Smoking promotes exacerbated inflammatory features in dendritic cells of Chilean rheumatoid arthritis patients

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ABSTRACT

Background: *The dual potential to promote tolerance or inflammation when facing self-antigens makes dendritic cells (DCs) fundamental players in autoimmunity. There is an association between smoking and DCs maturation in patients with rheumatoid arthritis (RA). However, ethnicity is a key factor in autoimmune disorders.* **Aim**: *To evaluate phenotypic and functional alterations of DCs obtained from Chilean patients with RA as compared to healthy controls (HC). In second term, to compare the inflammatory behaviour of DCs between smoker and non-smoker patients.* **Material and Methods**: *Monocyte-derived DCs and T-cells were obtained from blood samples isolated from 30 HC and 32 RA-patients, 14 of which were currently smokers and 18 non-smokers. Several maturation surface markers were evaluated in DCs, including HLA-DR, CD40, CD80, CD83 and CD86. Furthermore, autologous co-cultures of DCs and T-cells were carried out and then T-cell proliferation, and expansion of Th1, Th17 and Tregs were analysed.* **Results**: *Compared with HC, RA-patients displayed increased HLA-DR expression in DCs, which was manifested mainly in patients with moderate-to-high disease activity scores (DAS28). Furthermore, RA-patients presented a stronger Th17-expansion and a correlation between DAS28 and Th1-expansion. Both effects were mainly observed in patients in remission or with a low DAS28. Moreover, smoker RA-patients displayed enhanced HLA-DR and CD83 expression in DCs as well as an exacerbated Th17-expansion and a correlation between DAS28 and Th1-expansion.* **Conclusions**: *These findings suggest that smoking enhances the inflammatory behaviour of DCs and the consequent Th1 and Th17-mediated response in patients with RA*
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Key words: *Arthritis; Autoimmunity; Dendritic Cells; Smoking; T-Lymphocytes.*

El tabaco exacerba características inflamatorias en células dendríticas de pacientes chilenos con artritis reumatoide

Introducción: El potencial dual que poseen para promover tolerancia o inflamación ante antígenos propios, hace de las células dendríticas (CDs) actores fundamentales en el desarrollo de autoinmunidad. Existe una asociación

entre fumar y la maduración de las CDs en pacientes con artritis reumatoide (AR). No obstante, la etnicidad es un factor clave a considerar en desórdenes autoinmunes. **Objetivos:** *Comparar las alteraciones fenotípicas y funcionales de las CDs obtenidas desde pacientes Chilenos con AR y controles sanos (CS). Además, analizamos las diferencias en el comportamiento inflamatorio que existe entre las CDs obtenidas de pacientes fumadores y CS.* **Materiales y Métodos:** *Se obtuvieron CDs derivadas de monocitos y células T desde muestras de sangre aisladas de 30 CS y 32 pacientes con AR, 14 de los cuales eran fumadores y 18 no fumadores. Se evaluaron marcadores de maduración en la superficie de las CDs: HLA-DR, CD40, CD80, CD83 y CD86. Además, se realizaron co-cultivos autólogos de células T y CDs, analizando la proliferación de células T, y la expansión de células Th1, Th17 y Tregs.* **Resultados:** *En comparación con los CS, los pacientes AR mostraron un aumento de la expresión de HLA-DR en las CDs, principalmente en los individuos con DAS28 moderado-alto. Los pacientes con AR presentaron una mayor expansión de células Th17 y una correlación entre el DAS28 y la expansión de células Th1, ambos efectos manifestados principalmente en los individuos con un DAS28 bajo o en remisión. Además, los pacientes con AR fumadores mostraron un aumento en la expresión de HLA-DR y CD83 en las CDs y una expansión de células Th17 exacerbada así como una correlación entre el DAS28 y la expansión de células Th1.* **Conclusiones:** *Nuestros resultados sugieren que fumar favorece el comportamiento inflamatorio de las CDs y en consecuencia la inducción de respuestas mediadas por células Th1 y Th17 en los pacientes Chilenos con AR.*

To prevent autoimmune reactions, auto-reactive T-cells have to be eliminated or rendered tolerant, a process in which dendritic cells (DCs) play a fundamental role. In fact, constitutive depletion of DCs results in spontaneous fatal autoimmunity[1]. Moreover, during onset of autoimmunity DCs seem to be pivotal for priming of auto-reactive T-cells that have escaped of tolerance induction[2]. This dual potential of DCs promoting tolerance or inducing inflammation to auto-antigens, makes these cells fundamental players in the physiopathology of autoimmunity.

Evidence has shown that the duality of DCs functionality is defined by the expression of surface molecules and secreted mediators. In this regard, the mild expression of surface class II MHC (MHC-II), CD40, CD83 and of the co-stimulatory molecules CD80 and CD86 is associated with the tolerogenic potential, whilst high surface density of all these molecules is indicative of the inflammatory potential of DCs[3]. In addition, whereas tolerogenic DCs produce retinoic acid and the anti-inflammatory cytokine IL-10 that favour generation of regulatory T-cells (Tregs) with suppressive activity[4,5], DCs with inflammatory potential produce IL-12 and IL-23, which are required respectively to the acquisition of the inflammatory phenotypes T-helper-1 (Th1) and Th17 by naïve CD4+ T-cells[6,7]. Of note, previous evidence has shown that Th1 as well as Th17 cells

are the phenotypes of auto-reactive CD4^{+} T-cells involved in autoimmunity, including rheumatoid arthritis (RA) and its mouse model, the collagen-induced-arthritis (CIA)[8,9]. Indeed, due to that functional phenotype of DCs represent a key point of decision to promote tolerance or inflammation in response to specific antigens, the use of tolerogenic DCs loaded with auto-antigens as therapy for the treatment of RA is currently under clinical trials[10,11].

Accumulating evidence suggests that RA develops in genetically predisposed individuals after exposure to environmental triggers. In this regard, it has been shown that polymorphisms of various genes codifying for molecules involved in immunity, such as MHC-II, inflammasome or inflammatory cytokines are differentially distributed depending on the ethnicity[12-14]. Thus, these findings suggest that different ethnicity should affect the immune system behaviour in response to conditions representing risk factors triggering autoimmunity.

Among the environmental risk factors for RA one of the most documented is tobacco, a factor that favours the citrullination of self-constituents, which represent a major source of auto-antigens in RA[15,16]. Importantly, some studies have suggested that smoking has an impact on the functionality of DCs and their capacity to induce Th1/Th17-mediated responses in RA-patients[17-19]. Moreover, previous data has shown that nicotine promotes the activation of NF-κB, increasing the pro-inflammatory potential of antigen-presenting cells[20,21].

Despite there are previous studies analysing how DCs phenotype is altered in Chilean RA-patients[22], the impact of smoking in the phenotype of DCs in relation to the severity of disease manifestation in Chilean RA-patients has not been previously addressed. In this study, we evaluated how is the functional phenotype of DCs affected in Chilean RA-patients and how tobacco impacts in DCs functionality and disease manifestation in those patients.

Materials and Methods

Subjects

Thirty-two female RA-patients were included in the study, born in Chile with Chilean parents who met the 1987 or 2010 American College of Rheumatology (ACR) classification criteria for RA[23,24]. All were outpatients from one clinical network consisting in: University Hospital of the Pontificia Universidad Católica de Chile and San Joaquín Medical Center. Foreigners or patients with foreign parents, or those who had clinical overlap with other autoimmune diseases were excluded. Clinical description of our patients is shown in Table 1. Treatment of patients analysed included methotrexate with doses ranging between 10 and 25 mg weekly; prednisone with doses ranging between 0 and 20 mg per day; sulfasalazine with doses ranging between 2,100 and 700 mg daily; and hydroxychloroquine at a dose of 200 mg daily. No patients under therapy with biological agents were included in this study. All patients who agreed to participate in this study signed an informed consent and, once recruited, we proceeded to obtain demographic and clinical data by questionnaire and assessment by coordinators and rheumatologists. Thirty healthy female with an average age of 42 ± 13 years were included as HC,

Table 1. Demographic features, disease activity and laboratory parameters in Female Rheumatoid Arthritis (RA) patients and Healthy Controls (HC)

	RA patients (n = 32)
Age (years)	50,00 ± 12,63
Current Smokers (+/-)	14/18
Disease Duration (years)	6,03 ± 5,54
DAS28 (mean ± sd)	4,03 ± 1,88
DAS28 Frequency	
Remission, < 2,6 (n)	8
Mild, 2,6 < 3,2 (n)	6
Moderate, 3,2 < 5,1 (n)	7
Severe, > 5,1 (n)	11
ESR[1] (mm/1st h)	38,84 ± 3,07
CRP[1] (mg/dL)	848,4 ± 923,20
RF[1] (IU/mL)	387,8 ± 773,3
Treatment	
Methotrexate (+/-)	20/12
Prednisone (+/-)	14/18
Azulfidine (+/-)	08/24

[1]ESR: erythrocyte sedimentation rate, CRP: c-reactive protein, RF: rheumatoid factor.

which were summoned to an interview in order to explain the study. The study was conducted over a period of 18 months and was approved by the local University Ethics Committee.

Variables ascertained

Data about comorbidities and previous/currents treatments was obtained in the interview and confirmed in their medical record. Serum samples were stored at -80°C to complete study of antibodies. The ACR core of set of variables[25] including the number of swollen joints, the number of painful joints (ascertained by study physicians) and a pain scale were recorded at the visit. Functional status was assessed with the Health Assessment Questionnaire (HAQ); the HAQ is scored on a scale of 0-3, with higher scores indicating higher levels of disability[26]. The intensity of their pain was assessed with a 10 cm Visual Analog Scale (VAS) where 0 is no pain and 10 is the worst possible pain. The HAQ and VAS were self-administered during the patients' scheduled visits. Clinical data and disease duration were obtained, routine blood sample biochemistry, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were measured at the time of the study visit. These parameters also allowed calculating the score of disease activity (DAS28)[27]. Activity was classified as remission (DAS28 < 2.6), low-activity ($2.6 \leq DA28 \leq 3.2$), moderate-activity ($3.2 < DAS28 \leq 5.1$) and high-activity (DAS28 > 5.1). To define 2 groups, we merge remission with low activity and moderate with high activity. Exposure to DMARDs was defined as more than 3-months of use of them without modifications. Methotrexate, glucocorticoids, hydroxychloroquine, cyclophosphamide, azathioprine, mycophenolate mophetil were also recorded. Tobacco was recorded as present or absent in the last 3-months. The laboratory data evaluated the presence of rheumatoid factor (RF) and anti-CCP by immunoturbidimetry and ELISA, respectively.

Antibodies

All fluorochrome-conjugated monoclonal antibodies (mAbs) were purchased from Biolegend (San Diego, CA), including PE-anti-CD11c, allophycocyanin-anti-CD14, allophycocyanin-Cy7-anti-HLA-DR, PE-Cy7-anti-CD40, Brilliant-Violet421-anti-CD80, Alexa-Fluor488-anti-CD83, allophycocyanin-anti-CD86, Brilliant-Violet421-anti-CD3, allophycocyanin-anti-CD4, PerCP-anti-CD25, Alexa-Fluor647-anti-FoxP3, Brilliant-Violet510-anti-IFN-γ, allophycocyanin-Cy7-anti-IL-17, allophycocyanin-anti-Tbet, PE-anti-GM-CSF.

Generation of Monocyte-Derived DCs and autologous co-cultures

Monocyte-derived DCs were differentiated as described[28]. DCs immunophenotypes were analysed by flow cytometry as indicated in Figure S1. Autologous co-cultures were carried out with DCs (purity 88% ≤ CD11c⁺) and with the T-cell enriched fraction obtained from non-adherent PBMCs, as described before[29], and analysed as indicated in Figure S2.

Flow cytometry analyses

For immunostaining of cell-surface markers, cells were washed with PBS, resuspended at 2×10^6 cells/ml (50 µl/tube), stained with the ZombieAqua (Fixable-Viability kit, Biolegend) and incubated with fluorochrome-conjugated mAbs for 30 min at 4 °C. Cells were washed with PBS, fixed with 1% formaldehyde in PBS and analysed by flow cytometry. To analyse expression of transcription factors and production of cytokines, cells were restimulated with 1 µg/mL ionomycin and 50 ng/mL PMA for 4h in the presence of 5 µg/mL brefeldin A. Cells were stained with ZombieAqua, followed by staining for cell-surface markers. Intracellular staining was done with the Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA). Data were collected with a Canto II (BD) and analysed in the ZombieAqua-negative population using the FlowJo software (Tree Star).

Statistical analysis

All values were expressed as mean ± SEM. Differences in means between two groups were analysed by 2-tailed Student's *t*-test. When three or more experimental groups were compared, differences were analysed by one-way ANOVA followed by Dunnett's *post-hoc* test. Correlations between different parameters were analysed by Pearson's test when data was normally distributed or by Spearman's test when data was not normally distributed. Normal distribution was determined by Shapiro-Wilk test. *P* value ≤ 0.05 was considered significant. Analyses were performed with GraphPad Prism 6 software.

Results

DCs obtained from Chilean RA-patients display increased inflammatory features

To evaluate whether the functional phenotype of DCs is altered in RA-patients with respect to HC we aimed to compare key surface markers involved in the functional switch from tolerogenic to inflammatory DCs. For this purpose monocyte-derived DCs were generated and a pool of key surface maturation markers were analysed by flow cytometry in the CD11c^{+} population, including HLA-DR, CD40, CD80, CD83 and CD86. The results show a selective increase in the surface density of HLA-DR on DCs obtained from RA-patients in comparison with those DCs obtained from HC, whilst there were no significant alterations on the other surface markers analysed (Figure 1A). In addition of surface markers we also analysed the functionality of DCs with regard to their capability to stimulate inflammatory or anti-inflammatory CD4^{+} T-cell responses. Accordingly, we performed a set of co-culture experiments in which DCs were loaded with a superantigen (staphylococcal enterotoxin A; SEA), cultured with autologous T-cells and then the general proliferation or the expansion of the suppressive subset Tregs, and inflammatory populations, including Th1 and Th17, were analysed. Interestingly, when correlation analyses were performed (Table S1), a significant association between disease severity (DAS28) and the expansion of Th1 cells (CD3^{+} CD4^{+} Tbet^{+} cells) in co-cultures was found (Figure 1B). Furthermore, whereas the general proliferation (Figure 1C), expansion of Tregs (Figure 1D) or expansion of Th1 (Figure 1E) were unaltered, the generation of Th17 was significantly increased in co-cultures obtained from RA-patients in comparison to those obtained from HC (Figure 1F). Together, these results indicate that DCs obtained from Chilean RA-patients display an increased ability to promote an inflammatory CD4^{+} T-cell response.

Disease activity is not completely associated to the exacerbated inflammatory features of DCs obtained from RA-patients

To gain a deeper characterization of which are the subtypes of RA-patients displaying alterations in the phenotype and function of DCs, we next analysed the key surface maturation markers and the capability to promote expansion of different T-cell subsets on DCs obtained from those RA-patients displaying moderate-to-high-DAS28 (DAS28>3.2; 18 patients) and from those bearing remission or low-DAS28 (DAS28<3.2; 14 patients) in comparison to DCs obtained from HC. These results show that DCs obtained from moderate-to-high-DAS28 RA-patients display a selectively exacerbated up-regulation of surface HLA-DR in comparison to those DCs obtained from HC (Figure 2A). However, unexpectedly the increased capability of DCs to promote expansion of Th17 cells was confined to those DCs obtained from the subgroup of RA-patients displaying remission or low-DAS28 (Figure 2B). Similarly, positive correlations were observed between disease severity and the capability to promote expansion of Th1 cells (analysed as CD3^{+}CD4^{+}Tbet^{+} or CD3^{+}CD4^{+}Tbet^{+}IFN-γ^{+} populations) exclusively in DCs obtained from remission or low-DAS28 RA-patients (Figure 2C and Table S1). Taken together, these results indicate that exacerbated inflammatory features observed in Chilean RA-patients are not clearly associated to the disease severity. Thereby, these findings suggest that other parameters different to disease severity should be related to the higher inflammatory potential of DCs obtained from RA-patients.

Selective increase of inflammatory features of DCs obtained from smoker RA-patients

Since smoking has been associated to the alteration of inflammatory parameters in antigen-presenting cells obtained from RA-patients with other ethnicities[17,30], we next analysed whether the alterations observed in the inflammatory potential of DCs observed in Chilean RA-patients (Figure 1) were also related with this environmental risk factor. When we compared the extent of expression of surface maturation markers and the parameters determined in co-culture experiments between the subgroups of smoker RA-patients and non-smoker RA-patients we did not find significant differences (data not shown). However, co-cultures obtained from smokers RA-patients revealed an exacerbated expansion of Th17 cells with an evident trend (p = 0,0612) in comparison to those co-cultures obtained from non-smokers RA-patients (Figure 3A). In agreement with this observation, when the inflammatory features of DCs were compared between HC and the subgroup of smoker RA-pa-
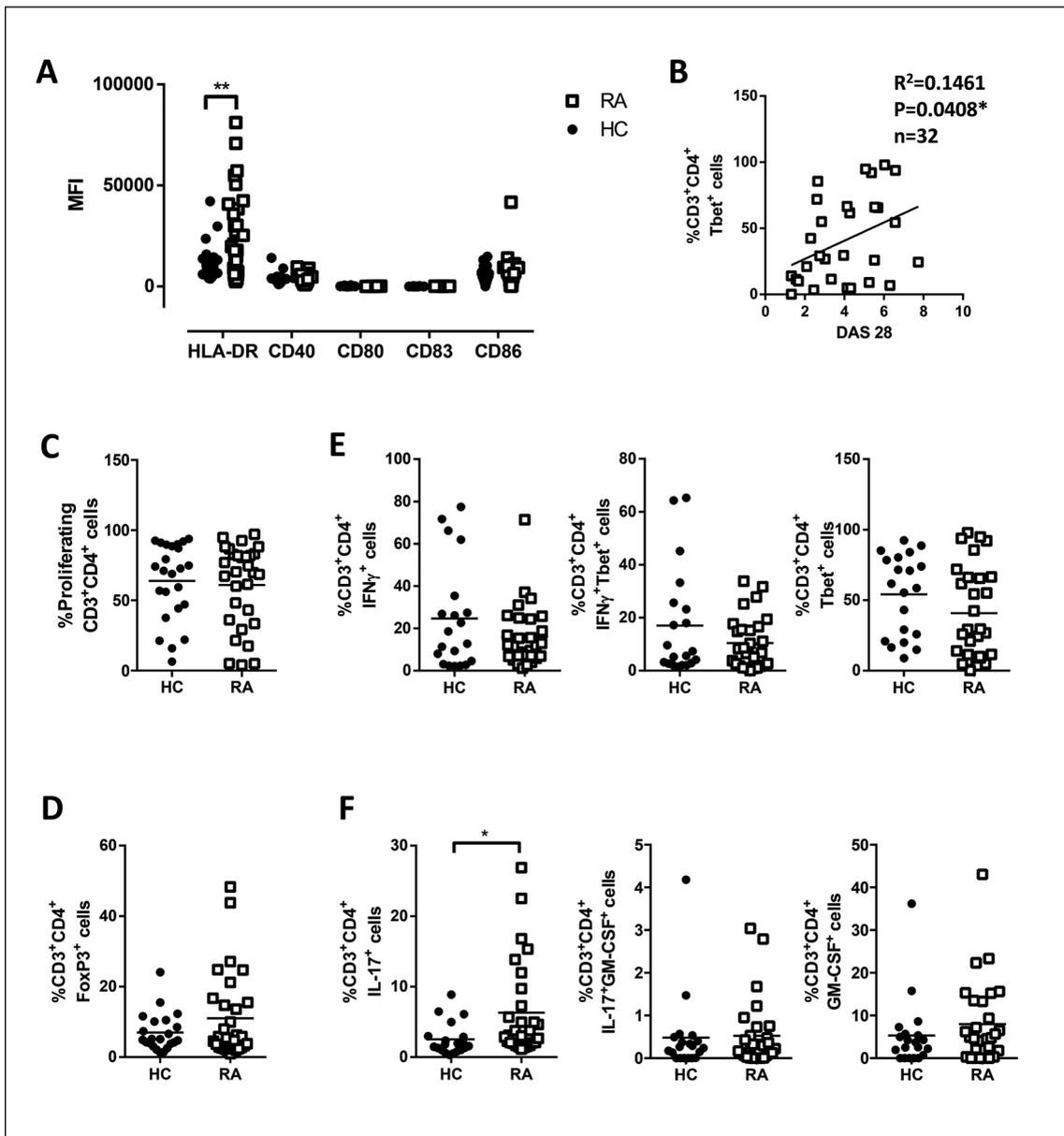
Figure 1. DCs obtained from RA patients display a selective up-regulation of HLA-DR on the cell surface and increased capacity to promote inflammatory phenotypes in CD4+ T-cells. Monocyte-derived DCs were obtained from peripheral blood from healthy control (HC) or Rheumatoid Arthritis (RA) patients. After 6 days of culture in presence of GM-CSF and IL-4, we determined the expression of several maturation markers by flow cytometry. (**A**) The surface density of maturation markers was quantified as the MFI of the marker confined to the CD11c+ DCs population. (B-F) DCs obtained from HC or RA patients were pulsed with *staphylococcus aureus* enterotoxin A (SEA) 1 ng/mL for 1h. Then DCs were washed and co-culture with autologous T-cells at 1:5 ratio for 7 days. (**B**) The correlation between DAS28 from all patients and Tbet+CD3+CD4+ cells frequency was analysed. (**C**) Dilution of Cell trace Violet (CTV) fluorescence was assessed in the CD3+CD4+ population by FACS. (**D**) Tregs generation was determined by expression of FoxP3, (**E**) Th1 polarization was determined by Tbet and IFN-γ expression and (**F**) Th17 polarization was determined by expression of GM-CSF and IL-17 in the CD3+CD4+ population. Values represent mean ± SEM, with 32 RA patients (grey symbols) and 30 HC (black symbols). Data was analysed by One-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test (A), Spearman's correlation analysis (B) and Student's *t*-Test (C-F).*, p < 0,05;**, p < 0,01.
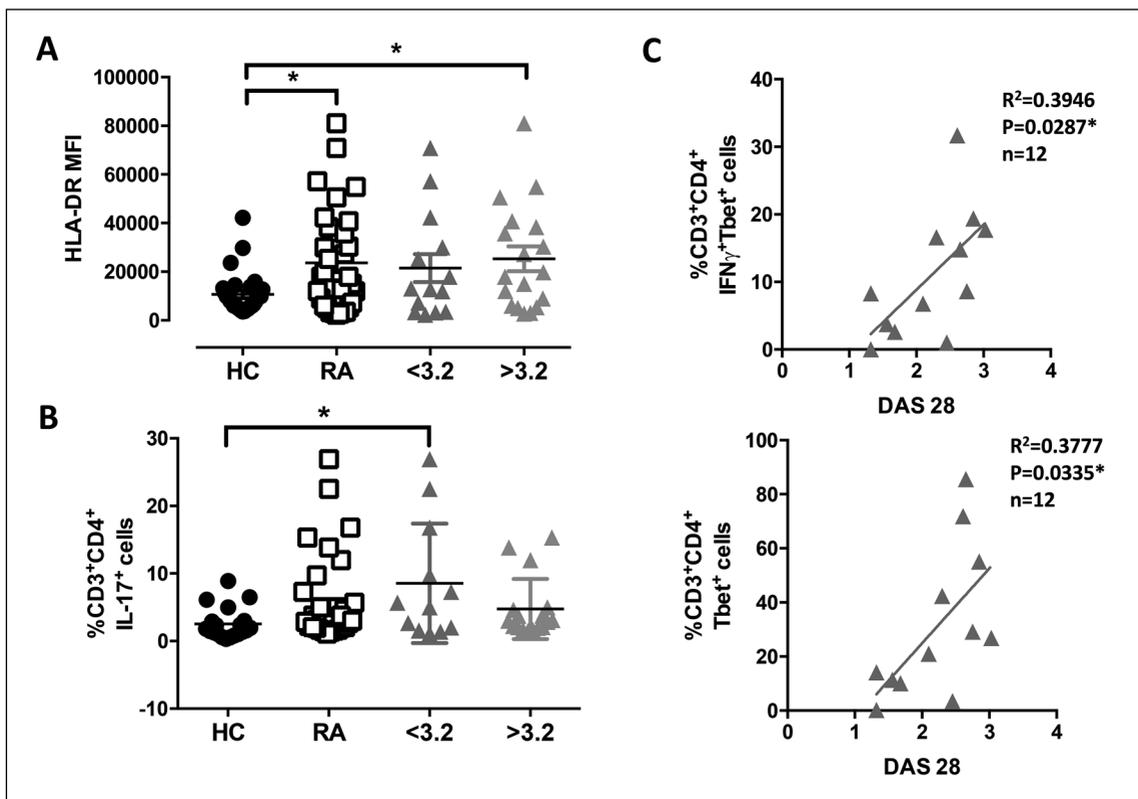
Figure 2. Up-regulation of HLA-DR in DCs from RA patients is explained by high-DAS28 patients, whilst increased capacity to promote inflammatory phenotypes in CD4+ T-cells is confined to low-DAS28 patients. Monocyte-derived DCS and autologous co-cultures of DCs with T-cells were obtained from peripheral blood from healthy control (HC) or Rheumatoid Arthritis (RA) patients as described in figure 1 legend. Data was analysed in a sub-group of RA patients with DAS28 lower than 3.2 (< 3.2, grey triangles) and higher than 3.2 (> 3.2, black triangles) and compared with total RA patients and HC. (**A**) The surface density of HLA-DR was quantified as the MFI of the marker confined to the CD11c^{+} DCs population. (**B**) Th17 polarization was determined by the expression of IL-17 in the CD3^{+}CD4^{+} population. (**C**) The correlation between DAS28 from low-DAS28 patients and Th1 cells was analysed as the frequency of Tbet^{+}CD3^{+}CD4^{+}IFN-γ^{+} cells (top panel) or Tbet^{+}CD3^{+}CD4^{+} cells (bottom panel). Values represent mean ± SEM, with 32 RA patients (grey squares), 14 low-DAS28 RA patients (light grey triangles), 18 high-DAS28 RA patients (dark grey triangles) and 30 HC (black symbols). Data was analysed by One-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test (A and B) and Spearman's correlation analysis (C).*, p < 0,05.

tients, we found a significant increased expansion of Th17 response (Figure 3B) and exacerbated surface density of the maturation markers HLA-DR and CD83 (Figure 3C-D). No differences were found when the other surface maturation markers and parameters determined in co-culture experiments were analysed (data not shown). Interestingly, when maturation surface markers were compared in HC and RA-patients in iDCs and mDCs, we observed that HLA-DR density was exacerbated in both smoker and non-smoker RA-patients in iDCs, whilst after DCs maturation

exacerbated HLA-DR density was maintained only in smoker RA-patients (Figure S3). It is noteworthy that after DCs maturation CD86 density becomes increased in both smoker and non-smoker RA-patients (Figure S3). Furthermore, we analysed the association of clinical score with the different parameters associated to the inflammatory potential of DCs in the subgroup of smoker RA-patients and we found a significant correlation of DAS28 with the induction of Th1 cells in autologous co-cultures (Figure 3E). To rule out the possibility that exacerbated
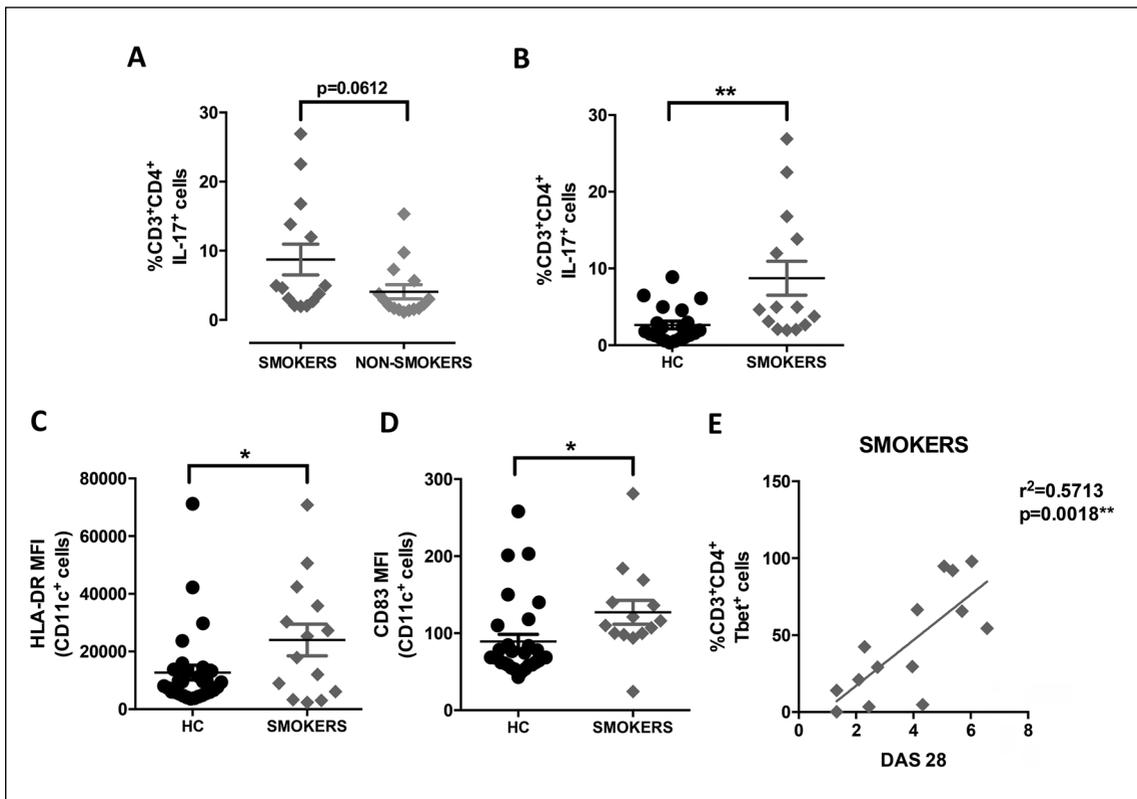
Figure 3. Selective increase in surface maturation markers in DCs and in the frequency of Th17 cells induced in autologous DC-T co-cultures obtained from smoker-RA patients. Monocyte-derived DCs and autologous co-cultures of DCs with T-cells were obtained from peripheral blood from healthy controls (HC) and Rheumatoid Arthritis (RA) patients as described in figure 1 legend. Data was analysed in the sub-group of smoker (+) or non-smoker (-) RA patients. (**A**) Th17 polarization was determined by the expression of IL-17 in the $CD3^+CD4^+$ population in co-cultures obtained from smoker or non-smoker RA patients. (**B**)Th17 polarization was determined by the expression of IL-17 in the $CD3^+CD4^+$ population in co-cultures obtained from HC or from the sub-group of smoker-RA patients. (C-D) The surface density of HLA-DR (**C**) and CD83 (**D**) was quantified as the MFI of the marker confined to the $CD11c^+$ DCs population in HC and smoker-RA patients. Values represent mean ± SEM, with 14 smoker-RA patients (open squares), 18 non-smoker RA patients (grey squares) and 30 HC (black circles). (**E**) The correlation between DAS28 from smoker RA patients and $Tbet^+CD3^+CD4^+$ cells frequency. Data was analysed by Student's t-Test (A-D) or Spearman's correlation analysis (E). *p < 0,05; **, p < 001.

inflammatory parameters observed in T-cells in co-culture experiments were due to alterations in CD4+ T-cells rather than DCs, we analysed how were the phenotypes of CD4+ T-cells obtained from fresh peripheral blood of RA-patients. The results show no significant differences in the frequencies of inflammatory and suppressive phenotypes of CD4+ T-cells among HC, smoker and non-smoker RA-patients (Figure S4). Thus, these results indicate that tobacco favours the exacerbation of the inflammatory potential of DCs in several aspects in Chilean RA-patients.

Discussion

This study shows a selective effect of tobacco favouring the inflammatory features of DCs in Chilean RA-patients. Of note, this is the first work analysing how smoking impacts on the inflammatory behaviour of DCs in a group of Chilean RA-patients.

Despite a recent study has shown no differences in HLA-DR expression in DCs obtained from Chilean RA-patients and HC[22], our data here shows a strong exacerbation of HLA-DR density

in DCs obtained from Chilean RA-patients in comparison to Chilean HC (Figure 1A). This discrepancy could be due to the different sources of monocytes obtained to be differentiated into DCs: Whereas García-González obtained monocytes from leukopheresis from RA-patients and from buffy-coats from HC[22], here we obtained monocytes from fresh blood samples from RA-patients and HC. Since smoke has been proposed to represent one of the main environmental factors exacerbating disease manifestations and triggering RA[15,16], we decided to analyse our results confined to the subgroup of smoker and non-smoker RA-patients. Despite smoker and non-smoker RA-patients present similar socio-demographic and clinical features (Table S2), we found that cigarette significantly favours an inflammatory phenotype on monocyte-derived DCs with a consequent increase in Th17 response in Chilean RA-patients (Figure 3). In contrast, a previous study addressing the link between smoke and the inflammatory potential of DCs found that cigarette reduced the production of IL-6 by monocyte-derived DCs, which was associated to a decreased Th17 response in New Zealander RA-patients[17]. Discrepancies obtained between this study and our present results might be due to different ethnicity of RA-patients in both studies, a key factor conditioning the response of immune system to environmental factors[12-14]. Supporting this hypothesis, a recent study performed in transgenic mice lacking endogenous MHC-II and bearing the human RA-associated alleles HLA-DRB1*0401 or DQA1*0301/DQB1*0302 showed that exposition of HLA-DRB1*0401-bearing mice to cigarette smoke reduces CIA severity, whilst DQA1*0301/DQB1*0302-bearing mice exposed to cigarette smoke exacerbated CIA manifestation[30]. Studies in Chilean RA-patients show high frequency of the HLA-DRB1*0403 allele[31], which could account for the differences observed.

Material suplementario en versión digital de la Revista Médica de Chile en https://scielo.conicyt.cl

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