mice immunized with a mixture of OVA and Peptide-25 in IFA, was abrogated by the anti-CD4 mAb treatment before immunization, and the level of CTL response, measured as LU, was similar to that mounted in spleen cells from OVA-immunized mice. Unexpectedly, an enhanced OVA-specific CTL response, mounted in a culture of spleen cells from OVA-immunized mice, was observed when anti-CD4 mAb mice had been treated before OVA immunization.

To examine the roles of IFN-γ-producing cells in the CTL response, we immunized both wild-type and IFN-γ$^{-/-}$ mice with a mixture of OVA and Peptide-25 in IFA. Spleen cells from each group of mice were then subjected to the *in vitro* CTL assay specific for OVA. The results revealed that an enhanced CTL response specific for OVA was mounted in spleen cells from wild-type mice immunized with a mixture of OVA and Peptide-25 in IFA, while the enhancement was not observed in spleen cells from IFN-γ$^{-/-}$ mice (Fig. 4c,d). These results indicate that Peptide-25-reactive IFN-γ-producing T cells contribute to the enhancing effect of Peptide-25 on OVA-specific CTL generation.

Co-immunization with a mixture of OVA and Peptide-25 increases proportions of OVA-specific CTL precursors

To examine the cellular mechanisms of the enhancing effect of coimmunization of mice with a mixture of OVA and Peptide-25 on CTL generation following *in vitro* OVA stimulation, we examined the frequencies of OVA-specific CTL precursors. Spleen cells from mice were immunized with either OVA in IFA or a mixture of OVA and Peptide-25 in IFA 10 days before the experiments. The cells were then stained with OVA peptide-loaded H-2K^b:Ig protein and anti-mouse IgG1–PE and analysed using FACSCalibur. The results revealed that frequencies for OVA-specific CTL precursors in spleen cells (0·42%) from mice immunized with a mixture of OVA and Peptide-25 were about two-fold higher than those in spleen cells (0·21%) from OVA-immunized mice (Fig. 5).

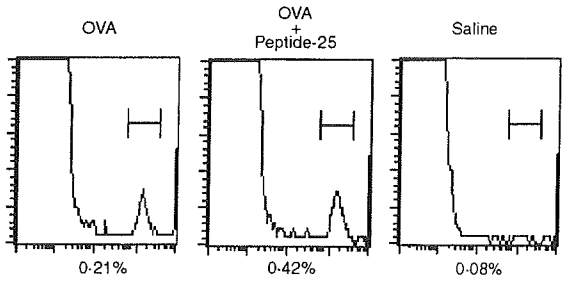

Figure 5. Frequency analysis of OVA-specific CTL. Two groups of mice were immunized with either OVA in IFA or OVA and Peptide-25 in IFA. Spleen cells from each group of mice were prepared 10 days after the immunization and stained with 4 μg of OVA peptide (SIINFEKL)-loaded H-2K^b:Ig protein and incubated for 60 min at 4°. After washing with staining buffer, cells were resuspended in 100 μl staining buffer containing appropriately diluted anti-mouse IgG1-PE and anti-CD8-FITC. After washing twice with staining buffer, cells were analysed using FACSCalibur.

Co-immunization of mice with a mixture of OVA and Peptide-25 can suppress E.G7 growth, leading to tumour rejection *in vivo*

We examined whether the immunization of C57BL/6 mice with a mixture of OVA and Peptide-25 was effective as a prophylactic intervention into the growth of E.G7. Three groups of 12 mice each were immunized with OVA in IFA, Peptide-25 in IFA, or a mixture of OVA and Peptide-25 in IFA. As a control, a fourth group of 12 mice was injected with IFA. Ten days after the immunization, we transplanted viable E.G7 tumour cells onto the backs of the mice and monitored tumour growth (Fig. 6a) and survival (Fig. 6b) after tumour challenge. As shown in the figures, the transplanted E.G7 established tumour masses within days of the tumour challenge in the abdomen of both the control group and the Peptide-25-immunized group of mice, leading to the death of all animals by day 30. Tumour growth in mice immunized with OVA in IFA was slightly delayed compared with that in the control mice however, all mice died by day 40 after the tumour challenge. Interestingly, mice immunized with a mixture of OVA and Peptide-25 in IFA showed significantly delayed tumour progression. Among these 12 mice, the E.G7 tumours of six of them were eradicated by Day 60 after the tumour challenge and the lifetimes of the mice were continued, tumour-free, for up to 100 days. By contrast, all mice in the other groups died within 40 days after the tumour challenge. These results indicate that coimmunization of Peptide-25 with OVA enhances not only an OVA-specific CTL response *in vitro* but also induces a potent antitumour immunity against OVA-expressing tumour cells *in vivo*.

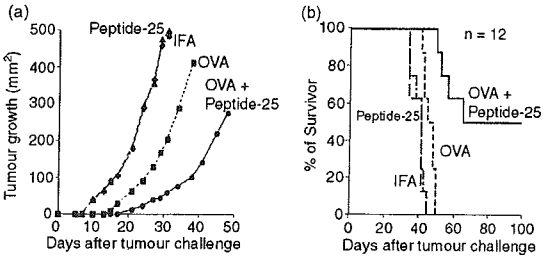

Figure 6. Enhancement of antitumour immunity by Peptide-25. (a) Suppression of E.G7 growth by augmented induction of E.G7-specific immunity. Three groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (10 μg) in IFA or Peptide-25 (10 μg) in IFA subcutaneously. As a control, a group of mice was injected with IFA. All groups of mice were challenged with 5×10^5 viable E.G7 cells subcutaneously 10 days after the immunization. Growth of E.G7 tumour was monitored by measuring its size periodically (2- to 3-day intervals) and expressed as mm^2. (b) Survival of E.G7-bearing mice. The percentages of survivors in the respective groups shown in (a) are displayed.

The enhancement of the Th1 response to OVA by coimmunization with Peptide-25

To understand the mechanisms of the enhancing effect of Peptide-25 on the OVA-specific CTL response, we examined the Th1 generation specific for OVA when C57BL/6 mice were immunized with OVA in IFA, Peptide-25 in IFA, or a mixture of OVA and Peptide-25 in IFA. First, we measured anti-OVA antibody in the serum of each group of mice by ELISA 10 days after immunization. As shown in Fig. 7(a), OVA-immunized mice produced predominantly anti-OVA IgG1 antibody and produced lower levels of the anti-OVA IgG2a antibody. In contrast, mice immunized with a mixture of OVA and Peptide-25 produced predominantly anti-OVA IgG2a antibody. We could not detect any anti-OVA antibody in sera from Peptide-25-immunized mice (Fig. 7a).

To evaluate the cytokine-producing profiles in CD4$^+$ T cells in the spleen, spleen cells from each group of immunized mice were stimulated *in vitro* with OVA for 48 hr, and the proportions of IFN-γ- and IL-4-producing cells were examined by intracellular cytokine staining. Cells stained were gated on live CD4$^+$ cells and analysed by FACS. The results revealed that we could detect IFN-γ-producing CD4$^+$ T cells but not IL-4-producing cells when spleen cells from mice immunized with a mixture of OVA and Peptide-25 were stimulated with OVA (Fig. 7b). In contrast, OVA stimulation of spleen cells from OVA-immunized mice could become both IFN-γ- and IL-4-producing cells. We confirmed the enhancement of OVA-induced IFN-γ production by ELISA (Fig. 7b). Co-immunization of OVA with Peptide-9 slightly enhanced the proportions

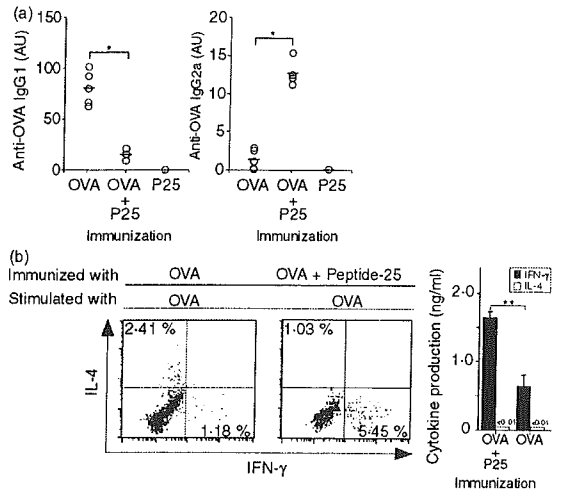

Figure 7. Enhancement of OVA-specific Th1 response by coimmunization of C57BL/6 mice with OVA and Peptide-25. Two different groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (P25) (10 μg) in IFA, or Peptide-25 (10 μg) in IFA subcutaneously. (a) Serum anti-OVA IgG1 and IgG2a were titrated by ELISA 10 days after the immunization. Each open circle represents the results of an individual mouse. The horizontal bar represents the mean value of six mice. $*P < 0{\cdot}01$ by Student's *t*-test. (b) Ten days after immunization, spleen cells were stimulated *in vitro* with OVA (10 μg/ml) for 4 days. Intracellular staining of IL-4 and IFN-γ was carried out to the recovered cells. Cells stained were gated on live $CD4^+$ cells and examined by FACSCalibur. The percentages of IL-4- and IFN-γ-producing $CD4^+$ T cells are presented in the upper left and lower right regions, respectively. IL-4 and IFN-γ produced in the culture supernatants were titrated by ELISA. The values represent the mean and standard deviation of the triplicate cultures. $**P < 0{\cdot}05$ by Student's *t*-test.

of IFN-γ-producing cells, while coimmunization with Peptide-18 did not (data not shown). We did not observe differences in proportion of $CD4^+$ $CD25^+$ T cells between Peptide-25-immunized and Peptide-9-immunized T cells. These results suggest that the weak Th1-inducing ability of Peptide-9 correlates with a weak ability to enhance OVA-specific CTL generation when coimmunized with OVA.

The involvement of Peptide-25-reactive T cells in the induction of an OVA-reactive Th1 response was examined by using the rat anti-mouse clonotypic TCR mAb KN7 that can recognize TCR expressed on the Peptide-25-reactive Th1 clone of C57BL/6 mice. Enhanced induction of an OVA-specific IFN-γ-producing T-cell response in mice immunized with a mixture of OVA and Peptide-25 was impaired when we injected KN7 intraperitoneally 1 day before the immunization. In contrast, the control group that received rat IgG injection in place of KN7 did not show this suppressive effect (data not shown).

Peptide-25-reactive T cells can activate DCs through Peptide-25 leading to enhanced induction of OVA-presenting activity

The enhancement of an OVA-specific CTL response by coimmunization of Peptide-25 with OVA may be because the Peptide-25 directly or indirectly affects the OVA-presenting activity of APCs. To investigate this issue, we first evaluated the antigen-presenting activity of DCs. We propagated immature DCs *in vitro* by culturing bone marrow cells with GM-CSF and IL-3 for 6 days. They were stimulated for 48 hr with Peptide-25 in the presence of splenic $CD4^+$ T cells from P25 TCR-Tg mice. As a control, DCs were also stimulated with lipopolysaccharide. The expression of surface markers such as CD40, CD80, CD86, MHC class I antigen and intracellular adhesion molecule-1 (ICAM-1) was analysed using FACSCalibur. The results revealed that Peptide-25 stimulation alone did not alter the expression of the above surface markers on DCs (data not shown). When we cocultured DCs with $CD4^+$ T cells from P25 TCR-Tg mice together with Peptide-25, the expressions of MHC class I and ICAM-1 were enhanced to a similar extent to that found under lipopolysaccharide stimulation and this led to the induction of IL-12p40 production (Fig. 8a). Such DCs showed more effective OVA presentation to $CD8^+$ T cells from OT-1 mice and enhanced OT-1 cell divisions (Fig. 8b). These results suggest that Peptide-25-reactive $CD4^+$ T cells directly activate DCs in the presence of Peptide-25, leading to effective OVA cross-presentation for the activation of $CD8^+$ T cells.

Discussion

Antitumour immune responses involve complex interactions among various immunocompetent cells. $CD8^+$ CTLs are major effector cells capable of direct tumour destruction both *in vivo* and *in vitro*, and they recognize MHC class I binding peptides derived from molecules with altered expression in tumour cells.[28–30] Although the need for $CD4^+$ Th cells in regulating $CD8^+$ T cells has been documented, their target epitopes and functional impact in antitumour responses remain unclear.

There has been a recent reappraisal of the role and importance of $CD4^+$ Th cells in antitumour responses. $CD4^+$ Th cells are considered to contribute to the activation of $CD8^+$ CTLs through the expression of the CD40 ligand and production of cytokines, such as IL-2 and IFN-γ that are essential for the proliferation and maturation of $CD8^+$ CTL precursors. $CD4^+$ Th cells recognize MHC class II binding peptides on APCs and their interaction may result not only in the activation and priming of $CD4^+$ Th cells, but also in the activation of APCs themselves.[31–33] Consequent to these mutual activations, APCs prime and activate $CD8^+$ CTLs specific for the

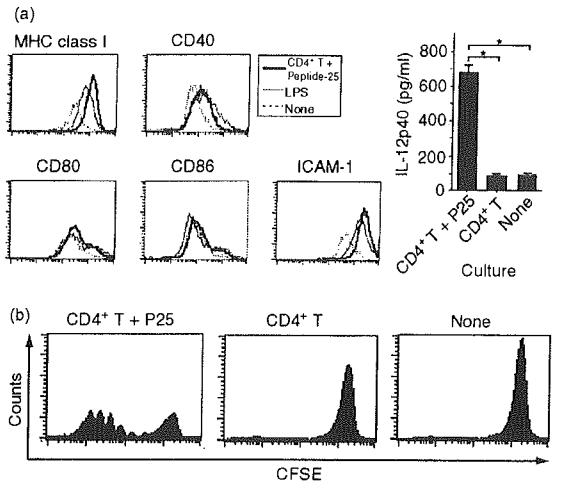

Figure 8. Activation of DCs by culturing with Peptide-25 in the presence of $CD4^+$ T cells from P25 TCR-Tg mice. (a) Immature DCs were propagated by culturing bone marrow cells with GM-CSF (20 ng/ml) and IL-3 (20 ng/ml) for 6 days. The cells recovered (5×10^5) were cultured with lipopolysaccharide (LPS; 5 μg/ml), Peptide-25 together with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice or left untreated for 48 hr. The expression of surface markers was assessed by FACS analysis. IL-12p40 in the cultured supernatant was assessed by ELISA. (b) CFSE-labelled $CD8^+$ T cells (5×10^5) from OT-1 mice were cultured with immature DCs (5×10^5) and OVA (10 μg/ml) for 4 days. The cells were cocultured with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice and Peptide-25 (P25) (10 μg/ml) (left panel) or $CD4^+$ T cells from P25 TCR-Tg mice (middle panel). Subsequently, cell division of the $CD8^+$ T cells was monitored by FACSCalibur.

tumour antigen or peptides. This scenario of cellular interaction assumes that $CD4^+$ Th cells and $CD8^+$ CTLs may not necessarily be in direct or close association. $CD4^+$ T cells are also implicated in the activation of tumoricidal macrophages that are involved in tumour clearance. Cytokines produced by $CD4^+$ T cells can recruit and activate macrophages and eosinophils, linking the T-cell response with the innate immune response.

In this study using OVA as neo-tumour antigen in a mouse model, we investigated the contribution of the Th1 epitope within the 15 amino acid residues of the Ag85B protein. We then examined the significance of the defined Th1 epitope regarding $CD8^+$ CTL generation and tumour eradication. We demonstrated that the coimmunization of Peptide-25 and the OVA CTL epitope resulted in a marked increase in the OVA-specific $CD8^+$ CTL response (Fig. 1). The selection of immunization site for Peptide-25 and OVA is important and both should be immunized at the same site (Fig. 3). The augmenting effect of Peptide-25 on $CD8^+$ CTL generation was cancelled by *in vivo* administration of the anti-CD4 mAb and was diminished in IFN-$\gamma^{-/-}$ mice (Fig. 4), indicating that the $CD4^+$ T cells and IFN-γ-producing T cells are required. It remains elusive why the enhancement of OVA-specific CTL activity was abrogated when IFN-$\gamma^{-/-}$ mice were immunized with the mixture of Peptide-25 and OVA. As we reported, the generation of Peptide-25-reactive $CD4^+$ T cells significantly decreases in IFN-$\gamma^{-/-}$ mice as compared with wild-type mice. Furthermore, Peptide-25 stimulation of Peptide-25-immunized cells induces IL-2 production that is also impaired in IFN-$\gamma^{-/-}$ cells.[22] IFN-γ-dependent IL-2 production may be indispensable to enhance the OVA-specific $CD8^+$ CTL generation and expansion in this particular system. Whilst we favour the possibility that impaired production of IFN-γ by $CD4^+$ T cells contributes to the reduction of CTL activity seen in mice primed with Peptide-25 and OVA we cannot exclude the possibility that ablation of IFN-γ production by $CD8^+$ T cells also plays a role.

Another explanation is that IFN-γ may play an important role in a cell-to-cell contact between Peptide-25-specific $CD4^+$ Th cells and APCs to enhance the induction of the OVA-specific $CD8^+$ CTL response. APCs are capable of processing and presenting exogenous antigens along with MHC class I molecules to $CD8^+$ T cells, which is termed antigen cross-presentation. The antigenic peptide generation for cross-presentation appears to be dependent on both the ubiquitin–proteasome system and the transporter associated with antigen processing.[34–37] Imai and his colleagues recently demonstrated that exogenously added OVA is accumulated in microsomal fractions, including the endoplasmic reticulum and late endosomes followed by retrograde transport to the cytoplasm through the Sec61 transporter complexes.[38] IFN-γ may facilitate these processes of OVA cross-presentation by APCs leading to enhancement of the induction of the OVA-specific $CD8^+$ CTL response.

Unexpectedly, our result showed that anti-CD4 mAb treatment before OVA immunization in mice enhanced the OVA-specific CTL response. Although we do not have concrete evidence, anti-CD4 mAb treatment may eliminate OVA-specific $CD4^+$ T cells that negatively regulate OVA-specific $CD8^+$ CTL generation.

Among I-A^b-binding peptides, Peptide-25 showed the most potent effect that correlated with potency for the *in vivo* induction of Th1 generation to each peptide. These results indicate that a Th1 epitope, regardless of the difference in its molecular origin, exhibits a helper activity for *in vivo* $CD8^+$ CTL generation. It is unclear why I-A^b binding Peptide-9 does not augment the induction of the OVA-specific CTL response. Peptide-9 showed lower immunogenicity for Th1 induction specific for its own compared with Peptide-25.[22] We analysed the Foxp3 expression in Peptide-9-reactive T cells by FACS and found no significant increase in the $Foxp3^+$ T regulatory cell population compared with Peptide-25-reactive T cells (data not shown). These results imply that the weak

potency of Peptide-9 to augment the OVA-specific CTL response is well correlated with its weak Th1-inducing potency instead of a T regulatory cell induction.

Both quantitative and qualitative changes may be involved in the Th effect of Peptide-25 in the CTL response. We think that a two-fold increase in the frequency of OVA-specific $CD8^+$ T cells and an increase of the lytic unit are qualitatively correlated with the Th effect of Peptide-25 (Figs 1–4). In addition, Peptide-25 stimulation of spleen cells from Peptide-25-immunized wild-type mice induces secretion of IL-2 that is impaired in IFN-$\gamma^{-/-}$ mice.[22] IL-2 produced by the Th1 cells may also enhance and expand the CTL maturation quantitatively. To evaluate qualitative changes more directly, we examined Granzyme B expression in OVA-reactive $CD8^+$ T cells. Results revealed that we did not observe significant increases in Granzyme B-expressing cells in the OVA-stimulated $CD8^+$ T cells in the presence of Peptide-25-primed Th cells compared with the precursors induced in the absence of the primed Th cells (data not shown). These results suggest that OVA-specific $CD8^+$ T-cell precursors induced in the presence of primed Th cells may differ quantitatively from these induced in the absence of primed Th cells.

The basis for the strong 'helper' function of Peptide-25 in the $CD8^+$ CTL response to E.G7 rejection is unknown. There are several possibilities to account for the enhancing effect of Peptide-25 on Th1 and CTL generation specific for OVA when Peptide-25 and OVA were coimmunized at the same site. Peptide-25 may directly activate APCs through unknown molecules resulting in effective cross-presentation of OVA molecules to both Th1 and CTL precursors. To address this issue, we propagated bone-marrow-derived DCs *in vitro*, stimulated them with Peptide-25 and analysed the surface expression of activation markers on DCs. The results revealed that expression of MHC class I, ICAM-1, CD40, CD80 and CD86 was not enhanced upon Peptide-25 stimulation (data not shown). We found that myeloid differentiating factor (MyD)88-deficient mice also showed enhanced CTL generation specific for OVA when coimmunized with Peptide-25 and OVA (data not shown). The MyD88-dependent signalling pathway may not be required for exerting helper activity by Peptide-25. On the whole, we do not support the hypothesis that Peptide-25 directly activates APCs.

As Peptide-25-reactive $CD4^+$ Th cells recognize MHC class II binding cognate Peptide-25 on APCs and their interaction may result in the activation of APCs, consequent to these mutual activations, APCs prime and activate $CD8^+$ CTLs specific for OVA peptides. The requirement for coimmunization of Peptide-25 and OVA in the same site suggests that the physical proximity of OVA and Peptide-25 is crucial for intracellular events in APCs. Cell-to-cell contact between Peptide-25-specific $CD4^+$ Th1 cells and APCs may be required or the two antigens need to be processed and presented by the same APC to augment the OVA-specific $CD8^+$ CTL response. To simplify the experimental system for further evaluation, we established an *in vitro* culture system using T cells from P25 TCR-Tg mice, CFSE-labelled T cells from OT-1 mice and bone-marrow-derived immature DCs propagated *in vitro*. As we reported, $CD4^+$ T cells from P25 TCR-Tg mice preferentially develop IFN-γ-producing T cells upon Peptide-25 stimulation in the presence of I-A^b splenic APCs under neutral conditions.[24] Surface expression of the MHC class I molecule, ICAM-1, CD40, CD80 and CD86 on DCs as well as IL-12p40 production was enhanced when DCs were cultured with $CD4^+$ T cells from P25 TCR-Tg mice and Peptide-25 (Fig. 8a). Furthermore, enhanced cell divisions of CFSE-labelled OT-1 T cells were observed when the cells were stimulated with OVA in the presence of DCs, Peptide-25 and $CD4^+$ T cells from P25 TCR-Tg mice (Fig. 8b). These results support the notion that $CD4^+$ Th1 cell-derived factor may augment antigen processing by APCs leading to the enhancement of the cross-priming of the antigenic peptide for $CD8^+$ T cells.

A number of approaches to augment $CD4^+$ T-cell help have been investigated.[2,10,13,39] One involves modifying the immunizing antigen itself by, for instance, haptenizing the antigen[40] or linking heterologous immunogenic peptides directly to the antigen.[17,41] The second involves coimmunization with tumour antigens and molecules with strong helper determinants.[42,43] The third, the discovery of a range of molecular signals, such as the CD40 ligand and other costimulatory signals[31–33] involved in the helper function of $CD4^+$ T cells, provides other ways to augment the $CD8^+$ T-cell response. Finally, broadly expressed wild-type molecules in murine tumour cells eliciting humoral immunity contribute to the generation of $CD8^+$ T cells and protective antitumour immune responses to unrelated tumour-specific antigen.[13]

It is important to ask whether the enhancing effect of Peptide-25 on $CD8^+$ CTL responses is beneficial, detrimental, or insignificant to the tumour-bearing host. It is likely that the phenomena that we have described in the mouse of a heightened $CD8^+$. CTL response to tumour antigens by corecognition of Peptide-25 has its counterpart in humans, occurring as a consequence of the simultaneous uptake of complex antigenic mixtures from disintegrating tumour cells by APCs. As coimmunization with Peptide-25 and tumour antigens also results in heightened resistance to tumour challenge in the mouse, this approach may be an attractive strategy for human cancer immunotherapy. In fact, there are ongoing clinical trials utilizing either heterologous helper antigens, such as keyhole limpet haemocyanin or tumour-derived helper antigens to augment antitumour immune responses by $CD8^+$ CTLs directed against tumour-derived CTL

epitopes.[44,45] As Ag85B and Peptide-25 are stimulatory to human $CD4^+$ T cells from PPD^+ healthy donors in a certain proportion of the Japanese population, Ag85B or peptide-25 may be applicable to augment antitumour immune responses by $CD8^+$ CTLs against tumour cells. Along with the scenario, we re-examined the effect of preimmunization with *M. tuberculosis* or Peptide-25 on the antitumour CTL response and found that preimmunization of mice with heat-killed *M. tuberculosis* or Peptide-25 followed by immunization with a mixture of OVA and Peptide-25 produced a significant increase in the number of $CD8^+$ CTL s (data not shown).

While it is obvious that $CD8^+$ T cells recognize MHC class I binding peptides derived from tumour target cells based on the subsequent tumour-specific destruction, peptide derivation from tumour cells may not be an absolute requirement for $CD4^+$ T cells because they do not directly interact with tumour cells that mostly lack MHC class II expression. Co-immunization of mice with TRP-2 of B16 melanoma and Peptide-25 in IFA enhances CTL generation specific for TRP-2 (Fig. 2). However, mice that had been immunized with a mixture of TRP-2 and Peptide-25 in IFA failed to eradicate B16 melanoma, a less immunogenic tumour, although B16 melanoma growth was reduced to a certain extent (data not shown), suggesting that enhancing the effect of Peptide-25 in the TRP-2-specific CTL may not be potent enough to eradicate a less immunogenic TRP-2-expressing tumour. We need another regimen together with Peptide-25 immunization that activates effector cells leading to the eradication of tumour cells with weak immunogenicity. This notion has become extremely important in the design of future vaccines aimed at the efficient activation of both T-cell populations involved in antitumour immune responses.

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Catalase Plays a Critical Role in the CSF-independent Survival of Human Macrophages via Regulation of the Expression of BCL-2 Family*

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M-colony-stimulating factor (M-CSF)-induced monocyte-derived macrophages (M-MΦ) required continuous presence of M-CSF for their survival, and depletion of M-CSF from the culture induced apoptosis, whereas human alveolar macrophages (A-MΦ) and granulocyte-macrophage (GM)-CSF-induced monocyte-derived macrophages (GM-MΦ) survived even in the absence of CSF. The expression of BCL-2 was higher in M-MΦ, and M-CSF withdrawal down-regulated the expression. The expression of BCL-X_L was higher in A-MΦ and GM-MΦ, and the expression was CSF-independent. The expression of MCL-1 and BAX were not different between M-MΦ and GM-MΦ and were CSF-independent. Down-regulation of the expression of BCL-2 and BCL-X_L by RNA interference showed the important role of BCL-2 and BCL-X_L in the survival of M-MΦ and GM-MΦ, respectively. Human erythrocyte catalase (HEC) and conditioned medium obtained from GM-MΦ or A-MΦ cultured in the absence of GM-CSF prevented the M-MΦ from apoptosis and restored the expression of BCL-2. The activity of the conditioned medium was abrogated by pretreatment with anti-HEC antibody. Anti-HEC antibody also induced the apoptosis of M-MΦ cultured in the presence of M-CSF and GM-MΦ and A-MΦ cultured in the presence or absence of GM-CSF and down-regulated the expression of BCL-2 and BCL-X_L in these MΦs. GM-MΦ and A-MΦ, but not M-MΦ, can produce both extracellular catalase and cell-associated catalase in a CSF-independent manner. Intracellular glutathione levels were kept equivalent in these MΦs, both in the presence or absence of CSF. These results indicate a critical role of extracellular catalase in the survival of human macrophages via regulation of the expression of *BCL-2* family genes.

Human tissue macrophages (MΦ)[2] play important roles for homeostasis, and *in vivo* alveolar (A)-MΦ acquire a strong antioxidant phenotype that contributes to prevention of the oxidant burst in an aerobic environment and can survive for long periods (1). In a previous study, we reported that human A-MΦ and GM-CSF-induced monocyte-derived macrophages (GM-MΦ) are resistant to hydrogen peroxide (H_2O_2) via their high basal and inducible levels of catalase activity and that M-CSF-induced monocyte-derived macrophages (M-MΦ) are sensitive to low levels of H_2O_2 with low levels of catalase activity (2). GM-MΦ is phenotypically identical to A-MΦ, whereas M-MΦ closely resembles peritoneal MΦ in respect to morphology, cell surface antigen expression, and several biological functions (2–7). *In vivo* A-MΦ express BCL-2 family proteins such as BCL-2 and BCL-X_L that prevent H_2O_2-induced apoptosis via inhibition of caspase-3 or -9 activation and cytochrome *c* release from mitochondria (8–11). These findings suggest that a high level of catalase activity enables long survival of GM-MΦ and A-MΦ with positive regulation of BCL-2 family protein.

In this study, we found that M-MΦ absolutely require CSF for their survival and express high levels of *BCL-2* gene and protein in the presence of M-CSF. In contrast to M-MΦ, GM-MΦ and A-MΦ can survive in the absence of CSF via high levels of *BCL-X_L* gene and protein. We further examined the relation between catalase activity and distinct expression of BCL-2 family protein and make clear the roles of CSF in the regulation of catalase activity and BCL-2 family protein in human tissue macrophages under the influence of oxidative stress.

EXPERIMENTAL PROCEDURES

Preparation and Culture of Macrophages—Monocytes (Mo) were obtained from peripheral blood mononuclear cells of normal healthy volunteers using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD14 monoclonal antibody-coated microbeads as described previously (7). $CD14^+$ Mo were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan), 3 mg/ml filtered glutamine (Sigma), 100 units/ml penicillin G potassium (Banyu Seiyaku Co., Ltd., Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika Co., Ltd., Tokyo, Japan), 10% autoclaved $NaHCO_3$, and 10% heat-inactivated fetal calf serum (Z. L. Bockneck Laboratories Inc., Ontario, Canada) with the following human recombinant cytokines at optimal concentrations: 5 ng/ml GM-CSF (Schering-Plough Japan, Osaka, Japan) or 50 ng/ml M-CSF (Morinaga Milk Industry Co., Ltd., Tokyo, Japan) at 37 °C in humidified 5% CO_2 for 7 days. During the culture, Mo differentiated to MΦ.

Human A-MΦ were obtained from healthy volunteers (non-smokers without diseases) by bronchial alveolar lavage method (2, 7). All volunteers gave informed consent to the use of their A-MΦ in part for this study.

Antisense Oligonucleotide (AS) Treatment—2′-*O*-methyl-modified oligoribonucleotide phosphorothioate 18-mer two CpG motifs targeted to the *BCL-2* initiation codon (G3139 (BCL AS): 5′-TCTCCCAGCGT-

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[2] The abbreviations used are: MΦ, macrophage; M-MΦ, M-CSF-induced monocyte-derived MΦ; A-MΦ, alveolar MΦ; GM-MΦ, granulocyte-macophage-CSF induced monocyte-derived MΦ; CSF, colony-stimulating factor; HEC, human erythrocyte catalase; CM, conditioned medium; Mo, monocytes; GSH, glutathione; AS, antisense oligonucleotide; BCL AS (G3139), 2′-*O*-methyl-modified oligonucleotide phosphorothioate 18-mer two CpG motifs targeted to the *BCL-2* initiation codon; BCL MS (G4126), G3139 variant with single base mismatch at each CpG motif; 5′-BCL-X AS, 2′-*O*-methyl-modified oligonucleotide phosphorothioate 18-mer to the 5′-splice site of BCL-X_L; MS, missense; anti-Cat ab, anti-HEC antibody.

GCGCCAT-3'), G3139 variant with single base mismatch at each CpG motif (G4126 (BCL missense (MS)): 5'-TCTCCCAGCATGTGCCAT-3') (12) and 18-mer to the 5'-splice site of BCL-X_L (5'-BCL-X AS, ACCCAGCCGCCGUUCUCC) (13) and *MCL-1* AS (ISIS 20408, TTG-GCTTTGTGTCCTTGGCG) (14) were synthesized by Proligo France SAS (Paris, France). Oligonucleotides with random sequences were used as negative controls. Cells were treated with oligonucleotides complexed with Lipofectin (5 mg/ml; Invitrogen) cationic lipid delivery agent according to the manufacturer's directions.

Assessment of Cell Number and Cell Viability—The number of adherent Mo and MΦ was determined by counting the liberated intact nuclei from lysed cells stained with 1% (w/v) cetyltrimethyl ammonium bromide (Cetavelone; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 0.1 M citric acid with 0.05% (w/v) naphthol blue black (Sigma). Cell viability was assessed by the trypan blue dye exclusion test.

Assessment of Apoptosis—DNA fragmentation was detected by immunohistochemical staining using the terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling method (Apo-tag kit; Oncor Co.) or visualized as DNA ladder formation (5). Cells were preincubated with 1% H_2O_2 in phosphate-buffered saline for inactivation of endogenous peroxidase for 5 min at room temperature. Incorporation of digoxigenin-conjugated dUTP to the terminal 3'-OH of fragmented DNA by exogenous terminal deoxynucleotidyl transferase was carried out at 37 °C for 1 h. The reaction products were incubated with horseradish peroxidase-linked anti-digoxigenin antibody at 37 °C for 30 min and visualized with the substrate 3–3' diaminobenzidine plus 0.6% H_2O_2.

Cells were lysed with hypotonic lysis buffer (10 mM Tris-Cl (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton-X), and crude DNA was extracted from the lysed cells by incubation with 40 μg/ml RNase and 40 μg/ml proteinase K for 1 h at 37 °C. The DNA was precipitated with final 50% propanol at −20 °C overnight and washed with 70% (w/v) ethanol. Electrophoresis was performed in 2% agarose at 50 V, and the migrated DNA was visualized by ethidium bromide staining.

Transmission Electron Microscopy—Cells were fixed by immersion in a 2.5% glutaraldehyde-2% paraformaldehyde mixture (10), followed by 1% glutaraldehyde-0.5% tannic acid diluted in 0.1 M cacodylate buffer (Wako Pure Chemical Industries, Ltd.) at 4 °C for 2 h. Samples were post-fixed with 1% OsO_4 (osmium tetroxide; Wako) at 4 °C for 2 h and then embedded in epoxy resin (Epok 812; Okenshoji. Co., Ltd., Tokyo). Thin sections were cut using a LKB-8800 ultratome (LKB, Uppsala, Sweden) and observed using a transmission electron microscope (Hitachi H-7000) after staining with uranyl acetate (Serva Electrophoresis GmbH)-0.2% lead citrate buffer (Wako).

Neutralization of Conditioned Medium—Conditioned medium was obtained from MΦ cultured in medium alone for 48 h at 37 °C and incubated for 60 min at 37 °C with 10 μg/ml rabbit anti-human erythrocyte catalase (HEC) IgG (lot PTC 9301; Athens Research and Technology, Inc., Athens GA) or with normal rabbit IgG (Organon Teknika Co.) as a control.

Measurement of Catalase Activity—Intracellular and extracellular catalase activity was measured according to Aebi's modified method as described previously (2). Purified HEC (5×10^4 units/mg, lot 643793; Calbiochem-Nova Biochem) was used for conversion of the catalase activity in samples into real catalase activity, and the activity was expressed as milliunits/ml per 2.5×10^5 cells or units/mg protein. Protein was measured using a protein assay kit (Bio-Rad Laboratories).

Measurement of GSH Level—The total GSH level was determined using a BIOXYTECH S. A. kit. Briefly, the cells were lysed with 5% metaphosphoric acid, and the chromophore formation catalyzed at pH 13.4 was measured at 400 nm as described previously (15). The intracellular GSH level was expressed as pmol/mg protein.

Isolation of RNA and Northern Blot Analysis—Isolation of total RNA and Northern blot analysis were performed as described previously (5). Blots were hybridized with cDNA probes against human catalase (kindly donated by Dr. K. Onozaki, Faculty of Pharmaceutical Sciences, Nagoya City University) and β-actin (Sigma). All the probes were labeled using a multiprime DNA-labeling system with [α-^{32}P]dCTP (New England Nuclear Research Products, Boston, MA). The blots were analyzed using a Fuji BAS 2000 bioimage analyzer (Fuji Photo Film Co., Ltd., Tokyo).

Immunoblot Analysis—Immunoblot analysis was performed as described in our previous report (7). The membrane was incubated overnight at 4 °C with 1 mg/ml rabbit anti-HEC antibody (anti-Cat Ab; Athens Research and Technology, Inc.), mouse anti-BCL-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-BCL-xL/S antibody (Santa Cruz Biotechnology), rabbit anti-BCL-X antibody (Transduction Laboratories, Lexington, KY), mouse anti-MCL-1 antibody (Santa Cruz Biotechnology), rabbit anti-BAX antibody (Santa Cruz Biotechnology), or normal rabbit or mouse IgG and then at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology). The blots were visualized with Amersham ECL reagent on Hyper ECL film (Amersham Biosciences).

Reverse Transcription and Polymerase Chain Reaction—Total RNAs (1 mg) were prepared by use of RNA Zol B (Cinna/Biotecx Laboratories, Friendswood, TX) and reverse transcribed by incubation in 50 μl of 10 mM Tris-HCl (pH 8.3), 6.5 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, each dNTP at 1 mM, 2 mM random primer, and 2.4 units/ml Moloney murine leukemia virus reverse transcriptase for 1 h at 42 °C (Takara Shyzo, Otsu, Japan). The conditions for PCR were as follows: in a 50-μl reaction, 0.15 mM each primer, each dNTP at 2.5 mM, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 1.25 units of *Taq* polymerase (Takara Shyzo). Primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase: sense, 5'-CCTTCATTGACCTCAACTAC-3' and antisense, 5'-AGTGATGGCATGGACTGTGGT-3'; *BCL-2*: sense, 5'-CATTTCCACGTCAACAGAATTG-3' and antisense, 5'-AGCACAGGATTGGATATTCCAT-3'; *BCL-X_L*: sense, 5'-TTG-GACAATGGACTGGTTGA-3' and antisense, 5'-GTAGAGTGGAT-GGTCAGTG-3'; *MCL-1*: sense, 5'-GAGGAGGAGGACGAGTT-GTA-3' and antisense, 5'-CAGCTTTCTTGGTTTATGGT-3'; *BAX*: sense, 5'-AAGAAGCTGAGCGAGTGTC-3' and antisense, 5'-CG-GCCCCAGTTGAAGTTGC-3'. Reactions were incubated in a PerkinElmer DNA thermal cycler for 25 cycles (with each cycle consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, and extension for 60 s at 72 °C) (5).

Statistical Analyses—Statistical analyses of the data were performed using Student's *t*-test. p values <0.01 were considered significant. The results shown are representative of three to seven independent experiments.

RESULTS

Requirement for Continuous Presence of CSF for the Survival of M-MΦ, but not of GM-MΦ and A-MΦ—M-MΦ, GM-MΦ, and A-MΦ were cultured with or without CSF, and then cell viability was determined. M-CSF withdrawal from M-MΦ induced cell death in a time-dependent manner; ~60% of the cells died by 2 days and almost all of the cells died by 8 days (Fig. 1*A*). In contrast, GM-CSF withdrawal from GM-MΦ or A-MΦ did not induce cell death significantly during the culture period (Fig. 1*A*). M-MΦ cultured in the absence of M-CSF

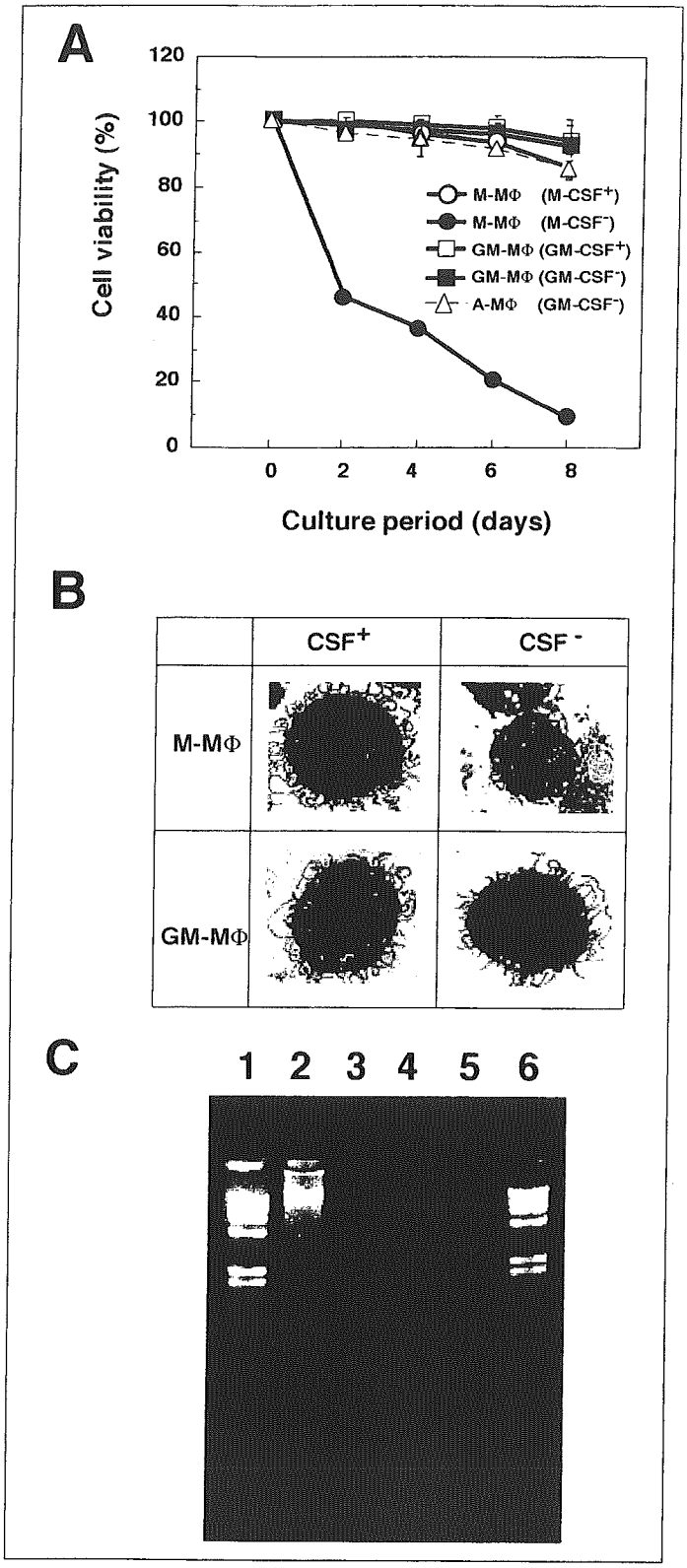

FIGURE 1. **Susceptibility of CSF-induced monocyte-derived MΦ and A-MΦ to CSF withdrawal.** M-MΦ and GM-MΦ (2.5×10^5/ml/well) were cultured in medium with or without M-CSF or GM-CSF, and A-MΦ were cultured in medium without GM-CSF. *A*, cell number and viability of MΦs were assessed using Cetavlon and trypan blue dye as described under "Experimental Procedures." Values are expressed as the means of triplicate cultures ± S.D. *B*, electron microscopic features of MΦ cultured in medium with or without CSF for 24 h (magnification ×10,000). *C*, electrophoresis of low molecular mass DNA from cultured MΦ. MΦ were incubated with M-CSF, GM-CSF, or medium alone for 24 h. Low molecular mass DNA was isolated from the cells, and electrophoresis was performed as described under "Experimental Procedures." HaeIII-digested I DNA fragment size standards were run in *lanes 1* and *6*. *Lane 2*, M-MΦ without M-CSF; *lane 3*, M-MΦ with M-CSF; *lane 4*, GM-MΦ without GM-CSF; *lane 5*, GM-MΦ with GM-CSF.

changed from spindle shaped to round and then detached from the dish and floated, with a shrunken cytosol and nucleus and with chromatin condensation and apoptotic vacuoles without microvilli (Fig. 1*B*). No such morphological change was observed in M-MΦ cultured with M-CSF or in GM-MΦ cultured with or without GM-CSF (Fig. 1*B*). A typical ladder pattern of internucleosomal DNA cleavage was detected in DNA of M-MΦ cultured for 24 h in the absence of M-CSF, whereas no significant DNA fragmentation was detected in DNA of M-MΦ cultured in the presence of M-CSF or that of GM-MΦ cultured in the presence or absence of GM-CSF (Fig. 1*C*). Apoptosis and DNA fragmentation of A-MΦ were similar to those of GM-MΦ (data not shown). These findings suggest that M-CSF withdrawal from M-MΦ induces apoptosis but both GM-MΦ and A-MΦ can survive in the absence of CSF.

Different Expression of BCL-2 and BCL-X_L Genes in M-MΦ, GM-MΦ, and A-MΦ and the Opposite Effect of CSF on Expression—*BCL-2* family genes play important roles in the apoptosis of many types of cells (10, 16–20). We therefore examined the expression of *BCL-2* family genes and the effect of CSF on their expression in M-MΦ and GM-MΦ by RT-PCR. In M-MΦ, the transcript of the *BCL-2* gene was stronger than that of the *BCL-X_L* gene, and M-CSF withdrawal decreased the expression of the *BCL-2* gene but induced a slight decrease in the expression of the *BCL-X_L* gene (Fig. 2*A*). In contrast to M-MΦ, GM-MΦ expressed strongly the *BCL-X_L* gene but weakly the *BCL-2* gene, and GM-CSF withdrawal had no significant effect on the expression of these two genes (Fig. 2*A*). In contrast to *BCL-2* and *BCL-X_L*, the transcriptional levels of the *MCL-1* and *BAX* genes were not significantly different between M-MΦ and GM-MΦ and were not affected by CSF deprivation (Fig. 2*A*). In accordance with the gene expression, the expression of the BCL-2 protein was higher in M-MΦ compared with GM-MΦ and M-CSF withdrawal decreased the expression of BCL-2 protein in M-MΦ (Fig. 2*B*). Similarly, the expression of the BCL-X_L protein was higher in GM-MΦ than in M-MΦ, but GM-CSF withdrawal had no significant effect on the expression of BCL-X_L protein in GM-MΦ (Fig. 2*B*).

The expression of BCL-2 family proteins in A-MΦ resembles that of GM-MΦ, and the expression was independent of CSF (Fig. 2*B*). As shown in Figs. 6 and 7, the expression of MCL-1 and BAX proteins in both MΦs was not changed in the presence or absence of CSF, in accordance with the expression patterns of the *MCL-1* and *BAX* genes.

HEC Stimulates the Survival and the Expression of BCL-2 in CSF-withdrawn M-MΦ, and Anti-HEC Antibody Abolishes the Survival-stimulating Activity of CM Obtained from CSF-withdrawn GM-MΦ and A-MΦ—The above findings suggest that GM-MΦ and A-MΦ, but not M-MΦ, can produce factor(s) that maintain their survival in the absence of CSF. To investigate this possibility, the conditioned medium (CM) obtained from these MΦ cultured for 48 h without CSF was used to examine its effect on the survival of M-CSF-withdrawn M-MΦ. CM of GM-MΦ or A-MΦ, but not of M-MΦ, prevented cell death of M-CSF-depleted M-MΦ (Fig. 3*A*).

A previous study showed that human CEM T cells can survive in serum-free medium supplemented with a low level of extracellular catalase (21). Therefore, we examined whether extracellular catalase prevents apoptosis in M-CSF-withdrawn M-MΦ and stimulates the expression of both *BCL-2* gene and protein. Addition of HEC prevented M-CSF-withdrawn M-MΦ from undergoing cell death in a dose-dependent manner, and 10 units/ml of HEC completely rescued the cells

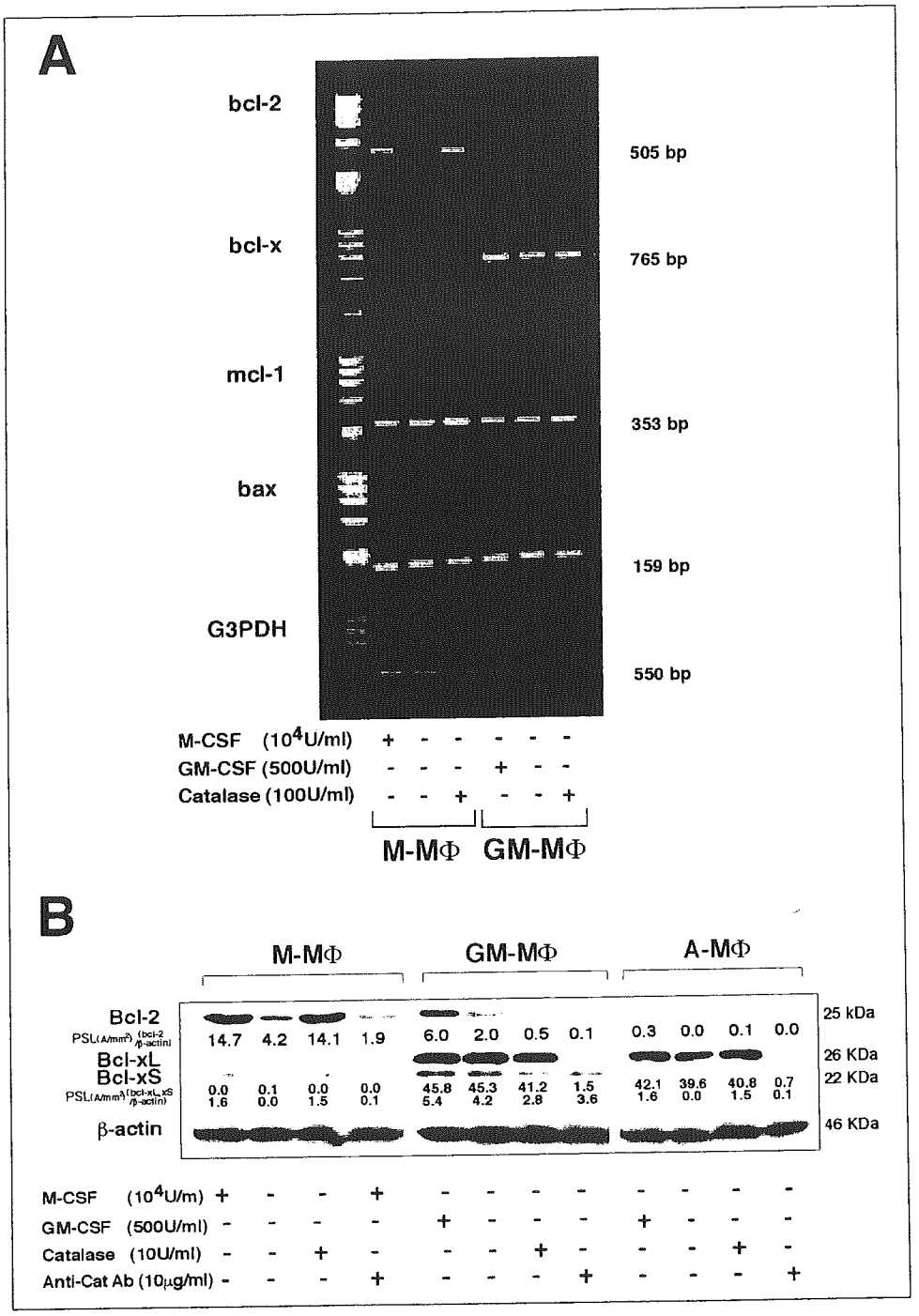

FIGURE 2. **Effects of CSF and catalase on the levels of protein and mRNA expression of *BCL-2* family genes in monocyte-derived MΦ and A-MΦ.** *A*, RT-PCR analysis of mRNA levels of *BCL-2* family genes and the glyceraldehyde-3-phosphate dehydrogenase gene in total RNA preparations (200 ng/lane) from these MΦs at 3 h of cultivation as described under "Experimental Procedures." M-MΦ and GM-MΦ were cultured in medium with or without CSF or supplemented with catalase. *B*, Western blot analysis of BCL-2, BCL-X_L/$_S$, or β-actin protein in cell lysates (25 μg/lane) probed using rabbit polyclonal BCL-2 or BCL-X antibody or mouse monoclonal β-actin antibody. M-MΦ were cultured for 48 h in medium with M-CSF or catalase or with M-CSF supplemented with anti-catalase antibody (*Anti-Cat Ab*), and GM-MΦ and A-MΦ were cultured in medium with GM-CSF or catalase or supplemented with Anti-Cat Ab. The relative amounts of these proteins in cells were measured using NIH image software, and the expression levels were corrected relative to those of β-actin (photo-stimulating luminescence (*PSL*), A/mm^2).

from apoptosis. Addition of 10 units/ml HEC to GM-CSF-withdrawn GM-MΦ had no such effect (Fig. 3*A*).

Then we examined the effect of anti-HEC antibody (anti-Cat Ab) on the activity of the CM of GM-MΦ. Pretreatment of the CM with anti-Cat Ab, but not with control IgG, completely abrogated the ability to rescue the cell death of M-CSF-withdrawn M-MΦ (Fig. 3*B*). Similar results were obtained in experiments using the CM of A-MΦ (data not shown).

Next, we examined the role of catalase in the expression of *BCL-2* family genes in M-CSF-withdrawn M-MΦ. Addition of catalase to M-CSF-withdrawn M-MΦ restored the expression of *BCL-2* but did not affect the gene expression of *BCL-X_L*, *MCL-1*, or *BAX* (Fig. 2*A*). Addition of catalase did not significantly change the gene and protein expression of *BCL-2* family genes in GM-CSF-withdrawn GM-MΦ (Fig. 2*A*). These results suggest that the active molecule in the CM of GM-MΦ and A-MΦ that can rescue the survival of M-CSF-withdrawn M-MΦ via restoration of *BCL-2* expression is catalase.

Anti-catalase Antibody Abolished the Expression of BCL-2 in M-MΦ Cultured with M-CSF and BCL-X_L in GM-MΦ or A-MΦ Cultured with or without GM-CSF and Induced Apoptosis of These MΦs—The above results indicate that catalase can stimulate the expression of *BCL-2* and rescue the survival of M-CSF-withdrawn M-MΦ. Therefore we next examined whether extracellular catalase also plays a critical role in the CSF-dependent survival of M-MΦ and in the survival of GM-MΦ and A-MΦ. Addition of anti-Cat Ab suppressed the expression of BCL-2 protein in M-MΦ cultured in the presence of M-CSF and of BCL-X_L

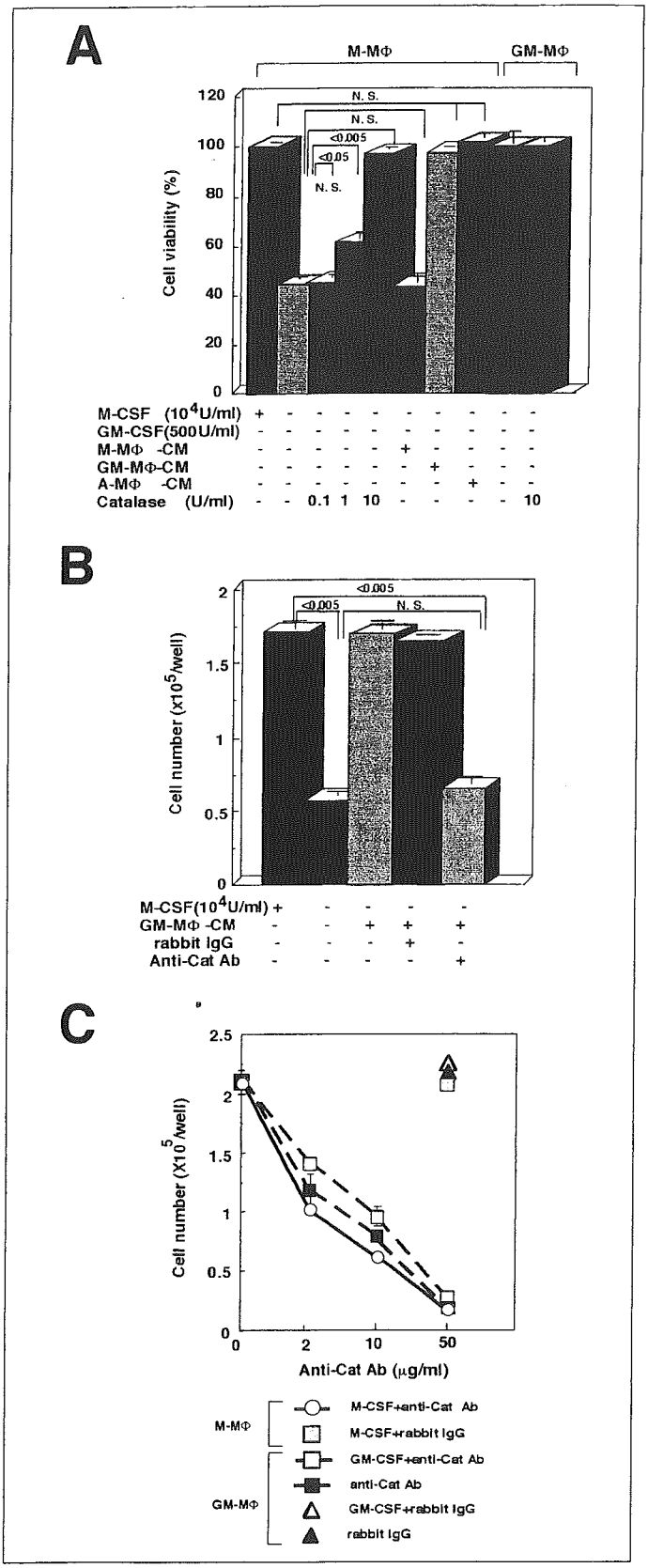

FIGURE 3. **Effect of endogenously produced or exogenously added catalase on the survival of M-MΦ and GM-MΦ.** *A*, M-MΦ were cultured for 48 h in the medium with M-CSF, medium containing the indicated concentrations of catalase, or 100% of conditioned medium (*CM*) obtained from M-MΦ, GM-MΦ, or A-MΦ. GM-MΦ were used as the control. Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. *B*, M-MΦ were cultured for 48 h in medium with M-CSF, CM pretreated with Anti-Cat Ab (*IgG*), or rabbit IgG as a control. Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. *C*, M-MΦ and GM-MΦ were cultured for 48 h in medium with or without CSF plus Anti-Cat Ab or rabbit IgG. Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D.

protein in both GM-MΦ and A-MΦ cultured without GM-CSF (Fig. 2*B*). In accordance with these data, addition of anti-Cat Ab induced cell death of M-MΦ cultured with M-CSF and GM-MΦ cultured with or without GM-CSF, and addition of control IgG had no effect on the cell viability of these MΦs (Fig. 3*C*). The cell death of these MΦ induced by anti-Cat Ab was due to apoptosis, as indicated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling staining (data not shown).

GM-MΦ and A-MΦ, but Not M-MΦ, Can Produce Enough Levels of Extracellular Catalase in the Absence of CSF—The above results suggest that GM-MΦ and A-MΦ, but not M-MΦ, can produce extracellular catalase in a CSF-independent manner and that the catalase supports the survival of those MΦs via maintenance of the expression of *BCL-2* family genes. To examine this possibility, we measured catalase enzyme activity and catalase protein levels in the CMs of M-MΦ, GM-MΦ, and A-MΦ cultured for 48 h in the presence or absence of CSF. The extracellular catalase activities in the CMs obtained from GM-MΦ and A-MΦ cultured both in the presence or absence of GM-CSF were not significantly different, and the levels were ~4-fold higher (~240 milliunits/ml/well) than that of M-CSF-treated M-MΦ (~60 milliunits/ml/well) (Fig. 4*A*). In contrast to GM-MΦ or A-MΦ, the extracellular catalase activity in CM of M-MΦ was dependent on CSF and that in CM of M-CSF-withdrawn M-MΦ was significantly lower than that in CM of M-CSF-treated M-MΦ, ~20 milliunits/ml/well (Fig. 4*A*). Western blot analysis of the extracellular catalase of CMs using anti-HEC antibody showed similar results to the data of the enzyme activity (Fig. 4*A*).

Next, we examined the cell-associated levels of catalase activity in these MΦs (Fig. 4*B*). The levels of catalase activity in GM-MΦ and A-MΦ lysates was ~5 units/mg protein both in the presence or absence of CSF, whereas that in M-MΦ in the presence of M-CSF was ~1 unit/mg. The catalase activity in M-CSF-withdrawn M-MΦ lysates (~250 milliunits/mg protein) was ~4-fold lower than that in M-CSF-treated M-MΦ lysates. In agreement with the measurements of enzyme activity, similar results were observed in Western blot analyses (Fig. 4*B*).

To further confirm the distinction between the regulation of extracellular and cell-associated catalase activity by CSF in M-MΦ *versus* GM-MΦ, we examined the levels of transcription of the catalase gene in these MΦs cultured with or without CSF (Fig. 4*C*). The level of the transcript of the catalase gene in M-CSF-withdrawn M-MΦ was ~3-fold lower than that in M-CSF-treated M-MΦ. In contrast, the level of the catalase transcript in GM-CSF-withdrawn GM-MΦ and A-MΦ was similar to that in GM-CSF-treated GM-MΦ and A-MΦ, and the level was 5-fold higher than that in M-CSF-treated M-MΦ.

Thus, the difference of total extracellular plus intracellular catalase activity and the difference in the levels of catalase gene expression between CSF-withdrawn M-MΦ and GM-MΦ reached ~15–20- and ~15-fold, respectively. The results indicate that extracellular catalase activity is regulated at the transcription levels by CSF-dependent M-MΦ but CSF-independent GM-MΦ and A-MΦ.

Thiol Derivatives Act as Additive Effectors That Rescue the Cell Death of MΦ—The above data suggest that extracellular catalase plays a major role in MΦ survival. However, several reports have demonstrated that thiol proteins and thiol compounds such as GSH, adult T cell leukemia-derived factor, and L-cysteine play important roles in the survival of

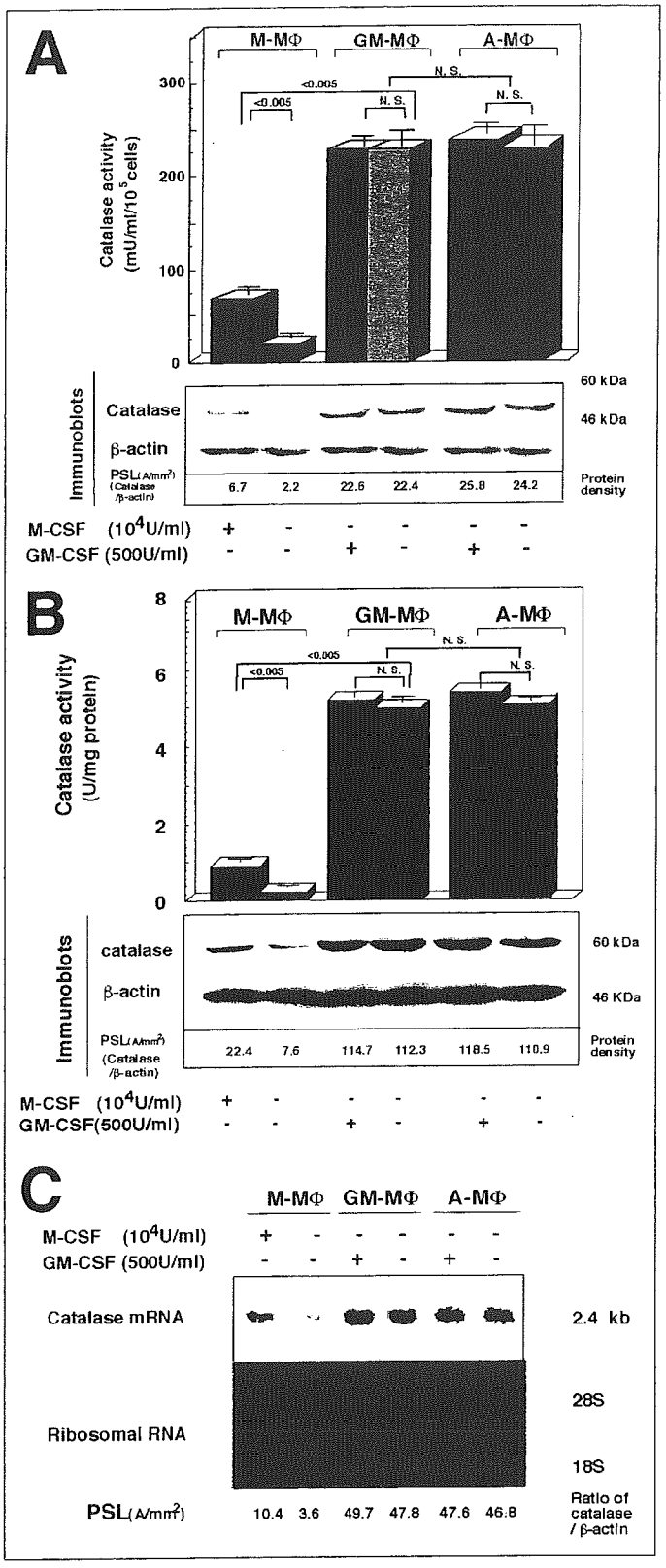

FIGURE 4. **Activities, protein levels, and mRNA levels of catalase in monocyte-derived MΦ and A-MΦ.** MΦ were cultured with or without CSF for 48 h (*A*, *B*) or for 3 h (*C*). *A* and *B*, enzyme activities (*upper panel*) and protein levels (*lower panel*) of catalase in the culture medium (25 μl/lane) (*A*) or cell lysates (25 μg/lane) (*B*) from MΦ were examined as described under "Experimental Procedures." Western blot analysis of catalase protein in the culture medium or cell lysates of MΦ was performed using anti-catalase antibody. The relative amounts of catalase protein in cells were measured using NIH image software, and the expression levels were corrected relative to those of β-actin (photo-stimulating luminescence (*PSL*), A/mm²). *C*, mRNA levels of catalase and the β-actin gene were examined in total RNA preparations (10 μg/lane) from these MΦ at 3 h of cultivation by Northern blot analysis. The relative transcript levels of catalase mRNA in cells were measured using NIH image software, and the expression levels were corrected relative to those of β-actin (photo-stimulating luminescence, A/mm²).

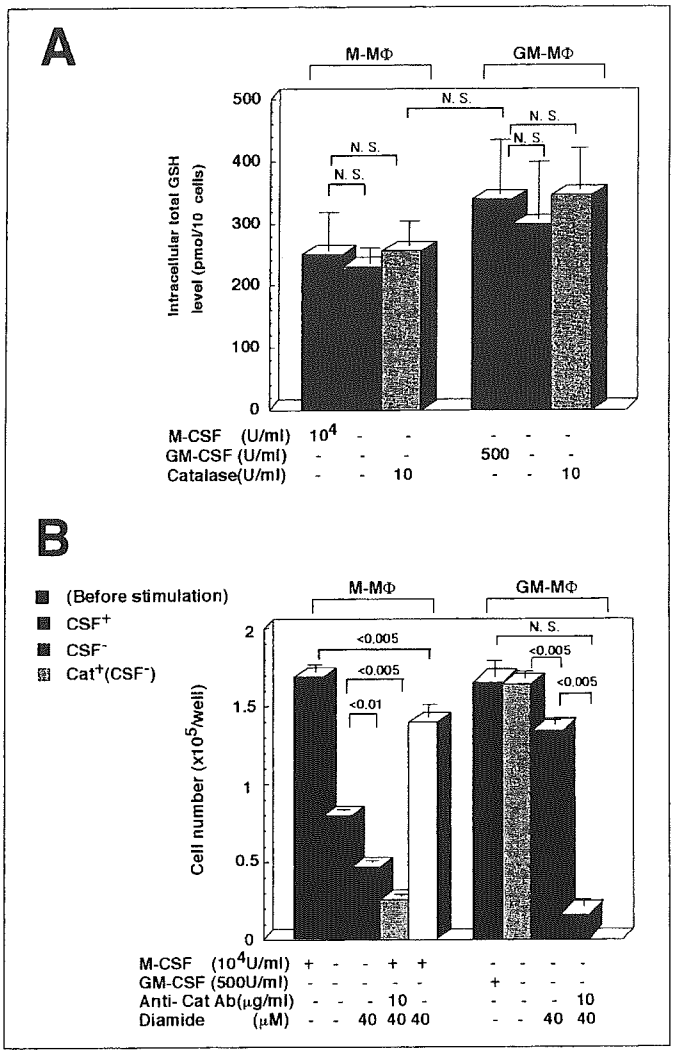

FIGURE 5. **Partial effect of GSH on the survival of monocyte-derived MΦ.** *A*, intracellular GSH levels in M-MΦ and GM-MΦ incubated with or without CSF for 24 h were measured as described under "Experimental Procedures." Values are expressed as the means of triplicate cultures ± S.D. *B*, M-MΦ or GM-MΦ were cultured for 48 h in medium supplemented or not supplemented with CSF, anti-catalase antibody (*Anti-Cat Ab*), diamide, or the combination of them, and cell number and viability were assessed as described above.

lymphocytes or neuronal cells in the absence of growth factors (22, 23, 24). Thus, thiol derivatives may help MΦ survival. The level of intracellular GSH, however, was almost the same in M-MΦ and GM-MΦ cultured with or without CSF or cultured with catalase (Fig. 5*A*), and this level (~300 pmol/10^5 cells) was similar to that in A-MΦ (data not shown). Diamide, which can bind the SH groups of reduced thiols and oxidize them (22), induced cell death of M-MΦ and GM-MΦ, but the levels of cell death were not very high (~20%) and no significant difference was observed between the effects of diamide on these two MΦs (Fig. 5*B*). 40 μM diamide and 10 μg/ml anti-Cat Ab showed synergistic effects on the cell viability of M-MΦ cultured with M-CSF and GM-MΦ cultured without CSF, causing reduction of their viability to <10% at 48 h (Fig. 5*B*). These results suggest that, in contrast to that of catalase,

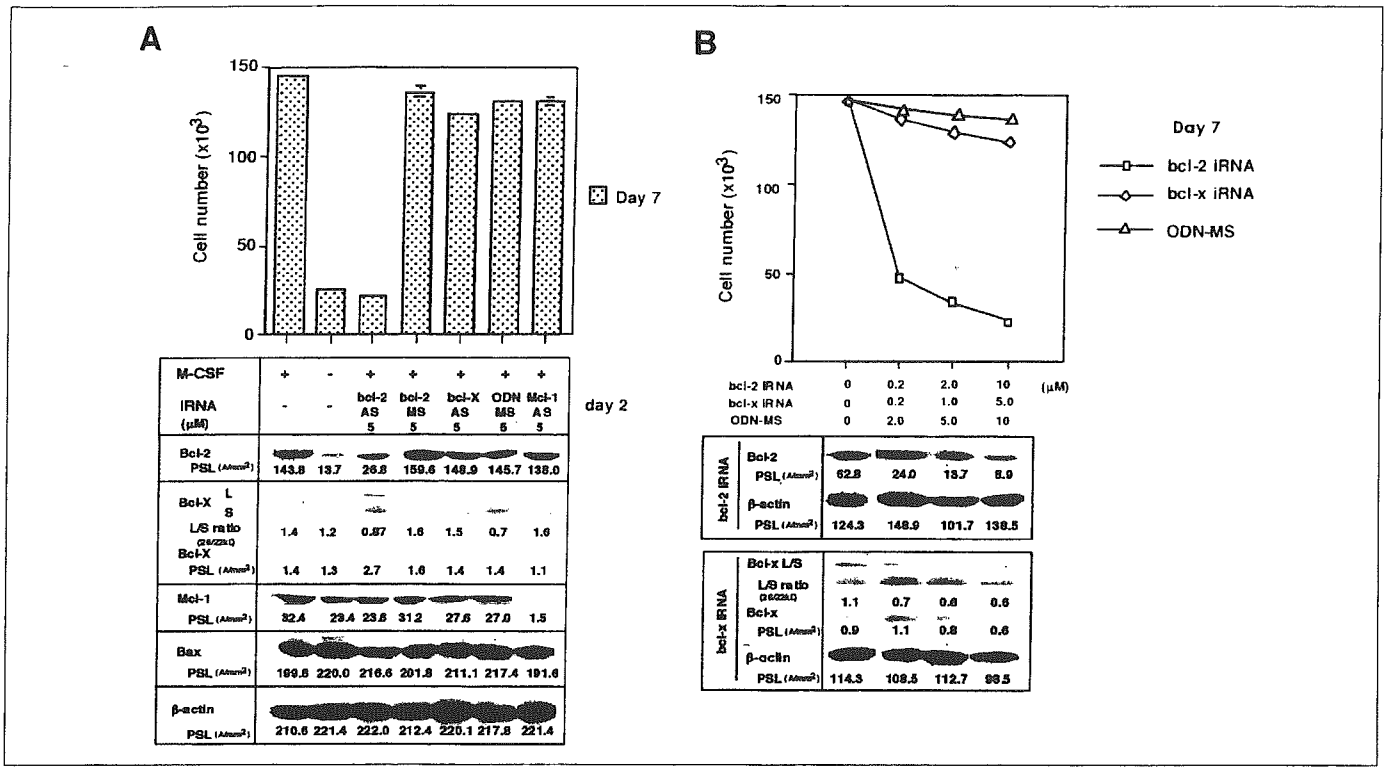

FIGURE 6. **BCL-2 AS dominantly induced cell death of M-MΦ.** *A*, cell number and viability of MΦ at 7 days after 5 μM AS treatment (*upper panel*) and immunoblot analysis at 2 days (*lower panel*). *B*, cell number and viability of MΦ at 7 days after the indicated concentrations of AS treatment (*upper panel*) and immunoblot analysis at 2 days (*lower panel*). M-MΦ were cultured in medium supplemented with the indicated concentrations of BCL-2 AS (G3139), BCL-2 MS (G4126), 5′-BCL-X AS (BCL-X AS), MCL-AS or control random sequence (oligonucleotide MS). Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. Western blot analysis of BCL-2, BCL-$X_{L/S}$, MCL-1, and β-actin proteins in cell lysates of MΦ probed using BCL-2, BCL-X (antibody from Santa Cruz Biotechnology for $X_{L/S}$ band, from Sinal transduction Labo for X_L band only), and β-actin antibody was performed as described in Fig. 4. The relative amounts of these proteins in cells were measured using NIH image software, and the expression levels were shown as photo-stimulating luminescence (*PSL*).

the levels of thiol derivatives were constant in these MΦs with CSF withdrawal-induced oxidative stress and that thiol derivatives have only a minor effect and cannot support the full survival of MΦ.

BCL-2 AS and BCL-X_L AS Dominantly Induce Cell Death of M-MΦ and GM-MΦ, Respectively—M-MΦ and GM-MΦ were treated with BCL-2 AS targeted to *BCL-2* initiation codon (G3139) and 5′-BCL-X AS targeted to the downstream alternative 5′-splice site of exon 2 of the *BCL-X* gene (12, 13). As controls, these MΦs were treated with BCL-2 MS (G4126, variant G3139), control oligonucleotides with random sequence (oligonucleotide MS), or Lipofectin alone. As shown in Fig. 6, BCL-2 AS (G3139) down-regulated the expression of BCL-2 protein in M-MΦ to 10–20% of that of control cells at 2 days after the oligonucleotide treatment and induced cell death in a dose-dependent manner. The cell viability markedly decreased to ~15% of that of control cells at 7 days of cultivation. In GM-MΦ, however, treatment with G3139 even in the high dose such as 10 μM induced only ~10% cell death, in agreement with the low expression of BCL-2 protein in this MΦ (Fig. 7).

In contrast, treatment of GM-MΦ with 5′-BCL-X AS down-regulated the expression of BCL-X_L protein to 20% of that of control cells treated with oligonucleotide MS at 2 days after the oligonucleotide treatment and induced the cell death in a dose-dependent manner (Fig. 7). The cell viability markedly decreased to ~25% of that of control cells at 7 days of cultivation. As shown in Fig. 6, however, ~90% of the cells are viable in M-MΦ treated with 5 mM 5′-BCL-X AS in agreement with the low expression of BCL-X_L protein.

MCL-1, a main molecule of BCL-2 family protein (14), is expressed in both M-MΦ and GM-MΦ, but down-regulation of this protein by treatment with MCL-1 AS did not stimulate the cell death of either MΦ or affect the expression levels of BCL-2 in M-MΦ and BCL-X_L in GM-MΦ. These findings suggest that BCL-2 and BCL-X_L expression supported by catalase prevents cell death of M-MΦ and GM-MΦ, respectively, in agreement with the dominant expression levels of gene and protein of BCL-2 in M-MΦ and BCL-X_L in GM-MΦ.

DISCUSSION

The present study showed that extracellular catalase has a novel role in the prevention of apoptosis in human MΦ through the dominant expression of BCL-2 in M-MΦ and BCL-X_L in GM-MΦ and that the regulation of catalase production is CSF-dependent in M-MΦ but CSF-independent in GM-MΦ and A-MΦ. Recently, H_2O_2 has been shown to enhance oxidative damage and apoptosis in C2-ceramide-pretreated HL-60 cells via a mechanism in which C2-ceramide inhibits catalase activity by increasing casapase-3-dependent proteolysis of catalase and down-regulation of catalase mRNA (25). Overexpression of catalase inhibits oxidation-mediated apoptosis through phosphorylated BCL-2, a reduced form of BCL-2 and BAX interaction (20). Thus, constant high activity of extracellular catalase plays an important role in the prevention of ceramide- and caspase-3-induced apoptosis in CSF independent of GM-MΦ and A-MΦ and CSF dependent of M-MΦ (11, 26).

GM-CSF and M-CSF stimulate catalase induction during the differentiation of Mo into MΦ, but GM-CSF alone establishes a CSF-independent autoregulatory system of catalase production in MΦ. We previously showed that human Mo-derived GM-MΦ resembles human A-MΦ in several respects (2, 5–7). In this study, we showed that GM-MΦ and A-MΦ have a similar phenotype of the resistance to apoptosis via CSF-independent expression of catalase and BCL-X_L. Con-

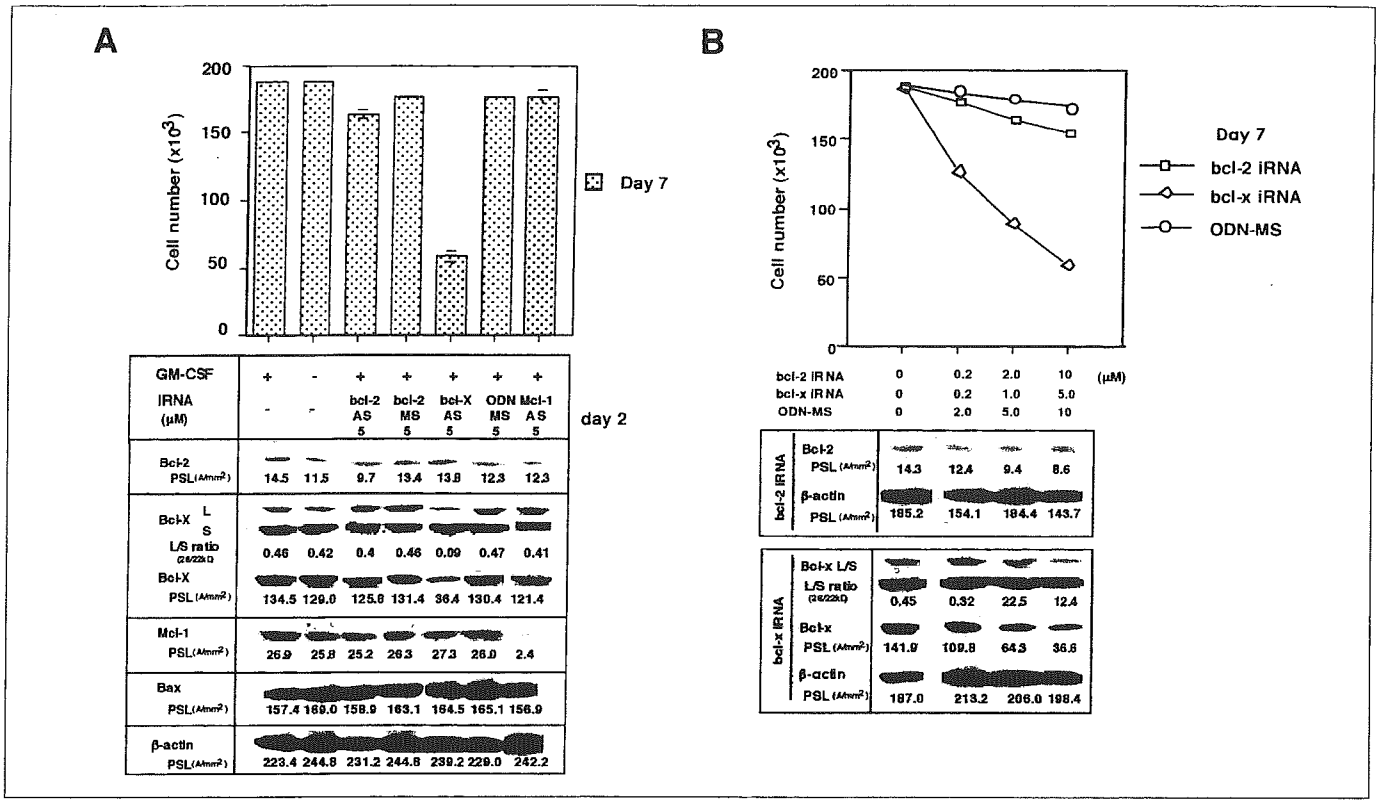

FIGURE 7. **BCL-X AS dominantly induced cell death of GM-MΦ.** *A*, cell number and viability of MΦ at 7 days after 5 μM AS treatment (*upper panel*) and immunoblot analysis at 2 days (*lower panel*). *B*, cell number and viability of MΦ at 7 days after the indicated concentrations of AS treatment (*upper panel*) and immunoblot analysis at 2 days (*lower panel*). The experimental procedure was performed as described in Fig. 6.

sistent with our present study, A-MΦ from human smokers express higher levels of $p21^{CIP1/WAF1}$ and BCL-X_L, but not BCL-2, and the former two molecules may reduce apoptosis (9). The autoregulatory mechanism of catalase induction in GM-MΦ and A-MΦ is not yet understood but may have a strong correlation with endogenously generated low levels of H_2O_2 (8), because we previously showed that GM-MΦ and A-MΦ, but not M-MΦ, can increase catalase expression at both the protein and mRNA levels when stimulated with H_2O_2 (2). Adequate low levels of H_2O_2 may keep catalase activity constant to prevent CSF deprivation-induced apoptosis in GM-MΦ and A-MΦ.

Thiol derivatives such as GSH, adult T cell leukemia-derived factor, and L-cysteine play an important role in the survival of lymphocytes or neuronal cells in the absence of growth factors (22–24). In our study, however, the activity of thiol derivatives was not significantly different between M-MΦ and GM-MΦ before and after CSF deprivation, and they had only a partial effect on MΦ survival. The reason for the differences between our study and previous studies might have been the differences in the experimental conditions (CSF-withdrawal *versus* serum depletion) or the cell type examined (MΦ *versus* lymphocytes/neuronal cells). Compared with MΦ, lymphocytes or neuronal cells may produce lower levels of catalase so that the thiol derivatives play a dominant role in their survival (21–24).

We demonstrated that catalase regulates apoptosis through the expression of BCL-2 and BCL-X_L in human MΦ used in the present study. In fact, down-regulation of these proteins by RNA interference treatment induced the cell death of the MΦs. Proapoptotic BCL-2 family protein, BAX, can induce apoptosis with permeabilization of mitochondrial membranes and cytochrome *c* release (10, 18, 27). In a recent study, induction of apoptosis of TF-1 cells by GM-CSF withdrawal is shown to be related to down-regulation of the *MCL-1* gene, and overexpression of *MCL-1* is shown to delay apoptosis (16). In our study, however, *BAX* and *MCL-1* were not associated with the regulation of apoptosis of MΦ, because both mRNA and protein levels of these genes were not significantly changed in M-MΦ and GM-MΦ after CSF deprivation. Moreover, we showed that down-regulation of the expression of MCL-1 protein by MCL-1 As treatment did not affect the viability of the MΦs.

Our interesting finding is that BCL-2 and BCL-X_L are differently expressed in MΦ during the Mo differentiation into MΦ in the presence of M-CSF and GM-CSF; the expression of BCL-2 is dominant in M-MΦ and that of BCL-X_L is dominant in GM-MΦ or A-MΦ. In accordance with such different expression patterns, we found by RNA interference experiments that BCL-2 and BCL-X_L play a critical role for the survival of M-MΦ and GM-MΦ, respectively. Similar differential expression of BCL-2 and BCL-X_L is also observed during the selection and maturation of mouse thymocytes toward splenic T cells (17, 19). At present, we do not know the mechanisms that control the different induction of BCL-2 and BCL-X_L in M-MΦ and GM-MΦ or A-MΦ, respectively. The distal promoter region of the BCL-X_L gene responds to very low levels of H_2O_2 at exon 1B-1D in rodent cardiac myocytes, and the expression of BCL-X_L protein is increased by H_2O_2 treatment (28). Thus, GM-MΦ and A-MΦ, which possess high catalase activity even in the absence of CSF, limit the levels of endogenously generated H_2O_2 to low levels that are suitable for BCL-X_L expression.

In conclusion, the present study is the first to reveal that CSF is a critical regulator of extracellular catalase activity that maintains selective expression of *BCL-2* family genes and prevents tissue-specific MΦ from apoptosis. These distinct patterns of CSF-induced regulation of catalase activity may greatly contribute to the oxidant stress-induced selection of tissue MΦs suitable for their respective microenvironments.

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Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages

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Objectives: Macrophages (Mφs) have various functions and play a critical role in host defense and the maintenance of homeostasis. Mφs exist in every tissue in the body, but Mφs from different tissues exhibit a wide range of phenotypes with regard to their morphology, cell surface antigen expression and function, and are called by different names. However, the precise mechanism of the generation of macrophage heterogeneity is not known. In the present study, the authors examined the functional heterogeneity of Mφs generated from human monocytes under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF).

Methodology: CD14 positive human monocytes (Mos) were incubated with M-CSF and GM-CSF for 6–7 days to stimulate the generation of M-CSF-induced monocyte-derived Mφs (M-Mφs) and GM-CSF-induced monocyte-derived Mφs (GM-Mφs), respectively. The expression of cell surface antigens and several functions such as antigen presenting cell activity, susceptibility to oxidant stress, and the susceptibility to HIV-1 and *mycobacterium tuberculosis* infection were examined.

Results: GM-Mφs and M-Mφs are distinct in their morphology, cell surface antigen expression, and functions examined. The phenotype of GM-Mφs closely resembles that of human Alveolar-Mφs (A-Mφs), indicating that CSF-induced human monocyte-derived Mφs are useful to clarify the molecular mechanism of heterogeneity of human Mφs, and GM-Mφs will become a model of human A-Mφs.

Key words: catalase, colony-stimulating factors, HIV-1, macrophages, *mycobacterium tuberculosis.*

INTRODUCTION

Macrophages (Mφs) have various functions beside phagocytosis such as bactericidal activity, antigen-presentation, tumor cytotoxicity, removal of aged or damaged cells, repair of injured tissue, bone resorption and special lipid metabolism. Mφs produce and secrete a large array of small- and macro-molecules that mediate the recruitment of hematopoietic or other cells, control cell growth, differentiation and function, influence vascular permeability in inflammatory sites and attack target microorganisms. Therefore, Mφs play a critical role in the host defense and the maintenance of homeostasis.

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Mφs exist in practically every tissue in the body, but Mφs from different tissues exhibit a wide range of phenotypes with regard to their morphology, cell surface antigen expression and function, and are called by different names. Mφs originate from hematopoietic stem cells in bone marrow, they are predominantly derived from circulating blood monocytes (Mos),[1] which enter various tissues and differentiate into tissue specific Mφ populations[2] under the influence of the microenvironment. The mechanisms mediating the terminal differentiation and heterogeneity of Mφs, however, are not well known but are of fundamental significance.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF) are two hematopoietic growth factors that are both implicated in regulating production and function of cells of Mo/Mφ lineage.[3–5] From the studies of mice, it is clear that GM-CSF regulates the phenotype and functions of Alveolar-Mφs (A-Mφs) critical to surfactant homeostasis and host defense.[6–8] In contrast, it is clear that

M-CSF plays an important role in the development, proliferation and differentiation of osteoclasts and certain tissue Mϕ populations such as peritoneal Mϕs.[9] Therefore, Mos recruited to the sites where either CSF is dominant might differentiate into a divergent phenotype of Mϕs, such as A-Mϕs and peritoneal Mϕs. In agreement with these studies, the authors' recent studies in human Mos indicate that M-CSF and GM-CSF stimulate the generation of two phenotypically distinct types of Mϕs (M-Mϕs and GM-Mϕs), and the phenotype of GM-Mϕs resembles that of human A-Mϕs.

METHODS

Cytokines

Recombinant human (rh) GM-CSF (1×10^8 U/mg) and rhM-CSF (1×10^8 U/mg) were provided by Schering-Plough Japan (Osaka, Japan) and Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), respectively.

Preparation of monocyte-derived macrophages

Mos were obtained from PBMC of normal healthy volunteers using a magnetic cell separation system (MACS: Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD14 mAb-coated microbeads. $CD14^+$ Mos were cultured in RPMI 1640 medium (Nissui Seiyaku Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS: Z. L. Bockneck Laboratories Inc., Ontario, Canada) in the presence of the following human recombinant cytokines at optimal concentrations: 5 ng/mL GM-CSF (Schering-Plough Japan, Osaka, Japan) or 50 ng/mL M-CSF (Morinaga Milk Industry Co. Ltd.) at 37°C under a humidified 5% CO_2 atmosphere for 7 days. During the culture, Mos differentiated to Mϕs. Human A-Mϕs were obtained from healthy volunteers (non-smokers without diseases) by the BAL method. All volunteers gave informed consent to the use of their A-Mϕs for part of this study.

Measurement of catalase activity

Intracellular and extracellular catalase activity was measured according to the method described by Aebi.[10]

HIV-1 infection and antisense treatment of Hck and C/EBPβ

Macrophage-tropic HIV-1 strains (HIV-1_{BaL}, HIV-$1_{JR\text{-}FL}$) were used. Phosphorothioate-modified antisense oligonucleotides for Hck (AS-Hck: 5′-TTCATCGACC CCATCCTGGC-3′) and C/EBPβ (AS-C/EBPβ: 5′-CAG GCGTTGCATGAACGCGG-3′), and their corresponding sense oligonucleotides (S-Hck: 5′-GCCAGGATG GGGTCGATGAA-3′ and S-C/EBPβ: 5′-CCGCGTTCAT GCAACGCCTG-3′), and their unrelated nonsense oligonucleotides (NS-Hck: 5′-CCATATTTCCCGCTCGC GTG-3′ and NS- C/EBPβ: 5′-CCAGAGAGGGCCCGTGT GGA-3′) were synthesized.

Mycobacterium tuberculosis (*mycobacterium tuberculosis*) infection and colony-forming unit assay

Mϕs were infected with single-cell suspension of *mycobacterium tuberculosis* H37Rv at Moi of 1–2. Infected Mϕs were cultured for 5–7 days in an antibiotics-free culture medium and the number of bacteria in the culture supernatant and within the cells were counted with a colony-forming unit (CFU) assay using Middlebrook 7H10 agar supplemented with OADC. For the determination of the number of intracellular bacteria, Mϕs were lysed by the addition of 1 mL of distilled water supplemented with 0.05% Tween 80.

T cell proliferation assay

PBMC (10^5 cells/well) with or without Mϕs were stimulated with 10 μg/mL PPD for 5 days in a 96-well microtiter plate at 37°C, and T cell proliferation was determined by the ^{3}H-thymidine incorporation assay.

Assay for IL-10 and γ-interferon activities

Culture supernatants were harvested from Mϕs and Mϕs plus PBMC stimulated with or without PPD for 24 h and 72 h for the assay of IL-10 and γ-interferon (IF), respectively. The levels of IFN-γ and IL-10 were determined by an ELISA (Endogen).

Statistical analysis

Statistical analysis was performed with the Student's *t*-test.

RESULTS

Monocyte-derived macrophages induced by M-CSF and GM-CSF are different in their morphology and cell surface antigen expression

When blood Mos were cultured in medium alone *in vitro*, Mos died, and CSF such as M-CSF or GM-CSF were necessary for their survival and differentiation into Mϕs.[11] Mϕs induced by M-CSF (M-Mϕs) had an elongated and spindle-like morphology, though some small and round cells remained, but Mϕs induced by GM-CSF (GM-Mϕs) were round and had a fried egg-like morphology. As shown in Figure 1, the expression of cell surface antigens were different between M-Mϕs and GM-Mϕs.[12–14] The phenotype of GM-Mϕs (fried egg-like shape and c-fms^{low}, $CD14^{low}$, $CD71^+$ and $710F^+$) closely resembled that of human A-Mϕs. In contrast, the morphology and the expression of cell surface antigens (c-fms^{high}, $CD14^{high}$) of M-Mϕs resembled that of human peritoneal Mϕs or influx Mϕs induced by inflammation.

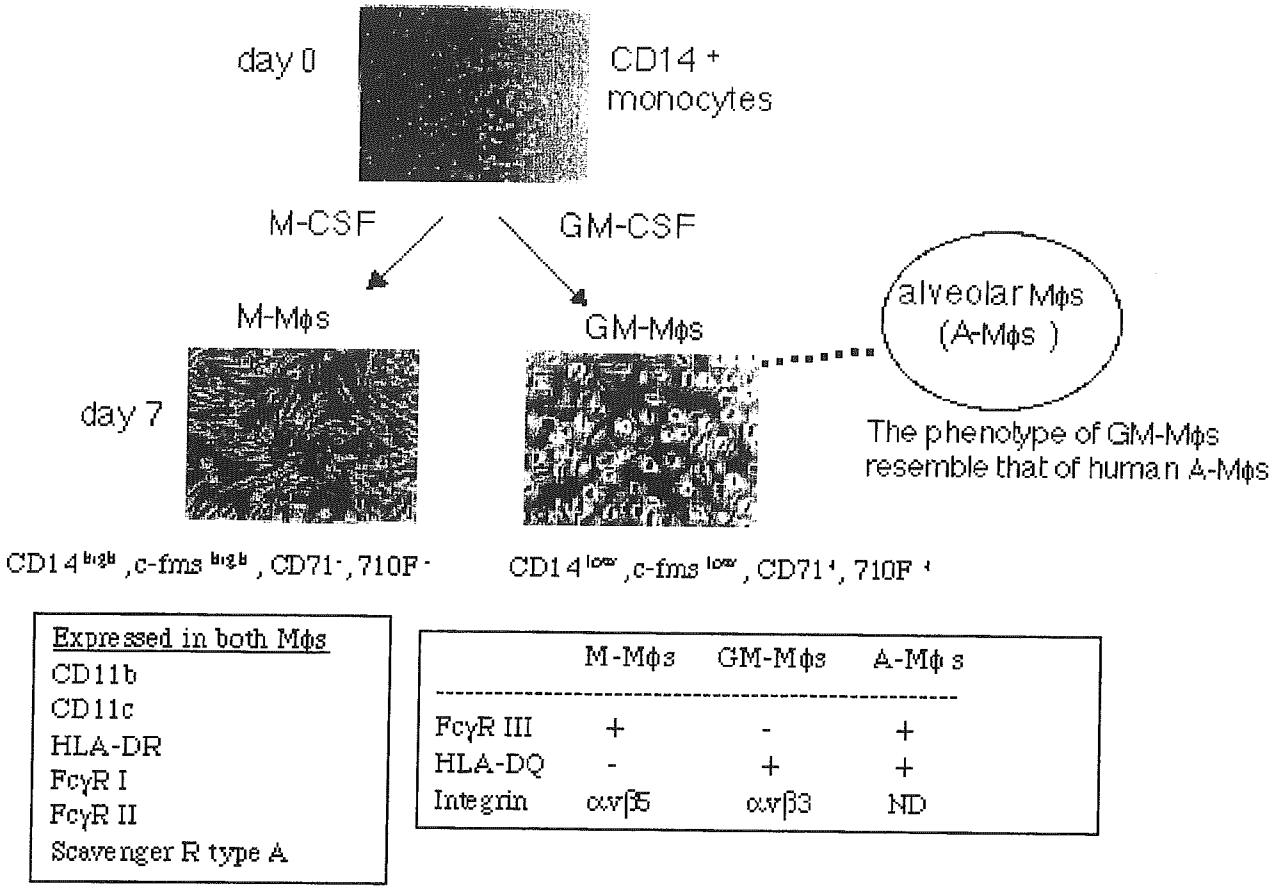

Figure 1 Effects of colony-stimulating factors on the differentiation of monocytes into macrophages.

Monocyte-derived macrophages induced by GM-CSF and M-CSF are different in their capacity for FcγR-mediated phagocytosis

Both M-Mφs and GM-Mφs ingested latex particles, but their capacity for FcγR-mediated phagocytosis was significantly different. More than 98% of M-Mφs phagocytized sensitized erythrocytes (EA), whereas only about 30% of GM-Mφs did.[13] Both types of Mφ could form rosettes in a similar manner with EA. Therefore, the defect in GM-Mφs may be located at the level of the signal transduction pathway need for ingestion after the binding of EA to FcγR.

Monocyte-derived macrophages induced by GM-CSF and alveolar macrophages but not monocyte-derived macrophages induced by M-CSF are resistant to H_2O_2 via their high basal and inducible levels of catalase activity

Mφs produce and release reactive oxygen species (ROS), such as H_2O_2, O_2^-, and OH, in response to phagocytosis or stimulation with various agents through activation of a multicomponent NADPH oxidase. ROS are essential for the killing of a wide variety of microbes. In addition, ROS have been implicated in inflammation and tissue injury. M-Mφs produced a large amount of H_2O_2 compared with GM-Mφs in response to zymosan or heat-killed *Staphylococcus aureus*.[15] GM-Mφs were highly resistant to H_2O_2, and expressed high levels of catalase activity. In contrast, M-Mφs expressed low catalase activity, and were about 50-fold more sensitive to H_2O_2 than GM-Mφs. The antioxidant state of A-Mφs was at a similar level to that of GM-Mφs by assessment of H_2O_2 sensitivity and catalase activity. About 1–10 mmol/L H_2O_2, similar to levels found on exhalation in cases of adult respiratory distress syndrome, did not induce cell death of either GM-Mφs or A-Mφs. GM-Mφs and A-Mφs but not M-Mφs could induce catalase expression in both protein and mRNA levels when stimulated with H_2O_2 or zymosan. These findings indicate that GM-Mφs and A-Mφs but not M-Mφs are strong scavengers of H_2O_2 via a high basal level of catalase activity and a marked ability of catalase induction.

Different susceptibility of monocyte-derived macrophages induced by M-CSF and GM-CSF to macrophage-tropic HIV-1

Recent studies indicate that human Mos/Mφs are a primary target for initial HIV-1 infection and a

major reservoir for latent infection.[16,17] M-Mφs had a great capacity to produce HIV-1, while GM-Mφs inhibited HIV-1 replication.[18,19] Similar levels of viral DNA formation were observed in both types of Mφ after HIV-1infection, indicating that the inhibition of HIV-1 replication in GM-Mφs occurs at post-transcriptional and translational levels but not at the viral entry. M-Mφs expressed a high level of Hck, which can bind HIV-1Nef and a large isoform of C/EBPβ, and HIV-1 infection increased the expression of Hck but not of C/EBPβ. GM-Mφs expressed a high level of C/EBPβ and a low level of Hck, and HIV-1 infection drastically increased the expression of a short isoform of C/EBPβ, which acts as a dominant negative transcription factor, but decreased that of Hck. Treatment of M-Mφs with antisense oligonucleotide for Hck not only suppressed the expression of Hck, but also stimulated the induction of the short isoform of C/EBPβ and inhibited viral replication. Treatment of GM-Mφs with antisense oligonucleotide for C/EBPβ not only preferentially inhibited the expression of the small isoform of C/EBPβ, but also stimulated the induction of Hck and stimulated the virus production at a high rate. These results suggest that the CSF-induced and HIV-1-mediated distinct regulation of Hck and small isoform of C/EBPβ represent the heterogeneous susceptibility of tissue Mφs to HIV-1 infection. The authors also found that A-Mφs from healthy volunteers dominantly expressed a low level of Hck and small isoform of C/EBPβ and hardly stimulated HIV-1 replication, indicating GM-Mφs resemble A-Mφs in their susceptibility to HIV-1 infection.

Monocyte-derived macrophages induced by M-CSF but not GM-CSF can inhibit the growth of *Mycobacterium tuberculosis* and kill the bacteria

M. tuberculosis is a major human pathogen that resides in the host lung as a facultative intracellular pathogen and is found primarily in A-Mφs. M-Mφs inhibited the growth of *M. tuberculosis* H37Rv and killed the bacteria, while GM-Mφs stimulated the growth. H_2O_2 and NO were not the effector molecules of M-Mφs against *M. tuberculosis*, because addition of catalase or a NO synthesis inhibitor did not significantly change the bacterial killing activity of M-Mφs, and the levels of NO produced by infected M-Mφs and GM-Mφs were very low and no significant difference was observed between them. When Mφs were infected with *M. tuberculosis*, augmented expression of NRAMP1 (SLC11A1) and strong activation of p38MAPK, ERK1/2 and JNK were observed in M-Mφs but not in GM-Mφs. These results indicate that the difference in the activation of MAPK and the augmented expression of NRAMP1 may be related to the distinct susceptibility of M-Mφs and GM-Mφs to *M. tuberculosis.*

Monocyte-derived macrophages induced by M-CSF but not GM-CSF produce IL-10 and inhibit the γ-interferon production by T cells

M-Mφs and GM-Mφs are distinct in their suppressor activity. M-Mφs and GM-Mφs equally suppressed T cell proliferation in PBMC stimulated with PPD, but only M-Mφs suppressed IFN-γ production by T cells in response to PPD.[20] M-Mφs but not GM-Mφs released a large amount of IL-10 in response to PPD, and the neutralizing antibody to IL-10 reversed the suppressor activities of M-Mφs on IFN-γ production but not lymphocyte proliferation. Production of IFN-γ by lymphocytes was markedly inhibited by exogenous IL-10, while exogenous IL-10 was not as potent as M-Mφs for suppressing lymphocyte proliferation. Therefore, the mechanism of M-Mφ mediated T cell suppression for IFN-γ production is largely attributed to IL-10. A-Mφs from rodents and humans selectively inhibit T-cell proliferation but permit cytokine secretion, such as interleukin-2 and IFN-γ.[21] In respect to the lacking of suppressor activity for IFN-γ production, but not for proliferation, by T cells, GM-Mφs resemble A-Mφs.

Down-regulation of IL-10 production by GM-Mφs may be beneficial for host defense mechanisms against bacterial and viral infection to augment Th1 response. In contrast, M-Mφs may play important roles in suppressing lymphocyte responses through potent immunosuppressive activity. Therefore, divergent macrophages play appropriate roles in different situations to maintain homeostasis in the host.

DISCUSSION

The present study shows that GM-CSF and M-CSF stimulate the differentiation of human monocytes into two phenotypically distinct types of macrophages *in vitro*. The phenotype and functions of GM-Mφs resembles that of human A-Mφs, indicating that GM-CSF plays an important role in the differentiation of human A-Mφs. In the serial analysis of gene expression of monocytes, GM-Mφs and M-Mφs identified the specific gene expression in these cells along with the expression of large and overlapping sets of genes.[22,23] In addition, recent studies indicate the critical role of transcription factor PU.1 in GM-CSF-dependent terminal differentiation of mouse A-Mφs.[24] However, the precise molecular mechanisms of the difference of GM-CSF- and M-CSF-dependent differentiation of Mφs are not yet fully known. A model system using CSF-induced human Mo-derived M-Mφs and GM-Mφs may contribute the precise analysis of the molecular mechanisms.

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