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Differential effect on TCR:CD3 stimulation of a 90-kD glycoprotein (gp90/Mac-2BP), a member of the scavenger receptor cysteine-rich domain protein family

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SUMMARY

We studied the effects of a 90-kD glycoprotein (gp90/Mac-2BP) belonging to the scavenger receptor family, present in normal serum and at increased levels in inflammatory disease and cancer patients, on some T cell function parameters. Whereas the lymphocyte proliferative response to non-specific mitogens such as phytohaemagglutinin (PHA) and concanavalin A (Con A), but not pokeweed mitogen (PWM), was strongly reduced, probably due to the lectin-binding properties of gp90/Mac-2BP, the response to T cell receptor (TCR) agonists such as superantigens and allogeneic cells was potentiated. When lymphocytes were stimulated with different anti-TCR:CD3 MoAbs, both in soluble and solid-phase form, gp90/Mac-2BP was able to down-regulate the proliferative response to anti-CD3 MoAb, whereas the response to anti-TCR $\alpha\beta$ MoAb was enhanced. A similar differential effect was observed when a MoAb against CD5 (another member of the scavenger receptor superfamily) was added to anti-CD3 or anti-TCR-stimulated cells; anti-CD5 MoAb strongly down-modulated the CD3-mediated response, whereas its presence in culture was associated with potentiation of the response to TCR $\alpha\beta$ agonists. gp90/Mac-2BP was able *per se* to up-regulate Ca^{2+} levels in freshly isolated lymphocytes; moreover, its presence in culture was associated with increased Ca^{2+} mobilization following stimulation with anti-TCR $\alpha\beta$, but not anti-CD3 MoAb. These data indicate that gp90/Mac-2BP could be able to influence some immune responses, possibly through multiple homologous interactions with other members of the scavenger receptor family; moreover, our findings suggest that signalling through the different components of the TCR:CD3 complex may follow distinct activation pathways into the cells.

Keywords CD3 gp90/Mac-2BP scavenger receptor TCR T lymphocytes

INTRODUCTION

A 90-kD glycoprotein (gp90) was originally identified in the culture supernatant of human breast cancer cells [1]; this protein was also found at low levels in the serum of healthy individuals, and at elevated concentrations in the serum of patients with cancers of various origins [2–4] and HIV infection [5–8]. Purification and cloning studies [9,10] revealed the identity of gp90 to the Mac-2 binding protein (Mac-2BP), a natural ligand of the soluble β-galactoside-specific S-type lectin Mac-2, now known as galectin-3 [11]. Galectin-3, which is highly expressed by cells of the macrophage/monocyte lineage [12], is a bifunctional secreted protein with a lectin domain capable of binding laminin; this interaction may serve as a bridge between macrophages and extracellular matrix proteins, microorganisms, or other cells bearing galactosylated proteins [13].

The gp90 glycoprotein (gp90/Mac-2BP) belongs to a newly described protein family, whose first described component was identified as the type I macrophage scavenger receptor [10,12,14,15]. The motif that characterizes this family, designated as scavenger receptor cysteine-rich (SRCR) domain, is highly conserved between evolutionarily distinct species [16]. Although no function has yet been attributed to this domain, it is noteworthy that all previously characterized mammalian SRCR domain-

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containing proteins are expressed or secreted by cells associated with the immune system, and known or thought to be involved in different immunological mechanisms [15–17].

Several lines of evidence suggest that gp90/Mac-2BP may play a role in host defence, shielding the body from invasion by bacterial and parasitic pathogens. Constitutively high gp90/Mac-2BP expression levels were found in tissues containing cavity-lining secretory epithelia, as well as in human breast milk [18]; in addition, *in vitro* exposure to gp90/Mac-2BP is associated with enhanced generation of cytotoxic effector cells [9]. Furthermore, observations in cancer patients as well as *in vitro* experiments demonstrated that gp90/Mac-2BP secretion is induced by interferon-alpha (IFN-α) and IFN-γ and tumour necrosis factor-alpha (TNF-α) [19,20], thus suggesting that the stimulatory action of these factors on cellular host defence systems could involve this glycoprotein. Finally, recent work showed that increased gp90/Mac-2BP expression in several tumour cell lines significantly reduced their tumourigenicity in athymic mice [21]; indeed, gp90/Mac-2BP expression was associated with strong induction of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression on tumour mass endothelium. In view of these properties, and of the possible role of gp90/Mac-2BP as a molecular alarm signal for immune surveillance and cellular defence [9], we explored the effects of this glycoprotein on some *in vitro* T cell function parameters.

MATERIALS AND METHODS

gp90/Mac-2BP protein source and preparation

The gp90/Mac-2BP glycoprotein was obtained from two different sources: cell culture medium of gp90/Mac-2BP-transfected NIH-3T3 cells, and ascites obtained by paracentesis of patients with ovarian or colorectal carcinoma. For preparation of recombinant gp90/Mac-2BP, NIH-3T3 cells were transfected according to the calcium phosphate method [22] with a human gp90/Mac-2BP cDNA expression vector, which was constructed by placing the entire gp90/Mac-2BP coding sequence under the control of a cytomegalovirus early promoter [9]. The gp90/Mac-2BP-transfected NIH-3T3 cells (4×10^8) were injected into the extracapillary space of a hollow fibre bioreactor (CELLMAX 100; Cellco Inc., Germantown, MD; 1·6 m^2 surface area, 4 kD molecular mass cut-off); the cells received nutrient support by diffusion from the growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 4·5 g/*l* glucose, 10% fetal calf serum (FCS; GIBCO, Grand Island, NY), 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and the flow direction within the hollow fibre lumens was automatically reversed every 10 min to provide uniform nutrient distribution. The extracapillary space was periodically drained to harvest gp90/Mac-2BP-containing culture medium, and the perfusion medium replaced when glucose concentration fell below 1·0 g/*l* (usually every 12–24 h). The culture supernatant was recovered by low-speed centrifugation, and stored for subsequent purification.

Purification of gp90/Mac-2BP

Tissue culture medium from gp90/Mac-2BP-transfected NIH-3T3 cells or ascites fluid was immediately adjusted to 1 mM PMSF, and stored at 4°C for up to 1 week, in the presence of 0·01% (w/v) NaN_3. Pooled fluid (10–20 *l*) was clarified by filtration through glass wool and centrifugation at 10 000 ***g*** for 15 min and precipitated with ammonium sulphate, followed by dialysis against PBS pH 7·8. The samples were then applied to a 3×12 cm column loaded with Sepharose CL-4B conjugated to an anti-gp90/Mac-2BP MoAb; details of this MoAb (SP-2) were previously reported [1]. After washings with 0·5 M NaCl, gp90/Mac-2BP was eluted with 20 ml of Actisep elution medium (Sterogene Bioseparation Inc., Arcadia, CA). The apparent molecular weight and purity of the protein were assessed by SDS–PAGE [23]; gp90/Mac-2BP purified from both cell culture medium and ascites fluid migrated as a major band of ~92 kD with a minor component at ~68 kD; biochemical analysis revealed that this minor band represented a different glycosylation intermediate (data not shown). Using the above purification procedure, yields were typically >80%, as estimated by immunoradiometric assay [2].

Endotoxin contamination of gp90/Mac-2BP was reduced by phase separation using Triton X-114 [24]. After treatment, the endotoxin level was evaluated by a commercial kit (Limulus amebocyte lysate; Endosafe, Biologic-Q, Grignano, Italy); the endotoxin level was estimated to be <30 pg/ml at a gp90/Mac-2BP concentration of 100 μg/ml, the highest concentration used in culture. For use in tissue culture, the protein was finally dialysed against PBS to remove PMSF and NaN_3, filter sterilized, and stored at 4°C until use.

Mitogens

The following stimuli were used: phytohaemagglutinin (PHA-P) and pokeweed mitogen (PWM) (both from GIBCO; 1 μg/ml and 1:100 final dilution, respectively); concanavalin A (Con A; Difco, Detroit, MI; 5 μg/ml); Streptococcal enterotoxin B (SEB; Sigma, St Louis, MO; 100 ng/ml); different anti-TCR:CD3 MoAbs or allogeneic cells (see below). The anti-CD3 MoAbs used were OKT3 (IgG2a; Ortho Diagnostics, Raritan, NJ) and CBT3 (IgG2b; kindly provided by Dr F. Malavasi, Ancona, Italy). The anti-TCR MoAbs used were WT-31 (IgG1; Becton Dickinson, San José, CA) and BMA-031 (IgG2b; Behringwerke AG, Marburg/Lahn, Germany). Unless otherwise specified, all the anti-TCR:CD3 MoAbs were used at a final concentration of 100 ng/ml. The anti-CD5 MoAb (IgG2a) was a generous gift of Dr Bice Perussia (Philadelphia, PA).

Cell preparation and in vitro *culture*

Peripheral blood mononuclear cells (PBMC) were isolated from preservative-free heparin-anticoagulated venous samples from healthy laboratory staff volunteers or blood donors by Ficoll–Paque (Pharmacia-LKB, Uppsala, Sweden) gradient centrifugation, as reported elsewhere [25]. The cells were washed three times with RPMI 1640 medium, counted, and resuspended to 1×10^6/ml in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% non-essential amino acids, 50 μg/ml gentamycin, and 2×10^{-5} M 2-mercaptoethanol (complete RPMI).

PBMC (1×10^5 cells/well) were cultured in triplicate as reported [26] in 96-well U-bottomed microtitre plates (Costar Data Packaging, Cambridge, MA) in a total volume of 0·2 ml of complete RPMI, in both the presence and the absence of various concentrations of purified gp90/Mac-2BP; after 1–2 h incubation at 37°C, the different stimuli were added to the cultures. In the case of allogeneic stimulation, PBMC were cultured in the presence of an equal number of 3000 Gy-irradiated PBMC from unrelated donors. The cultures were incubated for 72 h (144 h in the case of allogeneic stimulation) at 37°C in the presence of 5% CO_2 in air. ^{3}H-thymidine (^{3}H-TdR; Amersham, Aylesbury, UK; sp. act. 74 TBq/mM, 1 GBq/well) was added 12 h before culture

termination. The cultures were finally harvested with an automated cell-harvesting device, and the amount of ^{3}H-TdR incorporated into DNA was assessed as reported [27].

In a set of experiments, to discern whether gp90/Mac-2BP effects could be mediated through accessory cell function, T cells were stimulated in the virtual absence of accessory cells with solid-phase anti-TCR:CD3 MoAbs. To this end, PHA-generated T cell blasts were cultured in the presence of recombinant IL-2 (kindly provided by Dr M. Fumagalli, Chiron-EuroCetus, Milan, Italy; 50 U/ml) for 3–4 weeks; the cells were recovered, washed, and cultured *in vitro* in the presence and the absence of soluble and solid-phase anti-TCR:CD3 MoAbs. MoAb insolubilization was obtained by coupling OKT3 or BMA-031 MoAb to the wells of flat-bottomed microtitre plates (Falcon 3072; Grenoble, France) in carbonate/bicarbonate buffer pH 9·6 (10 μg/ml), as described elsewhere [25].

Cytofluorimetric analysis of Ca^{2+} influx

Changes in the concentration of intracellular free calcium were detected by cytofluorimetric analysis using the fluorescent indicator INDO-1 (Sigma), as described [28,29]. Briefly, freshly isolated PBMC were divided into two aliquots; one was treated with gp90/Mac-2BP (100 μg/ml per 10^6 cells), and the second was resuspended in complete RPMI. After 90 min at 37°C, the cells were resuspended to 5×10^6/ml in RPMI–1% FCS, and incubated with 3 μM INDO-1 for 30 min at 37°C. The cells were then washed, resuspended in fresh medium, and kept in the dark at room temperature for 15 min. For cytofluorimetric analysis, 1×10^6 cells were stimulated in the presence of 1 mM $CaCl_2$ with anti-CD3 (OKT3, 10 μg/ml) or anti-TCR MoAb (WT-31, 20 μg/ml), followed by cross-linking with a polyclonal goat anti-mouse IgG antiserum (Sigma). In each experiment, the baseline Ca^{2+} level measurement of unstimulated cells was followed by the addition of the above stimuli.

Flow cytometric analysis was performed using an Elite cytometer (Coulter, Hialeah, FL) after UV excitation by 5 W argon ion laser (Coherent, Palo Alto, CA) emitting at 351 nm and running at 70 mW. INDO-1 fluorescence was collected using 430 dichroic (DL)/395 band pass filters for saturated INDO-1 violet emission and 550 dichroic/525 band pass filters for free INDO-1 blue emission. In each experiment, data were collected for at least 10 min, and displayed in a cytogram in which the violet/blue fluorescence ratio was plotted *versus* time. As a positive control, at the end of each experiment the cells were treated with the calcium ionophore A23187 (Calcimycin; Sigma), and analysed as above.

Statistical analysis

The non-parametric Mann–Whitney and Wilcoxon tests were used where appropriate. Unless otherwise indicated, the results were expressed as mean values ± 1 s.d.

RESULTS

Effect of gp90/Mac-2BP on the proliferative response to polyclonal mitogens and allogeneic cells

We first studied the effects of gp90/Mac-2BP on the lymphocyte proliferative response to polyclonal mitogens. gp90/Mac-2BP was not able *per se* to stimulate PBMC proliferation (not shown). When the glycoprotein was added to PHA-stimulated cultures, a virtual abrogation of the lymphocyte response was observed (Fig. 1); the presence of gp90/Mac-2BP in culture was also associated with a less pronounced, but still significant decrease in the proliferative response to Con A (Fig. 1; $P<0{\cdot}05$). These findings were not surprising, in view of the well known lectin-binding properties of gp90/Mac-2BP [9–11]. On the other hand, the addition of up to 100 μg/ml of purified gp90/Mac-2BP to PWM-stimulated cultures did not affect the proliferative response of freshly isolated PBMC to this mitogen (Fig. 1); no significant effect as well was seen when freshly isolated PBMC or PHA-activated blasts were stimulated with rIL-2 in the presence and in the absence of gp90/Mac-2BP (data not shown).

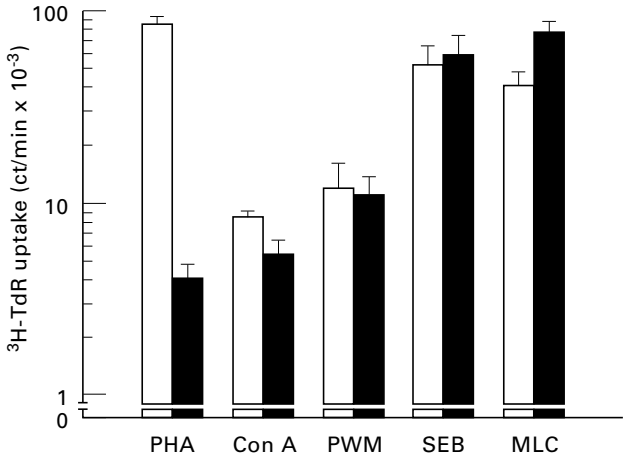

Fig. 1. Effect of gp90/Mac-2BP on the *in vitro* proliferative response of lymphoctyes to non-specific mitogens, superantigen (Streptococcal enterotoxin B (SEB)) and allogeneic cells (MLC). Freshly isolated peripheral blood mononuclear cells (PBMC) were incubated for 2 h at 37°C with 100 μg/ml of gp90/Mac-2BP, either recombinant or obtained from ascitic fluid of tumour-bearing patients; the cells were then stimulated with the different stimuli, as detailed in Materials and Methods. Results were expressed as ct/min of ^{3}H-TdR incorporation in the absence (□) and the presence (■) of gp90/Mac-2BP. Mean values ± 1 s.d. of 12 (phytohaemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM)) five (SEB) and eight (MLC) consecutive experiments are shown.

When PBMC were stimulated with SEB superantigen in the presence of gp90/Mac-2BP (Fig. 1), the mean proliferative response was not significantly different from that observed in its absence, due to the wide individual variability in the proliferative response to this mitogen; however, when the results were calculated as percent variation in individual experiments, compared with the results obtained in the absence of gp90/Mac-2BP, the presence of gp90/Mac-2BP in culture was consistently associated with a significant enhancement in the proliferative response to SEB (Table 1; $P<0{\cdot}05$). Finally, the presence of gp90/Mac-2BP in culture was associated with a significant increase in the proliferative response of PBMC to allogeneic stimulation (Fig. 1; $P<0{\cdot}001$). These data seemed to suggest that gp90/Mac-2BP could be able to potentiate the proliferative response triggered by TCR agonists; fully comparable effects were observed using either the recombinant gp90/Mac-2BP preparations or the protein purified from ascitic effusions (data not shown).

Effect of gp90/Mac-2BP on the proliferative response to MoAbs against the TCR:CD3 complex components

We next examined the effects of gp90/Mac-2BP on the proliferative response to different agonists of the TCR:CD3 complex. When

Table 1. Effect of gp90/Mac-2BP on the proliferative response of Streptococcal enterotoxin B (SEB)- and anti-CD3 MoAb-stimulated lymphocytes*

	SEB		Anti-CD3	
Exp. no.	RPMI	gp90/Mac-2BP	RPMI	gp90/Mac-2BP
1	112 450	118 290 (105)†	80 210	46 930 (58)
2	36 920	38 260 (104)	22 980	10 140 (44)
3	50 110	82 760 (165)	40 180	32 920 (82)
4	44 050	56 400 (128)	31 010	22 890 (74)
5	56 600	62 770 (111)	47 950	35 150 (73)

* Freshly isolated peripheral blood mononuclear cells (PMBC) were cultured *in vitro* in the absence (RPMI) and in the presence of gp90/Mac-2BP (100 μg/ml), and stimulated with either SEB superantigen or OKT3 MoAb. Results are expressed as ct/min of ^{3}H-TdR incorporation.

† Percent variation, compared with cultures without gp90/Mac-2BP (= 100%).

Table 2. Effect of gp90/Mac-2BP on the proliferative response of T cell blasts to solid-phase MoAbs directed against the TCR:CD3 complex*

	Soluble MoAb		Solid-phase MoAb	
	OKT3	BMA-031	OKT3	BMA-031
Exp. no. 1				
RPMI	360†	92	16 148	6313
gp90/Mac-2BP	417	123	10 083	9030
Exp. no. 2				
RPMI	249	85	77 043	4793
gp90/Mac-2BP	248	180	61 511	19 221

* Phytohaemagglutinin (PHA)-induced T cell blasts, obtained after 3-week culture in the presence of recombinant IL-2, were cultured in the presence of either soluble or solid-phase anti-CD3 (OKT3) and anti-TCR (BMA-031) MoAb, as detailed in Materials and Methods. Two representative experiments of five consecutive are shown.

† Results are expressed at ct/min of ^{3}H-TdR incorporation.

PBMC were stimulated with an anti-CD3 MoAb in the presence of different gp90/Mac-2BP concentrations, a dose-dependent decrease in the proliferative response was observed (data not shown); the down-modulating activity of gp90/Mac-2BP was consistently observed when PBMC were stimulated with two anti-CD3 MoAbs of different isotypes (Fig. 2). On the other hand, when PBMC were stimulated with two MoAbs directed against the TCR $\alpha\beta$ chains, a potentiating effect was invariably observed (Fig. 2). As previously observed for the down-regulating effect on anti-CD3 responses, the enhancing effect on anti-TCR-mediated responses was dose-dependent, and as few as 5 μg/ml of gp90/Mac-2BP were able to potentiate the proliferative response to the anti-TCR MoAb; moreover, the enhancing activity of gp90/Mac-2BP on TCR-mediated responses, and the down-modulating effect on CD3-mediated responses, were not due to the MoAb concentration employed, but were exerted over a wide range of MoAb concentrations (data now shown). Kinetic studies demonstrated that gp90/Mac-2BP had to be added within the first 12 h of culture to exert a down-modulating/potentiating effect on the proliferative response to the anti-CD3 or anti-TCR MoAb, respectively; PBMC pretreatment with gp90/Mac-2BP, followed by washing and anti-CD3 stimulation, did not change the lymphocyte proliferative response, compared with untreated cells (data not shown).

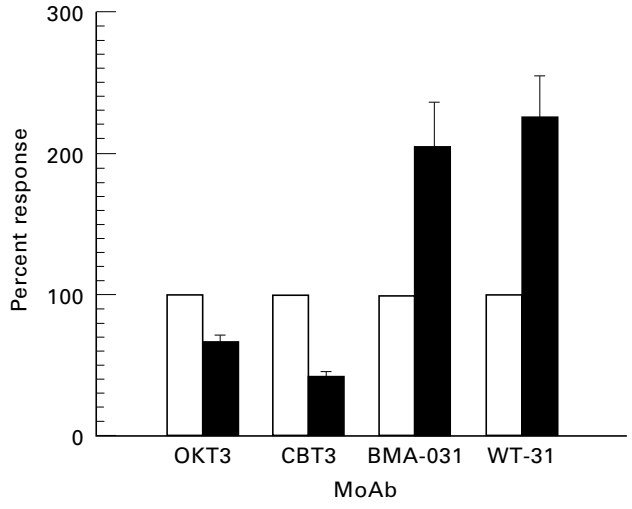

Fig. 2. Effect of gp90/Mac-2BP on the lymphocyte proliferative response to different anti-TCR:CD3 MoAbs. Freshly isolated peripheral blood mononuclear cells (PBMC) were cultured in the presence and the absence of gp90/Mac-2BP (100 μg/ml), and stimulated with anti-CD3 (OKT3, CBT3) or anti-TCR $\alpha\beta$ (BMA-031, WT-31) MoAbs, as detailed in Materials and Methods. Results were expressed as percent response in the presence of gp90/Mac-2BP (■) compared with the results obtained in its absence (□, = 100%). Mean values ± 1 s.d. of 36 consecutive experiments are reported.

Effect of gp90/Mac-2BP on the proliferative response to solid-phase TCR:CD3 complex agonists

Since the proliferative responses studied above were all dependent on the presence of accessory cells in culture, we wondered whether gp90/Mac-2BP could exert its effects directly on T lymphocytes or by modifying accessory cell function and hence T cell responses. To address this issue, we set up an accessory cell-independent assay, and studied the response to solid-phase anti-TCR:CD3 MoAb of IL-2-dependent T cell lines. As expected in view of the virtual absence of accessory cells, no response was observed to the soluble TCR:CD3 agonists (Table 2); on the other hand, when T cell blasts were cultured in the presence of solid-phase MoAb, the presence of gp90/Mac-2BP was associated with a down-regulation of the response to anti-CD3 MoAb, and a potentiation of the response to the anti-TCR MoAb (Table 2). Thus, although not completely ruling out an accessory cell-mediated effect of gp90/Mac-2BP, these data seem to indicate that this glycoprotein may exert its activity directly on T lymphocytes.

Effect of anti-CD5 MoAb on the proliferative response to TCR:CD3 agonists

The differential effect exerted by the gp90/Mac-2BP on the proliferative response to CD3 and TCR $\alpha\beta$ agonists was difficult to understand. Cytofluorographic experiments (data not shown) indicated that gp90/Mac-2BP did not modify CD3 or TCR expression on the cell surface. gp90/Mac-2BP belongs to the SRCR domain family, which also includes CD5 and CD6 [16]; since it is also known that members of the same protein family can non-specifically interact with each other [30], we wondered

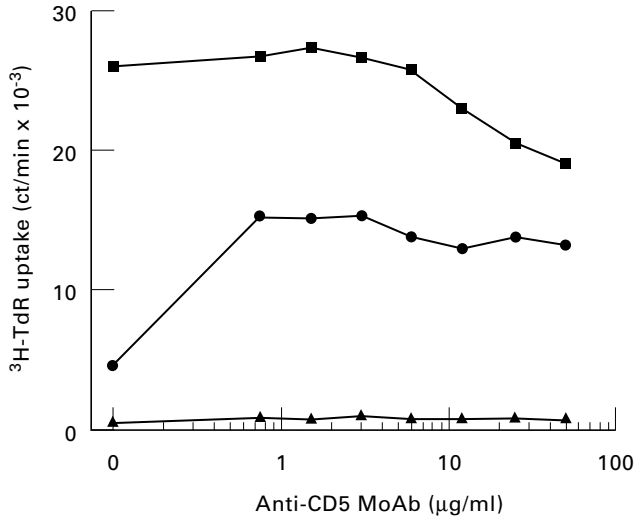

Fig. 3. Effect of anti-CD5 MoAb on the lymphocyte proliferative response to anti-TCR:CD3 MoAbs. Freshly isolated peripheral blood mononuclear cells (PBMC) were cultured in the presence of different anti-CD5 MoAb concentrations, and stimulated with anti-CD3 (CBT3, ■) or anti-TCR $\alpha\beta$ (BMA-031, ●) MoAb, ▲, Lymphocyte response to anti-CD5 MoAb in the absence of anti-TCR:CD3 MoAbs. Results were expressed as ct/min of ^{3}H-TdR incorporation; one representative experiment of four consecutive is reported.

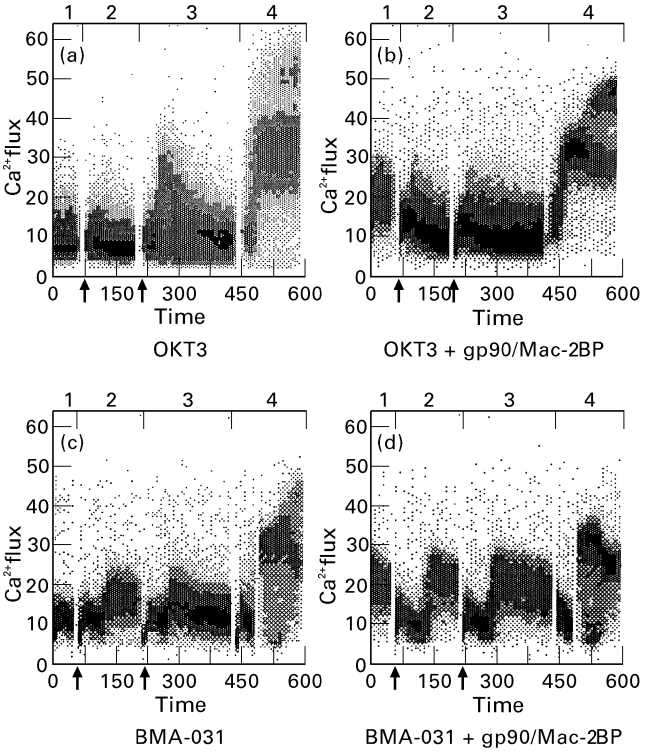

Fig. 4. Effect of gp90/Mac-2BP on Ca^{2+} influx in anti-CD3 (OKT3) and anti-TCR (BMA-031) MoAb-stimulated lymphoycytes. Freshly isolated peripheral blood mononuclear cells (PBMC) were incubated for 90 min in the absence (a,c) or in the presence (b,d) of gp90/Mac-2BP (100 μg/ml). The cells were then labelled with the fluorescent indicator INDO-1, and their intracytoplasmic Ca^{2+} levels followed over time, as detailed in Materials and Methods; the ordinate denotes the ratio between saturated (violet)/free (blue) INDO-1, as a measure of intra-cytoplasmic Ca^{2+} levels. The Ca^{2+} levels of unstimulated cells are reported in section 1 of each panel; the first arrow on the abscissa indicates the time of anti-CD3 or anti-TCR MoAb addition, while the second arrow indicates the addition of the anti-mouse immunoglobulin antibody. The effect of the ionophore on Ca^{2+} levels is indicated in section 4 in each panel. One representative experiment of four consecutive is shown.

whether the gp90/Mac-2BP differential effect on the proliferative response to the various TCR:CD3 agonists could be due to its interaction with some SRCR family receptors expressed on the T cell surface. We thus studied the effect of the presence in culture of an anti-CD5 MoAb on the proliferative response to TCR:CD3 agonists. As shown in Fig. 3, the anti-CD5 MoAb did not induce any ^{3}H-TdR incorporation in the absence of anti-TCR:CD3 MoAb at any of the concentrations used. When both anti-CD3 and anti-CD5 MoAbs were present in culture, the proliferative response to the anti-CD3 MoAb was down-modulated by anti-CD5 MoAb concentrations $\geq 10\,\mu$g/ml (Fig. 3); in contrast, the proliferative response to the anti-TCR MoAb was greatly enhanced in the presence of as little as $1\,\mu$g/ml of the anti-CD5 MoAb (Fig. 3). Thus, engagement of the CD5 antigen seemed to mimic closely the differential effect exerted by gp90/Mac-2BP on TCR/CD3 stimulation.

Effect of gp90/Mac-2BP on Ca^{2+} influx

We finally studied the effects of gp90/Mac-2BP on the Ca^{2+} influx induced by the different anti-TCR:CD3 MoAbs. As shown in Fig. 4, incubation of freshly isolated PBMC with gp90/Mac-2BP (section 1 in Fig. 4b,d) was associated with significantly higher Ca^{2+} levels, compared with untreated cells (section 1 in Fig. 4a,c); in four consecutive experiments, Ca^{2+} levels were $14{\cdot}6 \pm 1{\cdot}1$ nM in untreated cells, *versus* $19{\cdot}3 \pm 1{\cdot}0$ nM in gp90/Mac-2BP-treated PBMC ($P < 0{\cdot}05$). As previously reported [31], anti-CD3 stimulation of freshly isolated PBMC (Fig. 4a, section 2) was associated with a low or absent response in terms of Ca^{2+} increase; nonetheless, as expected [31], further CD3 ligation by anti-mouse immunoglobulin antibody was associated with a sharp increase in intracytoplasmic Ca^{2+} levels (Fig. 4a, section 3). When gp90/Mac-2BP-pretreated PBMC were stimulated with anti-CD3 MoAb, no response was observed (Fig. 4b, section 2); in addition, pretreatment of the cells with gp90/Mac-2BP was associated with poor response also to the addition of the second antibody (section 3 in Fig. 4b).

On the other hand, freshly isolated PBMC gave a measurable Ca^{2+} response to both anti-TCR MoAb and the cross-linking anti-mouse antibodies (Fig. 4c), and gp90/Mac-2BP pretreatment was associated with a change in the response profile. As shown in Fig. 4 (section 2 in Fig. 4c,d), gp90/Mac-2BP-treated cells showed a 27% increase in their response to the anti-TCR $\alpha\beta$ antibody, compared with untreated cells, and a 38% increase when the anti-TCR MoAb was further cross-linked by the addition of anti-mouse immunoglobulin (section 3, Fig. 4c,d). Thus, gp90/Mac-2BP is apparently able to exert complex effects on the Ca^{2+} homeostasis; on one hand, gp90/Mac-2BP is able *per se* to mobilize Ca^{2+} from intracellular stores, on the other it seems capable of potentiating Ca^{2+} responses when the TCR $\alpha\beta$ chains are engaged, while inducing a sizable down-modulation of Ca^{2+} recruitment from intracellular stores when the more distal chains of the TCR:CD3 complex are cross-linked.

DISCUSSION

These data demonstrate that gp90/Mac-2BP, a glycoprotein belonging to the SRCR domain superfamily [10–16], is able to potentiate strongly the lymphocyte proliferative response to TCR $\alpha\beta$ agonist, such as the SEB superantigen, alloantigens and anti-TCR MoAbs; on the other hand, gp90/Mac-2BP is capable of down-modulating the PBMC proliferative response to stimuli that by-pass the TCR $\alpha\beta$ chains, and directly act on the CD3 signal-transducing unit. The implications of our observations are manifold, and include the reason(s) for the differential effect of gp90/Mac-2BP on TCR:CD3 agonists, the mechanism(s) through which this effect is exerted, and the pathophysiologic significance of this protein in health and disease.

First, it is unclear which kind of interaction between gp90/Mac-2BP and lymphocyte membrane may occur. A specific receptor for gp90/Mac-2BP on the T cell surface has not yet been identified; in fact, Scatchard plot analysis experiments with ^{125}I-labelled gp90/Mac-2BP did not give evidence for the existence of a definite receptor (S. Iacobelli, unpublished observations). Even though our data did not provide direct evidence in this sense, it is reasonable to speculate that gp90/Mac-2BP could interact with other members of the SRCC domain family; the fact that gp90/Mac-2BP is able *per se* to mobilize Ca^{2+} from intracellular stores (Fig. 4) does indicate that some kind of interaction between gp90/Mac-2BP and lymphocyte membrane may occur. This hypothesis is not in conflict with previous observations on the interaction between different members of the same superfamily [30,32,33]. Indeed, when PBMC were stimulated in the presence of a MoAb directed against CD5, also a member of the SRCR domain family [16], we were able to reproduce the differential effect exerted by gp90/Mac-2BP on the proliferative response to anti-TCR and anti-CD3 MoAb. Thus, it is conceivable that complex homologous interactions of gp90/Mac-2BP with membrane proteins belonging to the SRCR domain superfamily may occur; indeed, gp90/Mac-2BP may also find other partners belonging to the SRCR family on the lymphocyte membrane, such as CD6 [34–36], and these interactions might interfere with the signal transduction pathways triggered by the different TCR:CD3 agonists, and translate into different responses, depending on which component of the TCR:CD3 complex is engaged.

In this regard, out data stress the important notion that the pathways of signal transduction from the TCR:CD3 complex could be more complex than previously thought, and that agonists that engage the TCR $\alpha\beta$ chains or the CD3 subunits may switch on different activation pathways. Anti-CD3 MoAbs have been long considered as a physiologic way of activating T lymphocytes. Our data stress the possibility that this assumption may not be completely true; indeed, our data corroborate a previously raised speculation, based on a separate body of evidence. We recently demonstrated [31] that, while in long-term T cell cultures anti-CD3 antibodies are able to induce intracellular Ca^{2+} increases without requiring further cross-linking, a measurable Ca^{2+} influx into freshly isolated PBMC is only obtained when subsequent ligation of the anti-CD3 MoAb with anti-mouse immunoglobulin is achieved, thus suggesting a different coupling of CD3 to more distal activation steps in cells at different stages [31]. In addition, it was shown that TCR $\alpha\beta$ chains and CD3 transduce different signals in immature T cells, compared with mature lymphocytes [37], and under particular experimental conditions anti-CD3 MoAb may signal distinctly from TCR $\alpha\beta$ ligation [38]. Finally, when CD4 is separately ligated, TCR $\alpha\beta$ but not CD3 stimulation is able to trigger the apoptotic process into the cell [39]. Altogether, these observations seem to indicate that stimuli that by-pass the TCR $\alpha\beta$ chains and act directly on the CD3 signal-transducing unit may translate into a sequence of events that, although leading in any case to cell activation, may follow different pathways, compared with those utilized by the physiologic interaction of the TCR:CD3 complex with its peptide–MHC ligand. As an obvious implication of this, it would be opportune to re-analyse signal transduction events using probes other than anti-CD3 MoAbs, that could more closely mimic the interaction of the natural ligand with the distal end of the TCR $\alpha\beta$ heterodimer.

Nonetheless, our data leave mostly unclear the mechanism through which gp90/Mac-2BP could exert its activities on lymphocyte responses. Data obtained with solid-phase MoAbs (Table 2) seem to indicate that accessory cell function is not involved in the observed phenomena, and that gp90/Mac-2BP may exert its effect directly on T lymphocytes. On the other hand, T cell activation involves a complex cascade of coordinated events, and any of the steps of this sequence could be affected by the gp90/Mac-2BP protein. We have found some effects of gp90/Mac-2BP on the pattern of Ca^{2+} influx in stimulated cells; while these data do not contradict previous observations on the differential effects of CD3 and TCR agonists on the Ca^{2+} response of lymphocytes [37,38], further studies will be needed to assess at which level(s) gp90/Mac-2BP could exert its activity on lymphocyte function.

Finally, our data cast new light on the possible biologic significance of gp90/Mac-2BP. The effects observed here were obtained with a range of gp90/Mac-2BP concentrations that may be encountered *in vivo* in pathologic conditions, such as cancer, inflammatory diseases, and HIV infection [3–8]. The significance of the gp90/Mac-2BP elevations in these conditions is debated. While some investigators advanced that gp90/Mac-2BP could be included within the array of molecules able to mediate the immunosuppressive status that characterizes these patients, our data on the enhancing effect of gp90/Mac-2BP on TCR-mediated responses would rather suggest an opposite idea; gp90/Mac-2BP could be in fact an immunopotentiating molecule, whose levels are increased in serum whenever an augmented immune response would be required. Recent data seem to confirm this hypothesis. It was shown that tumours that constitutively secrete gp90/Mac-2BP are invariable rejected, and that transfection of tumourigenic cells with a gp90/Mac-2BP construct is able to revert their phenotype from tumourigenic to non-tumourigenic [21]. To validate the possible role of gp90/Mac-2BP in the acute-phase responses in man, it will be necessary to define better its cellular source(s), receptor(s) or acceptor(s) on the different cell membranes, and precise mode(s) of action.

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