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PHENOTYPE CHANGES INDUCED BY IMMUNIZATION WITH ENCEPHALITOGEN AFFECTED THE FUNCTIONS OF PERITONEAL MACROPHAGES IN TWO RAT STRAINS WITH DIFFERENT SENSITIVITY TO EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) INDUCTION

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We have investigated the phenotype of peritoneal cells and the functions of peritoneal macrophages obtained from experimental autoimmune encephalomyelitis (EAE)-susceptible Dark Agouti (DA) and EAE-resistant Albino Oxford (AO) rat strains on days 1, 3 and 7 post immunization with encephalitogen. Resident peritoneal cells from immunized and non-immunized rats of both strains were subjected to flow cytometric analyses and after adherence were tested for zymosan phagocytosis, hydrogen peroxide (H_2O_2) and nitric oxide (NO) production. In non-immunized rats, macrophages from the DA rat strain phagocytosed more zymosan but produced less H_2O_2 than cells from the AO strain, while both strains produced comparable amounts of NO. Immunization increased phagocytosis in DA rats' cells, but decreased both phagocytosis and H_2O_2 production in cells from AO rats. Overall higher phagocyte ability in DA rats was associated with a significantly larger population of $ED1^+$ cells (macrophages and dendritic cells), in contrast to a more pronounced expression of ED2 antigen (resident macrophages) on cells from AO rats. Immunization also increased the expression of CD11b molecule on non-resident $ED2^-$ macrophages of DA, but not of AO rats. The early and subtle phenotype changes in peritoneal cells of both rat strains might mirror the mechanism contributing to their different sensitivity to the induction of autoimmunity.

Key words: experimental autoimmune encephalomyelitis (EAE), hydrogen peroxide (H_2O_2), nitric oxide (NO), phagocytosis, peritoneal cells phenotype, rat strains

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is widely used as an experimental model resembling multiple sclerosis, the most common demyelinating disease of the central nervous system (CNS) in humans (Hafler *et al*., 2005). EAE can be induced in susceptible Dark Agouti (DA) rats by

sensitization with myelin proteins (including myelin basic protein and myelin oligodendrocyte glycoprotein; Stefferl *et al*., 1999), co-administered with complete Freund's adjuvant (CFA), or with a mixture of myelin proteins from a syngeneic or allogeneic source, such as rat and guinea pig spinal cord (GPSC), respectively, in the presence of CFA (Dimitrijević *et al*., 2000). In DA rats EAE can be induced also by a single injection of spinal cord tissue without any adjuvant (Stošić-Grujičić *et al*., 2004). However, EAE can not be induced in the Albino Oxford (AO) rat strain (Mostarica-Stojković *et al*., 1982; Radulović *et al*., 1994), even with different encephalitogens and adjuvants other than CFA (Miljković *et al*., 2006). Higher production of proinflammatory cytokines in immune cells derived from DA relative to AO rats (Arsov *et al*., 1995; Banović *et al*., 1997; Miljković *et al*., 2006; Stošić-Grujičić *et al*., 1995) undoubtedly have an effect on high susceptibility to autoimmune inflammatory diseases in this strain. Effective apoptotic clearance in rats of AO strain, in contrast to the resistance to early apoptosis of the invading cells in target tissue of DA rats are amongst the proposed mechanisms that can contribute to striking differences in the susceptibility of these rat strains to EAE (Lukić *et al*., 2001; Mensah-Brown *et al*., 2005).

EAE is mediated by $CD4^+$ T cells producing interferon-gamma and interleukin (IL)-17, in response to the CNS – specific peptide components (Langrish *et al*., 2005; Swanborg, 2001). It is now established that T lymphocytes are activated at the periphery and then enter CNS to stimulate resident microglia. Both microglia stimulation and peripheral macrophages migration to CNS throughout the blood-brain barrier are required for subsequent recognition of myelin sheets target peptides by T cells and their reactivation in the spinal cord and brain (Juedes and Ruddle, 2001). It has been shown that elimination of blood-borne macrophages infiltrating CNS markedly suppressed the expression of clinical signs of EAE, probably by the lack of macrophage stimulatory activity for $CD4^+$ T cells (Huitinga *et al*., 1990; Tran *et al*., 1998).

Besides being potent antigen-presenting cells that contribute to adaptive immune response by generation of encephalitogenic T lymphocyte clones at the periphery and their reactivation in CNS, macrophages are also directly implicated in tissue damage in EAE, as they phagocyte myelin via Fc and CR3 receptors (Smith, 1993; van der Laan *et al.*, 1996) and produce pro-inflammatory cytokines, reactive oxygen radicals and nitric oxide (NO) (Hendriks *et al*., 2005; Gilgun-Sherki *et al*., 2004; Touil *et al*., 2001).

Our previous study revealed significant differences in peritoneal macrophage activity between rats of DA and AO strains at the peak of disease in DA animals (Mitić *et al*., 2007). Considering that upon EAE induction innate immune activation (priming mostly cells of the mononuclear phagocytic system) precedes the expansion of encephalitogenic $CD4^+$ T lymphocytes (Billiau and Matthys, 2001), we sought to determine if strain differences in macrophage activity, measured by phagocytosis, hydrogen peroxide (H_2O_2) and NO production at the early stages post-immunization before the onset of clinical signs of EAE in DA rats, might reflect the differences in the susceptibility to EAE in AO and DA rats. In addition, we sought to explore whether the changes in peritoneal

macrophage activities are caused by immunization-induced changes in their phenotypes. Our results reinforced the opinion that the early nonspecific immune activation can define the level of susceptibility to specific immune priming, and thus direct the immune response towards autoimmune pathology or disease resistance.

MATERIALS AND METHODS

Animals

Seven months old male inbred rats of two rat strains, DA and AO, were used in the experiment. Rats were derived from our breeding colony at the Immunology Research Center "Branislav Janković", Belgrade. Animals were housed in standard cages with free access to food pellets and tap water. All procedures involving animals and their care were approved by our Institutional Animal Care and Use Committee and followed principles described in the European Community's Council directive of 24 November 1986 (86/609/EEC).

Chemicals and immunoconjugates

Complete Freund's adjuvant (CFA, containing 1 mg/mL of Mycobacterium tuberculosis, strain H37Ra, ATCC 25177), zymosan A (from Saccharomyces cerevisiae), nitro blue tetrazolium chloride (NBT, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]ditetrazolium chloride), lipopolysaccharide (LPS, from *E. coli*, serotype 0111:B4), phorbol myristate acetate (PMA), horseradish peroxidase, phenol red and RPMI 1640 medium (with L-glutamine, without bicarbonate) and sodium azide were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum was obtained from Gibco (Grand Island, NY, USA). Phenol red-free minimal essential medium (MEM), phosphate buffered saline (PBS) and Bordetella pertussis vaccine were acquired from the "Torlak" Institute in Belgrade. For immunolabelling the following monoclonal antibodies (mAbs) were used: R-phycoerythrin (PE)-conjugated mouse anti-rat macrophage subset (anti-ED2, clone HIS 36) obtained from BD Biosciences Pharmingen (Mountain View, CA, USA); fluorescein-isothiocyanate (FITC)-conjugated mouse anti-rat CD11b (anti-CD11b, clone ED8) and biotin-conjugated mouse anti-rat – CD68 (anti-ED1, clone ED1) obtained from Serotec (Oxford, UK). The appropriate IgG isotype controls were purchased from BD Biosciences Pharmingen (Mountain View, CA, USA).

Induction of EAE

Animals were immunized with a single intradermal injection of 100 μL GPSC emulsified in CFA in the right hind footpad (DA n=18 and AO n=18). In addition, rats received a subcutaneous injection of 250 μL Bordetella pertussis vaccine (concentration $2x10^9$ microorganisms/mL) in the dorsum of the same foot. Non-immunized rats of DA (n=9) and AO (n=9) strains served as controls. Rats were weighed prior to and on days 1, 3 and 7 (d1, d3 and d7) post immunization.

Peritoneal cell harvest

On days 1, 3 and 7 post immunization (d1, d3 and d7) resident cells from immunized and non-immunized rats of both strains were obtained by washing the peritoneal cavity with sterile PBS. Lavages were centrifuged 10 min at 250 g and resulting cell pellets were resuspended in RPMI. The cells were counted, and the concentrations of individual cell suspensions were adjusted under laminar flow to a density of $1x10^7$ mL, $2.5x10^6$/mL and $1x10^6$/mL with regard to the assay. The cells obtained from 3 animals were pooled according to experimental groups. The results present data from two independent experiments.

Peritoneal cells phenotyping using flow cytometric analysis

Aliquots of $1x10^6$/mL peritoneal cells in 100 μL ice-cold medium (PBS pH 7.4 containing 2% foetal calf serum and 0.09% sodium azide) obtained from both strains on d1, d3 and d7 post-immunization, as well as from non-immunized animals, were centrifuged at 350 g for 5 min at 4°C to yield a pellet. The cells were incubated for 30 min on ice in the dark with anti-ED2-PE and anti-CD11b-FITC mAbs. For lysosomal membrane staining, cells were fixed in 1% paraformaldehyde in PBS and permeabilized for 15 min with a medium containing 0.2% Tween-20 and then incubated for 30 min with biotin-conjugated anti-ED1 mAbs followed by Sav PerCP as a second step reagent for 30 min. The cells were washed twice with the medium, and kept at 4°C in the dark until analysis. Ten thousand cells per sample were analyzed using FACS can flow cytometer (Cell Quest software, Becton Dickinson, Mountain View, CA, USA). Analysis was performed on macrophages gated according to side and forward scatter, while dead cells and debris were excluded from analysis by selective gating based on anterior and right-angle scatter. Non-specific isotype-matched controls were used for each fluorochrome type to define background staining. The percentage of positive cells for each labelling was determined using Cell Quest software (Becton Dickinson).

Phagocytosis (NBT reduction assay)

Suspensions of peritoneal cells ($2.5x10^6$ cells/mL) were plated at 100 μL/well in 96-well flat-bottomed tissue culture plates (NUNC). Plates were incubated 2h at 37°C in 95% air-5% CO_2. Non-adherent cells were removed by washing the plates twice with warm phenol red-free MEM. Adherent macrophages were stimulated with zymosan (125 μg/mL) in the presence of NBT (0.5 mg/mL) for 60 min at 37°C in 95% air-5% CO_2 (Pick *et al*., 1981). The cells were fixed with methanol, plates were air-dried overnight and optical densities (ODs) were measured at 545 nm. NBT reduction assay can be used an indirect measure of zymosan phagocytosis as the amount of engulfed zymosan particles was proportional to the reduction of yellow NBT to blue formazan. Results are expressed as ODs (545 nm) x 1000.

Hydrogen peroxide (H_2O_2) production assay

H_2O_2 release was determined by the method based on horseradish peroxidase-dependent conversion of phenol red by H_2O_2 into a compound with

increased absorbance at 600-610 nm (Pick and Mizel, 1981). The OD was read after adjusting the pH of the reaction to 12.5, in order to eliminate changes in the absorbance of phenol red due to its behavior as a pH indicator. Cell suspensions were prepared in the same manner as for the NBT assay. Macrophages (adhered on 96-well flat-bottomed tissue culture plates) were stimulated for peroxide production with 100 μL of 25 nM PMA in phenol red solution (PRS, 40 mM NaCl, 10 mM potassium phosphate buffer, pH 7, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/mL of horseradish peroxidase). PMA was prepared from stock solutions 10^{-2} M in DMSO, stored at -70°C. The plates were incubated for 1h at 37°C in 95% air-5% CO_2. The reaction was stopped with 10 μL of 0.5 M NaOH and ODs were determined at 620 nm. The concentration of H_2O_2 (μM) in the samples was calculated using 1-40 μM H_2O_2 as a standard.

Nitric oxide (NO) production assay

Peritoneal cells were adjusted to $1x10^6$/mL and plated 100 μL/well in 96-well flat-bottomed tissue culture plates (NUNC). The plates were incubated for 2h at 37°C in 95% air-5% CO_2. Non-adherent cells were removed by washing the plates twice with PBS under laminar flow. The adherent macrophages were stimulated with 1 μg/mL LPS in RPMI supplemented with 5% FCS and incubated for 48h at 37°C in 95% air-5% CO_2. Nitrite concentrations in the culture medium were measured by a method based on the Griess reaction (Green *et al*., 1982). The concentrations of nitrite (μM) in the samples were calculated using $NaNO_2$ standard curve in 1-40 μM range.

Statistical analysis

Statistical analysis of the data was performed using the SPSS 10.0 computer programme. The results were analyzed by one-way ANOVA and Student's independent samples t-test. LSD and Scheffe tests were used for *post hoc* analysis for the evaluation of the differences between independent groups. In the figures all data are displayed as mean $\pm$ (S.E.M.).

RESULTS

Peritoneal cells yield

Considering that rats of the AO strain on average weighed more than rats of the DA strain (AO: NIM=333.17$\pm$7.98 g; DA: NIM=244.83$\pm$ 9.33 g), the number of peritoneal cells ($x10^4$) recovered in 10 mL of MEM was expressed per gram of rat body weight. No changes in body weight (data not shown) or in peritoneal cells yield were observed in either of the strains over the 7 days post-immunization period. However, the peritoneal cell number was substantially higher in rats of the AO strain before immunization and at day 1 than in rats of the DA strain (Figure 1).

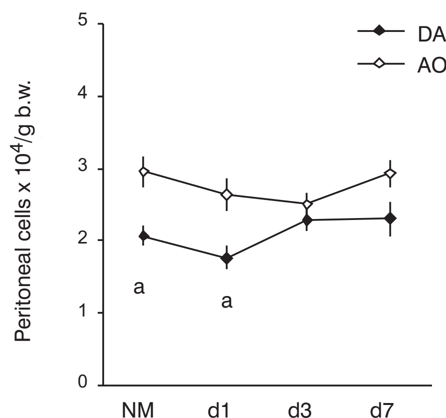

Figure 1. The number of peritoneal cells harvested from non-immunized (NIM) rats of Dark Agouti (DA) and Albino Oxford (AO) strains, and from DA and AO rats on days 1, 3 and 7 (d1, d3, d7) following immunization with GPSC/CFA. The values are given as mean ± S.E.M. Statistically significant differences: [a], $p<0.01$ vs. respective AO group

The influence of immunization with GPSC/CFA on macrophage functions

Peritoneal macrophages isolated from non-immunized DA strain rats phagocytosed more zymosan than macrophages isolated from non-immunized AO animals (Figure 2A). Immunization increased zymosan phagocytosis in cells obtained from DA rats on d1. In EAE-resistant AO strain immunization significantly diminished zymosan phagocytosis on d1 and increased it on d7.

Peritoneal macrophages isolated from non-immunized AO animals produced more H_2O_2 than cells isolated from control DA rats (Figure 2B). Immunization with encephalitogen did not influence H_2O_2 production in macrophages isolated from DA strain rats, but significantly decreased H_2O_2 production in macrophages isolated from AO rats on d1 and moderately diminished it on d3.

Peritoneal macrophages isolated from non-immunized and immunized rats of DA and AO strains produced comparable levels of NO (Figure 2C). Immunization slightly, but not significantly, suppressed NO production on d1 in AO rats and did not change NO production in macrophages from DA rats.

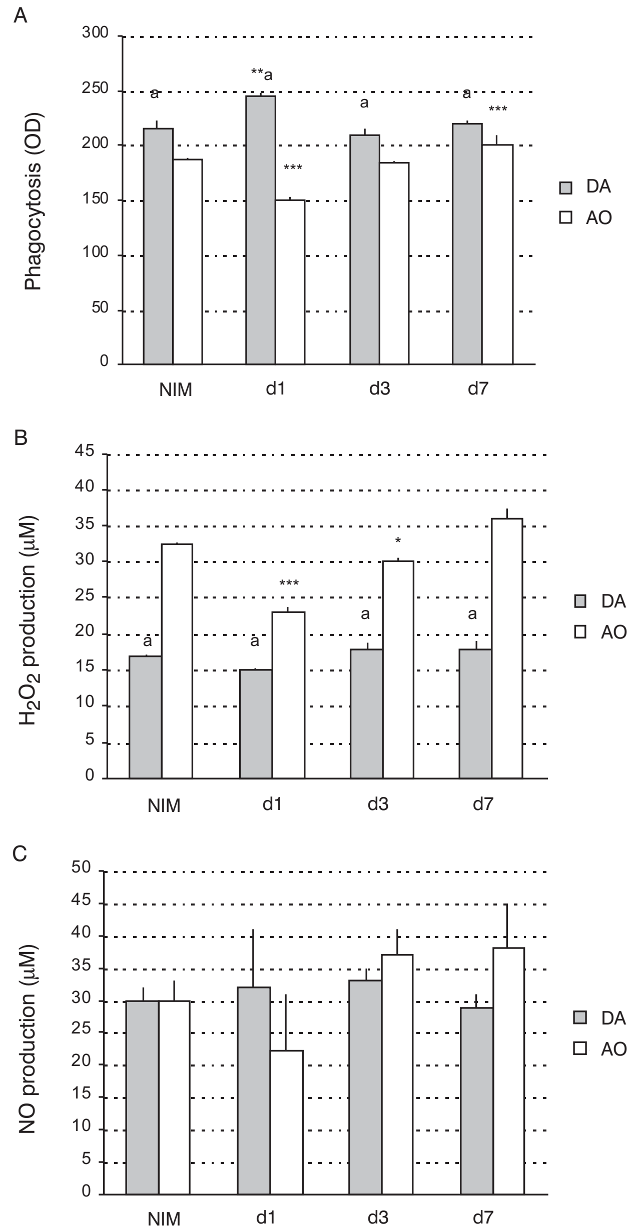

Figure 2. (A) Zymosan phagocytosis (B) production of H_2O_2 and (C) production of NO by macrophages isolated from non-immunized (NIM) rats of Dark Agouti (DA) and Albino Oxford (AO) strains, and from DA and AO rats on days 1, 3 and 7 (d1, d3, d7) following immunization with GPSC/CFA. The values represent mean ± S.E.M. Statistically significant differences: *, $p<0.05$; **, $p<0.001$; and ***, $p<0.0001$ vs. respective NIM group. [a], $p<0.0001$ vs. respective AO group

The influence of immunization with GPSC/CFA on peritoneal cell phenotype

In rats of DA strain there was no change in the percentages of $ED1^+ED2^+$ cells over the 7 day post-immunization period, while percentages of single positive cells changed in the opposite directions on d3, i.e., $ED1^+$ decreased and $ED2^+$ increased (Figure 3, left). The percentages of cells bearing $ED2^+$ antigen additionally decreased on d7 in DA rats (Figure 3, left). In AO rats percentages of $ED1^+$ cells decreased on d1 and d3 and increased on d7, while the percentage of $ED1^+ED2^+$ cells decreased only on d1. Contrary, percentages of $ED2^+$ significantly increased on d1 (Figure 3, right). Striking strain differences were observed before and at all days following immunization in the percentages of $ED1^+$ cells, ranging from 27.3 to 32.0% in DA and 14.3 to 19.3% in AO rats. In contrast, percentages of cells expressing ED2 antigen (either $ED2^+$ or $ED1^+ED2^+$) were significantly higher before and at all days following immunization in AO rats (Figure 3).

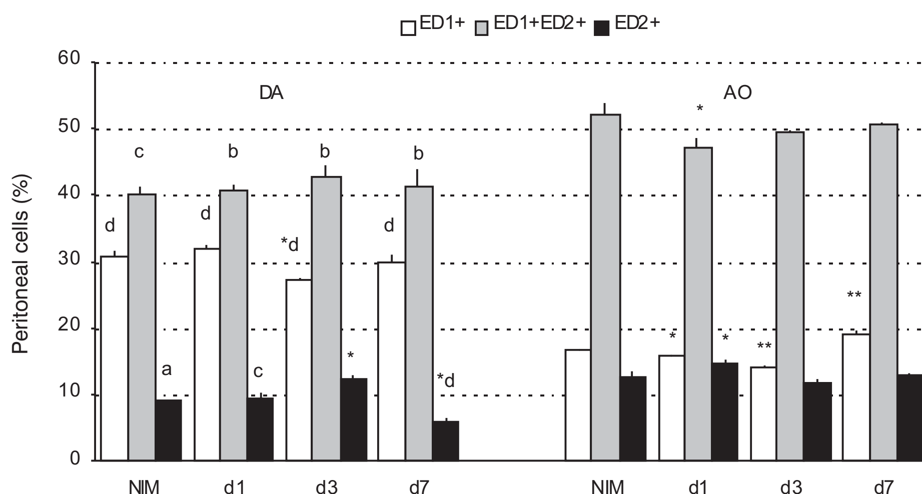

Figure 3. The expression of ED1 and ED2 antigens by peritoneal cells isolated from non-immunized (NIM) rats of Dark Agouti (DA, left) and Albino Oxford (AO, right) strains, and from DA and AO rats on days 1, 3 and 7 (d1, d3, d7) following immunization with GPSC/CFA. The values represent mean ± S.E.M. Statistically significant differences: *, $p<0.05$; and **, $p<0.01$ vs. respective NIM group. [a], $p<0.05$; [b], $p<0.01$; [c], $p<0.001$;and [d], $p<0.0001$ vs. respective AO group

The cells bearing ED1 antigen most likely belong to the populations of newly recruited monocyte-derived macrophages, tissue resident macrophages and dendritic cells (Dijkstra *et al.*, 1985; Vasilijić *et al.*, 2005), while those expressing ED2 antigen represent mature resident peritoneal macrophage subpopulation (Dijkstra *et al.*, 1994; Polfliet *et al.*, 2006). Further on, we have investigated the presence of CD11b antigen expressed on $ED2^+$ and $ED2^-$ macrophage

subpopulations. Firstly, results showed that roughly 50% of peritoneal cells from non-immunized DA rats expressed ED2 antigen and, except for the slight increase in its expression observed on d3, no change was found over the 7 days period following the immunization (Figure 4, left). In contrast, around 60% of cells from AO rats expressed ED2 antigen regardless of immunization (Figure 4, right).

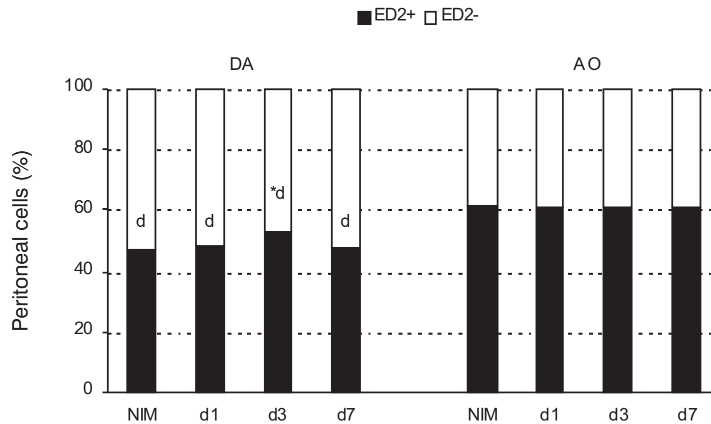

Figure 4. The expression of ED2 antigen by peritoneal cells isolated from non-immunized (NIM) rats of Dark Agouti (DA, left) and Albino Oxford (AO, right) strains, and from DA and AO rats on days 1, 3 and 7 (d1, d3, d7) following immunization with GPSC/CFA. The values represent mean ± S.E.M. Statistically significant differences: *, $p<0.05$ vs. respective NIM group. [a], $p<0.0001$ vs. respective AO group

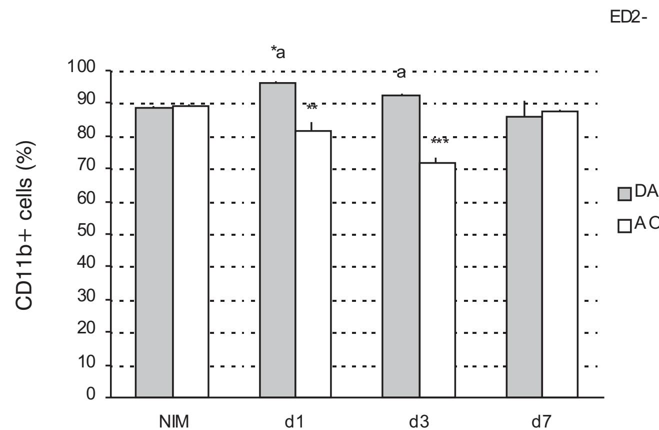

Figure 5. The expression of CD11b molecule by ED2^{-} peritoneal cells isolated from non-immunized (NIM) rats of Dark Agouti (DA) and Albino Oxford (AO) strains, and from DA and AO rats on days 1, 3 and 7 (d1, d3, d7) following immunization with GPSC/CFA. The values represent mean ± S.E.M. Statistically significant differences: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.0001$ vs. respective NIM group. [a], $p<0.0001$ vs. respective AO group

CD11b was detected on nearly 100% of $ED2^+$ cells from both rat strains and immunization did not change the level of its expression in either of the strains (ranging from 98.7% to 99.6% in DA, and from 98.4% to 99.6% $ED2^+$ cells in AO rats; data not shown). However, CD11b was present on around 90% of $ED2^-$ cells from both rat strains and its expression significantly decreased on d1 and d3 in AO rats and increased on d1 in DA rats (Figure 5).

DISCUSSION

It has been shown that macrophages can alter their phenotype upon stimulation with certain environmental stimuli with no change in their respective number, showing the macrophage properties are dependent on microenvironment and the activating signals they receive (Porcheray *et al.*, 2005; Stout and Suttles, 2004). Indeed, active immunization for EAE induction in DA and AO rats did not change the peritoneal exudate cell number *in vivo*, while it significantly affected their phenotype, as well as their functions.

Macrophages isolated from EAE-susceptible DA rats phagocytosed more zymosan, but produced less H_2O_2 than cells isolated from EAE-resistant AO animals. It is in line with the concept that predisposition to a given autoimmune response requires the requisite allele(s) that controls antigen presentation by antigen-presenting cells for T cell recognition (Atassi and Casali, 2008), of which phagocytosis is the first step. Phagocytic ability and H_2O_2 release represent the key macrophage activities, but the tests we used (i.e. reduction of NBT following zymosan phagocytosis, and phenol red assay following PMA stimulation, respectively) in fact both detect oxidative products after the respiratory burst (Pick and Mizel, 1981; Pick *et al.*, 1981). The main difference derives from the divergence in the route of macrophage activation. Zymosan particles (composed of α-mannan and β-glucan) initiate phagocytosis through the activation of mannose and β-glucan receptors, as well as CR3 complement receptors (Le Cabec *et al.*, 2000) and consequently commence the oxidative burst through NADPH oxidase-dependent mechanisms (Fallman *et al.*, 1993; Park 2003). In contrast, PMA directly activates protein kinase C and stimulates the oxidative burst with no phagocytosis (Chanok *et al.*, 1994; Johnston and Kitagawa, 1985). We have previously shown that when PMA was used as a stimulant in NBT reduction assay, macrophages isolated from non-immunized AO rats more efficiently reduced NBT, therefore showing higher oxidative capacity than cells from non-immunized DA rats (Miletić *et al.*, 2007). Accordingly, even though the immunization of DA rats in our study transiently increased zymosan phagocytosis on d1, no changes in H_2O_2 production were observed. Thus, immunization with encephalitogen increases the production of reactive oxygen species when phagocytosis is involved in the test assay, but does not otherwise influence the oxidative capacity of macrophages in DA rats.

Overall higher phagocytic ability in peritoneal cells from DA rats was associated with a significantly bigger (sometimes even doubled) population of ED1 antigen-expressing cells compared to peritoneal cells from AO rats, obtained before and over the period of 7 days after immunization. ED1 identifies a single

chain glycoprotein expressed predominantly on the lysosomal membranes specialized for interactions with phagosomes, and its expression correlates with the phagocytic capacity of the cell (Damoiseaux *et al.*, 1994). ED1 is present on newly recruited monocyte-derived macrophages, tissue resident macrophages and dendritic cells (Dijkstra *et al.*, 1985; Vasilijić *et al.*, 2005).Phenotype changes in cells from AO rats observed on d1 relative to NIM (decrease in percentages of $ED1^+$ cells) might account for their decrease in phagocytic ability on d1 and subsequent functional recovery paralleled by the increase of the ED1 expression on d7. However, the expression of ED1 remained unchanged in cells from DA rats on days following immunization with encephalitogen, but the expression of CD11b on non-resident macrophages $ED2^–$ cells, also comprising $ED1^+$ cells, possibly macrophages and dendritic cells (Butler and McMenamin, 1996) paralleled the phagocytic capacity rise on d1. CD11b represents the α subunit of adhesion molecule Mac-1 (CR3) and its up-regulation is required for most of the monocytes activities (Mazzone and Ricevuti, 1995). Mac-1 (CR3) binds zymosan (Le Cabec *et al.*, 2000) and is involved in phagocytosis (Chen *et al.*, 2008; Vachon *et al.*, 2007). Intravenous injections of antibodies directed against Mac-1 suppressed clinical signs of EAE (Huitinga *et al.*, 1993). Expression of Mac-1 is essential for the adhesion and transmigration of activated macrophages out of the peritoneum via mesothelial lymphatics to the lymph nodes (Cao *et al.*, 2005). In that sense, the rise in the expression of CD11b on $ED2^–$ subpopulation of peritoneal cells from DA rats may facilitate antigen presentation and development of the immune response. In contrast, $ED2^–$ cells from AO rats failed to up-regulate the expression of CD11b upon immunization. A significant decrease in the expression of CD11b on these cells on d1 paralleled the decrease in the phagocytic capacity when compared to the non-immunized controls. The decrease in CD11b expression was even more pronounced on d3, when a recovery of phagocytic function in macrophages of AO rats was observed, suggesting that some of the $ED2^+$ $CD11b^+$ (probably $ED1^+ED2^+$) macrophages compensated for the reduction in phagocytosis.

It is noteworthy that expression of ED2 antigen (labelling $ED2^+$and $ED1^+ED2^+$ cells) was more pronounced, roughly around 10% more, in AO than in DA rats, but almost all $ED2^+$ cells from both strains expressed CD11b. ED2 is a differentiation antigen (CD163) on resident mature macrophages (Polfliet *et al.*, 2006) not exhibiting phagocytic ability (McLennan, 1993), or at least exhibiting reduced capacity for phagocytosis (Hirata *et al.*, 1999), and is not expressed on monocytes (Dijkstra *et al.*, 1994; Polfliet *et al.*, 2006). ED2- expressing macrophages from EAE-resistant AO rats produce larger amounts of H_2O_2 than DA rats (both basally and early after immunization), which is in agreement with our previous results obtained with the same rat strains immunized with CFA for the induction of adjuvant arthritis (Miletić *et al*., 2007). It was suggested that the increased production of reactive oxygen radicals by macrophages from CFA-immunized AO rats could contribute to lower proliferation of T cells isolated from lymph nodes of this rat strain (Miletić *et al.*, 2006). Hydrogen peroxide produced at high concentrations inside cells can induce cell apoptosis (Um, 2001), and rapid apoptotic clearance of infiltrating mononuclear cells in AO rats is one of the

proposed mechanisms of their resistance to autoimmune disease induction (Lukić *et al.*, 2001, Mensah-Brown *et al.*, 2005). The increased oxidation state in immunized AO rats could abrogate antigen processing by decreasing capacity of antigen presenting cells (Preynat-Seauve *et al.*, 2003). It could also diminish T cells antioxidative capacity (Lahdenpohja *et al.*, 1998), and after sustained exposure decreases T lymphocyte functions (Flescher *et al*., 1994). Although $ED2^+$ macrophages produce H_2O_2 (Fukuda *et al.*, 2004), ED2 is also implicated in the prevention of oxidative damage (Kristiansen *et al.*, 2001). Relatively high expression of ED2 antigen on macrophages from AO rats might also protect the surrounding tissue from oxidative injury and in that sense contributes to the mechanisms of resistance to autoimmunity, since oxidative insult might break-up different tissue proteins and generate additional epitope targets for the immune attack (Preynat-Seauve *et al.*, 2003). In contrast, low production of H_2O_2 in non-immunized and immunized DA rats, and the fact that there were no detectable changes in PMA-stimulated H_2O_2 production at the peak of disease (Mitić *et al.*, 2007), dispute the significant contribution of macrophage H_2O_2 to the EAE induction in this rat strain. Indeed, it was reported that a reducing environment is optimal for T cell activation (Lahdenpohja *et al.*, 1998.) and that H_2O_2, if produced at low concentrations inside cells, can potentiate the survival pahway instead of apoptosis (Um, 2001).

Our study revealed that NO does not correlate with the sensitivity of AO and DA rats to EAE, in agreement with previous reports (Miljković *et al.*, 2006). Although NO products are formed early during EAE development and are connected to disease severity (Farias *et al.*, 1997; van der Veen *et al*., 1997), our results argue against their deleterious effects in DA rats. It has been also shown that resistance of PVG rats to actively induced EAE appears to be directly related to the systemic levels of reactive nitrogen intermediates and an enhanced NO production in splenic macrophages following sensitization for EAE, when compared to the highly susceptible Lewis rat (Staykova *et al.*, 2002). However, in AO rats, in contrast to rats of other EAE-resistant strains such as BN, PVG and F344 strain (Cowden *et al.*, 1998; Gold *et al.*, 1997; Staykova *et al.*, 2005), enhanced NO production could not be considered as a possible mechanism of resistance to EAE.

It is noteworthy that all three macrophage function parameters tested (although NO production was not statistically significant) were substantially decreased in rats of AO strain shortly after immunization (d1), but returned to control values by d7. As early events after immunization with GPSC/CFA comprise nonspecific immune activation that is driven by CFA rather than by specific encephalitogen (Billiau and Matthys, 2001), it could be speculated that components of CFA exerted suppressive effects on AO rats macrophages. However, the immunoprotective effect of CFA requires production of large quantities of NO (Kahn *et al.*, 2001) which is not the case in AO rats, or Th2-directed skewing of the immune response (Heeger *et al.*, 2000; Yip *et al.*, 1999). The latter is not supported by the finding that immune cells from both strains produce large amounts of IL-10 (Lukić *et al.*, 2001). Transient fall in macrophage functions on d1 after immunization of AO rats is paralleled by the phenotype

changes in all subpopulations, i.e. decrease in $ED1^+$ and $ED1^+ED2^+$ cells and decrease in the expression of CD11b on $ED2^-$ but not on $ED2^+$ cells, and also by the increase in the number of $ED2^+$ cells. The percentages of cells expressing $ED2^+$ and $ED1^+ED2^+$ on d3 returned to values comparable to those observed in non-immunized AO rats. However, percentages of $ED1^+$ were still decreased and CD11b was even less expressed in $ED2^-$ on d3. It could be speculated that diminutive disturbances in the proportion between single positive $ED2^+$ macrophages and double positive $ED1^+ED2^+$ cells induced depression on d1, and that functional recovery on d3 was mostly due to normalization in their balance. Phenotype changes that were still observed on d3 relative to NIM might be connected to other cell activities beside the functions we tested.

Altogether, our results propose that early an increase in phagocytosis following immunization with encephalitogen, accompanied by low level of H_2O_2 production, is permissive for EAE induction in DA rats. However, a defect in the up-regulation of CD11b and phagocytic ability upon immunization, in combination with a constantly augmented oxidation state in cells obtained from AO rats, might reflect their resistance to the induction of EAE. The early and subtle phenotype changes in peritoneal cells of both rat strains might mirror the mechanism contributing to their different sensitivity to the induction of autoimmune diseases.

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FENOTIPSKE PROMENE IZAZVANE IMUNIZACIJOM ENCEFALITOGENOM MENJAJU FUNKCIJE PERITONEALNIH MAKROFAGA U DVA SOJA PACOVA RAZLIČITE OSETLJIVOSTI PREMA INDUKCIJI EKSPERIMENTALNOG AUTOIMUNSKOG ENCEFALOMIJELITISA (EAE)

MITIĆ KATARINA, MILETIĆ TATJANA, KOVAČEVIĆ-JOVANOVIĆ VESNA, KUŠTRIMOVIĆ NATAŠA, KOSEC D, DIMITRIJEVIĆ MIRJANA i STANOJEVIĆ STANISLAVA

SADRŽAJ

Ispitivan je fenotip peritonealnih ćelija, kao i funkcije peritonealnih makrofaga, izolovanih od pacova Dark Agouti (DA) soja osetljivog na indukciju eksperimentalnog autoimunskog encefalomijelitisa (EAE) i pacova Albino Oxford (AO) soja koji je rezistentan prema EAE-u, 1, 3. i 7. dana nakon imunizacije encefalitogenom. Rezidentne peritonealne ćelije su ispitivane metodom protočne citofluorometrije, a zatim je nakon adherence testirana njihova sposobnost fagocitoze čestica zimozana i kapacitet produkcije vodonik peroksida (H_2O_2) i azot monoksida (NO). U neimunizovanih pacova makrofage DA soja su intenzivnije fagocitovale čestice zimozana i imale nižu sposobnost produkcije H_2O_2 nego ćelije pacova AO soja, ali nije bilo sojnih razlika u sposobnosti produkcije NO. Imunizacija je dovela do povećanja fagocitne sposobnosti makrofaga DA pacova, ali i do smanjenja fagocitoze i produkcije H_2O_2 makrofaga pacova AO soja. Generalno veću sposobnost fagocitoze u DA pacova prati i značajno veća zastupljenost $ED1^+$ ćelija (koje čine uglavnom makrofage i dendritične ćelije) nasuprot većoj zastupljenosti ED2 antigena (marker rezidentnih makrofaga) na ćelijama pacova AO soja. Imunizacija encefalitogenom je takođe dovela do povećanja ekspresije CD11b molekula na nerezidentnim $ED2^-$ ćelijama pacova DA, ali ne i AO soja. Rane i diskretne fenotipske promene na peritonealnim ćelijama pacova oba soja verovatno odslikavaju mehanizme koji doprinose njhovoj različitoj osetljivosti prema indukciji autoimunskih oboljenja.