

The polarization of T_h1/T_h2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production

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Keywords: adoptive transfer, glutathione, IFN- γ , IL-4, IL-6, IL-10, IL-12, innate immunity, reductive and oxidative macrophages, T_h1/T_h2 development

Abstract

We have been proposing the functional discrimination of two classes of macrophages (Mp), i.e. reductive macrophages (Rmp) with a high intracellular content of glutathione and oxidative macrophages (Omp) with a reduced content. In this paper we will present the evidence that the T_h1/T_h2 balance is regulated by the balance between Rmp and Omp due to the disparate production of IL-12 versus IL-6 and IL-10. Rmp were induced by *in vivo* application of *N*-acetyl-L-cysteine or glutathione monoethylester and Omp by L-cystine derivatives, diethyl maleate or L-buthionine-[*S,R*]-sulfoximine. The Mp arbitrarily called Omp showed elevated IL-6 and IL-10 production, and reduced NO and IL-12 production. The Rmp elicited a reciprocal response, i.e. elevated IL-12 and NO production, and reduced IL-6 and IL-10 production. The cytokine propensities of Omp or Rmp were inter-converted to each other. The results were also confirmed by using auto-MACS purified F4/80⁺ Mp without adherence. Interestingly, IFN- γ induced Rmp and augmented NO generation with decreased production of IL-6, whilst IL-4 induced Omp and augmented IL-6 production. CD4⁺CD44⁻ naive T_h0 cells were differentiated preferentially either to T_h1 or T_h2 cells, depending on the presence of Rmp or Omp during the initial 24 h of culture, from ovalbumin-specific TCR-transgenic mouse spleen cells in the presence of IL-2. Taken together, Rmp induction may generate the amplification loop of a Rmp/ T_h1 circuit and Omp that of Omp/ T_h2 . The findings implicate that the alteration in Mp functions because altered intracellular glutathione may play a relevant role in the pathological progression of inflammation.

Introduction

Macrophages (Mp) play diverse and relevant roles in the host defense mechanism against invasive and noxious insults (1,2). In primitive organisms, Mp are the main cells responsible for host defense. Recently, the important role of innate immune responses relative to adaptive immune responses has been re-evaluated based on the findings of essential roles of IFN- γ produced by Mp and of the Toll-like receptor (TLR) family–MyD88–NF- κ B system (3,4). In line with increasing evidence claiming central roles of innate over adaptive immune responses, a paradigm has been proposed on the presence of the functional heterogeneity of Mp (5–8).

IL-12 is secreted physiologically by monocytes, Mp and dendritic cells (DC) in response to pathogens, and plays a

pivotal role in regulating cell-mediated immunity (9). IL-12 also plays an important role in maintaining the *in vivo* balance between T_h1 and T_h2 responses (10). The development of T_h1 cells from T_h0 cells requires IL-12, and is prevented by prostaglandin E_2 and IL-10 (11), whereas differentiation into T_h2 cells requires IL-4 or IL-6 (12,13).

Accumulating evidence suggests that intracellular redox status regulates various aspects of cellular function (14). Typical is the impact on the regulation of NF- κ B activity relevant for gene activation of cytokines (15).

Glutathione (GSH) is abundant in virtually all cells, and constitutes the first line of cellular defense mechanism against oxidative injury and is the major intracellular redox buffer in

ubiquitous cell types (16). Our group and others demonstrated that GSH depletion, in murine antigen-presenting cells (APC) or in peritoneal Mp, decreased the secretion of IL-12 (17, 18). We further reported that exposure of human alveolar Mp to IFN- γ or IL-4 either increased or decreased the GSH/GSSG ratio, respectively (19). Accordingly lipopolysaccharide (LPS)-induced secretion of IL-12 in alveolar Mp was enhanced by IFN- γ but inhibited by IL-4. Thus, the ability to generate a T_h1- or T_h2-type response has turned out to depend not only on T cells but also on the intracellular thiol redox status of Mp. In this paper we will provide evidence that the T_h1/T_h2 balance is certainly regulated by the balance between RMp and OMp, and that the skewed RMp or OMp induction may generate the amplification loop of a RMp/T_h1 or an OMp/T_h2 circuit respectively.

Methods

Animals

C57BL/6, DBA/2 and BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). DO11.10 TCR-Tg mice [I-A^d-binding ovalbumin (OVA) 323–339-specific TCR-transgenic mice] were originally donated by Professor S. Habu (Tokai University) and bred in our own animal facility for >5 years. They were maintained under specific pathogen-free conditions.

Reagents

N-acetyl-L-cysteine (NAC, A-8199), glutathione monoethyl ester (GSH-OEt, G-1404), L-buthionine-[S,*R*]-sulfoximine (BSO, B-1525) and diethyl maleate (DEM, M-5887) were purchased from Sigma (St Louis, MO), and *N,N'*-diacetyl-L-cystine [(NAC)₂] and *N,N'*-diacetyl-L-cystine dimethyl ester [(NACOMe)₂] were from Bachem (Bubendorf, Switzerland; E-1770). Glutathione diethyl ester [GSH-(OEt)₂] and γ -L-glutamyl-L-cysteine diethyl ester [γ GC-(OEt)₂] were synthesized by Peptide Research Institute (Minoo, Japan) on our request and purity >98.3% confirmed by HPLC. LPS was derived from *Escherichia coli* 055:B5 (3120-25-0; Difco, Detroit, MI), and GSH and GSSG were from Sigma. NO₂ was measured using a commercial Griess-Romijn reagent (077-01852; Wako Pure Chemical, Tokyo, Japan). Monochlorobimane (MCB, M-1381; Molecular Probes, Eugene, OR) was used to stain intracellular GSH. Recombinant mouse IFN- γ was purchased from R & D Systems (Minneapolis, MN; MG-IFN). Mouse IL-10 and human IL-12 were from Genzyme (Cambridge, MA; 2488-01) and PharMingen (San Diego, CA; PM-19361V) respectively. Recombinant human IL-2, IL-6 and lentinan [LTN; a β (1–3)-glucan from edible mushroom, *Lentinus edodes* Berk. (Sing)] are the products of Ajinomoto (Tokyo, Japan). Anti-CD4, anti-CD3 and phycoerythrin (PE)-conjugated rat anti-mouse CD44 antibodies were purchased from PharMingen (PM-09421D, PM-01081D and 01225A).

Biochemical determination of intracellular GSH and GSSG

The determination was carried out according to the method previously described (19). Briefly, peritoneal Mp adherent to dishes were collected by scraping with a rubber policeman and washed 3 times with cold washing buffer. The cell pellet

was immediately lysed with 100 μ l of lysis buffer. Thereafter, 15 μ l of 0.1 N HCl and 15 μ l of 50% sulfosalicylic acid were added. After centrifugation, supernatants were collected for GSH and GSSG assay. The total cellular GSH concentration was assayed using the GSH reductase-DTNB recycling procedure. The change in the absorbance of 412 nm was measured using an Arvo multilabel counter (Wallac, Turku, Finland). The GSSG concentration was also assayed. Then 2 μ l of 2-vinylpyridin was added to the cell lysate supernatant, mixed for 1 min and the pH was adjusted to 7.5. Subsequently, the reaction mixture was allowed to stand at room temperature for 60 min. The content of GSH was obtained by subtracting the amount of GSSG from the total GSH content.

Nitrite assay

The accumulation of NO₂ was taken as a parameter for NO production. NO production by Mp was measured in supernatants collected after 48 h of culture. Briefly, cell-free supernatants were incubated with the Griess reagent for 10 min at room temperature and absorbance at 550 nm was measured using an Arvo multilabel counter. The concentration of NO₂ was determined by the square linear regression analysis of a sodium nitrite standard that was measured in each experiment.

Peritoneal macrophages

Peritoneal cells (PC) were harvested by injecting 5 ml of an ice-cooled phenol red-free RPMI 1640 medium (Nikken Seibutsu, Kyoto, Japan) without antibiotics into a peritoneal cavity. The collected PC from five or more mice were added to a microplate (Nunc, Roskilde, Denmark; 167008) at 1–3 \times 10⁵ cells/200 μ l in phenol red-free RPMI 1640 without antibiotics. Cells were plated in quadruplicate wells. The adherent cells after a 3 h incubation were used as resident peritoneal Mp for production of cytokines and NO by culturing for 48 h. OMp were induced by administering i.p. 20 μ g of LPS 4 days before or 10 μ g/head of DEM 3 h before the harvest of Mp. RMp were induced by administering i.p. 100 μ g of LNT 3 times every 2 days until 2 days before the harvest of Mp or by administering either a single shot of 20 mg/head of GSH-OEt 1 day before the collection of Mp or 10 mg/head of NAC 3 times every 2 days until 2 days before the collection. Unless indicated, Mp were stimulated with 100 U/ml IFN- γ and/or 100 ng/ml LPS for cytokines production. For some experiments peritoneal Mp were purified with an auto-MACS (Miltenyi Biotech, Bergisch-Gladbach, Germany) instead of adherence procedures. PC were first reacted with Fc block (PharMingen; 01241D) for 30 min and with FITC-conjugated anti-IgM antibody (PharMingen; 02084D) for 45 min, and selected for IgM-negative cells to remove B cells using FITC-coated magnetic microbeads (Miltenyi Biotech; 487-01). To the further purification, the resultant suspension was reacted with PE-conjugated anti-F4/80 antibody (Caltag, Burlingame, CA; RM2904-3) for 45 min and positively selected using PE-coated magnetic microbeads (Miltenyi Biotech; 488-01) with an auto-MACS according to the manufacturer's instructions. Thus purified Mp preparations consistently showed >98% F4/80⁺ cells purity by FACScan (Becton Dickinson, Franklin Lakes, NJ) analysis.

Qualitative determination of intracellular GSH with ACAS

An aliquot of 300 μ l of a PC suspension adjusted to a density of 3×10^5 cells/ml in phenol red-free RPMI 1640 were charged into a Lab-Tek Chamber Slide (Nunc; 136439) and incubated at 37°C for 3 h. After non-adherent cells were removed, the culture was washed 3 times with the same medium, and 300 μ l of MCB adjusted to 10 μ M in the same medium was added thereto and the reaction was conducted for 30 min. The fluorescent intensity implicating the amount of intracellular GSH was monitored by an argon-ion laser cytometry with a Meridian ACAS 470 work station (Meridian Instruments, Okemos, MI). Intracellular GSH levels were detected with an excitation wavelength of 350 nm and an emission wavelength of 460 nm.

CD4⁺ T cell culture

Spleens from five to 10 mice were pooled and provided for further fractionation of the cell population. CD8⁺ T cells and B cells were first removed using magnetic microbeads (Polyscience, Warrington, PA; 8-4330G, 8-4340G) coated with anti-CD8 antibody (53-6.7) and anti-HSA antibody (M16/9), both gifts of Professor T. Saito (Chiba University). CD4⁺ T cells were further positively purified with an auto-MACS using microbeads coated with anti-CD4 antibody. The purity of fractionated CD4⁺ T cells was consistently >99%. Thus purified CD4⁺ T cells were seeded in a microplate (Falcon 3915; Becton Dickinson) pre-coated with anti-CD3 antibody at a cell density of 5×10^5 /well and incubated for 48 h. For some experiments peritoneal Mp were added at a cell density of 1×10^5 /well onto the microplate, adhered for 3 h followed by washing and co-cultured together with purified CD4⁺ T cells to gain an efficient production of cytokines. The recovered culture supernatant was frozen for ELISA assay of IFN- γ , IL-10 and IL-4.

Determination of IL-12, IL-10, IL-6, IFN- γ and IL-4 by ELISA

The IL-12, IL-10, IL-6 IFN- γ and IL-4 concentration was determined using an ELISA kit (Opt EIA; PharMingen, for IL-12, IFN- γ , IL-6, IL-4 and IL-10) according to the indication of the manufacturer.

T_h1/T_h2 differentiation and intracellular fluorescence staining of cytokines

CD4⁺CD44⁻ naive T cells were purified from spleen cells from DO11.10 TCR-Tg female mice using cell sorting (FACS Vantage SE; Becton Dickinson). CD8⁺ T cells and HSA⁺ B cells were removed as described above. The resultant cell suspension was labeled with FITC-conjugated anti-CD4 (PharMingen; 01064D) and PE-conjugated anti-CD44 (PharMingen; 01225A) in Hank's solution for 45 min, and provided for cell sorting. Purified CD4⁺CD44⁻ T cells (2×10^5 /2 ml/well) were stimulated with 6 μ M OVA323-339 peptide (Peptide Research Institute), in the presence of X-ray (30 Gy)-irradiated BALB/c spleen cells, 2 ng/ml IL-12 and 100 U/ml IL-2 for T_h1 development. T_h2 cells were induced in the presence of 25 ng/ml IL-4 and 100 U/ml IL-2. To evaluate the distinct role of RMp or OMp in the differentiation of naive T_h0 cells, peritoneal Mp of BALB/c mice were cultured at a cell density of 1×10^5 /well for the initial 24 h in the presence of redox

modifying agents followed by extensive washing. The non-adherent cells recovered were added back to the corresponding original wells after washing to remove the agents. Thereafter, purified CD4⁺ CD44⁻ T cells were added and cultured for 6 days together with 6 μ M OVA323-339 peptide without BALB/c spleen cells in the presence of only IL-2 (100 U/ml). After 7 days culture, cells were harvested, washed twice with PBS and viable cells were recovered with Lympholyte M (Cedarlane, Hornby, Ontario, Canada; CL5030). For the detection of cytoplasmic cytokine expression, cells were stimulated with immobilized anti-CD3 mAb for 6 h in the presence of 2 μ M monensin (Wako Pure Chemicals, Osaka, Japan), fixed with 4% paraformaldehyde and treated with permeabilizing solution (150 mM NaCl, 5 mM EDTA, 0.02% NaN₃ and 50 mM Triton X-100, pH 7.5). Then the fixed cells were stained with PE-conjugated rat anti-mouse IL-4 mAb (PharMingen; 18195A) and FITC-conjugated rat anti-mouse IFN- γ (PharMingen; 18114A) for 45 min. For flow cytometric analysis, data of 10,000 cells were analyzed using a FACScan and CellQuest software.

Statistical analysis

Results are expressed as means \pm SE. Significant differences were calculated using Student's *t*-test. A value of *P* < 0.05 was considered statistically significant.

Results

Redox regulation of NO and IL-6 production and redox status of distinct Mp

The Mp with elevated levels of intracellular GSH compared to Mp were arbitrarily called RMp and the Mp with reduced intracellular content called OMp. One of the authors reported that LNT reduced spontaneous production of prostaglandin E₂ by peritoneal Mp (20). LPS is well known to augment prostaglandin E₂ production. RMp were induced by LNT and OMp by LPS (17). Alternatively, RMp were induced by either GSH-OEt or NAC and OMp by DEM. The OMp induced by LPS priming showed elevated IL-6 production with reduced production of NO. On the contrary, RMp induced with LNT elicited reduced IL-6 production with elevated production of NO (Fig. 1a and b). As expected, GSH-OEt-primed Mp shared the functional phenotype of RMp induced by LNT, the dose-dependent augmentation of NO production upon *in vitro* stimulation with IFN- γ and/or LPS (Fig. 1c and d) and the reduced production of IL-6 upon *in vitro* stimulation with IFN- γ (Fig. 1e). DEM-primed Mp turned out to share the phenotype of OMp induced by LPS (Fig. 1c, d and e). It was shown that the intracellular GSH content in Mp was certainly up- or down-modulated by NAC, GSH-OEt or DEM respectively, as were monitored both by cytometric detection with MCB staining (Fig. 2b) and by biochemical determination (Fig. 2a).

Redox regulation of IL-10 production

IL-10 is well known to prevent the differentiation of naive T_h0 to T_h1, thereby promoting the development of T_h2 (11). However, nothing is been known so far on the redox regulation of IL-10 production by Mp. NAC (10mg), GSH-OEt (20 mg), GSH-(OEt)₂ (2 mg) or γ -GC-(OEt)₂ (2 mg) was i.p. injected to

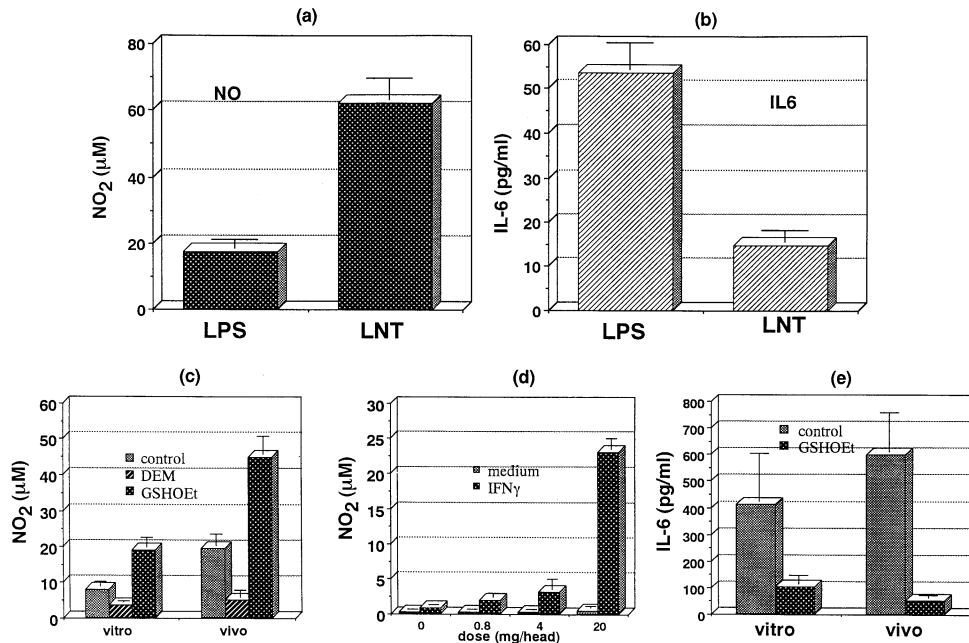


Fig. 1. Distinctive production of NO and IL-6 by RMP and OMP. DBA/2 female mice were used. OMP were induced by 20 μg of LPS (a and b) or by 50 μg of DEM (c). RMP were induced by 100 μg of LNT (a and b) or by 10 mg of GSH-OEt (c and e; vivo). Varying doses of GSH-OEt were similarly applied (d). The adherent Mp were stimulated with IFN-γ plus LPS for NO production and with IFN-γ for IL-6 production. For *in vitro* induction adherent cells were stimulated in the presence of either 5 mM GSH-OEt or 100 μM DEM (c and e; vitro). Results shown are representative of three (a, b, c and e) and two (d) separate experiments. (a, b and e, $P < 0.01$; c, $P < 0.05$; d, 20 mg/head, $P < 0.01$.)

induce RMP and PC were harvested 20 h later. The whole PC or PC adherent to dishes were cultured in the presence of LPS for 48 h. Cytine derivatives, (NAC)₂ (200 mg) or (NAC-OMe)₂ was applied to induce OMP. It is clear from the results shown in Fig. 3 that IL-10 production by LPS stimulation was reduced consistently in RMP compared to resident Mp, while it was elevated in OMP. The induction of OMP and RMP with these compounds was confirmed by argon-ion laser cytometry (data not shown).

Distinct impact of IFN-γ and IL-4 on NO and IL-6 production

The treatment of OMP with IFN-γ augmented NO generation upon subsequent *in vitro* LPS stimulation, while IL-4 augmented IL-6 production with no augmentation of NO production (Fig. 4a and c). The treatment of Mp with IL-4 prior to exposure to IFN-γ reduced the augmented production of NO and restored the suppressed IL-6 production (Fig. 4b and d). The priming effects were abrogated in the presence of an anti-IL-4 receptor antibody (1688-01; Genzyme), while an anti-IL-2 receptor γ chain antibody (TuGm3, the gift of Professor Sugamura, Tohoku University) had no effect (Fig. 4e). The results indicated the distinct impact of IFN-γ and IL-4 on the redox status of Mp, i.e. the former may induce RMP and the latter OMP. The conclusion was consistent with our observations that IFN-γ or IL-4 either increase or decrease the GSH/GSSG ratio of human alveolar Mp respectively (19).

Redox regulation of IL-12 production

IL-12 production was also regulated by the intracellular redox status of Mp. *In vivo* GSH-OEt-primed RMP produced IL-12 upon IFN-γ plus LPS stimulation *in vitro*, whereas neither

resident Mp nor DEM-elicited OMP did (Fig. 5a). Likewise LNT, molecularly distinct from GSH-OEt, endowed Mp with the capability to produce IL-12, whereas LPS-induced Mp were devoid of the capability to produce IL-12 (data not shown). The results indicate that RMP had the potential to produce IL-12, but OMP were devoid of the potential. The production of IL-12 by RMP was reduced by *in vitro* treatment with IL-4 prior to the stimulation with IFN-γ plus LPS (Fig. 5b). This may be due to the function of IL-4 to convert RMP to OMP as described.

Depriving GSH-OEt-elicited Mp of GSH by 3 h DEM or 24 h BSO exposure completely ablated the potential to produce IL-12 (Fig. 6a and b), concomitant with the reduction of intracellular GSH content from 7.56 ± 0.81 to 1.22 ± 0.39 or 2.32 ± 0.27 nmol/10⁶cells. The capability to produce IL-12 was restored by converting OMP to RMP with re-exposure to GSH-OEt (Fig. 6b). This re-exposure showed the repletion of intracellular GSH from 1.22 ± 0.81 to 6.55 ± 0.62 nmol/10⁶cells and the repletion was confirmed also by cytometry. This implies that IL-12 production is actually dependent on the intracellular GSH content in Mp.

Functional phenotypes of F4/80⁺ Mp distinctive in their intracellular GSH

PC include not only Mp but also B cells, neutrophils, and a minor population of NK cells, NKT cells and dendritic cells. The adherence to dishes does not exclude the contamination of cells other than Mp and the possibility of the artificial change of redox status during adherence procedure. Therefore we next purified PC Mp with MACS and treated them with GSH-OEt or BSO. The results illustrated in Fig. 7 are all consistent

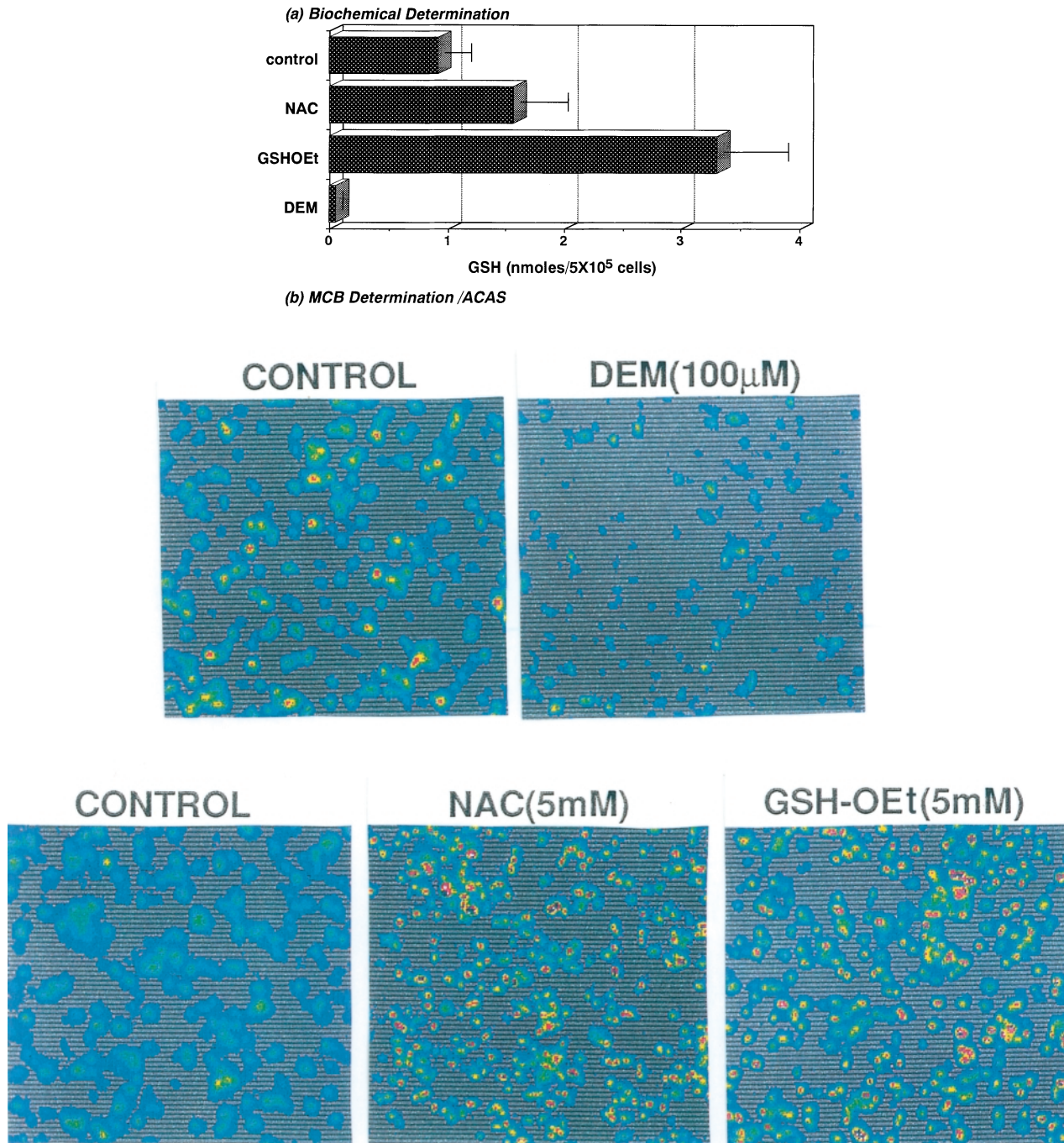


Fig. 2. Intracellular GSH content of RMp and Omp. Adherent PC Mp of DBA/2 female mice were treated with 5 mM NAC, 5 mM GSH-OEt for 24 or 100 µM DEM for 3 h and presented for biochemical determination of intracellular GSH (a). Alternatively the amount of intracellular GSH was monitored by argon-ion laser cytometry (b). The yellowish or red color means the abundant presence of intracellular GSH. Results shown are representative of two separate experiments.

with those described above. The ratio of IL-12/IL-10 is reduced in Omp and considerably elevated in RMp (Fig. 7).

Polarization of T_H1/T_H2 balance by RMp/Omp inducers or by adoptive transfer of RMp/Omp

In the succeeding experiments we investigated the *in vivo* polarization of T_H1/T_H2 balance after administration of a RMp

(GSH-OEt) or Omp inducers ((NACOME)₂), both applicable for clinical use. The results indicated contrasting effects between a RMp and an Omp inducer to induce either T_H1 or T_H2 polarization (Fig. 8a). BSO elicited a comparable IFN- γ /IL-4 ratio to that elicited by (NACOME)₂. Next the RMp or Omp induced by *in vivo* priming with GSH-OEt or BSO were transferred (2.5×10^5 cells) adoptively to recipient mice. Then

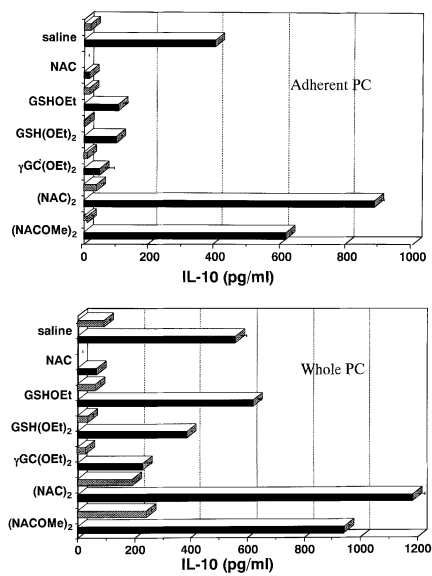


Fig. 3. Distinctive production of IL-10 by RMp and OMp. C57BL/6 female mice were used. NAC (10 mg), GSH-OEt (20 mg), GSH-(OEt)₂ (2 mg) or γ-GC-(OEt)₂ (2 mg) was injected to induce RMp and the PC were harvested 20 h later. Cyttine derivatives, (NAC)₂ or (NACOMe)₂ (200 μg) was applied to induce OMp. The whole PC or PC adherent to dishes were cultured either in the absence (light bars) or presence (dark bars) of LPS for 48 h. Results shown are representative of two separate experiments.

cytokines produced by CD4⁺ T cells and Mp of recipient mice stimulated with anti-CD3 antibody or IFN-γ and/or LPS were analyzed (Fig. 8b). The results showed that T_h1 polarization of recipient mice by the RMp transfer (elevated IFN-γ/IL4) and T_h2 polarization (reduced IFN-γ/IL4) by the OMp transfer. At the same time it was clarified that PC-Mp of recipient mice elicited the functional phenotypes of RMp or OMp, corresponding to the transfer of RMp or OMp respectively, as indexed by the change of IL-12/IL-10.

Regulation of T_h1 and T_h2 cell development by Mp at distinct redox status

The priming of purified CD4⁺CD44⁻ T cells stimulated with OVA323–339 peptide in the presence of irradiated BALB/c spleen cells, IL-12 and IL-2 resulted in the development of IFN-γ single-producing T_h1 cells (18.9% T_h1 versus 6.9% T_h2). The presence of IL-4 and IL-2 induced IL-4 single-producing T_h2 cells (0.17% T_h1 versus 21.2% T_h2). To evaluate the distinct role of RMp or OMp in the differentiation of naive T_h0 cells, peritoneal Mp of BALB/c mice were cultured for the initial 24 h in the presence of GSH-OEt or BSO and non-adherent cells were recovered. After extensive washing the recovered cells were added back to the original well to further culture for 6 days together with 6 μM OVA peptide in the presence of only IL-2 without irradiated BALB/c spleen cells. The priming in the presence of RMp and IL-2 resulted in a polarized development of T_h1 cells (32.6% T_h1 versus

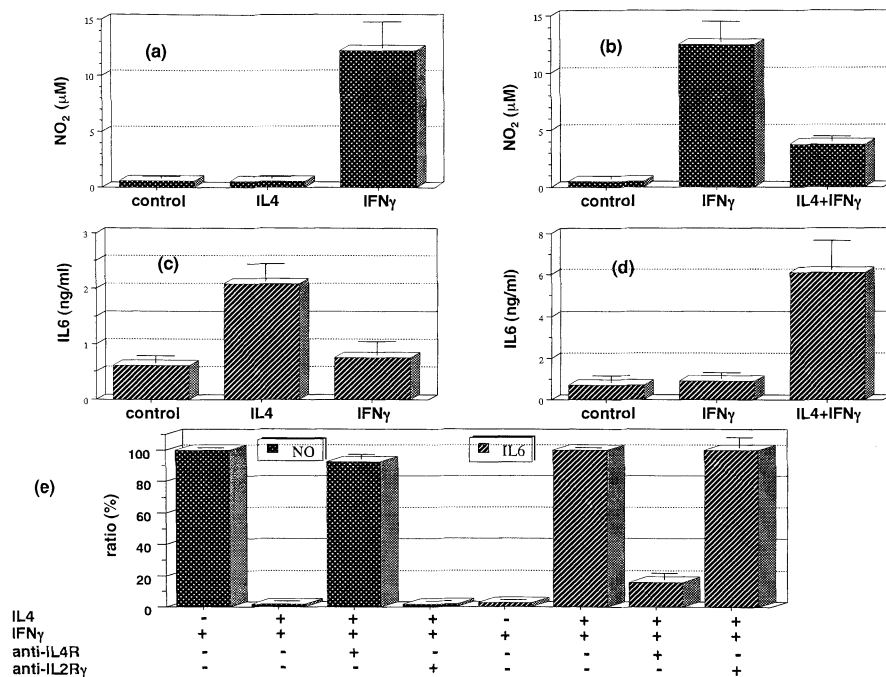


Fig. 4. Distinctive production of NO and IL-6 by IFN-γ and IL-4. DBA/2 female mice were used. The LPS-primed PC were harvested and seeded to microplates at a cell density of 2×10⁵/well. The adherent Mp were treated either with 100 U/ml IFN-γ or with 50 ng/ml IL-4 for 48 h in the presence of 100 ng/ml LPS, and the recovered culture supernatants were assayed for NO and IL-6 (a, c and a, IFN-γ, P < 0.01; c, IL-4, P < 0.01). In (b and d), adherent Mp were cultured initially in the absence or presence of 50 ng/ml IL-4 for 20 h and stimulated with 100 U/ml IFN-γ for 48 h in the presence of 100 ng/ml LPS (b and d, IFN-γ versus IL-4 + IFN-γ, P < 0.01). The results in the presence of either anti-IL-4R antibody (10 μg/ml) or anti-IL-2Rγ antibody (10 μg/ml) during a IL-4 pulse are shown (e).

10.8% T_h2). The presence of Omp and IL-2 induced a polarized development of T_h2 cells (4.9% T_h1 versus 40.4% T_h2) (Fig. 9). The extent of polarization varied from experiment to experiment (Rmp: T_h1 23–34.8%, T_h2 6.7–12.2%; Omp:

T_h1 2.5–5.4%, T_h2 28.2–40.4% in three separate experiments). However, the direction of polarization (Rmp to T_h1, Omp to T_h2) was always consistent, even in the absence of addition back of the non-adherent cells.

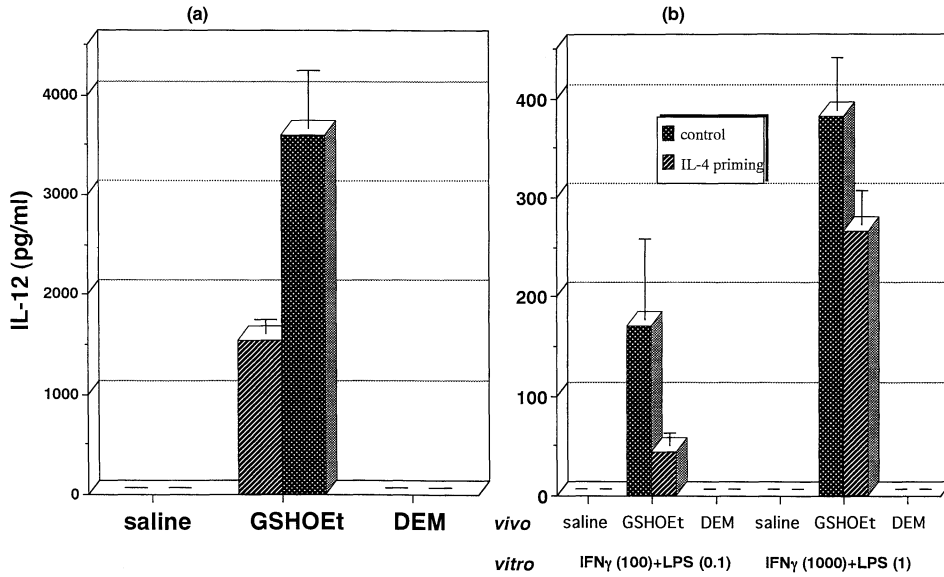


Fig. 5. Distinctive production of IL-12 by Rmp and Omp. DBA/2 female mice were used. Omp were induced by 50 μ g of DEM and Rmp were induced by 10 mg of GSH-OEt. Mp were stimulated either with 100 U/ml IFN- γ plus 100 ng/ml LPS (light bars) or 1000 U/ml IFN- γ plus 1000 ng/ml LPS (dark bars) for (a). To test the IL-4 priming effect adherent Mp were cultured initially in the absence or presence of 50 ng/ml IL-4 for 20 h and then stimulated (b). Results shown are representative of two separate experiments. (Control versus IL-4 priming; $P < 0.05$.)

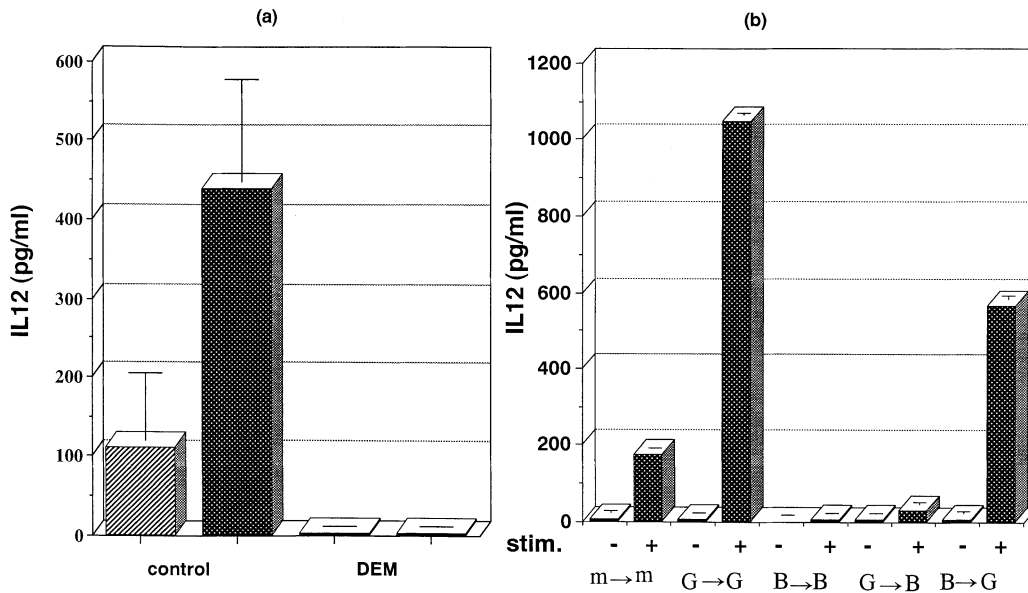


Fig. 6. IL-12 production depends on the intracellular content of GSH. Rmp was induced as in Fig. 5. Depriving Rmp of GSH was carried out by exposure to 100 μ M DEM for 3 h (a) before *in vitro* stimulation either with 100 U/ml IFN- γ plus 100 ng/ml LPS (light bars) or 1000 U/ml IFN- γ plus 1000 ng/ml LPS (dark bars) for 48 h (a). Results shown are representative of three separate experiments. (Control versus DEM, $P < 0.01$.) In (b), 1×10^5 /well PC were first pulsed with either 10 mM GSH-OEt or 0.5 mM BSO for 24 h, washed extensively, and then pulsed again with them and then stimulated. g, GSH-OEt; b, BSO. Results shown are representative of two separate experiments. (m \rightarrow m versus g \rightarrow g, m \rightarrow m versus b \rightarrow g, g \rightarrow g versus b \rightarrow g, g \rightarrow b, b \rightarrow b versus b \rightarrow g, $P < 0.01$.)

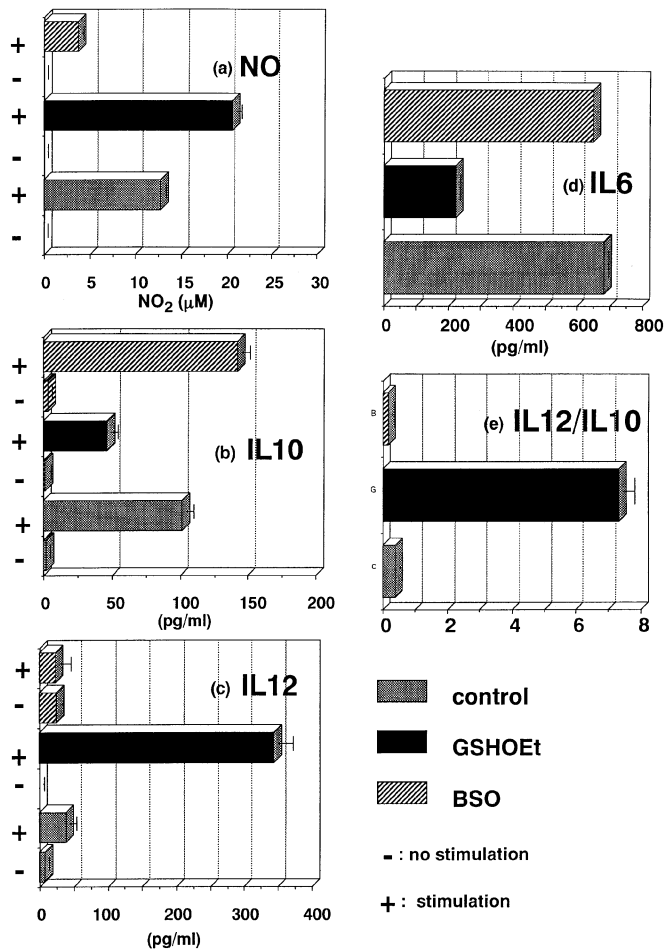


Fig. 7. Distinctive functional phenotypes of Rmp and Omp induced from purified F4/80⁺ PC. Peritoneal Mp of DBA/2 female mice were purified with an auto-MACS instead of adherence procedures. The purified Mp were stimulated with IFN- γ and/or LPS (for IL-6, IFN- γ ; for IL-10, LPS; for NO and IL-12, IFN- γ + LPS). Results shown are representative of two separate experiments.

Discussion

GSH is a non-protein tripeptide, and plays significant roles in many biological processes and the preservation of the intracellular redox balance (21,22). The depletion of GSH restores depressed T cell responses, and a decrease in IL-2 and IFN- γ secretion by T cells (23,24). NAC, GSH and other thiols reduced the production of T_h2 -derived IL-4 (25), although the indirect regulation by the redox status of contaminating APC cannot be ruled out. DEM and BSO, known to induce intracellular reactive oxygen intermediates, inhibited nitrite secretion by IFN- γ plus LPS, whereas a cell permeant GSH-OEt augmented this in a dose-dependent manner (Figs 1 and 7). GSH is a cofactor needed for full activity of NO synthase of Mp (26). A close correlation was observed between levels of intracellular GSH and nitrite secretion (21).

IL-6 and IL-10 are cytokines produced by T_h2 and Mp, and share pleiotropic biological activities on Mp (27,28). IL-10 suppresses a wide scope of Mp functions stimulated with

IFN- γ , including NO production (29). LPS-stimulated Mp were shown to directly produce IL-10 (30), although nothing was resolved on redox regulation of IL-10 induction. LPS-stimulated Mp produced higher amounts of IL-10 by modulating the redox equilibrium to the oxidative status by pharmacological agents (Fig. 3). A similar modulation of the redox equilibrium by pharmacological thiols turned out to evoke contrasting effects on IL-12. Only the skewing to a reductive status endowed Mp with the ability to produce IL-12 upon IFN- γ plus LPS (Fig. 4), consistent with our previous observations (17,19). Peterson *et al.* also reported that GSH depletion in murine APC decreases the secretion of IL-12 (18). Our finding of IL-12 production by Mp skewed to a reductive thiol redox status may be indicative of the presence of a subpopulation of Mp to produce IL-12. In fact, ~20% of splenic Mp precursors appear to produce IL-12 (31). DC, by their production of IL-12, also play a direct role in the development of IFN- γ -producing cells (32). The redox regulation of the cytokine propensities of DC is now under extensive study and preliminary results indicate the presence of such regulation.

APC involving Mp, DC and B cells are central to the development of either T_h1 or T_h2 from naive T_h0 cells. IL-12 has a major effect on the inductive phase of priming by enhancing commitment to IFN- γ production (33). The availability of IL-12 and IFN- γ as opposed to IL-4 is decisive for the maturation to T_h1 or T_h2 (12,34). Recent reports have shown that other factors such as IL-6 (7) and IL-10 (11,35) as well as MIP-1 α and MCP-1 can selectively direct differentiation of T_h0 cells (36). CD4⁺CD44⁻ naive T_h0 cells were differentiated preferentially either to T_h1 or T_h2 cells, depending on the dominance of either Rmp or Omp respectively (Fig. 9). Rmp induction may drive polarization to T_h1 , which in turn generates a skewing to Rmp through production of IFN- γ . On the contrary, Omp induction may drive polarization to T_h2 , which in turn results in a skewing to Omp through production of IL-4. This indicates the possible presence of an amplification loop of a Rmp/ T_h1 circuit and that of Omp/ T_h2 in local inflammatory sites. Mp are one of the main sources of IL-12, which drives IFN- γ production, and might favor the development of T_h1 cells (37). The Mp depletion *in vivo* shifts an expected T_h1 response to a T_h2 response (38). IL-10 inhibits IL-12 production and thereby drives IL-4 production (39). Rincon *et al.* pointed out the possibility of IL-6-triggered initial IL-4 production by naive T_h0 cells and addressed the key role in a link between innate immunity and the T_h2 response (13). Coffmann *et al.* immediately raised the question that the specific conditions or pathogens that would favor T_h2 over T_h1 induction by this pathway are not clear (40). The issue presented in this paper may provide the indices that the altered intracellular redox status of APC triggered by pathogens will direct the specific condition to produce IL-6.

A paradigm has been proposed on the presence of functional heterogeneity of Mp either in the metabolic activities or cytokine propensities (5–8,41). Studies presented here show that intracellular GSH levels in Mp affect the cytokine propensities of Mp and influence the T_h1/T_h2 balance. The distinctive production of cytokines and NO between Rmp and Omp was confirmed with purified F4/80⁺ Mp (Fig. 7), although it is still elusive whether the minor (<2%) population, such as DC, interferes with the observation. The adherent cell population

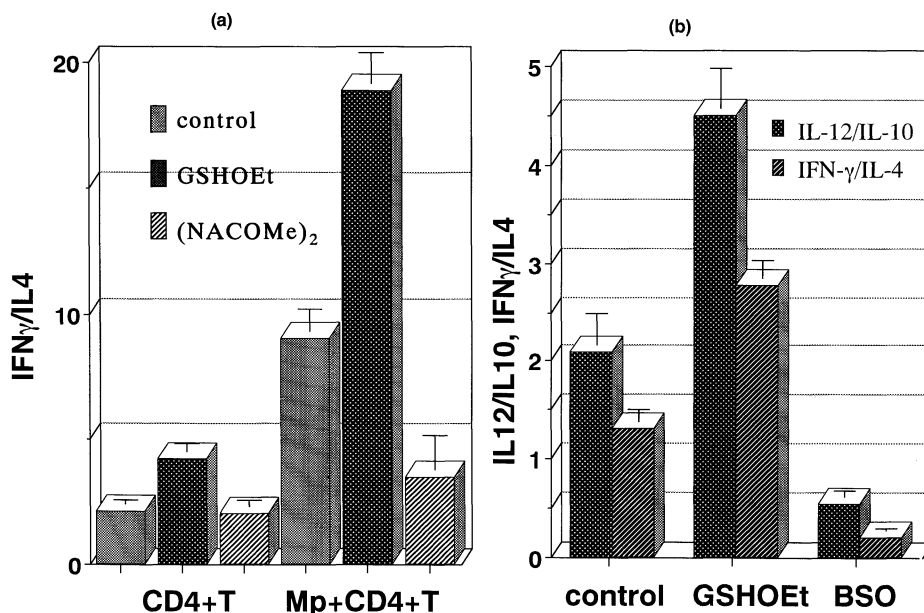


Fig. 8. Polarization of T_H1/T_H2 balance by RMp/OMp inducers or by adoptive transfer of RMp/OMp. (a) GSH-OEt (10 mg) or (NACOMe) $_2$ (200 μ g) was i.p. injected on day 1 and 4, and on day 5 spleens and PC were harvested. The culture supernatants of purified splenic CD4 $^+$ T cells, stimulated with anti-CD3 antibody either in the absence or presence of PC-Mp, were analyzed for IFN- γ and IL-4. Results shown are representative of three separate experiments. BSO (1 mg)-elicited CD4 $^+$ T cells showed an almost comparable IFN γ /IL-4 ratio (M ϕ $^+$ CD4 $^+$ T: 2.8 ± 1.3). (b) RMp induced by GSH-OEt or OMp induced by BSO (1 mg) (2.5×10^5 cells) were transferred to recipient mice, and PC and splenocytes were harvested 4 days after the transfer. The cytokines produced by purified CD4 $^+$ T cells or M ϕ of recipient mice upon *in vitro* stimulation were analyzed. Results shown are representative of two separate experiments.

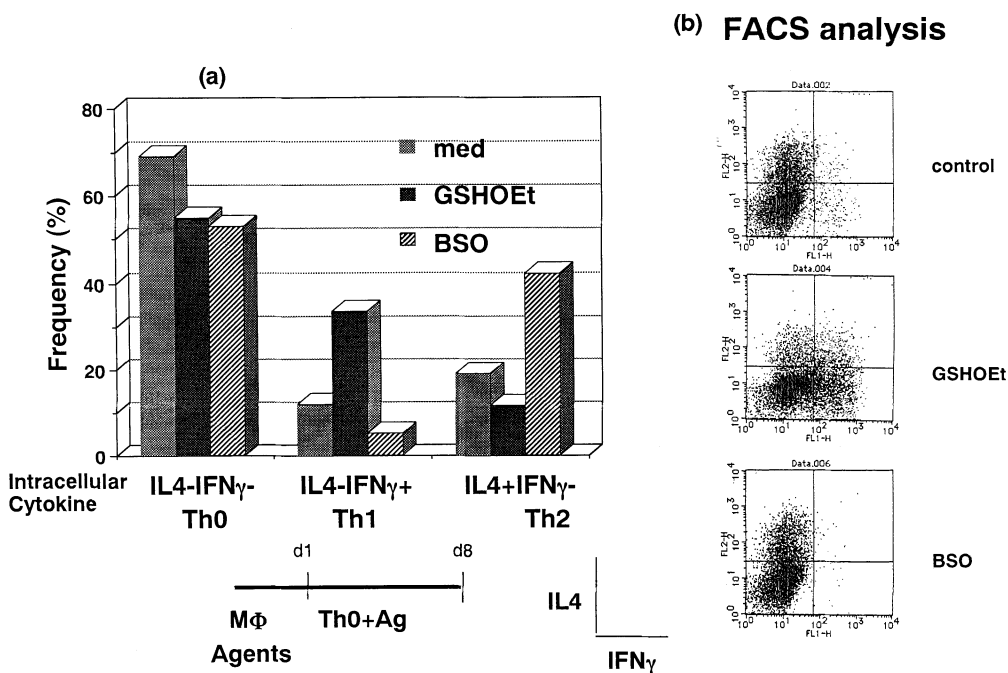


Fig. 9. T_H1 and T_H2 cells development in the presence of RMp or OMp. Peritoneal M ϕ of BALB/c mice were cultured for the initial 24 h in the presence of GSH-OEt (10 mM) or BSO (0.5 mM) and non-adherent cells were recovered. After extensive washing the recovered cells were added back to the original well and purified CD4 $^+$ CD44 $^-$ T cells were added to further culture for 6 days together with 6 μ M OVA peptide in the presence of only IL-2 without irradiated BALB/c spleen cells. The detection of cytoplasmic cytokine expression was pursued as detailed in Methods. Results shown are representative of three separate experiments.

involved ~0.7% CD11c⁺ cells (on average) besides F4/80 single-positive cells by immunofluorescence studies.

Our proposal to classify propensities of Mp into RMp and OMp in terms of intracellular content of GSH may not be absolute but rather relative, but useful to conceptualize the convergence of diversified immunological outcomes initiated by a variety of stimuli. RMp and OMp classification may simply mean the distinct metabolic states or the intracellular redox status of Mp and there may be a continuum of phenotypes between these two Mp in the absence of clonally separable phenotypes. Mp from T_h1-like mice strains (T_h1 Mp) are more easily activated to produce NO than those from T_h2-like strains (8). Considering the elevated NO production by T_h1 Mp and RMp, and the IL-4- or IL-10-mediated reduction of NO production (24,42,43), the T_h1 Mp may well correspond to our RMp and their T_h2 Mp to OMp. IL-4, IL-10 and prostaglandin E₂ inhibit the production of NO from Mp through depletion of arginine and up-regulated arginase I (44). The regulated substrate competition between arginase and iNOS has implications for understanding the physiological regulation of NO production (45). T_h1 cells lead to an exclusive induction of iNOS, whereas T_h2 cells up-regulate arginase without inducing iNOS (5). LPS stimulates T_h2, but not T_h1, Mp to increase arginine metabolism to ornithine (8). All of these observations could be incorporated into a unity if a linkage between intracellular content of GSH and the regulation of two metabolic enzymes, arginase and iNOS, can be resolved. A dichotomy in monocytes (M1 and M2) was also hypothetically proposed (41). M1 cells function as pro-inflammatory Mp, whereas M2 cells, by virtue of IL-10 production, act as anti-inflammatory Mp. In disease models, including insulin-dependent diabetic mellitus, inflammatory bowel diseases and hepatitis, RMp dominates at the stage of tissue destruction and the dominancy is tuned to OMp at the healing stage (Murata *et al.*, manuscript in preparation). Stein *et al.* introduced the concept of T_h2-associated alternative Mp activation (46). In contrast to classical Mp activation by IFN- γ and LPS, activation of Mp by IL-4 was classified as alternative (47). Our observations that IL-4-induced OMp and IFN- γ -induced RMp phenotypes (19) (Fig. 3) strongly favor the argument that RMp may be classically activated Mp and OMp alternatively activated. Recently, Desmedt *et al.* argued that Mp efficiently elicit cellular immunity, selectively suppress an already generated T_h2-dependent response and hence behave in the organism as T_h1-dedicated APC (48). In this context it is of interest to clarify the impact of the redox status of Mp on ongoing T_h1 or T_h2 responses. Our preliminary results indicate the successful modulation of ongoing T_h1 or T_h2 responses in insulin-dependent diabetes and inflammatory bowel diseases models (Murata, unpublished data). Our data of the adoptive transfer of RMp shown in Fig. 8 support their notion in part, but did not coincide with their observation inasmuch as OMp transferred the T_h2 prototype in recipient mice (Fig. 8). Although the resident Mp involved ~1–5% of RMp in DBA/2 mice, it is difficult to define resident Mp as T_h1-dictated RMp.

Finally, it should be noted that LPS and LNT did elicit distinct effects on the intracellular redox equilibrium of Mp (Fig. 1), although both LPS and β (1–3)-glucans are known as cell wall components of either Gram-negative bacteria or

fungi. LPS elicits the activation of an intracellular signaling cascade via TLR4, while one β (1–3)-glucan, zymosan, via TLR2 (49). Although the skeletal structure of LNT is similar to zymosan, most of the immunological activities are distinct. LNT may provide a useful tool to further clarify the role of the intracellular redox status in a signaling cascade, TLR family–MyD88–NF- κ B system (3,4), and to resolve a link between innate and adaptive immunity coordinated by TLR.

The findings in this paper may present a new insight, in terms of redox regulation of Mp physiology, into the altered immune response in a variety of inflammatory and autoimmune diseases under diverse oxidative stresses. Further, the concept of RMp and OMp potentially offers a better understanding to the controversial observation of dual effects of cytokines on Mp (40,50,51), and to the interplay between Mp/DC and T cells in local peripheral inflamed tissues. It is of note that the altered functions of Mp, distinct in their intracellular redox status, may indirectly influence the T_h1/T_h2 balance through modulating DC functions relevant in initiating antigen-triggered immune responses.

Acknowledgements

The authors thank Drs T. Saito, S. Taki and H. Arase (Chiba University Medical School), H. Fujiwara (Osaka University Medical School), S. Habu (Tokai University Medical School), L. Herzenberg (Stanford University), and H. Dvorak (Harvard University) for continuous encouragement and helpful advice, and Ms Yoko Hamuro for secretarial assistance.

Abbreviations

ACAS	adherent cell analyzing system
APC	antigen-presenting cells
BSO	L-buthionine-[S,R]-sulfoximine
DEM	diethyl maleate
GSH	glutathione
GSSG	oxidized glutathione
GSH-OEt	glutathione monoethylester
LNT	lentianin
LPS	lipopolysaccharide
MCB	monochlorobimane
Mp	macrophages
NAC	N-acetyl-L-cysteine
NO	nitric oxide
OMp	oxidative macrophage
OVA	ovalbumin
PC	peritoneal cell
PE	phycoerythrin
RMp	reductive macrophage
TLR	Toll-like receptor

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