Altered Phenotype and Functionality of Circulating Immune Cells Characterize Adult Patients with Nonalcoholic Steatohepatitis

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Abstract Nonalcoholic steatohepatitis (NASH) is a chronic inflammatory liver disease associated with insulin resistance and its metabolic consequences. Leukocyte mobilization, intrahepatic activation, and an exacerbated production of reactive oxygen species (ROS) and cytokines contribute to the development of NASH. Though alterations in peripheral blood (PB) T cell proportions and functionality remain unidentified, they might play a main role in NASH progression. We have compared the phenotype and Th1/Th2 commitment of peripheral immune cell reservoirs in adult patients and controls as well as the ability of neutrophils and monocytes to handle an ex vivo challenge. Also, we correlated those parameters with the main histological characteristics in NASH. Compared with controls, patients showed increased numbers of CD4$^+$ cells and both CD4$^+$ and CD8$^+$ CD45RO subsets together with a higher frequency of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells. We also found a decreased number of CD4$^+$ and CD8$^+$ CD45RA subsets. The distinctive production of IFN-γ highlights the significance of the observed skewed frequencies of PB T cells. Whereas ROS production by monocytes from NASH patients did not differ from controls, circulating neutrophils displayed a particularly higher phorbol myristate acetate-induced production of ROS. A negative correlation between oxidative burst and fibrosis grade was observed. This study reveals the presence of a characteristic profile of peripheral immune cells in NASH. We also discuss the probable influence of obesity on some of our present findings.

Keywords Memory and naïve T cell subsets · polymorphonuclear cells · oxidative stress · cytokines

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Introduction

The spectrum of nonalcoholic fatty liver diseases (NAFLD) ranges from simple fatty liver (hepatic steatosis) to nonalcoholic steatohepatitis (NASH) which is its more progressive form [1, 2]. The generation of intrahepatic oxidative stress and the activation of immune cells are important factors contributing to the pathogenesis of NAFLD [3, 4].

NASH occurs in a context defined by the presence of risk factors for the metabolic syndrome (Met S) and with insulin resistance as a well-identified etiological factor [5]. To be diagnosed as Met S, individuals may have central obesity plus two of four additional factors [6]. Visceral adipose tissue (VAT) from obese individuals is a source of soluble factors delivered to the liver via the portal vein. VAT is inflamed due to the influx of monocytes and the local activation into polarized proinflammatory macrophages termed M1. Also, the local expression of T cell chemokines is involved in the recruitment of inflammatory Th1 cells and effector CD8$^+$ T cells. Inflammation causes insulin

resistance, increased lipolysis, serum-free fatty acids flux, alteration of adipokine secretion, and consequently hepatic fat accumulation (steatosis) and hepatic insulin resistance.

NASH is histologically characterized by the presence of hepatic steatosis, ballooning, cell damage, and a mild portal and lobular infiltration of a small number of lymphocytes and polymorphonuclear leukocytes [7, 8]. Fibrosis progression on serial biopsies has been documented in approximately 30% of the cases [9, 10]. Even though the pathogenesis of NASH and especially the mechanisms involved in liver injury remain not fully understood, its current model is based on the "two-hit" hypothesis [11]. According to this hypothesis, an excessive accumulation of fat within hepatocytes represents the first hit [12]. This condition is believed to augment the sensitivity to a series of second multiple hits given by the complex interactions between liver resident cells, inflammatory mediators and reactive oxygen species (ROS) leading to cell death, inflammation, and fibrosis [13, 14]. Both hepatocytes and nonparenchymal cells, such as classically activated Kupffer cells (KC), along with newly recruited neutrophils and monocytes augment the sensitivity to second hits owing to their ability to secrete cytokines and to promote the influx of inflammatory cells and to produce ROS. As a consequence, the generation of oxidative stress speeds up the development and progression of NASH acting in a cooperative manner with proinflammatory cytokines [15]. In fact, the presence of by-products of lipid peroxidation within the liver in patients with NASH provides evidences for the involvement of the oxidative stress [16]. Those by-products promote the influx of inflammatory cells including lymphocytes as an additional source of proinflammatory cytokines in detriment to liver homeostasis [14]. The progression of NASH is assured because cytokines enhance the oxidative burst in resident and/or newly recruited cells [17].

The present study was designed to investigate the potential immunological alterations involving PB lymphocytes, monocytes, and neutrophils to gain information on the immunopathogenic aspects of NASH development. We aimed to examine the phenotype and Th1/Th2 commitment of PB T cells and the presence of hyper-responder monocytes and/or neutrophils in the blood. Also, we aimed to evaluate their potential correlation with histological features of NASH. To this end, we have studied the distribution of naïve (CD45RA) and memory (CD45RO) LT helper (CD4^{+}) and cytotoxic (CD8^{+}) peripheral subpopulations and assessed IFN-γ and IL-4 production by intracellular flow cytometry. We stimulated circulating monocytes and neutrophils with a protein kinase C activator to which cells respond in vitro [18] and evaluated the generation of ROS as an early marker of their inflammatory commitment [19].

Materials and Methods

Patients

Twenty adult patients with NASH (>18 years) from the Hepatology division of the Gastroenterology Hospital "Dr. C.B. Udaondo" and the Italian Hospital of Buenos Aires were enrolled in this study. The diagnosis of NASH was based on the following criteria: (1) absence of other causes of liver disease such as alcoholic hepatitis, chronic viral hepatitis, toxic hepatitis, primary biliary cirrhosis, or autoimmune hepatitis, (2) daily alcohol consumption minor to 20 g, and (3) typical characteristic features of NASH in the liver biopsy, including macrovesicular steatosis, lobular inflammation together with ballooning degeneration [20, 21]. Thirty age-matched (52 years; range, 43–69) lean healthy individuals were recruited among members of the staff of Immunogenetics at the Clinical Hospital "José de San Martin". To ensure the voluntary participation, all the staff was given information of the study and those who intended to participate were told to make contact with the investigators. None of the controls had used medications; the alcohol ingestion was less of 20 g/day; and the body mass index (BMI) was less than 25 kg/m^2. They stated neither to suffer from any metabolic condition such as diabetes, hypertension, or insulin resistance nor to have abnormal values of total cholesterol within 6 months previous to the study. All subjects were informed of the aim of the study and gave their written informed consent. The study was approved by the respective local Ethics Committees informed of internationally endorsed standards for the application of the Helsinki Declaration. A clinical evaluation, an ultrasonography and laboratory tests including liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), triglycerides, and cholesterol were performed to all patients.

Histologic Analysis

The histologic diagnosis was established using hematoxylin and eosin (H & E) and Masson's trichrome stain of formalin-fixed paraffin-embedded liver samples according to the nonalcoholic fatty liver disease scoring system [20]. Slides from all biopsies were examined by two pathologists that scored the liver histology by using previously published criteria. Activity grade was semi quantitatively assessed using a 9-point scale (steatosis=0–3; lobular inflammation=0–3; and ballooning=0–2). Fibrosis stage was measured using a 5-point scale.

Reagents and Antibodies

Ficoll-Hypaque was purchased from Pharmacia Biotech (Uppsala, Sweden). RPMI 1640 was obtained from Life

Technologies (Gaithersburg, MD, USA). Gentamicin, glutamine, gelatin, ionomycin, brefeldin A, and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Lysis buffer was made up of 50 ml distilled water, 0.415 g of ammonium chloride, 0.084 g of sodium bicarbonate, and 0.1 ml of 0.5 M EDTA (pH 7.3). 2′7′-Dichlorofluorescin diacetate (DCFH-DA; Sigma) was made up daily by dissolving 0.8 mg of DCFH-DA in 84 μl of methanol and 750 μl of phosphate-buffered saline (PBS) containing 5 mM glucose and 0.1% gelatin (PBSg). PMA was dissolved in dimethyl sulfoxide to give a 25-μg/ml stock solution and stored at −20°C. Monoclonal antibodies (mAbs), Cytofix/Cytoperm and Perm Wash buffer were purchased from Becton–Dickinson (BD; Mountain View, CA, USA).

Cell Isolation Procedures

Blood samples were collected by venous puncture in heparin at the time of diagnosis and immediately processed to obtain monocytes and neutrophils. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque density gradient centrifugation at 2,000 rpm at room temperature for 20 min. PBMC were washed twice in PBS, counted, and resuspended in RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 50 μg/ml gentamicin. For neutrophil assays, 10 ml of lysis buffer were added to propylene tubes and 1 ml of whole blood was then added to each tube. Tubes were mixed by inverting and after 10 min were spun at 1,200 rpm for 10 min. Total leukocytes were washed twice in PBS and resuspended in RPMI medium to yield a final concentration of 10^6 cells/ml.

Monoclonal Antibodies and Cell-Surface Staining

The following mAbs conjugated with fluorescein isothiocyanate (FITC) or Peridinin-Chlorophyll-Protein Complex (PerCP) were used for flow cytometry analysis of peripheral blood subpopulations: CD4 (Leu3a/PerCP), CD8 (Leu2/PerCP), CD45 RA (HI100/FITC), and CD45 RO (UCHL1/FITC). IgG1 isotypes (G1CL–FITC) were used at the same concentration to control for nonspecific binding. After incubation with surface-specific mAbs for 30 min at 4°C, PBMC were washed and resuspended in PBS.

Intracellular Cytokine Detection

PMBC were stimulated with 25 ng/ml of PMA and 1 mM ionomycin, in the presence of 1 mM Brefeldin A for 4 h at 37°C. After stimulation, cell-surface staining was performed using PerCP anti-CD4 or anti-CD8 as describe above. After that, the cells were fixed during 20 min at 4°C

with a fixation kit (BD) and then washed with the suitable permeabilization buffer (BD). PE-conjugated anti-IFN-γ or anti-IL-4 or the corresponding isotypes were added to the cells and incubated at 4°C for 40 min in the presence of 50 μl of permeabilization buffer.

DCFH Oxidation

PBMC or total leukocytes (10^6 cells per milliliter) were incubated with 10 μl of DCFH-DA in PBSg, yielding a final concentration of 5 μM, for 15 min at 37° with shaking. After washing the cells twice with PBS, cell stimulation was initiated with the addition of 100 ng/ml of PMA at 37°C for 20 min (polymorphonuclear cells) or 60 min (mononuclear cells). Basal ROS production by resting cells was evaluated by analyzing saline-treated preparations. Following stimulation, PBMC were incubated with anti-CD14 (M5E2/PE; 5 μl/10^6 cells) for 20 min at 4°C.

Fluorescence Cytometric Analysis

Gates were separately set on lymphocytes, monocytes, or neutrophils according to their forward- and side-scatter properties and used to collect events from tubes stained with the control isotypes or the different fluorochrome-conjugated mAbs.

For lymphocytes analysis, cells were gated on the basis of side-scatter and simultaneous CD4 or CD8 expression. The expression of CD45RO and CD45RA isoforms was analyzed in a similarly selected CD4 or CD8 gates.

For monocytes analysis, cells were gated in a FSC/SSC dot plot (R1). In population R1, CD14-expressing monocytes were further gated in the SSC/FL2-PE dot plot (R2). Finally, this R2 population was analyzed regarding its DCF fluorescence in the FL1-FITC histogram. On the other hand, neutrophils were gated in a FSC/SSC dot plot and then analyzed for their DCF fluorescence. An isotype-matched negative control antibody was used in all cases to assess background fluorescence intensity. Acquisition and analysis were performed on a FACSCalibur flow cytometer (BD) using the WinMDI software version 2.9.

Statistical Analysis

GraphPad Prism© software (GraphPad, San Diego, CA, USA) was used for all analyses. Normality was assessed by the Kolmogorov–Smirnov test. Because the majority of the values showed a distribution different from normal, a nonparametric Mann–Whitney U test was used. All values are expressed as median±standard deviation. Spearman's rank correlation coefficients were used to test the association between immunological parameters measured and

histological characteristics in patients with NASH. Those patients with no signs of fibrosis were excluded from the correlation analysis between immunological parameters and stage of fibrosis. The level of significance was fixed in all cases at $p < 0.05$.

Results

Demographical Features and Clinical and Histological Characterization of Patients with NASH

A complete characterization of the enrolled patients is shown in Table I. Patient's median age was 55 years (range, 43 to 72 years), and gender distribution showed 30% of male and 70% of female. According to reference values [22], all 20 adult patients with NASH showed elevated BMI, being 85% obese (85% showing obesity type II) and 15% overweighted. The serum AST and ALT levels were elevated in 35% and 80% of the samples, respectively. Abnormal lipid profile (increased cholesterol and/or triglycerides) was found in 55% of patients with NASH. In addition, about 55% of NASH patients suffer from diabetes while 45% have hypertension (data not shown). The histological features of the patient population are described in Table I. The degree of steatosis was generally severe (40% of patients) or moderate (40% of patients). Mild lobular inflammation was observed in about 75% of patients while moderate inflammation was seen in 20% of patients with NASH. Only one patient showed a severe pattern of inflammation. Mild degree of ballooned hepatocytes was exhibited by 85% of patients while 10% have a moderate degree. Only one patient showed no evidence of ballooning. Fifty percent of NASH patients demonstrated signs of fibrosis, 40% showing mild, 40% moderate, and 20% severe stage of fibrosis.

Table I Individual characterization of patients with NASH

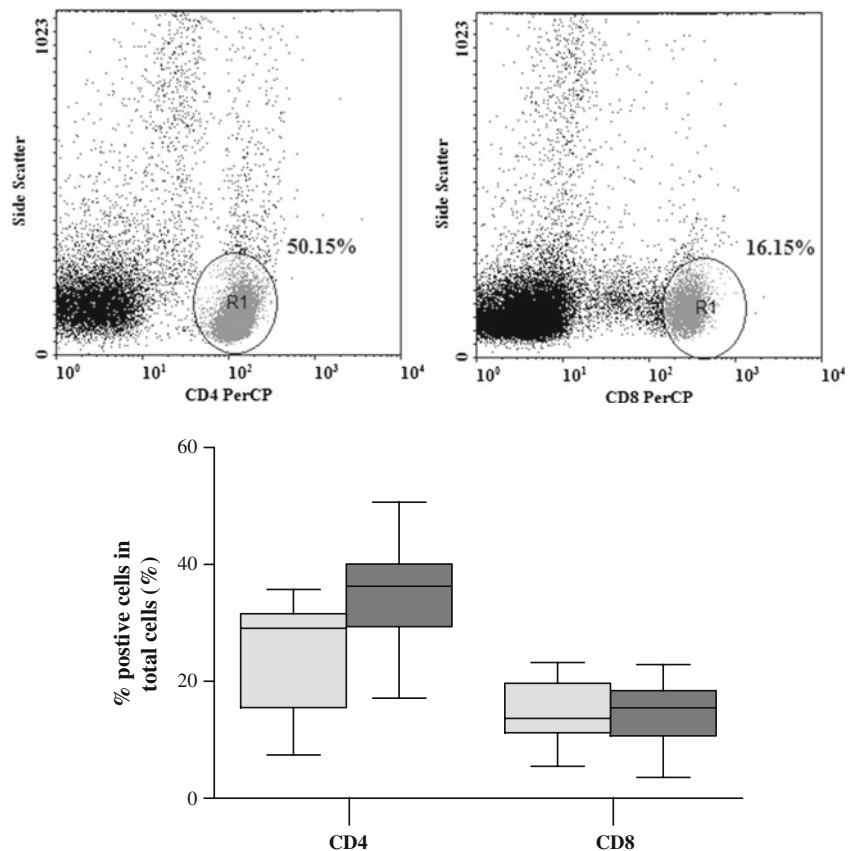| Patient	Gender (age)	BMI	Serum transaminases		Lipid profile		Histological characteristics				
			AST	ALT	TC	TG	St	Inf	Ball	Fibrosis	AG
1	F (61)	39.2	36	41	247	273	2	1	1	0	4
2	F (46)	28.0	46	51	239	365	3	1	1	1	5
3	M (43)	26.0	53	79	156	144	1	1	1	1	3
4	F (55)	38.5	87	78	151	112	1	3	2	3	6
5	M (63)	27.2	25	34	247	111	2	2	1	2	5
6	F (55)	30.8	30	45	161	111	2	1	1	1	4
7	F (61)	31.0	35	39	196	206	1	1	1	1	3
8	F (54)	31.2	45	68	249	102	3	1	1	0	5
9	M (52)	36.5	32	52	256	213	3	2	1	0	6
10	F (50)	39.6	16	23	224	116	3	2	1	0	6
11	F (55)	37.8	31	26	218	252	3	1	1	2	5
12	M (55)	34.9	38	50	228	442	2	2	1	2	5
13	F (45)	37.4	112	127	186	175	3	1	1	2	5
14	F (49)	33.3	26	54	145	60	2	1	1	0	4
15	F (72)	39.0	65	81	200	63	1	1	2	4	4
16	M (50)	35.1	35	75	147	103	2	1	2	0	5
17	F (58)	36.1	22	31	165	274	2	1	0	0	3
18	F (46)	32.5	46	43	199	158	3	1	2	0	6
19	F (53)	29.1	36	50	191	180	3	1	2	0	6
20	F (55)	30.7	30	31	167	186	3	1	1	0	5

A BMI from 25 to 29.9 was considered overweight, from 30 to 34.9, obesity type I, and from 35 to 39.9, obesity type II. Normal ALT and AST levels are ≤32 and ≤48 IU/L, respectively. The reference ranges for TC and TG are 150 to 199 mg/dL and 31 to 119 mg/dL, respectively. St grade: score 0 (5% cells), 1 (5–33%), 2 (33–66%), and 3 (66%). Inf grade: score 0 (0 foci), 1 (2 foci), 2 (2–4 foci), and 3 (4 foci). Ball grade: score 0 (none), 1 (few balloon cells), and 2 (many cells/prominent cells). Fibrosis stage: 0 (none), 1 (mild/moderate zone 3 perisinusoidal fibrosis), 2 (zone 3 and portal/periportal fibrosis), 3 (bridging fibrosis), and 4 (cirrhosis)

Abbreviations: *BMI* body mass index (kg/m^{-2}), *AST (UI/L)* aspartate aminotransferases, *ALT (UI/L)* alanine aminotransferases, *TC (mg/dL)* total cholesterol, *TG (mg/dL)* triglycerides, *St* steatosis, *Inf* lobular inflammation, *Ball* ballooning, *AG* activity grade: 0–3 (St)+0–3 (Inf)+0–2 (Ball)

Differential Expression of CD45RO and CD45RA Within CD4$^+$ and CD8$^+$ Subpopulations

The size of CD4$^+$ and CD8$^+$ lymphocyte subpopulations was measured at the peripheral compartment. While total percentage of CD4$^+$ cells was increased in patients with NASH (36.33±9.23 vs. 29.08±9.61; p=0. 02; vs. Co), no differences between the study groups were found for the CD8$^+$ subpopulation (16.73±5. 22 vs. 13.68±5.60; p= 0.93) (Fig. 1). The percentage of the CD45RO within the CD8$^+$ subpopulation was higher in patients than controls (56.66±10.21 vs. 47.14±6.76; p=0.01). Reciprocally, CD45RA cells were significantly decreased within the CD8$^+$ subpopulation from patients with NASH (42.23± 9.73 vs. 66.04±10.24; p<0.001; vs. Co). Similarly, when assessing the same naïve and memory markers on the CD4$^+$ subpopulation, an increased percentage of CD45RO (72.99 ±5.09 vs. 60.38±5.88; p=0.013; vs. Co) and, reciprocally, a decreased percentage of CD45RA (29.45±8.90 vs. 43.29± 6.62; p=0.008; vs. Co) were evidenced in patients with NASH (Fig. 2). No significant correlations were found between these immunological parameters and histological features in NASH.

An Increased Percentage of CD4$^+$ IFN-γ^+ and CD8$^+$ IFN-γ^+ T Cells in Patients with NASH

We used intracellular staining to study the expression of the most representative cytokines of Th1 (IFN-γ) and Th2 (IL-4) profiles in PBMC (Fig. 3). An increased frequency of IFN-γ-producing CD4$^+$ T cells was detected in patients with NASH (21.97±7.61) related to Co (3.78±3.61; p= 0.011). In contrast, no differences were found for the frequency of IL-4-producing CD4$^+$ cells between both groups. A higher frequency of IFN-γ-producing CD8$^+$ T cells was also found in patients with NASH (35.15±4.81 vs. 16.66±3.75; p=0.001; vs. Co). No significant correlations were found between the increased percentages of CD4$^+$ IFN-γ^+ and CD8$^+$ IFN-γ^+ T cells and histological features in NASH.

Increased ROS Production in Circulating Neutrophils but Not in Monocytes from Patients with NASH

We compared the mean channel fluorescence intensities of the signals generated by monocytes or neutrophils on the basis of a stimulation index (SI) defined by the ratio

Fig. 1 Distribution of CD4$^+$ and CD8$^+$ T cell subpopulations in peripheral blood. Patients 2–5, 8–12, and 19 (Table I) were studied. Representative dot plots of CD4 or CD8 expression vs. SSC for gating purpose (*upper panels*). The relative proportion of CD4$^+$ in total cells in patients (*NASH*; n=10) was higher than in healthy controls (*Co*; n=10). The relative proportion of CD8$^+$ in total cells did not differ between patients and controls. The *box and whiskers* show non-parametric statistics: median, lower and upper quartiles and confidence interval around the median (*lower panel*). The Mann–Whitney U test was used to determine significant differences between the two groups. *p<0.05 was considered statistically significant

Fig. 2 Distribution of naïve (*CD45RA*) and memory (*CD45RO*) T cell subsets within CD4$^+$ and CD8$^+$ peripheral blood subpopulations. Patients 2–5, 8–12, and 19 (Table I) were studied. Representative dot plots of CD45RA or CD45RO expression on CD4 or CD8 T cells in a NASH patient (*upper panels*). The proportion of CD4$^+$ T cells expressing CD45RO was higher in patients (*NASH*; n=10) than in healthy controls (*Co*) and, reciprocally, proportion of CD4+ T Cells expressing CD45RA was lower in NASH compared with controls. Likewise, the proportion of CD8$^+$ T cells expressing CD45RO was higher in patients whereas the proportion of CD8$^+$ expressing CD45RA was decreased in comparison to healthy controls. The *box and whiskers* show non-parametric statistics: median, lower and upper quartiles, and confidence interval around the median (*lower panels*). The Mann–Whitney *U* test was used to determine significant differences between the two groups. *$p<0.05$ was considered statistically significant
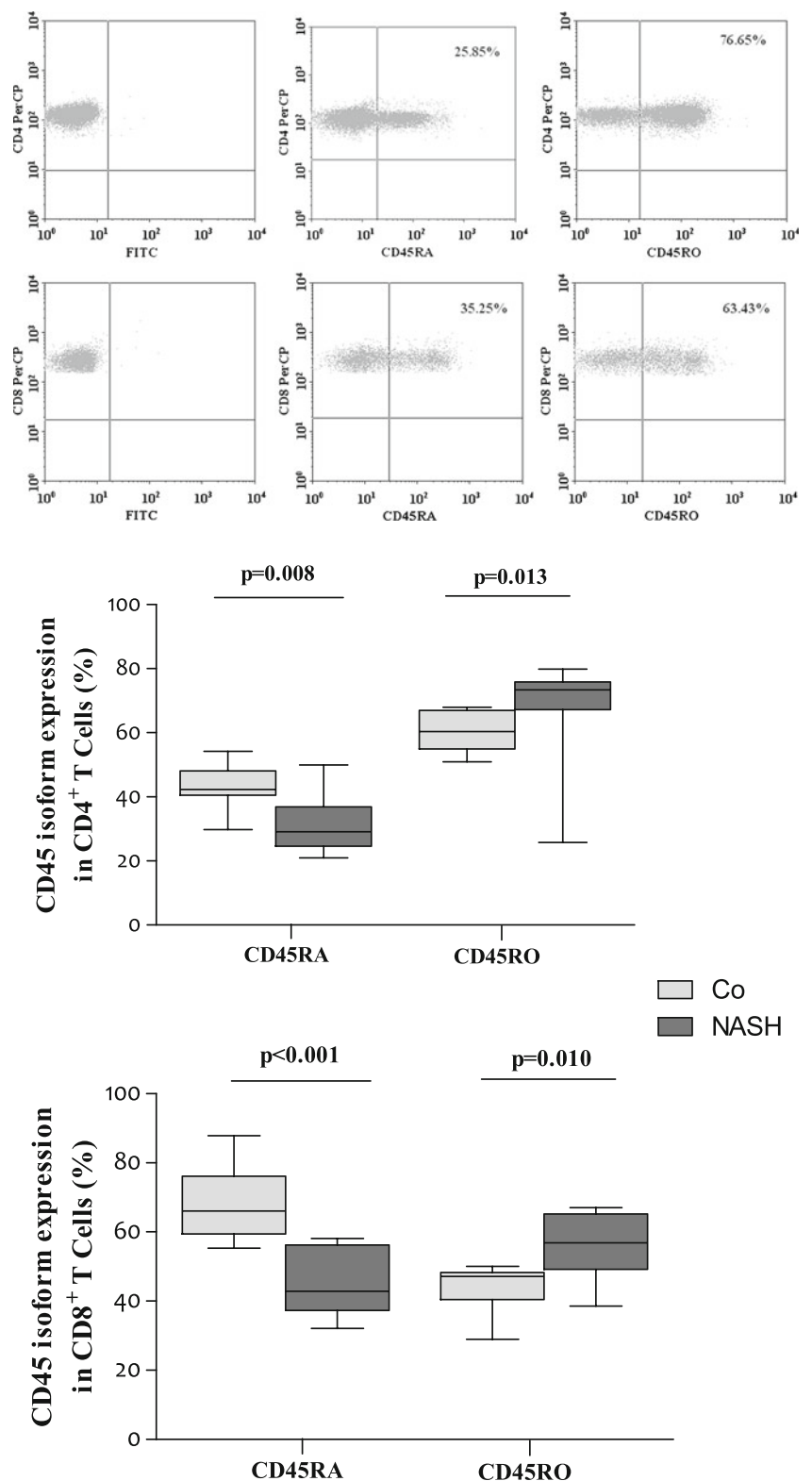
between PMA-stimulated monocytes (or neutrophils) and non-stimulated monocytes (or neutrophils). After PMA stimulation, monocytes showed similar mean SI in patients and controls (2.99±2.86 vs. 4.42±3.02; p=0.562) (Fig. 4). On the contrary, neutrophils from patients with NASH produced a higher amount of ROS as evidenced by their increased mean SI (21.01±6.41 vs. 3.6±1.45; p<0.001; vs. Co) (Fig. 5). We found a negative correlation between SI generated by neutrophils and the stage of fibrosis (r=−0.91; p=0.016). No significant correlations were found between

Fig. 3 Percentage of IFN-γ-
and IL-4-producing CD4⁺ and
CD8⁺ cells in peripheral blood.
Patients 8–10, 13, and 15–20
(Table I) were studied. Cells
were gated on CD4 or CD8 vs.
side-scatter dot plots. Represen-
tative dot plots of CD4 or CD8
cells showing intracellular IFN-
γ and IL-4 in a NASH patient
(*upper panel*). The percentages
of IFN-γ-producing cells in total
CD4⁺ and IFN-γ-producing
cells in total CD8⁺ cells, re-
spectively, were higher in
patients (*NASH*; n=10) com-
pared with controls (*Co*; n=10).
No differences were found be-
tween both groups regarding IL-
4-producing cells in CD4⁺ cells.
The *box and whiskers* show
non-parametric statistics: medi-
an, lower, and upper quartiles
and confidence interval around
the median values (*lower panel*).
The Mann–Whitney *U* test was
used to determine significant
differences between the two
groups. *p* value of <0.05 was
considered statistically
significant
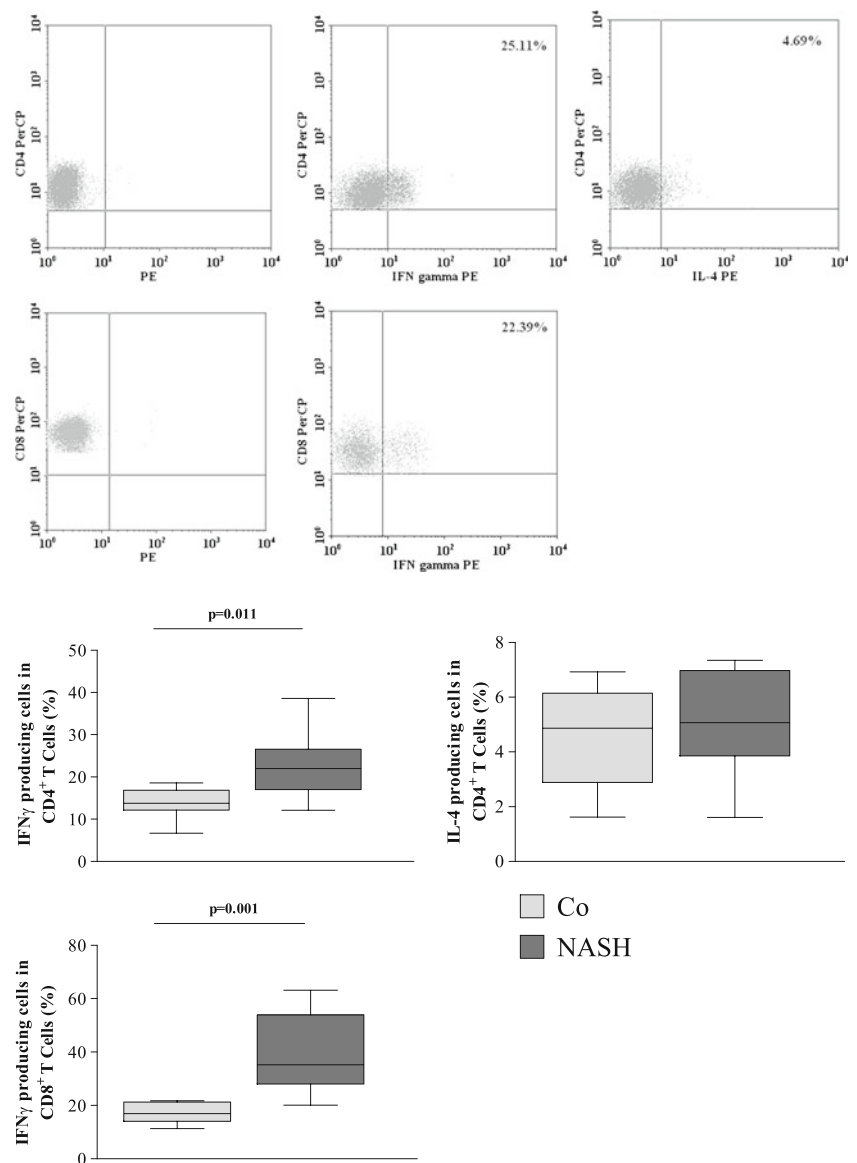
SI generated by neutrophils and other histological features
in NASH.

Discussion

Some immunological alterations involving circulating lym-
phocytes and neutrophils were found in patients with
NASH revealing the presence of a particular profile of
peripheral immune cells. Concerning PB we found in first
place, a similar percentage of total CD8⁺ LT but increased
CD8⁺ CD45RO and decreased CD8⁺ CD45RA subpopula-
tions; second, increased percentages of total CD4⁺ and
CD4⁺ CD45RO together with decreased CD4⁺ CD45RA
subpopulation and third, an increased percentage of IFN-γ-
producing CD4⁺ and CD8⁺ T cells. Concerning neutrophils,
patients with NASH were characterized by an augmented

production of ROS following stimulation with PMA. In
those patients with NASH characterized by the presence of
fibrosis, a negative correlation was found between the SI of
neutrophils and fibrosis stage. Conversely, monocytes were
not committed to an inflammatory phenotype in NASH.

Naïve and memory T cells can be phenotypically distin-
guished based on the expression of CD45 isoforms. Whereas
memory T cells display CD45RO after the stimulation by
antigens, naïve T cells carry CD45RA [23, 24]. The expression
of CD45 isoforms on the surface of LT helpers and cytotoxic
cells was extensively studied in healthy individuals [25, 26]
and also searching for the establishment of the immunologic
background in a large range of diseases. For instance, an
altered profile of CD45 isoforms expression has been
associated with metabolic diseases such as hypothyroidism
and diabetes type 2 [27] and in many chronic hepatitis
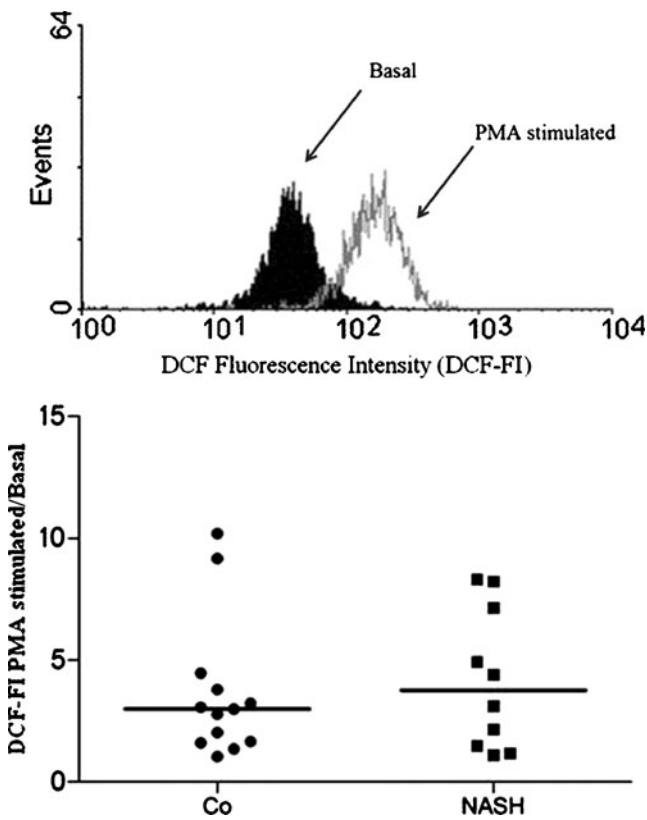including hepatitis C and alcoholic cirrhosis [28, 29]. An

Fig. 4 Stimulation Index in peripheral blood monocytes after challenge with PMA. Patients 1–8, 14, and 16 (Table I) were studied. PBMC were stimulated with PMA (100 ng/ml) for 60 min and stained with anti-CD14 to gate the monocyte population in forward-side scattering. Monocytes were selected in a FSC vs. SSC dot plot, and CD14$^+$ monocytes were further gated in the SSC-H vs. CD14-PE dot plot (not shown). The resulting population was analyzed regarding its DCF fluorescence in the FL1-FITC histogram (upper panel, gated on monocytes). A Stimulation Index for monocytes was defined by the ratio between DCF fluorescence intensity (DCF-FI) in PMA-stimulated and non-stimulated (basal) monocytes. No differences were found for the stimulation index between patients (NASH; $n=10$) and controls (Co; $n=13$). Horizontal bars indicate median values (lower panel). A two-tailed Mann–Whitney U test was used to determine significant differences between the two groups
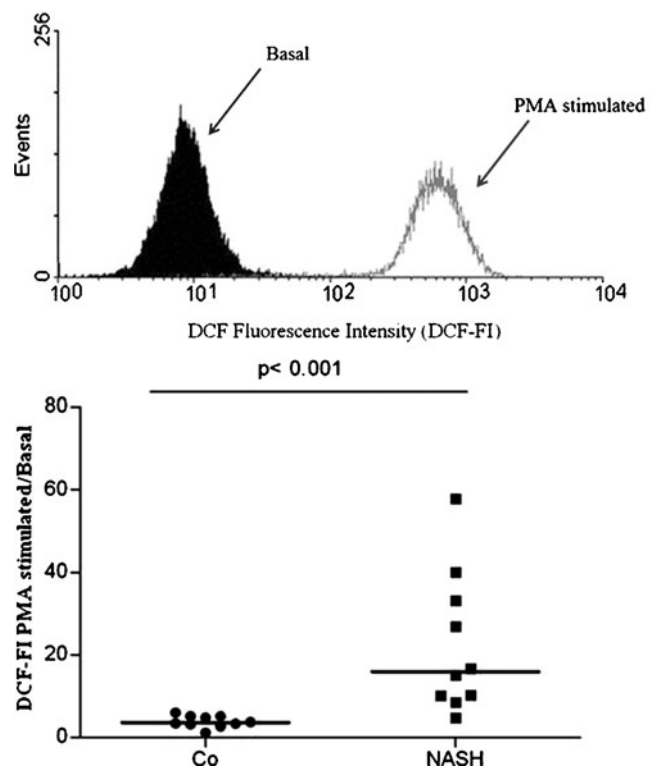

Fig. 5 Stimulation Index in peripheral blood neutrophils after challenge with PMA. Patients 6–14 and 17 (Table I) were studied. Neutrophils were stimulated with PMA (100 ng/ml) for 20 min. A gate was made for the neutrophil subpopulation in a FSC vs. SSC dot plot and then analyzed for DCF fluorescence intensity (DCF-FI; upper panel, gated on neutrophils). A Stimulation Index for neutrophils was defined by the ratio between DCF-FI in PMA-stimulated and non-stimulated (basal) neutrophils. A higher median value for the stimulation index was found in patients (NASH; $n=10$) than controls (Co; $n=10$). Horizontal bars indicate median values (lower panel). A two-tailed Mann–Whitney U test was used to determine significant differences between the two groups

abnormal distribution of circulating naïve and memory CD4$^+$ T cells was also found in patients with primary biliary cirrhosis compared with controls. Notwithstanding the concomitantly analyzed liver infiltrating lymphocytes showed the same abnormality than PB lymphocytes, differences between patients and controls were markedly greater in the liver of those patients [30]. It remains as a possibility that the local proportion of lymphocyte subsets in patients with NASH might also differ from control livers.

NAFLD are considered the hepatic manifestation of the MetS. Accordingly, previously reported correlation between increased numbers of CD4$^+$ and CD4$^+$ CD45RO PB cells with clustered features of the MS [31] agrees with our present observations in patients with NASH. The increases of CD45RO together with the reciprocal decreases of CD45RA

proportions at both CD4$^+$ and CD8$^+$ peripheral compartments might reflect an intrahepatic stimulatory process concerning the transition of CD45RA into CD45RO, which is well matched with the prevalence of autoimmune phenomena reported in NAFLD [32]. Alternatively, our results might reflect an imbalanced migration of lymphocyte subsets into the liver, but a complete analysis of signals that govern the lymphocyte recruitment into the liver remain to be performed.

The faint division between obesity and liver disease was demonstrated in a study of the hepatic expression patterns of inflammatory/immune response genes associated with obesity without steatosis (OWS), obesity with severe steatosis (OSS) and obesity in the context of NASH. The upregulation of a number of specific genes in NASH and not in OWS and OSS was revealed. Other genes are upregulated in OWS and OSS, and further increased in NASH. This study clearly indicates that some hepatic characteristics of obesity non NASH extends beyond

established NASH. The majority of those NASH markers identified in the liver did not show a differential expression in the VAT of NASH patients vs. OWS and OSS [33]. This finding underlines the notion that the VAT constitutes an autonomous source of regulator molecules that might influence hepatic and/or systemic metabolic and immunological functions. Given that our study patients were obese or overweighed, we cannot completely rule out that our findings are influenced by an overall increase in body fat.

The important role ascribed to IFN-γ in hepatic damage is based on its capability to prime KC upon the co-recruitment of monocytes and lymphocytes into the inflamed liver, and also on its increased expression observed in experimental models of TNF-α related liver injury [34]. NASH is not considered a Th1-polarized disease; however, several studies have shown that an excessive production of Th1 proinflammatory cytokines and a deficit of anti-inflammatory cytokines are key players in its development [35, 36]. Among adaptive cells of the immune system, T lymphocytes play a major role in the inflammatory response and thus we hypothesized that an unregulated function might be critical for NASH. Our present data showing an increased Th1 phenotype within the peripheral CD4$^+$ T cell population in NASH is intriguing. T cells as well as macrophages are recruited into human VAT [37]. Given that IFN-γ promotes macrophage activation in human adipose tissue [38], we can hypothesize that a potential vicious circle could be established, in which IFN-γ produced by the incoming CD4$^+$ T cell population and probably also by effectors CD8$^+$ T cells into the VAT might be capable to further sustain the M1 phenotype development of macrophages [39]. In addition, insulin sensitivity might be attenuated and human adipocyte differentiation might be suppressed by IFN-γ [40]. Thus, our results suggest that an intriguing interplay between M1 and Th1 polarization states might represent an interaction between the innate and adaptive immune systems in NASH.

IL-4 production, on the other hand, appeared to be related to a constitutional feature of the investigated individuals. Activation of innate immunity in NASH involves sequestration of both neutrophils and monocytes from blood into the liver [41–43].

The observed infiltration of neutrophils [44] led us to consider the probable involvement of PMN cells as potential causative factor within the complex pathophysiology of NASH. Innate cytokines like TNF-α and IL-1α are inducers of inflammatory chemokines produced not only by local sources but also by peripheral blood cells. These cytokines were recognized as sensitizing factors acting upon leukocyte infiltration of the liver [44]. Neutrophil recruitment can also be promoted by factors of the complement cascade triggered by hepatocyte apoptosis. These factors can further stimulate neutrophil respiratory burst thereby causing formation and diffusion of ROS into nearby cells. The resulting intracellular

oxidative stress induces mitochondrial dysfunction and cell death amplifying hepatocytes damage and cytokines release [45–47] and contributes to stellate cell activation and hepatic fibrosis [48].

Our results suggest for the first time that the ability of peripheral neutrophils to handle the challenge provided by PMA is less in NASH patients than in control subjects. The negative correlation between SI generated by neutrophils and the stage of fibrosis demonstrates a relationship between a greater activation status of peripheral neutrophils and early fibrosis. In agreement, smaller amount of ROS were observed in liver cirrhosis patients compared with healthy subjects [49].

Through a functional study based on ROS generation we showed that oxygen metabolism of circulating monocytes was within the same range of values in patients with NASH and controls. The evaluation of additional markers of monocyte activation was out of our scope and remains to be examined. Once within the liver, the pivotal role reported for KC in NASH is not only the consequence of ROS production, but also by one or more steps of KC activation [50]. By instance, the lipid composition of the liver probably in addition with extra factors associated with obesity in NASH influences the oxidative burst in KC.

Conclusions

We clearly demonstrated the presence of an altered phenotype and functionality of circulating immune cells as a distinctive characteristic in adult patients with NASH. However, we cannot completely exclude the influence of an overall increase in body fat on some of our present findings.

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