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CHRONIC AND ACUTE ALCOHOL EXPOSURE PREVENTS MONOCYTE-DERIVED DENDRITIC CELLS FROM DIFFERENTIATING AND MATURING

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Increasing evidence suggests that alcohol abuse may be linked to adverse immunomodulatory effects on immune responses. Our study was undertaken to clarify the immunological consequences of chronic and acute alcohol exposure on differentiation and maturation of human dendritic cells (DCs). Using immunochemical and cytofluorimetric analysis we determined the phenotype and functions of monocyte-derived DCs from alcoholics and healthy subjects and analyzed their ability to respond to lipopolysaccharide (LPS) in the presence or absence of ethanol (EtOH) exposure. Our results showed that alcoholics' monocytes differentiated to immature DCs with altered phenotype and functions (alc-iDCs). Alc-iDCs showed fewer CD1a$^+$ cells, weaker CD86 expression and higher HLA-DR expression associated with lower endocytosis and allostimulatory functions than iDCs from healthy subjects (control-iDCs). Despite these impairments, alc-iDCs produced TNF-α and IL-6 in large amounts. LPS stimulation failed to induce full phenotypical and functional alc-iDC maturation. *In vitro* acute EtOH exposure also prevented alc-iDCs and control-iDCs from maturing in response to LPS. T-cell priming experiments showed that EtOH treatment prevented LPS-stimulated control-iDCs from priming and polarizing naïve allogeneic T cells into Th1 cells, thus favouring a predominant Th2 environment. Collectively, our results provide evidence that chronic and acute alcohol exposure prevents DCs from differentiating and maturing in response to a microbial stimulus.

Emerging evidence shows an increased incidence of infectious diseases, as well as a greater susceptibility to cancer in humans chronically abusing alcohol (1-5). Evidence from human and animal studies *in vivo,* as well as from *in vitro* experiments suggests that alcohol abuse may exert adverse immunomodulatory effects on innate and adaptive immune responses (4, 6-9). Alcohol-induced immune dysfunctions depend on various factors including the dose and duration of alcohol exposure (chronic *vs* acute) and on the presence and characteristics of additional stimuli such as microbial molecules.

In chronic alcoholics and in a chronic ethanol-feeding model in mice, impaired antigen-specific T cell proliferation and a delayed-type hypersensitivity

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response were associated with immune abnormalities (10-13). Chronic alcoholism is associated also with numerical, phenotypical, and functional changes in circulating peripheral blood dendritic cells (DCs). In chronic alcoholics without liver disease, the major DC change is an increased secretion of inflammatory cytokines (14).

DCs are the most potent antigen presenting cells (APCs). The functional state of DC maturation and their activation degree control immunity and tolerance (15). Like chronic alcohol abuse, acute alcohol abuse also has immunoregulatory potential. Precisely how chronic and acute alcohol abuse alters human innate immunity, thus causing immune dysfunction, remains unclear. A need for more information is important in designing new therapeutic strategies for improving immune responses to pathogens, tumours and vaccinations in alcohol abusers.

To clarify the immunological effects exerted by alcohol abuse on human monocyte-derived DCs, we investigated whether *in vivo* chronic alcohol exposure and *in vitro* acute ethanol (EtOH) treatment alters the ability of monocytes to differentiate and mature into functional DCs. For this purpose immature DCs (iDCs) were generated *in vitro* from chronic alcoholics and healthy control subjects. Using immunochemical and cytofluorimetric analysis we determined the phenotype and functions of DCs and analyzed the ability of iDCs to respond to the microbial product lipopolysaccharide (LPS). We also wanted to find out whether EtOH-treated LPS-stimulated DCs correctly primed naïve T lymphocytes, thus inducing Th1 cell polarization.

MATERIALS AND METHODS

Blood donors

The procedures for enrolment and study protocols were fully approved by the institutional review board of the Sapienza University of Rome. Seven alcohol-dependent patients (five men and two women; aged 18-60 years) consecutively admitted for treatment to the Alcohologic Reference Center for the Lazio Region, Sapienza University of Rome, were eligible for participation. To be included, the patients had to be drinking more than 90 g of EtOH/day until the day of study entry, and to be constantly negative for hepatitis B surface antigen and antibodies to hepatitis C virus and to human immunodeficiency virus. None of the patients had signs or symptoms of infection at clinical evaluation, and this finding was confirmed by biochemical data: CBC with normal WBC count; ESR and Alpha1- Alpha2 glycoprotein within normal range; urine analysis negative; normal chest X-ray, especially without signs of tuberculosis (tuberculin test or PPD test negative). Exclusion criteria were polydrug abuser patients with a diagnosis of chronic liver disease confirmed by clinical and ultrasonographic studies, and patients with a diagnosis of malnutrition confirmed by anthropometric measurements according to published criteria (16). The mean duration of alcohol abuse was 17.71 years ± 6.32. Alcohol dependence was assessed following the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV-R). Blood samples from seven sex- and age-matched healthy blood donors from the Alcohologic Reference Center at the Sapienza University of Rome, were used as controls. Control subjects had no previous liver disease, ongoing infection or autoimmune processes and consumed less than 15 g of ethanol/day. Alcohol user's habits in healthy donors were determined by a health assessment questionnaire that incorporates the Alcohol Use Disorders Identification Test (AUDIT) and health screening survey to identify the frequency and quantities of their alcohol use. Blood alcohol levels of alcoholics and healthy subjects were determined at admission through breath alcohol concentration (BAC) levels. The BAC values in patients were 185, 85, 0, 170, 0, 264, and 253 (mean ± SD: 137 ± 110), whereas in controls they were always negative. Written informed consent was obtained from patients and controls, and the study was conducted in accordance with the Helsinki Declaration of 1975 and 1983.

Generation of DCs and T lymphocytes

Dendritic cells were generated from peripheral blood monocytes, as described previously (17). In brief, PBMCs were isolated by density gradient separation (Lympholyte, Cedarlane Oxford, UK). $CD14^+$ monocytes were purified from PBMC by incubation with anti-CD14-coated microbeads (Miltenyi Biotec Belgish, Gladbach, Germany), followed by sorting with a magnetic device (MiniMacs Miltenyi Biotec), according to the manufacturer's instructions. Monocytes were incubated for 5 days in RPMI 1640 supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO, Life Technologies, Paisley, UK), 5×10^{-5} M 2-mercaptoethanol (Merck Milano, Italy) and 10% Fetal Calf Serum (Hyclone Laboratories Logan, UT) containing 100 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (rGM-CSF; R&D System MN, USA) and 1000 U/ml of recombinant human interleukin

4 (rhIL-4; R&D System) to obtain iDCs. iDCs untreated and treated with 0.5, 10, 25, 50, or 100 nM EtOH (Merck), were cultured for 18 hours with or without 100 ng/ml of phenol-purified LPS (strain 0111:B4 Escherichia coli, Sigma-Aldrich Milano, Italy). Phenotypic DC maturation was assessed by flow cytometric analysis. $CD4^+$ T cells were purified from PBMCs by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Negatively selected $CD4^+$ T cells were depleted of $CD45RO^+$ cells using anti-CD45RO-coupled magnetic beads and LD negative selection columns (Miltenyi Biotec) to obtain negatively-selected $CD45RA^+$ T cells. The purity of negatively selected $CD4^+CD45RA^+$ cells was analyzed by direct staining for membrane expression of CD45RA and of CD4 using phycoerythrin (PE)-conjugated monoclonal antibody (mAb) to CD45RA and fluorescein isothiocyanate (FITC)-conjugated mAb to CD4 (BD-Biosciences, San Josè, CA, USA). A portion of the negatively selected $CD4^+CD45RA^+$ T cells was cryopreserved for later use in T cell priming experiments.

DC phenotype

For flow-cytometric analysis of surface molecule expression, DCs were harvested, and 2×10^5 cells per sample were resuspended in phosphate-buffered saline (PBS). Cells were then incubated with saturating concentrations of the various fluorochrome-conjugated mAbs for 30 min at 4°C. The stained cells were washed twice in PBS and analyzed by flow cytometry on a FACSCanto using CellDIVA (BD-Biosciences). The following mouse anti-human mAbs were used: PE-conjugated mAbs to CD1a, CD80, CD86, CCR5 and HLA-DR, and FITC-conjugated mAbs to CD14, CD83, CCR7 and CD40 (BD-Biosciences). Typically, iDC cultures contained > 90% $CD1a^+$ $CD14^-$ cells. Flow cytometric results were expressed as median and interquartile ranges of the positive cell percentages (CD1a, CD14, CD83) and of the mean fluorescence intensity (MFI) (CD80, CD86, CD40 and HLA-DR) and as arithmetic mean ± SD (CCR5 and CCR7). Apoptosis was determined by ApoAlert Annexin V-FITC Apoptosis Kit (annexin V-FITC with propidium iodide) according to the manufacturer's recommendations (Clontech Laboratories Palo Alto, CA, USA).

DC endocytosis

Because DCs lose their high endocytic activity during maturation, we assessed the effect of *in vivo* and *in vitro* alcohol exposure on mannose receptor-mediated endocytosis measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. Cells (2×10^5 per sample) were incubated in RPMI 1640 medium with the addition of FITC-dextran (1 mg/ml; molecular mass 40,000, Sigma) for 30 min at 37°C or at 4°C (for control binding). After incubation, DCs were washed twice with PBS and fixed with 1% formaldehyde. At least 5×10^3 cells per sample were analyzed.

DC migration

We investigated the ability of DCs to migrate in response to the chemokines MIP-1α/CCL3 (a CCR5 ligand) and MIP-3β/CCL19 (a CCR7 ligand) (18) (R&D System). The chemokines were diluted in migration media RPMI 1640 + 0.5 % wt/vol BSA and 600 µl were placed in each well for measurement of chemotaxis. Transwells (Costar, Cambridge, MA) with 8 µm polycarbonate membrane pore size were loaded with 100 µl DCs (1×10^5 cells/well), placed in the 24-well plates, and incubated in a humidified atmosphere of 5% CO_2 in air for 3 hours at 37°C. The numbers of migrating DCs harvested from the lower chambers were counted by FACS (60 second counts). Cells gated into the region of the large, granular cell population in the forward and side scatter dot plot were counted as migrating DCs. Results were expressed as migration indices, that were calculated as follows: [(mean number of cells migrating in response to chemokine)/(mean number of cells migrating in response to control medium)].

DC cytokine production

Because phenotypic DC maturation increases cytokine production, we determined by ELISA the cytokine content in stimulated and unstimulated DC culture supernatants collected at 18 h. Culture supernatants were collected, immediately divided into aliquots to avoid repeated freezing and thawing and stored at -80°C until tested in ELISA. All samples were assayed in duplicate and the assays for the same cytokine were run on the same day. Levels of IL-12p70, TNF-α, IL-10 and IL-6 were determined by ELISA (OptEIA kits; BD-Biosciences) following the manufacturer's instructions. The ELISA sets that we used in our study were validated in preliminary experiments designed to determine the detection limits as well as the linearity and range of ELISA, essentially in accordance with the ICH Q2A and Q2B guidelines (International Conference on Harmonization, CPMP, European Medicines Agency). The intra-assay variation ranged from 3 to 6% and the inter-assay variation from 4 to 9%. The limits of detection were as follows: IL-10, TNF-α: 16 pg/ml; IL-12p70: 7.8 pg/ml; IL-6: 2.2 pg/ml.

DC allostimulatory ability

Because features of DC function *in vivo* are critical for antigen presentation and T cell activation, we evaluated the allostimulatory ability of stimulated and unstimulated DCs in a standard mixed lymphocyte reaction (MLR).

Although this assay is not specific for a given antigen, it provides adequate information on the overall antigen-presenting function of DCs. The mismatch between DCs and T cells was routinely checked with a bidirectional allogeneic MLR strategy. Allogeneic MLR between irradiated healthy control PBMC used as stimulators and alcoholics'/healthy subjects' PBL used as responders were determined before doing MLR experiments using alcoholics' and healthy subjects' DCs as stimulators. All alcoholics' and healthy subjects' responder PBL yielded similar alloproliferative reactions. In MLR experiments control wells contained a single cell population (PBL, PBMC and DCs) left unstimulated or stimulated with phytohaemagglutinin (PHA: 10 μg/mL, Sigma). The ability of DCs to stimulate allogeneic T cells was assessed and irradiated DCs (30 Gy) were used as stimulator cells. To exclude a possible toxic suppressive effect of EtOH on T cells, EtOH-stimulated DCs were extensively washed before being used in co-culture experiments. Allogeneic T cells ($1x10^5$ cells/well) were incubated with the irradiated DCs for 3 days at a different responder/stimulator ratios (1:4 to 1:32 DC:T) in a 96-well round bottom plate. On day 2, 0.5 μCi/well ^{3}H-methyl-thymidine (Amersham, Life Science, Milan, Italy) was added to each well. After a further 18 h at 37°C, cells were harvested on glass fiber filter paper (Wallac, EG&G Company, Turku, Finland), using an automatic cell harvester (Harvester 96, MACH III M, TOMTEC Orange, CT, USA). ^{3}H-methyl-thymidine uptake into cell DNA was measured by reading samples in a β counter (1450 Microbeta Plus, Wallac). Net counts per minute (cpm) of triplicate cultures were measured.

Nuclear factor-κB (NF-κB) translocation

The NF-κB p65 transcription factor assay kit (Active Motive Carlsbad, CA, USA) was used to monitor NF-κB activation. Unstimulated DCs and DCs stimulated for 45 min at 37°C, in 5% CO_2 with EtOH (0.5, 10, 25 and 50 nM) and LPS (100 ng/ml) were lysed, protein was quantified, and equal amounts of lysates were used to test activated levels of p65 subunits with the antibodies directed against the subunits bound to the oligonucleotide containing the NF-κB consensus binding site. As a positive control, we used a HeLa cell extract, and to monitor the specificity of the assay, we used NF-κB wild-type and mutated consensus oligonucleotides, according to the manufacturer's instructions.

T-cell priming assay

To find out whether EtOH-treated DCs primed naïve T lymphocytes, negatively selected naïve allogeneic T cells were cultured with EtOH-treated DCs at a ratio of 20:1. LPS-matured DCs were used as positive control to prime IL-4- or IFN-γ-expressing T cells. To exclude a possible toxic suppressive effect of EtOH on T cells, EtOH-stimulated DCs were extensively washed before being used in co-culture experiments. Activated T cells were expanded for 10 days with recombinant IL-2 (30 U/ml; Roche Molecular Biochemicals Indianapolis Indiana, USA), added on day 5, in a 24-well plate in complete medium to obtain polyclonal T cell lines to be analysed for IL-4 and IFN-γ expression, by flow cytometry. In brief, 10^6 cells were stimulated with 10^{-7} M phorbol 12-myristate 13-acetate plus 1 μg/ml ionomycin for 4 h in the presence of 10 μg/ml brefeldin A (all reagents Sigma Aldrich). Cells were labelled with anti-CD3 PerCP (BD-Biosciences) (5 μl/10^4 cells, 30 minutes on ice), and then cells were fixed with FACS lysing solution, treated with FACS permeabilizing solution (BD-Biosciences), stained with a predetermined optimal concentration of anti-cytokine mAb or appropriate isotype mAb control and analyzed on a FACSCanto (BD-Biosciences). Variables evaluated were the pattern of cytokine expression on the $CD3^+$ population. Cells were gated according to light scatter properties to exclude cell debris. A minimum of 10,000 viable cells was analyzed for each sample. Results were processed using Diva software (BD-Biosciences).

Statistical Analysis

Mean values, standard deviations, medians and ranges, as well as the 25th and 75th percentiles, were calculated for each variable under study. All the statistical procedures were performed by STATA 8.1 statistical package. The Mann-Whitney U and Kruskal-Wallis non-parametric tests were used to evaluate the statistical significance of intergroup differences in all the tested variables. P values < 0.05 were considered statistically significant.

RESULTS

In vivo chronic alcohol exposure impairs in vitro monocyte differentiation to DCs

Flow cytometric analysis showed that whereas healthy control monocytes cultured with GM-CSF and IL-4 differentiated to the typical immature DC phenotype ($CD1a^+$, $CD86^+$, $CD80^+$, $CD40^+$, $HLA\text{-}DR^+$, $CD83^-$) (control-iDCs), monocytes obtained from alcoholics differentiated to an altered immature DC phenotype (alc-iDCs) (Fig. 1A, B, C). Alc-iDCs contained fewer CD1a-immunoreactive cells than control-iDCs ($P = 0.0012$ by Mann-Whitney non parametric test, $n = 7$ experiments) (Fig.1A). They also expressed CD86 more weakly than control iDCs ($P = 0.0040$) and showed significantly higher HLA-DR expression ($P = 0.0060$) (Fig. 1C).

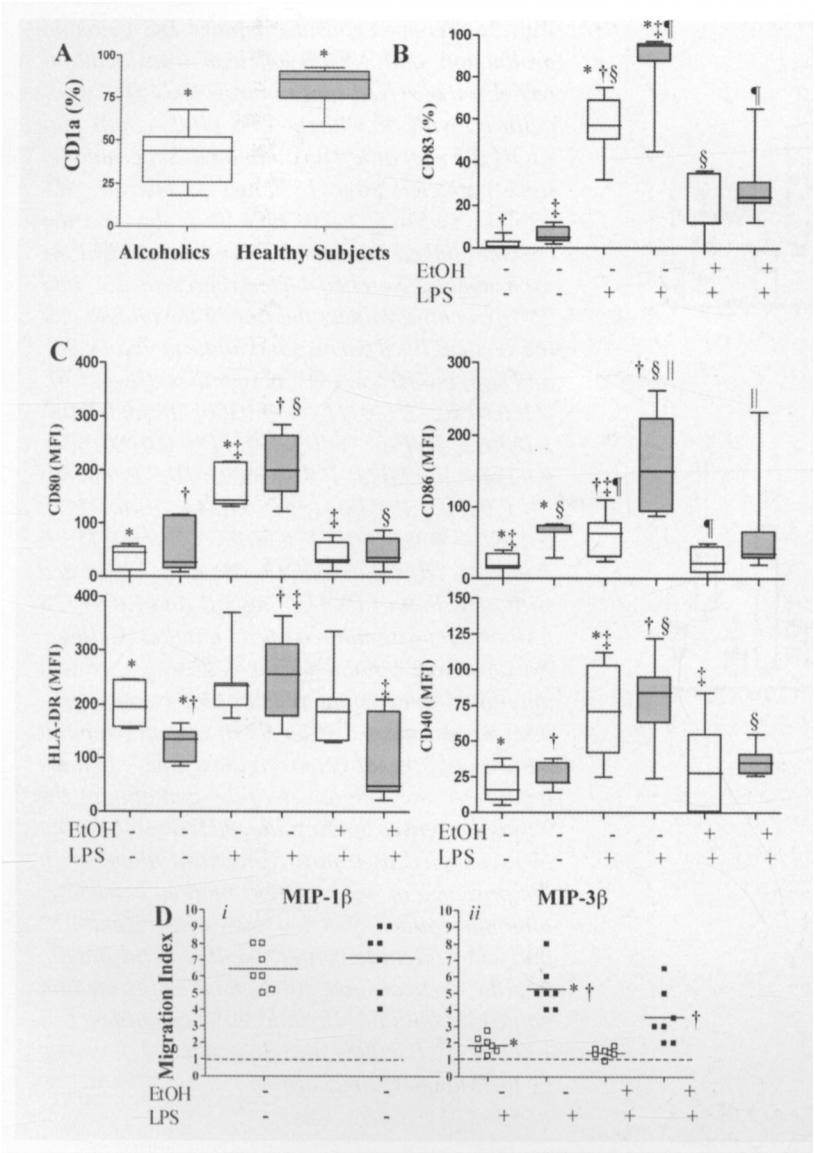

Fig. 1. *In vivo and in vitro alcohol exposure impairs the ability of monocytes to differentiate and mature into functional dendritic cells (DCs). Human immature DCs (iDCs) were generated from alcoholics (□) and from healthy blood donors (■). Five-day iDCs were cultured with or without LPS (100 ng/ml) in the presence or not of EtOH (25 nM). DC surface molecule expression and migratory capacity were analyzed by flow cytometry, as described in the "Materials & Methods" section. (**A**) The appearance of DC differentiation marker CD1a (* P = 0 .0012) was analyzed in five-day iDCs generated from alcoholics and healthy blood donors. (**B**) The appearance of DC maturation marker CD83 (*†‡P =0.0180, § P = 0.0023, ¶ P = 0.0012), (**C**) the upregulation of costimulatory and activation molecules CD80 (* P 0.0180, ‡ § P 0.0006), CD86 (* P 0.0040, † P 0.0060, ‡ § P 0.0180, ¶ P = 0.0111, ‖ P 0.0175), HLA-DR (* P 0.0060, † P = 0.0180, ‡ P 0.0111) and CD40 (* P 0.0180, † P = 0.0379, § P 0.0262) were analyzed after 18 hours of culture. Results are expressed as positive cell percentages (**A, B**) and mean fluorescence intensity (MFI) (**C**) of seven independent experiments. Notched boxes represent 25th and 75th percentile values; the horizontal lines and the vertical lines correspond to the median value and the 10th and 90th percentiles. (**D**) Migration of DC derived from alcoholics (□) and from healthy blood donors (■) towards the chemotactic stimuli MIP-1β and MIP-3β. (* P = 0.0006). The horizontal lines correspond to the arithmetic means.*

*Samples were analyzed on a FACSCanto cytofluorimeter using CellDIVA software (BD Biosciences). P values by the Mann-*Whitney test.

Flow cytometric analysis of CCR5 and CCR7 chemokine receptor expression detected similar CCR5 levels in control- and alc-iDCs (MFI: 25±15 and 20±15). Neither control iDCs nor alc-iDCs expressed CCR7 (data not shown). As chemokine receptor expression analysis suggested, alc-iDCs and control-iDCs both migrated in response to MIP-1α/CCL3 and did so with similar migration index (Fig.1D). Conversely, neither alc-iDCs nor control-iDCs migrated in response to MIP-3β/ CCL19.

In flow cytometry investigating antigen endocytosis, alc-iDCs took up significantly smaller amounts of FITC-dextran antigen than control-iDCs (MFI: 2235±502 vs 2875± 547, P = 0.0420, n = 7 experiments) (data not shown).

In vivo chronic alcohol exposure impairs in vitro full DC maturation in response to LPS

Experiments investigating whether in vitro LPS-induced DC maturation overcame the iDC defects related to chronic alcohol exposure detected a significantly lower percentage of CD83-immunoreactive cells and significantly weaker CD86 expression in LPS-stimulated alc-iDCs (alc-DCs) than in LPS-stimulated control-iDCs (control-DCs) (CD83: P = 0.0180; CD86 P =

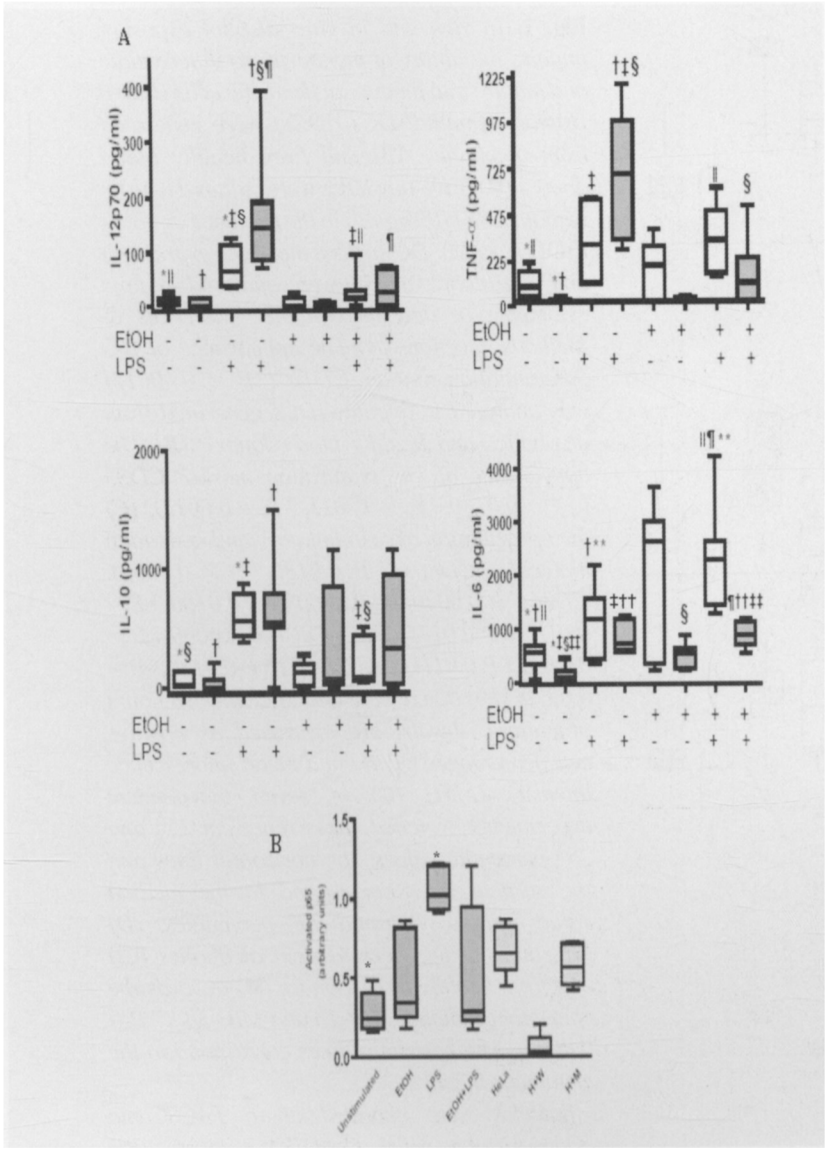

Fig. 2. *Alcohol exposure impairs DC cytokine production and NF-kB nuclear translocation. (A) Alc-iDCs (□) and control-iDCs (■) were cultured with or without LPS (100 ng/ml) and EtOH (25 nM) or left unstimulated. Supernatants were collected after 18 hours to measure IL-12p70, TNF-α, IL-10 and IL-6 by specific enzyme linked immunosorbent assay (ELISA) experiments. Notched boxes represent 25th and 75th percentile values; the line in the middle and the vertical lines correspond to the median value and both the 10th and 90th percentiles. (IL-12p70: *† P 0.0180; ‡ P 0.0111; § P 0.0350; ‖ P = 0.0280; ¶ P 0.0012; TNF-α: * P 0.0310; † P = 0.0180; ‡ P = 0.0253; § P 0.0023; P 0.0425; IL-10: * P 0.0180; † P 0.0280; ‡ P 0.0041; § P 0.0343; IL-6: * ‡ § ‖ P 0.0181; † P 0.0280; ¶ P 0.0017; ** P 0.0041; †††‡‡ P 0.0006. (B) Control-iDCs (■) were stimulated with or without LPS (100 ng/ml) and EtOH (25 nM) or left unstimulated for 45 minutes. Cellular extracts were obtained after cell lysing, protein quantified, and equal amounts of lysates were used to test activated levels of the p65 subunit with antibody directed against the subunits bound to the oligonucleotide containing the NFkB consensus binding site. A HeLa cell extract (■) was used as a positive control alone or in the presence of wild-type or mutated consensus oligonucleotide. Notched boxes represent 25th and 75th percentile values; the line in the middle and the vertical lines correspond to the median value and both the 10th and 90th percentiles. (* P = 0.0043; †‡ P 0.0087; n 5 experiments). P values by the Mann-Whitney test.*

0.0060 by Mann-Whitney non parametric test, n = 7 experiments) (Fig. 1B, C). CD83-immunoreactive cells were nevertheless more numerous and CD80, CD86 and CD40 expression was stronger in alc-DCs than in alc-iDCs (P = 0.0180), suggesting their partial maturation.

Chemokine receptor analysis showed that CCR7 was expressed in smaller amounts in alc-DCs than in control-DCs (MFI: 46±20 vs 23±18, P = 0.0430) (data not shown). In accordance with this finding, fewer alc-DCs than control-DCs migrated in response to MIP-3β/CCL19 (P = 0.0006, n = 7 experiments) (Fig.1D). Flow cytometric analysis of antigen endocytosis showed that alc-DCs and control DCs took up similar amounts of FITC-dextran antigen (data not shown). Immunological measures were similar in patients with negative and positive BAC values and we found no correlation between BAC values and immunological variables (data not shown).

In vitro ethanol treatment impairs full DC maturation

In alc-iDCs and control-iDCs, in vitro alcohol exposure further impaired final LPS-stimulated DC maturation (Fig. 1 B, C, D). In dose-response experiments the lowest EtOH dose able to impair control-iDC maturation was 25 nM, equivalent to

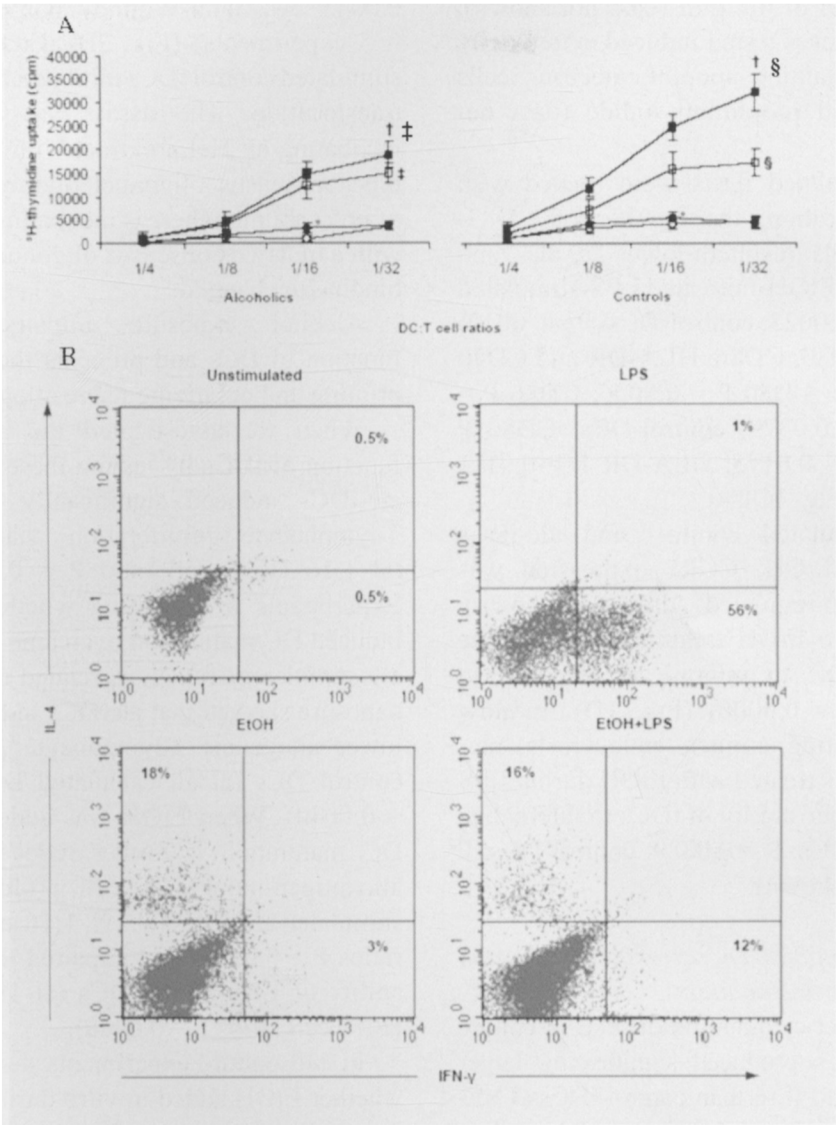

Fig. 3. *Alcohol exposure impairs allostimulatory function of DCs and prevents them from correctly priming and polarizing naïve allogeneic T cells. (**A**) Five-day human DCs were stimulated with LPS (100 ng/ml) (■), EtOH (25 nM) (●), LPS plus EtOH (□) or left unstimulated (○). After 18 hours, DCs were extensively washed and cultured with allogeneic T lymphocytes (1 x 10^5 cells/well) for 3 days at various stimulator-responder ratios (1:4 to 1:32 DC/T). Proliferation of allogeneic T cells was measured by ^{3}H-methyl-thymidine incorporation. Data are presented as mean cpm ± SD of seven independent experiments. (at 1:4, 1:8 and 1:16 DC/T cell ratio, alc-iDCs vs control-iDCs: $^*P = 0.0027$; at all DC/T cell ratio, alc-DCs vs control-DCs: $^\dagger P = 0.0040$; at 1:8, 1:16 and 1:32 DC/T cell ratio, EtOH-treated alc-DCs vs alcohol-DCs and EtOH-treated control-DCs versus control-DCs: $^{\ddagger\,\S}P = 0.0280$). P values by the Mann-Whitney test. (**B**) DCs derived from healthy subjects were stimulated with LPS (100 ng/ml) and/ or EtOH (25 nM) or left unstimulated for 18 hours. A total of 5 x 10^4 DCs were used to stimulate 1 x 10^6 allogeneic naïve negatively selected CD4+CD45RA+ T cells. Activated T cells were expanded with rhIL-2 (30 U/ml). On day 10, T-cell lines were stimulated with PMA and ionomycin for 4 hours in the presence of brefeldin A. Cells were stained with anti-hu-CD3PerCP and processed for intracellular labelling with anti-hu-IFN-γ-FITC and anti-hu-IL-4-PE. The numbers show the percentage of activated CD3+ cells producing the cytokine. Samples were analyzed on a FACSCanto cytofluorimeter using CELLDiva software (BD Biosciences). The figure shows a representative experiment from five with similar results.*

a blood alcohol level of 0.1 g/dl (data not shown). None of the EtOH doses tested induced cytotoxicity as determined by staining apoptotic/necrotic cells with annexin V and propidium iodide (data not shown).

When LPS-stimulated iDCs were treated with EtOH during maturation, the number of CD83-immunoreactive cells resulted lower in alc- and control-DCs than in EtOH-untreated LPS-stimulated DCs (alc-DCs: P = 0.0023; control-DCs: P = 0.0012) (Fig. 1B), as did CD80, CD86, HLA-DR and CD40 expression (alc-DCs: CD80 P = 0.0006; CD86 P = 0.0111; CD40 P = 0.0379; control-DCs: CD80 P = 0.0006; CD86 P = 0.0175; HLA-DR P = 0.0111 CD40 P = 0.0262) (Fig. 1C).

When LPS-stimulated control- and alc-iDCs were treated with EtOH, CCR7 expression was not significantly up-regulated (data not shown). Accordingly, in vitro EtOH treatment reduced the ability of control-DC to migrate in response to MIP-3β/CCL19 (P = 0.0006) (Fig. 1D). In flow cytometry investigating antigen endocytosis, alc-DCs and control-DCs treated with EtOH during LPS maturation retained the ability of iDCs to internalize FITC-dextran (alc-DCs: P = 0.0389; control-DCs: P = 0.0389) (data not shown).

Alcohol exposure impairs DC cytokine production and NF-κB nuclear translocation

In ELISA to detect cytokine production in culture supernatants, alc-iDCs produced significantly larger amounts of TNF-α and IL-6 than control-iDCs (TNF-α: P = 0.031 and IL-6: P = 0.0181) (Fig. 2A). When alc- and control-iDCs were stimulated with LPS, the production of all cytokines increased but alc-DCs produced significantly lower amounts of IL-12p70 and TNF-α than control-DCs (P = 0.0350 and P = 0.0253). Alc-iDCs stimulated with LPS and treated with EtOH produced significantly lower amounts of IL-12p70 and IL-10 and higher levels of IL-6 than EtOH-untreated alc-DCs (P = 0.0111, P = 0.0041 and P = 0.0041). TNF-α production remained unchanged. Control iDCs stimulated with LPS and treated with EtOH produced lower amounts of IL-12 and TNF-α and higher amounts of IL-6 than EtOH-untreated control DCs (P = 0.0012, P = 0.0023 and P = 0.0006).

LPS-stimulated control-iDCs showed significant massive NF-κB p65 nuclear translocation (P = 0.0043 by Mann-Whitney non-parametric test, n = 5 experiments) (Fig. 2B). EtOH added to LPS-stimulated control iDCs inhibited NF-κB p65 nuclear translocation. The assay was specific, because incubating an HeLa extract with an unbound wild-type consensus oligonucleotide abolished binding of p65 subunit, whereas incubating the HeLa extract with a mutated consensus oligonucleotide left NFκB binding unchanged.

Alcohol exposure impairs allostimulatory function of DCs and prevents them from correctly priming and polarizing naïve allogeneic T cells

When we investigated the antigen-presenting function of iDCs by testing these cells in an MLR, alc-iDCs induced significantly lower allogeneic T-lymphocyte proliferation than control iDCs (at 1:16 DC/T cell ratio P = 0.0027) (Fig. 3A). Experiments investigating whether in vitro LPS-induced DC maturation overcame the iDC defective allostimulatory functions related to chronic alcohol exposure showed that alc-DCs induced significantly lower allogeneic T-lymphocyte proliferation than control DCs (at all calculated DC/T cell ratios P = 0.0040). When EtOH was added *in vitro* during DC maturation it further reduced the degree of alloantigen-induced T cell proliferation in LPS-stimulated alc-iDCs (at 1:8, 1:16 and 1:32 DC/T cell ratios P = 0.0280) and impaired the allostimulatory ability of LPS-control-DCs (at 1:8, 1:16 and 1:32 DC/T cell ratios P = 0.0280).

In cell culture experiments designed to find out whether EtOH added in vitro during DC maturation impaired the ability of DCs to prime and polarize allogeneic naïve CD4+ CD45RA+ into typical T helper type 1 (Th1) cells, cytofluorimetric analysis showed a smaller number of IFN-γ-producing T cells and a larger number of IL-4-producing cells in control-iDCs stimulated with LPS and treated with EtOH than in samples stimulated with LPS and EtOH-untreated (12% *vs* 56% and 16% *vs* 1%) (Fig. 3B). Most naïve T çells (18%) co-cultured with EtOH-treated control-iDCs turned into typical IL-4 producing T cells (Th2). Only a small percentage (3%) differentiated into IFN-γ-producing T cells.

DISCUSSION

In this study we provide new information showing

that *in vivo* chronic alcohol exposure prevents monocytes from differentiating and maturing into functional DCs. We also provide *in vitro* evidence that acute ethanol treatment acts directly on differentiated iDCs generated from alcohol-dependent patients and healthy controls, preventing them from maturing correctly in response to the microbial stimulus LPS. An unexpected finding was that, instead of correctly priming and polarizing naïve T lymphocytes into Th1 cells, *in vitro* EtOH–treated control iDCs stimulated with LPS turned them into Th2 cells.

iDCs from alcoholics and healthy controls differed in both phenotype and function. Despite the phenotypical and functional impairments, characterized by reduced allostimulatory and endocytic ability, alc-iDCs produced the proinflammatory cytokines TNF-α and IL-6 in large amounts in accordance with the high circulating levels of proinflammatory cytokines found in alcoholics (19-20). Alcohol may contribute to proinflammatory activation of monocytes (20-23) by regulating cytokine secretion at a post-transcriptional level and by increasing intestinal permeability to bacterial LPS. Microbial LPS stimulation of alc-iDCs only partially restored the alc-iDC impairment, thus inducing a semi-mature phenotype (24). In accordance with their semi-mature phenotype, alc-DCs were less able than control-DCs to migrate in response to the chemokine MIP-3β/CCL19, a typical feature of mature DCs (18).

Several findings in our study provide evidence that *in vitro* acute alcohol exposure directly prevents DCs from maturing. These findings partially agree with results from Mandrekar et al (8). After *in vitro* EtOH exposure, maturation was more severely impaired in DCs from chronic alcoholics than in DCs from healthy subjects. The high inflammatory response (IL-6 production) shown by DCs from alcoholics probably reflects *in vivo* chronic alcohol exposure of monocytes. The degree of endotoxemia in alcoholics would also have a profound effect upon their immune status.

T-cell priming experiments showed that DCs, treated with EtOH during maturation, polarized naïve T lymphocytes into IL-4-producing Th2 cells. Evidence that EtOH polarizes the immune response toward Th2-driven humoral immunity is reported in alcoholics (25). We hypothesize that phenotypical and functional changes induced by *in vitro* EtOH exposure of control iDCs, even though quantitatively small, together with other possible ethanol-induced DC changes, may be responsible for the observed Th2 cell polarization. A possible mechanism to explain why Th2 cells increased is that EtOH inhibited IL-12 production (26), thus inducing an IL-12/IL-10 imbalance that may promote a tolerogenic state in alcoholics.

A limitation of our study is the small number of patients recruited. Unfortunately, the large amount of blood needed for the study (50-60 ml) makes it hard to find patients who give their consent to enrolment. Furthermore, most alcohol dependent-patients were excluded from the study because they had infections or liver disease, or both, and many were polydrug abusers. Another limitation is that we assessed the effects of acute alcohol exposure only in *in vitro* experiments. Although in our study the immunological measures in chronic alcoholics were similar in patients with negative and positive BAC values and we found no correlation between BAC values and immunological variables, we cannot exclude the probability that also acute alcohol intake may in part contribute to immunological impairments. To fully clarify the effects of chronic alcohol intoxication further studies are also necessary to characterize DCs derived from alcoholics who had stopped alcohol intake at least one year previously.

In conclusion we provide evidence that chronic alcohol exposure affects the ability of monocyte-derived DCs to respond properly to a pathogen-related molecule such as LPS confirming that alcoholics are less able than other individuals to control infections and cancer (26-27), and respond to vaccinations (13, 28). Our evidence that EtOH polarizes the immune response toward Th2-driven immunity, deregulating the Th1 response may explain the immunological impairments observed in alcohol abusers (1, 3, 29-30).

Overall, our results in this study, along with previous findings, again underline the immunological effects that alcohol exposure exerts on innate immune response, thus leading to immune deregulation. Knowing the mechanisms of immune deregulation caused by chronic and acute alcohol exposure may help to design innovative strategies for manipulating immune responses thus improving

immunity to pathogens or tumors, or responses to vaccinations in alcoholics.

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