Increased, but Functionally Impaired, CD14⁺ HLA-DR⁻/low Myeloid-Derived Suppressor Cells in Psoriasis: A Mechanism of Dysregulated T Cells

David C. Soler¹, Andrew B. Young¹, Lori Fiessinger¹, Fabrizio Galimberti¹, Sara Debanne², Sarah Groff¹, Thomas S. McCormick¹,³ and Kevin D. Cooper¹,³,⁴

The clinical extent of psoriasis pathology is regulated in part by defects in immune networks, including a defect in the suppressive actions of regulatory T cells. Recently, CD14⁺ HLA-DR⁻/low monocytic myeloid-derived suppressor cells (Mo-MDSCs) have been shown to suppress T-cell activation as one of their suppressive mechanisms. However, little is known about the role of Mo-MDSCs and their functional relationship to T-cell suppression in relation to human chronic immune-mediated inflammatory diseases, including psoriasis. Despite psoriasis being a hyperinflammatory condition, Mo-MDSCs were elevated in psoriatic patient peripheral blood mononuclear cells compared to nonpsoriatic healthy controls (2.6% vs. 0.9%, P < 0.002). Freshly isolated psoriatic Mo-MDSCs directly suppressed CD8 T-cell proliferation less efficiently than healthy control Mo-MDSCs. In addition, psoriatic Mo-MDSCs expressed reduced surface expression of programmed cell death protein 1 compared to healthy controls. Additional in vitro assays also demonstrated that psoriatic and control Mo-MDSCs both induce regulatory T-cell conversion from naive T effector cells, but, importantly, the regulatory T cells induced by psoriatic Mo-MDSCs displayed decreased suppressive functionality. These results suggest that aberrations in psoriatic Mo-MDSCs prevent proper suppression of effector T-cell expansion and hamper the immune system’s ability to correctly self-regulate.


INTRODUCTION
Psoriasis is a highly prevalent (1–3% of Caucasian populations) chronic immune-mediated inflammatory disease of the skin that is modified by susceptibility genes and environmental triggers. In addition to an enormous negative impact on quality of life, psoriasis has co-morbidities, including destructive psoriatic arthritis, stigmatization, depression and anxiety, inflammatory bowel disease, lymphoma, obesity, metabolic syndrome-associated conditions, and increased risk of early death from cardiovascular disease (Davidovici et al., 2010; Gelfand et al., 2010, 2011; Mehta et al., 2010, 2011a, 2011b). The advanced state of validation of specific psoriasis pathogenesis pathways via biologic therapies provides a unique opportunity to link pathomechanisms of an immune-mediated inflammatory disease with cellular mediators. Thus, psoriasis is dependent on activated memory effector T cells [alefacept (Ellis and Krueger, 2001; Sugiyama et al., 2008), DAB(389)IL-2 (denileukin diftitox, ONTAK) (Martin et al., 2001), cyclosporin A (Ellis et al., 1986), lymphocyte function-associated antigen 1–expressing leukocytes (myeloid cells and T cells) (Boehncke, 2007; Giblin and Lemieux, 2006; Hodulik and Hadi, 2006; Shear et al., 2006), tumor necrosis factor-producing cells (monocytes, T cells, others), IL-23 (monocytes and dendritic cells), and IL-17A and IL-17F and their receptor (Lebwohl et al., 2012; Leonardi et al., 2008; Papp et al., 2012)]. However, the immunocellular mechanisms implicated in psoriasis T-cell hyperactivation have not been precisely characterized. We have previously demonstrated that psoriasis impairs the suppressive fitness of regulatory T cells (Tregs) (Sugiyama et al., 2005), particularly through the action of IL-6/signal transducer and activator of transcription-3 (STAT3) (Goodman et al., 2009, 2011). Interestingly, these are also key factors promoting survival and expansion of myeloid-derived suppressor cells (MDSCs) (Wu et al., 2011, 2012).

The association of MDSCs and cancer has been known for more than 2 decades (Strober, 1984; Young et al., 1999), and their increased numbers have been reported to correlate with a poor disease prognosis (Gabittas et al., 2011; Greten et al., 2011; Kitano et al., 2014). Furthermore, recent reports...
have suggested that they may also be abnormal in chronic immune-mediated inflammatory diseases such as arthritis (Fujii et al., 2013), asthma (Boomer et al., 2013; Zhang et al., 2013), and inflammatory bowel disease (Haile et al., 2008). MDSCs represent an intrinsic part of the myeloid cell lineage and are described as a heterogeneous population (Gabrilovich and Nagaraj, 2009; Gabrilovich et al., 2007; Talmadge and Gabrilovich, 2013). Traditionally, MDSCs are classified into granulocytic (CD33+, CD15+, CD14-, CD11b+; HLA-DRlow) or monocytic (CD33+, CD15-, CD14+, CD11b+, HLA-DRlow) subpopulations (Condamine and Gabrilovich, 2011; Gabrilovich and Nagaraj, 2009; Solito et al., 2014).

Myeloid cells are produced in the bone marrow, and in healthy individuals they quickly differentiate into mature granulocytes, macrophages, or dendritic cells. However, under pathological conditions such as cancer, myeloid differentiation can be altered, resulting in an expanded MDSC population found in peripheral blood. Several transcription factors have been linked to MDSC function and regulation (Lechner et al., 2011a, 2011b), including signal transducer and activator of transcription-3, hypoxia-inducible factor 1-alpha, HIF-1α, and CCAAT/enhancer binding protein beta, but unique monocyte MDSC-specific transcriptional factor(s) have not been identified. Several regulatory mechanisms are used by MDSCs to modulate T-cell proliferation, but we focused on the capacity of MDSCs to suppress CD8 T-cell proliferation (Kim et al., 2011; Noman et al., 2014) and the ability of MDSCs to induce regulatory T cells (Hoechst et al., 2011). Interestingly, human monocyte myeloid-derived suppressor cells (Mo-MDSCs), defined as CD14+ HLA-DRlow have been recently shown to induce regulatory T cells (Hoechst et al., 2008, 2011; Jitschin et al., 2014). Elevated levels of this type of Mo-MDSC defined solely by CD14+ and HLA-DRlow expression has also been described to represent a poor prognosis for melanoma and other malignancies (Condamine et al., 2015; Greten et al., 2011; Hoechst et al., 2008; Jitschin et al., 2014; Poschke et al., 2010). The ability of Mo-MDSCs to induce Tregs introduces a unique link to psoriasis pathology, because psoriatic Tregs have been previously shown by us and others to be defective (Cai et al., 2012; Furuhashi et al., 2013; Goodman et al., 2009, 2011; Quaglino et al., 2009; Sugiyama et al., 2005; Zhang et al., 2008). The upstream cell populations leading to the defect in psoriatic Tregs have not been defined. In this study, we present evidence that psoriasis patients display an elevated number of circulating Mo-MDSCs in their peripheral blood. However, in contrast to MDSCs observed in cancer patients (Castella et al., 2015; Dubinski et al., 2015; Greten et al., 2011; Hoechst et al., 2008; Wang et al., 2013), psoriatic Mo-MDSCs display a decreased suppressive profile compared to Mo-MDSCs from healthy control subjects. Whereas Mo-MDSC-mediated conversion of naïve effector T cells into induced regulatory T cells (iTregs) was observed in psoriatic as well as control Mo-MDSCs, converted Tregs from psoriatic origin displayed diminished suppression of autologous proliferating CD8 T cells. These observations suggest an effect of psoriasis on circulating myeloid monocytic cells and a previously unrecognized cellular contributor to compromised control of immune regulation in psoriasis patients, as well as a potential target for immune restitution.

RESULTS

Mo-MDSCs are increased in the peripheral blood of patients with psoriasis

Based on previously published reports on other auto-inflammatory illnesses and the overactivity of signal transducer and activator of transcription-3—mediated signaling in psoriasis, which modulates MDSC, we hypothesized that the frequency of Mo-MDSCs in psoriatic patients would be abnormal compared to healthy controls (Haile et al., 2008; Yeager et al., 2012). Patient demographics for subjects participating in this study are listed in Table 1. Representative images demonstrating the calculation of Mo-MDSCs as a percentage of CD14+ selected cells from blood of healthy control or psoriatic individuals are shown in Figure 1a and b, respectively. The average proportion of Mo-MDSCs presented as a percentage of total CD14+ cells is shown in Figure 1c (23.4% in psoriasis vs. 9.7% in controls, n = 22 and n = 18, respectively, P < 0.002), representing an elevated level in 63% of psoriasis patients. We also determined the proportion of CD14+ HLA-DRlow Mo-MDSCs calculated as a percentage of total peripheral blood mononuclear cells (PBMCs) as shown in Figure 1d for psoriatic patients versus healthy controls (2.6% vs. 0.9%, n = 17 and 19, respectively, P < 0.002). CD14+ bead-enriched cells from both healthy controls and psoriatic patients were CD33+, CD11b+ (100%), and CD15− (0%) (see Supplementary Figure S1a and b, respectively, online). To determine the relative intensity of HLA-DR on CD14+ HLA-DRlow populations, we further analyzed the median fluorescence intensity and found that psoriatic patients displayed a significantly lower mean median fluorescence intensity than controls (21,486 vs. 27,107, n = 16 and n = 13 respectively, P < 0.02; Figure 1e).

We next asked whether the number of circulating Mo-MDSCs is correlated with psoriasis severity. Indeed, the psoriasis area and severity index (PASI) for each patient correlated significantly with circulating Mo-MDSCs among psoriasis patients with psoriasis severity (r = 0.72, R² = 0.53, P = 0.00091; Figure 1f). Although psoriasis treatments may affect the number of circulating Mo-MDSCs, only psoriasis patients washed out or naïve to treatment were enrolled in the current study.

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<th>Table 1. Patient demographics</th>
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<td><strong>Type of patient</strong></td>
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<td>Psoriasis area and severity index</td>
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Abbreviation: NA, not applicable. Values are given as number, median (range), or median (range) ± standard deviation.

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Psoriatic Mo-MDSCs suppress less proliferating CD8 T cells than healthy controls

Next, we tested whether Mo-MDSCs from psoriatic patients suppressed autologous T-cell responses at the same level as Mo-MDSCs isolated from healthy controls. CD14⁺ monocytes were selected by magnetic separation and sorted on lack of HLA-DR expression. Mo-MDSCs were added at a 1:2 ratio to autologous anti-CD2/CD3/CD28-stimulated e670-labeled CD8 T cells, and proliferation was analyzed by dye dilution assay (Figure 2a and b). Psoriatic Mo-MDSCs have a statistically significant lower suppressive capacity toward their proliferating CD8 T cells compared to the suppression exhibited by healthy control Mo-MDSCs (106% proliferation vs. 47% proliferation, respectively, relative to their respective CD8 T cells stimulated without MDSC present [100%], P < 0.0001). When CD14⁺ HLA-DR⁺ monocytes were used as controls, they failed to suppress proliferation of the responding CD8 T cells, as expected (data not shown).

To address whether the defect in suppression in the psoriatic cultures lay within the MDSCs or the responding T cells...
(i.e., lack of suppression by MDSC vs. overproliferation by psoriatic CD8 T cells), we also undertook a criss-cross experiment using healthy Mo-MDSCs mixed with proliferating psoriatic CD8 T cells and vice versa (Figure 2c). Whereas healthy Mo-MDSCs effectively suppressed psoriatic CD8 T-cell expansion (Figure 2c, first panel and Figure 2d, first bar), psoriatic Mo-MDSCs failed to suppress healthy proliferating CD8 T cells (Figure 2c, second panel, and Figure 2d, second bar). Note that although the proliferation of stimulated CD8 T cells was slightly higher in psoriatic individuals, as we reported previously (Sugiyama et al., 2005), the difference in accelerated proliferation did not reach statistical significance ($P = 0.07$) and was not accountable for the observed decrease in suppression among psoriatic MDSCs, although this may contribute to the ultimate suppressive capacity of psoriatic MDSCs.

Psoriatic Mo-MDSCs exhibit decreased levels of programmed cell death protein 1 and programmed death-ligand 1 compared to healthy control Mo-MDSCs

To address potential mechanisms of Mo-MDSC suppression, we examined known functional mediators of MDSC suppression. Surface expression of programmed cell death protein 1 (PD-1), a molecule associated with suppression through release of IL-10 in monocytes (Said et al., 2010) was assessed by flow cytometry. Interestingly, we found that Mo-MDSCs from psoriasis patients have a decrease in PD-1 expression compared to healthy Mo-MDSCs (13% vs. 28%, $n = 14, n = 9$ respectively, $P = 0.028$; Figure 3a and b). In conjunction with this reduction in PD-1, IL-10 was produced by psoriatic MDSC at about half the level of control MDSC, with IL-10 levels of 25, 36 and 64 pg/ml in psoriasis Mo-MDSCs (Average expression, 42 pg/ml) and 101 and 109 pg/mL (Average expression 105 pg/ml) in control MDSC (see Supplementary Figure S2a online). The programmed death-ligand 1 (PD-L1), is associated with suppression of CD8 T cells by direct contact phagocytosis (Kim et al., 2011), and MDSC have also been described to suppress CD8 T-cell proliferation through expression of PD-L1

![Figure 2. Psoriatic peripheral blood monocytic myeloid-derived suppressor cells (Mo-MDSCs) are less potent direct inhibitors of T-cell proliferation.](image)
Psoriatic and control Mo-MDSCs induce conversion from CD4$^+$ effector T cells to induced Tregs

We next investigated the capacity of psoriatic and control Mo-MDSCs to generate iTregs. CD2/CD3/CD28 bead-stimulated effector CD4 T cells isolated from healthy controls were mixed with healthy control or psoriatic Mo-MDSCs (1:2) for 3 days, and iTregs (CD4$^+$) were detected using CD25$^+$ and forkhead box p3$^+$ (Foxp3$^+$) staining (see Supplementary Figure S3 online). Supplementary Figure S3 shows the results of two independent experiments for the conversion of iTreg by either psoriatic or healthy control.
Mo-MDSCs effectively suppressed CD8 T-cell proliferation by >50% (44% of control proliferation, n = 5; Figure 4b, last bar). Preliminary findings indicate that this did not appear due to aberrant expression of either cytotoxic T lymphocyte-associated protein-4 (CTLA-4) or glucocorticoid-induced tumor necrosis factor receptor-family related gene (GITR) on the psoriasis MDSC-iTregs (see Supplementary Figure S4a and b online).

Mo-MDSCs and psoriasis skin
Mo-MDSCs may have limited ability to enter skin, as both control and psoriasis MDSC expression of skin-homing chemokine receptors such as CCR4 and CCR10 is quite limited (see Supplemental Figure S2c and d). Despite this, Mo-MDSCs were visualized (Figure 5a) in psoriatic skin using a combination of mouse-anti-human CD14 (Abcam, Cambridge, MA) and Alexa Fluor 647-conjugated goat anti-mouse IgG (Life Technologies, Grand Island, NY) as well as FITC-conjugated mouse anti-human HLA-DR (BD Biosciences, San Jose, CA). Monocytes positive for CD14-Alexa Fluor 647 but negative for HLA-DR–FITC expression (white arrows) were marked as Mo-MDSCs and can be visualized in the dermis of psoriasis patients as well as in the dermal epidermal junction region (dashed white line). Double-positive CD14+ HLA-DR+ monocyte/macrophage lineage cells (orange arrows) and CD14+ HLA-DR+ dendritic cell lineage and potentially activated endothelium endothelial (gray arrows) cells in psoriasis tissue. Mo-MDSC cells are also detectable in healthy control skin, although quantitative differences between psoriasis and control tissue are not noted (Figure 5b).

DISCUSSION
Treatments of psoriasis patients that achieve a stably rebalanced immune system with relatively durable treatment-free remissions remain a major challenge in dermatology (Nestle, 2008; Nestle et al., 2009). Escape of T helper type 17 (Th17) inflammatory pathways from natural regulatory mechanisms, such as the reduced functionality of regulatory T cells in psoriasis (Sugiyama et al., 2005), has emphasized the intricate cellular immune regulation responsible for balancing inflammatory response with timely resolution in healthy skin. Identifying mechanisms of immunoregulatory dysfunction in psoriasis are expected to result in new therapeutic approaches that leverage the ability of the immune regulatory balance to be restored and then maintained.

Mo-MDSC modulation of Treg production, direct suppression of T-cell activation, and myeloid differentiation status in the background of psoriatic pathology have not been examined to date. Currently, the best characterization of human Mo-MDSCs are cells expressing CD14 with low or absent HLA-DR expression, a recognized shortcoming, albeit the only currently available discriminating marker combination (Gabrilovich and Nagaraj, 2009).

In this study we show that psoriatic Mo-MDSCs exhibit a capacity to convert naïve T cells into iTregs compared to healthy Mo-MDSCs, although the psoriatic Mo-MDSC–converted iTregs have diminished suppressive functions, an observation that provides insight to our previously published report that Tregs in psoriasis are not decreased in number but have diminished suppressive capacities.
However, it is possible that rather than creating iTreg cells, the Mo-MDSC co-cultured CD4 T cells may have differentiated directly into another lineage (e.g., T helper type 2) that would also be suppressive and may account for the observed inhibitory effect on CD8 T cells seen afterward. Importantly, it has also been recently shown that Mo-MDSCs attract Tregs in a C-C chemokine receptor type 5-dependent manner in murine skin (Schlecker et al., 2012), an observation that complements our recent results demonstrating that psoriatic patient Tregs have decreased expression of C-C chemokine receptor type 5, which could explain the decreased number of high potency C-C chemokine receptor type 5+ Tregs in the skin (Soler et al., 2013).

In this study, we also show that Mo-MDSC numbers in psoriatic patients are increased compared to healthy controls, as reported for other skin conditions such as chronic contact eczema (Marhaba et al., 2007) and squamous cell carcinoma (Vasquez-Dunddel et al., 2013). We also show that HLA-DR median fluorescence intensity on CD14+ cells is decreased in
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MATERIALS AND METHODS

Cell isolation and sorting

PBMCs were isolated from freshly obtained blood by Histopaque density gradient centrifugation (Sigma-Aldrich, St. Louis, MO) as described previously (Soler et al., 2013). CD14+ monocytes were positively selected from PBMCs using magnetic CD14 microbeads (Miltenyi Biotec, San Diego, CA) with a magnet according to the manufacturer's instructions. CD14+ cells were then flow sorted into Mo-MDSCs using a Becton Dickinson FACS Aria flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The purity of the Mo-MDSC cells after sorting was >93%. All studies of human subjects were approved by the Institutional Review Board of University Hospitals Case Medical Center (Cleveland, OH). Peripheral blood samples were obtained from volunteer healthy controls or psoriasis patients after informed written patient consent was obtained according to the principles of the Declaration of Helsinki.

Antibodies and flow cytometry

To determine the frequency and phenotype of Mo-MDSCs in PBMCs, multicolor fluorescence-activated cell sorting was done using the following antibodies: anti-CD14–APC (Invitrogen, Carlsbad, CA) and HLA-DR-FITC (BD Biosciences, San Jose, CA). Regulatory T cells were sorted using CD4-APC (Life Technologies, Grand Island, NY), CD25-PE, and Foxp3-PE. Analysis of FACS data was done using Winlist software, version 7.0 (Verity, Topsham, ME). Isotype-matched antibodies were used with all the samples as controls. Specifically, the population defined as HLA-DR+Foxp3− was based on isotype staining with gating to include, at a minimum, 95% of the CD14+ population.

Suppression assay

Mo-MDSC cells were purified and sorted as described earlier. Autologous CD8 T cells were isolated from PBMCs using anti-CD8 receptor-family related gene and cytotoxic T lymphocyte-associated protein-4, two of the key effector proteins used by Tregs, on the induced Tregs. We found that although glucocorticoid-induced tumor necrosis factor receptor-family related gene was highly expressed in both psoriatic and control induced Tregs, cytotoxic T lymphocyte-associated protein-4 was decreased in iTregs from psoriatic origin.

Thus, we demonstrate an in vitro model for examining the ability of Mo-MDSCs to induce Tregs and show that the functionality of iTreg induced by psoriatic Mo-MDSCs is deficient in suppressive capacity. Despite an increase in circulating Mo-MDSCs in psoriasis, which would be expected to exert regulatory control over T-cell activation and expansion (Albeituni et al., 2013; Dugast et al., 2008; Gros et al., 2012), psoriasis patient Mo-MDSCs are inferior in direct suppressive capacity as well as ability to induce iTregs that are capable of suppressing proliferation of expanding CD8 T cells (Hoechst et al., 2008). The combination of inducing a Treg phenotype while creating a dysfunctional Treg is consistent with observations that psoriasis patients, despite having normal levels of Tregs, exhibit dysfunctional suppression of activated T cells (Goodman et al., 2009; Sugiyama et al., 2005).

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microbeads and a magnetic column (Miltenyi Biotech). Psoriatic or healthy control Mo-MDSCs were seeded 2:1 in a 96-well round-bottom plate with CD8 cells previously labeled with 5 μM of e670 (eBiosciences, San Diego, CA). CD8 T-cell proliferation was induced by anti-CD2/CD3/CD28 stimulation beads (Miltenyi Biotech), and the suppressive capacity of Mo-MDSCs was measured using a BD C6 flow cytometer after 5 days of co-culture. Controls included a positive T-cell proliferation control (CD8 T cells alone), an induction negative control (CD8 T cells with medium only), and labeled but unstimulated CD8 cells. The CD8 T-cell proliferative capacity was titrated by decreasing the ratio of anti-CD2/CD3/CD28 bead to effector CD8 cell to reach approximately 60% proliferation (normalized to 100% proliferation) to allow for detection of either increased or decreased proliferation following cell-cell stimulation. Criss-cross experiments were performed in the same manner as described earlier with the modification of psoriatic Mo-MDSCs onto healthy control CD8 T cells stimulated with anti-CD2/CD3/CD28 bead and vice versa (control Mo-MDSCs onto psoriatic proliferating CD8 T cells).

**iTreg induction assay**

For analysis of in vitro-generated CD4+ CD25+ Foxp3+ iTregs, Mo-MDSCs were sorted as described, and CD4 T cells were purified using a negative isolation kit (Miltenyi Biotech). Additionally enriched CD4 T cells were stained with CD4-APC and CD25-PE and sorted for CD4+ bottom 20% of CD25+ cells. The CD4+ CD25+ T cells were stimulated with anti-CD2/CD3/CD28 beads (Miltenyi Biotech) following the manufacturer’s instructions with a modified bead to cell ratio (1 bead per 8 cells) in the presence or absence of either psoriatic or healthy control Mo-MDSCs for 3 days in round-bottom 96-well plates. T cells were analyzed after 3 days and gated on a CD4+ CD25+ Foxp3+ population. Alternatively, to functionally test in vitro Mo-MDSC–induced Tregs after 3 days as described earlier, Mo-MDSCs were removed magnetically with anti-CD14 microbeads (Miltenyi Biotech), and the negatively isolated iTregs were co-cultured 1:4 with e670-stained bead-activated CD8 T cells for 5 additional days. Suppression was measured using a BD C6 flow cytometer as described earlier.

**Oxidative stress measurements**

ROS were measured by flow cytometry using 2′,7′-dichlorofluorescein diacetate, a fluorogenic dye that measures hydroxyl, peroxyl, and other ROS species within isolated Mo-MDSCs. Psoriatic or healthy control PBMCs were incubated at room temperature in the presence of 300 pM 2′,7′-dichlorofluorescein diacetate for 10 minutes, washed with phosphate buffered saline (PBS), then labeled with anti-CD14–APC, anti–HLA-DR–FITC to allow for electronic selection of Mo-MDSCs. After incubation on ice for 20 minutes, cells were washed with PBS and analyzed using a BD C6 flow cytometer. After diffusion into Mo-MDSCs, 2′,7′-dichlorofluorescein diacetate fluorescence was reduced through deacetylation to a nonfluorescent compound, which was then oxidized by ROS into fluorescent 2′,7′-dichlorofluorescein and measured by flow cytometry.

**IL-10 enzyme-linked immunosorbent assay**

IL-10 from normal and psoriatic MDSCs was quantified using the human IL-10 enzyme-linked immunosorbent assay kit (R&D). MDSCs and CD8 were mixed at a 2:1 ratio, and 200 μl of supernatant was collected from day 5 co-cultures according to the manufacturer’s recommendations. Absorbance 450 nm with wavelength correction to 540 nm was measured on a Perkin Elmer (Waltham, MA) Victor X3 spectrophotometer.

**Immunohistochemistry**

Tissue sections 7 μm thick were cut and fixed as described previously with some modifications (Toichi et al., 2006). Briefly, slides were dried for 20 minutes at room temperature, then fixed in cold acetone for 20 minutes. Rehydration was performed using DAKO (Carpinteria, CA) buffer for 1 minute. Slides were blocked with 10% goat and mouse serum for 30 minutes at room temperature. Mouse anti-human CD14 (Abcam clone 2Q1233, Cambridge, MA) was incubated overnight at 4 °C. Slides were then rinsed 5 minutes three times with PBS and stained for 60 minutes at room temperature using Alexa Fluor 647-conjugated goat anti-mouse IgG (Life Technologies, Grand Island, NY). Slides were then further rinsed for 5 minutes three times with PBS and stained with FITC-conjugated mouse-anti-human HLA-DR (BD Biosciences clone G46-6 San Jose, CA) for 2h at room temperature. Slides were then rinsed again 3 times 5 min each with PBS and mounted using Vectashield with DAPI (Vector Labs, Burlingame, CA). Images were acquired using an UltraView VoX spinning disk confocal system (PerkinElmer, Waltham, MA) mounted on a Leica DMi6000B confocal microscope (Leica Microsystems, Bannockburn, IL) equipped with a HCX PL APO 20X/1.4 objective. All confocal images were analyzed using Volocity software (PerkinElmer).

**Statistical analysis**

The statistical significance between values was determined by Student t test or nonparametric Mann-Whitney test and equality of medians when samples were not distributed normally. All data are expressed as mean ± standard error of the mean. Probability values of P ≤ 0.05 were considered significant.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.12.036.

**REFERENCES**


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