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Down-regulation of Macrophage Immune Activity by Natural CD8+ Regulatory T Cells*

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To evaluate the influence of natural regulatory CD8+ T cells on macrophages we investigated *in vitro* production of cytokines, reactive oxygen intermediates (ROIs) and expression of CD80 surface costimulatory molecules by macrophages (MF) of wild type (WT) B10PL and syngeneic knock-out (KO) strains, TCRα-/-, β_2m-/- and CD1d-/- mice. MF of TCR α-/- (CD4- and CD8-) and β_2m-/- (CD8-) animals produced higher levels of TNF-α, IL-6, IL-12 and ROIs and showed increased expression of CD80 costimulatory molecules in comparison to MF of WT or CD1d-/- (NKT-) mice. When MF of these strains were conjugated with TNP hapten and injected i.v. into WT mice to test either induction of contact sensitivity (CS) or tolerance, only TNP-MF of TCRα-/- and β_2m-/- animals induced a significant CS reaction, while cells of WT and CD1d-/-strains were tolerogenic. MF of the tested strains can be classified functionally as resembling either proinflammatory (TCRα-/- and β_2m-/-mice) or immunosuppressive (WT and CD1d-/-) phenotypes. We suggest the presence of an *in vivo* regulatory loop in which innate CD8+ Treg cells control the transition between MF phenotypes and thus adjust the magnitude of the inflammatory response to strictly local requirements.

Key words: Macrophages, CD8+Tregs, contact sensitivity, cytokines, reactive oxygen intermediates.

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Skin painting with haptens such as trinitrophenyl (TNP) leads in mice to the development of a contact-sensitivity (CS) reaction mediated by Ag/MHC class II restricted Th1 CD4+ cells (ASHERSON & PTAK 1968) or class I restricted $CD8^+$ Tc cells (SAINT-MEZARD *et al*. 2004). A similar form of CS reaction can be induced by subcutaneous injection of hapten-conjugated peritoneal macrophages (TNP-MF). However, when TNP-MF are administered intravenously (i.v.) they induce antigen-specific CD8+ suppressor T cells (Ts) that not only inactivate immune CD4+ lymphocytes that mediate CS (GREENE *et al*. 1978; PTAK *et al*. 1980; SZCZEPANIK *et al*. 1993) but also suppress the antigen-presenting activity of MF making them unable to induce immunity (PTAK & GERSHON 1982). This results in a long lasting state of tolerance (unresponsiveness). Similar antigen-specific adoptive regulatory T cells were described in several experimental systems (JIANG & CHESS 2004; VLAD *et al*. 2005).

In the absence of antigenic stimulation the immune system of naive mice is equipped with several populations of down-regulatory cells such as natural CD4+CD25+ FoxP3+ Treg cells and less well characterized natural CD8+ regulatory T cells (JIANG & CHESS 2004; RIFA'i *et al*. 2004), which inhibit immune responses in a antigen-nonspecific, MHC-non-restricted way and play a key role in the suppression of self-reactive T lymphocytes and in preservation of self-tolerance (RIFA'i *et al*. 2004).

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However, the question of whether natural regulatory CD8+ T cells can influence MF immune activity, as adoptive antigen-induced CD8+ suppressor cells do, is still open (PTAK & GERSHON 1982). Thus, our experiments were aimed to test this possibility.

In this study we describe the outcome of i.v. immunization of B10PL (H-2^u) wild-type mice with TNP-substituted peritoneal oil-induced MF isolated from syngeneic, wild-type (WT) mice or from their histocompatible deletion mutants (knock-out animals, KO) lacking certain subpopulations of T lymphocytes, or with MF fractions obtained by density gradient centrifugation. Our *in vitro* studies on cytokines and oxygen radical production and *in vivo* experiments on immunogenicity show that MF of WT and KO mice lacking CD8+ T cells differ functionally.

Material and Methods

All experiments were approved by the Ethics Committee for Animal Research of Jagiellonian University in Kraków (dated 16.03.2004).

Abbreviations

CS – contact sensitivity, DPBS – Dulbecco phosphate buffered saline, FCS – fetal calf serum, iTreg – induced regulatory T cells, KO – knock-out mouse, mAb – monoclonal antibody; MF – macrophages, nTreg – natural regulatory T cells, PCL – picryl chloride (TNP – chloride), ROIs – reactive oxygen intermediates, TLR – Toll-like receptors, TNBSA – trinitrobenzene sulfonic acid, TNP – trinitrofenyl, Treg – T regulatory lymphocyte, WT – wild type mouse.

Animals

Eight to ten week old inbred B10PL (H-2^u) male mice wild type (WT) and syngeneic deletion mutants TCRα-/- (lacking $CD4^+$, $CD8^+$ and NKT cells), β_2m-/- (lacking $CD8^+$ and NKT cells), CD1d-/- (lacking NKT cells) mice from the Department of Human Developmental Biology Medical College Jagiellonian University, Kraków, Poland were used. The mice were fed with autoclaved food and water, and kept under sterile conditions.

Reagents

The following reagents were used:

Lucigenin (bis-N-methylacridinum nitrate), zymosan A, heparin sodium salt, o-phenylenediamine (OPD), recombinant murine TNF-α, (all from Sigma, St. Louis, MO); Ficoll 400 (Pharmacia, Uppsala, Sweden); RPMI 1640, fetal calf serum (FCS) (Gibco Life Technologies, Grand Island, NY); trinitrobenzene sulphonic acid (TNBSA) (Eastman Kodak, Rochester, NY); twice recrystallized picryl chloride [2,4,6-trinitrophenyl (TNP) chloride, PCL] (Chemica Alta, Edmonton, Alberta, Canada); recombinant mouse IL-6 (Pepro-Tech, Rocky Hill, NY); recombinant TGF-β, biotinylated anti-mouse, -human, -pig TGF-β1 antibodies, IL-10 OptEIA^{TM} ELISA Set and mouse IL-12p40 ELISA kit (all from BD Pharmingen, San Diego, CA); paraffin oil Marcol 152 (Exxon Corp. Hutson TX); horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA); pyrogen-free distilled water (Polish-American Institute of Paediatrics, CM UJ, Kraków), granulated milk (Marvel, Chivers LTD, Coolock, Ireland). For cell cultures Nunc labware (Roskilde, DK) was used.

Monoclonal Antibodies

Rat anti-mouse IL-6 (MP5-20F3), biotinylated rat anti-mouse IL-6 (MP5-32C11), rat anti-mouse TNF-α (G281-2626), biotinylated rat anti-mouse TNF-α (MP6-XT3), rat anti-mouse TGF-β1 (A75-2.1), rat anti-mouse FITC-conjugated anti-Mac3 and anti-CD40 mAbs and rat anti-mouse PE-conjugated anti-CD32/16, anti-CD23, anti-CD11b, anti-CD14, anti-CD54, anti-CD80, anti-CD86, anti-CD95L mAbs (all from BD Pharmingen, San Diego, CA); rat-anti-mouse CD8 (clone TIB105) was received by courtesy of Yale University (New Haven, CT).

Isolation of peritoneal oil-induced macrophages

Peritoneal macrophages (MF) were collected using 5 mL of DPBS containing 5U heparin/mL from the peritoneal cavity of mice which had been injected i.p. with 1 mL of Marcol 152 mineral oil 4 days earlier. Four to six mice were used per group. Peritoneal exudate cells contained over 90% macrophages ($Fc\gamma R^+$ and $esterase^+$ cells) and were not purified further (PTAK *et al*. 1980, SZCZEPANIK *et al*. 1993). To test the influence of $CD8^+$ T cells on MF activity, B10PL wild type mice were injected intravenously (i.v.) with 0.25 mg of anti-CD8 mAbs one day before harvest of oil-induced MF.

Discontinuous gradient centrifugation

Macrophages were separated into fractions by centrifugation on a 9% Ficoll gradient as described previously (SZCZEPANIK *et al*. 1993). 0/9% Ficoll

interface forms the borderline between immunogenic and tolerogenic MF. Cells recovered from the 0/9% interface and pellet were examined for expression of several important surface markers or were TNP-labeled and injected i.v. into naïve recipients to test the induction of contact hypersensitivity.

Lucigenin-dependent chemiluminescence

Macrophages were resuspended at a concentration of 5 x 10^5 viable cells /well in 0.2 mL RPMI 1640 medium supplemented with 10% FCS in 96-well flat bottom dark plates (Nunc, Roskilde, Denmark) and 1 μM lucigenin was added and cells were incubated for 15 min at 37°C in dark adaptation (GYLLENHAMMER 1987). Then, some MF groups were stimulated by zymosan opsonized with mouse serum (in a ratio of 10 particles per cell) and plates were immediately transferred to a Lucy 1 luminometer (Anthos, Salzburg, Austria) and photon emission was measured for 75 to 100 min. Each experiment was run in duplicate.

Flow cytometry

Macrophages were resuspended in PBS supplemented with 1% FCS and FcγR were blocked with 2.4G2 mAb (except the groups stained for CD32/16 and CD23). Then, MF were stained with appropriately diluted FITC-conjugated anti-Mac3, or anti-CD40 mAbs or with PE-conjugated anti-CD32/16, anti-CD23, anti-CD11b, anti-CD14, anti-CD54, anti-CD80, anti-CD86, anti-CD95L mAbs. Cells surface markers were analysed by flow cytometry using an Ortho Cytorone Absolute flow cytometer collecting 1 x 10^4 cells (Ortho Diagnostic Systems, Raritan, NJ). Immunocount II software was used for data acquisition and analysis.

Cytokine immunoassay

Macrophages were cultured in 24-flat bottom plates at a concentration of 5 x 10^5/mL in RPMI 1640 medium supplemented with 5% FCS for 24 or 48h in an atmosphere of 5% CO_2 at 37°C. Supernatants for TNF-α and IL-6 estimation were collected after 24 h but for IL-10, IL-12 and TGF-β after 48 h and frozen at -80°C until further use. Concentrations of IL-6, IL-10, IL-12p40, TNF-α, TGF-β, were estimated as described previously in capture ELISA test (BRYNIARSKI *et al*. 2003, 2004).

Induction of contact sensitivity by TNP-derivatized MF and its measurement

MF or their fractions were labeled with 10 mM TNBSA in PBS and then washed extensively in DPBS supplemented with 2% FCS (PTAK *et al*. 1980; SZCZEPANIK *et al*. 1993). Cell viability exceeded 95%. Hapten conjugated MF ($1x10^6$) in 0.2 mL of PBS were injected i.v. into mice. Seven days later ear thickness was measured with a digital engineer's micrometer (Mitutoyo, Tokyo, Japan) before the application of 10 μL of 0.4% PCL solution in a mixture of acetone and olive oil (1:1) to both sides of the ears. The increment of ear thickness was measured 24 h later and expressed in units of 10^{-2} mm ± SD (ASHERSON & PTAK 1968). In each experiment a separate group of non-immunized controls was challenged simultaneously with hapten and their resulting 24 h ear swelling responses were subtracted from that of the immunized group to obtain the net increase. To test the tolerogenic capacity of TNP-MF, mice which received i.v. cell –injection 7 days earlier were skin sensitized by topical application of 150 μL of 5% PCL in an acetone:ethanol mixture (1:3) to the shaved abdomen (ASHERSON & PTAK 1968). Four days later animals were tested for the CS reaction as described above. Mice that did not receive TNP-MF and were only actively immunized with PCL were used as a positive control.

Statistical analysis

All experiments were carried out three to four times. A double-tailed Student's *t*-test was used to assess the significance of the differences between positive control and the experimental groups, with $p<0.05$ accepted as a minimum level of significance.

Results

Macrophages of TCRα-/- and β_2m-/- mice produce higher levels of proinflammatory cytokines and express more CD80 molecules than MF of other strains

The data presented in Table 1 shows that MF of TCR α-/- and β_2m -/- mice produce a significantly higher level of proinflammatory cytokines such as TNF-α, IL-6 and IL-12 (p40) and slightly more anti-inflammatory cytokine IL-10 than WT or CD1d-/- MF. Production of inhibitory cytokine TGF-β was similar in all tested groups.

Simultaneously, MF of TCRα-/- and β_2m-/- mice showed significantly increased expression of CD80 co-stimulatory molecules which play a central role in the induction of the immune response by activation of naive T cells. In TCRα-/- and β_2m-/- mice almost 70% of MF constitutively expressed CD80 while in WT and CD1d-/- animals the expression of this molecule was 27.4% and

Table 1

Production of cytokines by MF of B10PL WT mice and their syngeneic deletion mutants (KO). $5x10^5$ MF in 1 ml RPMI medium supplemented with 5% FCS were cultured at 37°C in a 5% CO_2 atmosphere. For TNF-α and IL-6 measurements supernatants were collected after 24h culture for other cytokines after 48h. The concentrations of cytokines were measured in a capture ELISA test (see Materials and Methods) and are expressed in pg/ml

Mf isolated from	TNF-α [pg/ml]	IL-6 [pg/ml]	IL-10 [pg/ml]	IL-12(p40) [pg/ml]	TGF-β [pg/ml]
αTCR-/-	50±7	1667±97	47±6	1026±25	240±52
β_2m-/-	78±8	1228±63	42±2	1025±31	305±28
CD1d-/-	5±1	875±60	17±4	429±17	270±56
B10PL	7±2	813±35	25±5	599±23	267±21

38.8%, respectively (Fig. 5). Other surface molecules such as CD86, CD14 or CD54 were only slightly and insignificantly increased in TCRα-/- and β_2m-/- mice.

Lack of all T lymphocytes or only $CD8^+$ lymphocytes results in higher production of ROIs by peritoneal macrophages

Fig. 1 shows that MF of TCRα-/- and β_2m -/- mice stimulated with zymosan produced significantly more ROIs than MF of WT and CD1d-/- animals. The data presented in Fig. 2 shows that MF of WT mice depleted of $CD8^+$ cells by prior *in vivo* treatment with anti-CD8 mAb produced significantly more ROIs after zymosan treatment than MF of control mice that received the isotype control. Anti-CD8 mAb by itself had no direct influence on MF activity.

MF of TCRα-/- and β_2m -/- mice induce a CS response while MF of WT and CD1d-/- mice are tolerogenic

The data presented in Fig. 3 shows that TNP-MF from TCRα-/- and β_2m-/- induce a significant CS response after i.v. injection into naïve syngeneic WT recipient mice. In contrast, TNP-MF from WT and CD1d-/- donors did not induce a CS immune response in WT recipients which may result from

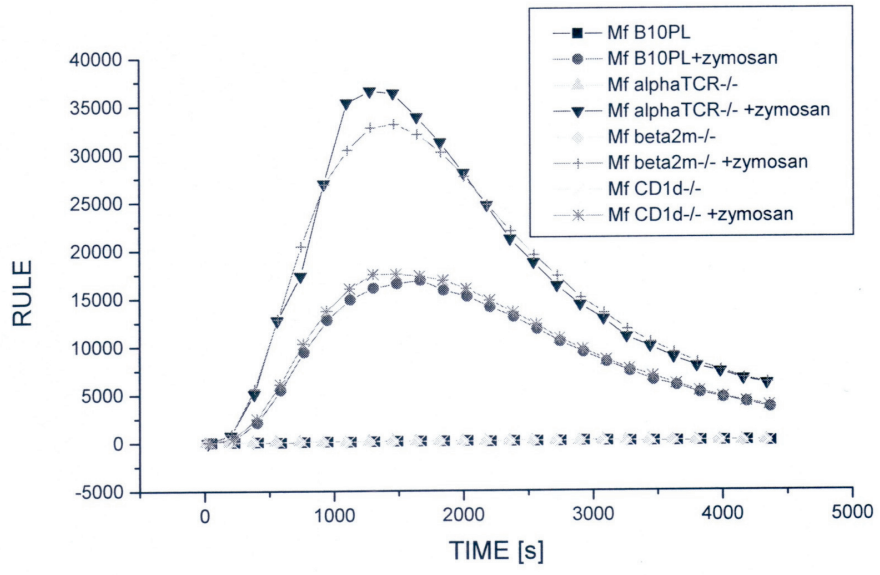

Fig. 1. Production of reactive oxygen intermediates (ROIs) by MF of B10PL WT mice and their syngeneic deletion mutants (TCRα-/-, β2m-/- and CD1d-/- KO mice).
To $5x10^5$ MF in 220 microlitres of nutrient medium lucigenin and zymosan were added and photon emission was measured over 75-100 min (abscissa). For details see Materials and Methods. The results of one out of three experiments are demonstrated. Chemiluminescence (ordinate) is expressed in arbitrary units (RULE).

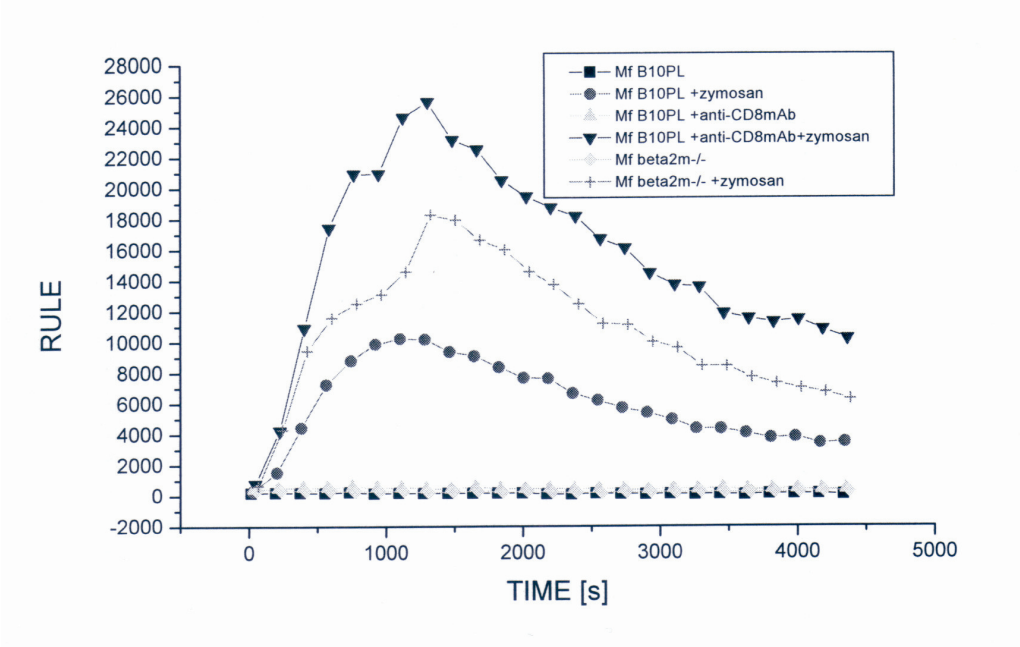

Fig. 2. Production of reactive oxygen intermediates (ROIs) by macrophages of wild type B10PL mice treated *in vivo* with rat anti-mouse CD8 mAb compared with MF of β_2m-/- mice.
To 5x10^5 Oil-MF in 220 microlitres nutrient medium lucigenin and zymosan were added and photon emission was measured over 75-100 min (abscissa). For details see Materials and Methods. The results of one out of three experiments are demonstrated. Chemiluminescence (ordinate) is expressed in arbitrary units (RULE).

Donors of TNP-macrophages injected into B10 PL recipients		Contact sensitivity reaction in units x10^{-2} mm±SE
Gr. A	WT	1±0.9
Gr. B	CD1d-/-	0±0.2
Gr. C	TCRα-/-	4.5±0.5
Gr. D	β_2m-/-	4.7±0.5

Fig. 3.Induction of contact sensitivity by TNP-macrophages isolated from B10PL wild-type and knock-out mice.
Macrophages of from wild type B10PL mice and KO animals of syngeneic strains: CD1d-/-, TCRα-/- and β_2m-/- were labeled with TNP (see Materials and Methods) and used as antigen-presenting cells in passive transfer of TNP-CS into naïve recipients. and 1x10^6 viable cells were injected i.v. into B10PL recipients (6 mice per group). (CS reaction was elicited seven days later by TNP-Cl challenge and manifested as ear swelling, measured with a micrometer and expressed in units x10^{-2} mm ± SE.)
Statistical significance: group A vs groups C and D $P<0.01$; group B vs groups C and D $P<0.01$.

their low immunogenicity or alternatively on their ability to induce tolerance rather than immunity. To determine which of these two possibilities is true, WT mice were i.v. injected with TNP-MF isolated from different knock out or WT mice and subsequently sensitized with homologous TNP hapten by the highly immunogenic epicutaneous route. Figure 4 shows that the recipient mice which

Donors of TNP-macrophages injected into B10 PL recipients		Sensitization with PCl after 7 days
Gr. A	B10 PL	+
Gr. B	CD 1d -/-	+
Gr. C	TCRá -/-	+
Gr. D	β_2m -/-	+
Gr. E	none	+

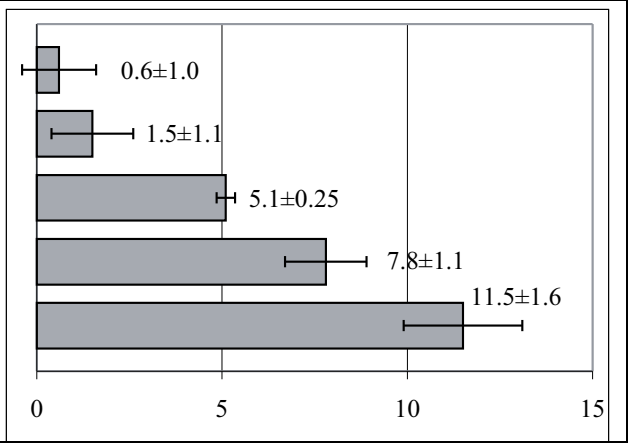

Fig. 4. Induction of tolerance by antigen-presenting MF from B10PL WT mice and their CD1d-/- deletion mutants. Macrophages from wild type B10PL mice and KO animals of syngeneic strains CD1d-/-, TCRα-/- and β_2m-/- were labelled with TNP (see Materials and Methods) and $1x10^6$ viable cells were injected i.v. into B10Pl recipients (6 animals per group). Seven days after TNP-MF injection, mice were additionally skin sensitized by topical application of picryl chloride (PCL, TNP-chloride) (see Materials and Methods) on the shaved abdomen and 4 days later were ear-challenged and tested for CS reaction. Mice were PCl sensitized only in the positive control (group E). Statistical significance: group A vs groups C and D $P<0.01$, group B vs groups C and D $P<0.01$, group E vs groups C and D $P<0.05$.

Donors of TNP-macrophages		Mf Ficoll fraction	Percentage of CD80 positive MF
Gr. A	WT	non-separated	27
Gr. B	WT	0/9% Ficoll interface	65
Gr. C	WT	Ficoll pellet	22
Gr. D	β_2m -/-	non-separated	69
Gr. E	β_2m -/-	0/9% Ficoll interface	81
Gr. F	β_2m -/-	Ficoll pellet	65

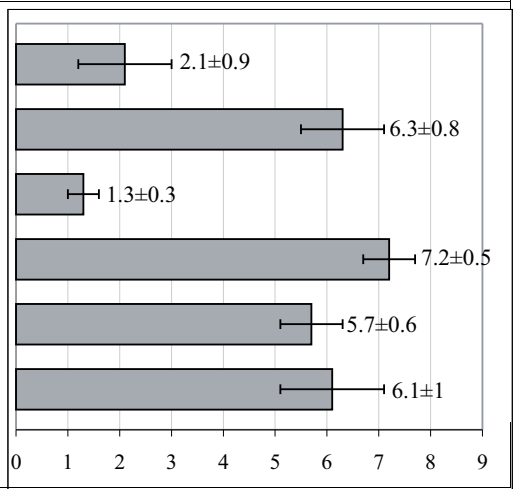

Fig. 5. Induction of contact sensitivity (CS) reaction in recipient B10PL WT mice by MF fractions from WT and β2m mice. Macrophages from B10PL WT and β2m-/- animals were separated on Ficoll gradient into light (0/9% interface) and heavy (pellet) fractions. Unseparated cells and their fractions were labeled with TNP and $1x10^{-6}$ TNP – MF was injected i.v. into B10PL WT recipients which after 7 days were ear-challenged and tested for CS reaction. Results are expressed in units$x10^{-2}$ mm±SE. Statistical significance A vs B $P<0.05$; A vs D $P<0.05$; C vs F $P<0.05$. The table also presents the percentage of CD80-positive cells in tested fractions.

received CD1d-/- or WT TNP-MF before skin sensitization failed to produce any significant CS reaction upon antigen challenge, indicating that MF of CD1d-/- and WT mice are not only nonimmunogenic but in fact highly tolerogenic. In contrast, animals injected with TCRα-/- or β_2m -/- TNP-MF prior to epicutaneous hapten sensitization developed a significant CS reaction, although lower than in mice only PCL sensitized (compare groups C, D and E).

Next, using Ficoll gradient centrifugation we compared the Ag-presenting activity of TNP-substituted light (0/9% Ficoll interface) and heavy (pellet) MF fractions from WT and β2m-/- mice with non-separated TNP-MF populations of both strains. Figure 5 shows that light MF fractions of both strains were equally immunogenic while only heavy MF fraction of β2m-/- animals upon TNP-labeling induced a significant CS reaction. It is necessary to add that cells in the light fraction of both strains formed only a minority of the total cell gradient load, not exceeding 10 per cent. Immunogenicity of the indicated MF fraction was paralleled by a high percentage of cells expressing the CD80+ marker.

Discussion

Macrophages localized in different tissues and body cavities are heterogenous and differ morphologically and functionally. Their heterogeneity is additionally enhanced by the actual microenvironmental influences such as non-specific inflammatory stimuli ("danger" signals) e.g. PAMPs or cytokines. These signals recognized by cell surface recognition molecules lead to reprogramming of the cell machinery, dependent on the quality of signal, to alternative MF polarization. Polarized macrophages have been classified into two groups, pro- and anti inflammatory (M1 and M2 respectively) although intermediate forms also exist (i.e. "mixed" or "overlapping" phenotypes) (MARTINEZ *et al*. 2009; BENOIT *et al*. 2008; MOSSER & EDWARDS 2009; MURRAY & WYNN 2011a; MURRAY & WYNN 2011b).

The main goal of our study was to characterize *in vitro* the possible functional differences between MF isolated from WT and several strains of KO mice, deficient in certain T cell subsets and compare the ability of TNP-substituted MF to induce an immune response or tolerance when given i.v. into naïve syngeneic WT recipients. As MF donors we used TCRα-/- mice missing all Tαβ+ cells (CD4+, CD8+ and NKT), β_2m -/- animals defective in expression of MHC class I antigens and also class Ib, such as CD1d and Qa antigens which results in the lack of CD8+ and NKT cells and finally CD1d-/- mice with absent NKT cells. In all *in vivo* experiments B10.PL (H-2^u) wild-type histocompatible animals were used as MF recipients and their MF were used as control antigen presenting cells.

Our experiments show that MF of mice lacking CD8+ lymphocytes: TCRα-/- and β_2m-/- differed in many aspects from Mf of WT and CD1d-/- animals. They produced higher levels of proinflammatory cytokines (TNF-α, IL-6 and IL-12) (Table 1) as well as reactive oxygen intermediates (ROIs) (Fig. 1) and showed increased expression of CD80 costimulatory molecules. Moreover, MF of TCRα-/- and β_2m-/- donors conjugated with TNP-hapten and injected into WT recipients induced a significant contact sensitivity reaction; under these conditions WT and CD1d-/- MF not only were non-immunogenic but instead induced tolerance (Fig. 3). It is well known that IL-12 plays an important role in the induction of Th1 lymphocytes that mediate the CS reaction. This cytokine upregulates the expression of CD80/86 costimulatory molecules and in cooperation with TNF-α and IL-6 enhances induction of CS (USHIO *et al*. 1998). Other studies showed that IL-6 makes T cells refractory to suppression mediated by Treg lymphocytes (PASARE & MEDZHITOW 2003).

Our *in vitro* and *in vivo* studies might suggest that MF of the tested mouse strains belong to two different activation phenotypes (MARTINEZ *et al*. 2009; BENOIT *et al*. 2008; MOSSER & EDWARDS 2008). However, our further experiments using cells separated by gradient centrifugation indicate that MF of the tested strains were most likely a mixture of different proportions of two MF phenotypes. Macrophages of WT mice contained low numbers of light fraction MF which contain a high proportion of CD80-positive cells able to induce CS, while MF present in the heavy fraction were tolerogenic in WT animals and contained low numbers of CD80 positive cells. In contrast, both gradient fractions of β2m-/- MF induced CS and showed a high proportion of CD80-positive cells. Despite this, as shown in Fig. 3, MF of these two strains still induced a marginal suppression apparent when comparing the magnitude of the CS reaction in groups of mice (C and D) which received TNP – MF and then were skin-sensitized.

The high immunogenicity of MF, apparent in the absence of CD 8^+ lymphocytes is functionally determined by their ability to produce a potpourri of proinflammatory cytokines (IL-6, IL-12, TNFα) creating a favourable milieu for the formation of immunological synapse between TCR of responding cells and MHC class II antigen on APC and on the presence of CD80 marker. In contrast, the mechanism of action of natural $CD8^+$ cells on MF activity and on their phenotype is unclear at pres-

ent. Whether it depends on reciprocal recognition by receptors on cell membranes or on soluble mediators released by Treg cells can be at present only a matter of speculations. Our former experiments in which contacts of T cells with isolated, metabolically inactive MF cell membranes led to down-regulation of T cell activity may suggest that in a reverse situation surface mechanisms are responsible (PTAK & GERSHON 1975).

In summary, the presence of an *in vivo* regulatory mechanism by which innate regulatory $CD8^+$ cells supervise the activity of MF may have an important biological sense, providing adjustment of the size and character of MF activity to local requirements.

Naturally present CD8+ lymphocytes described here, controlling the MF phenotype in a non-specific manner, are only a fraction of the total CD8+ population and are certainly different from antigen-specific CD8+ T cells generated by i.v. administration of hapten-substituted MF and responsible for tolerance (PTAK *et al*. 1980). Multiple subtypes of naturally occurring regulatory CD8+ T cells with different mechanisms of action and attributed functions have been described by several authors. This seems to be a heterogeneous cell group since some express CD25 and FoxP3 (BIENVENU *et al*. 2005) or alternatively CD122 (RIFA'i *et al*. 2004) or CTLA 4 antigens. Some show a low level of CD45RC (XYSTRAKIS *et al*. 2004) or are either CD28 positive or negative (VLAD *et al*. 2005, 2008). It was suggested that the target of natural CD8+ regulatory cells may be the APCs (JIANG & CHESS 2004; XYSTRAKIS *et al*. 2004; BIENVENU *et al*. 2005; VLAD *et al*. 2008) but the experimental proof was lacking. Our experiments demonstrate that this indeed might be the case. Future experiments are aimed at establishing the phenotype of natural-regulatory CD8+ T cells that control MF activity and the mode of their action.

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