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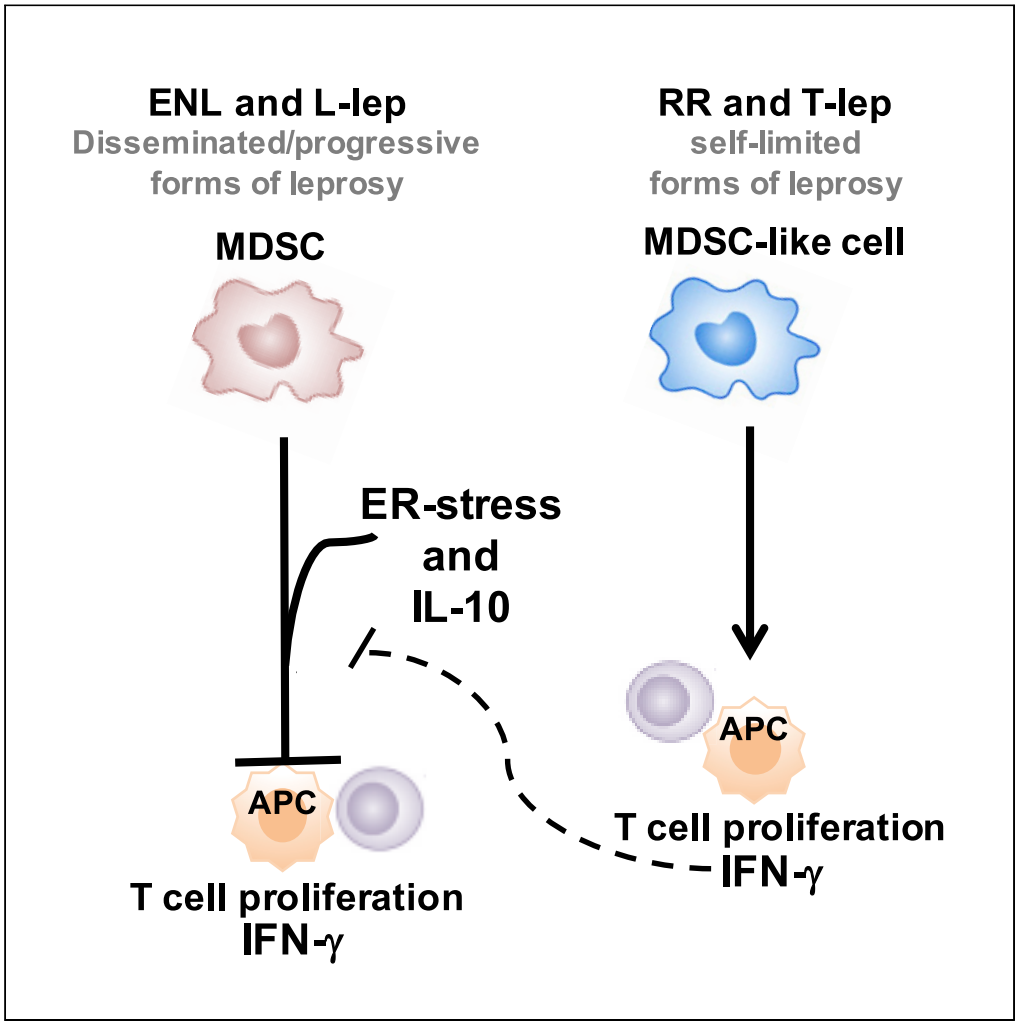

Kindra M. Kelly-Scumpia, Aaron Choi, Roksana Shirazi, ..., Feiyang Ma, Matteo Pellegrini, Robert L. Modlin

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HIGHLIGHTS

Cells with an MDSC phenotype are increased in blood and skin of patients with leprosy

Only MDSCs from patients with leprosy with disseminated infection suppress T cell function

MDSC function is dependent on increased ER stress and IL-10 production

MDSC function can be reversed in the presence of IFN-γ

DATA AND CODE AVAILABILITY

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ER Stress Regulates Immunosuppressive Function of Myeloid Derived Suppressor Cells in Leprosy that Can Be Overcome in the Presence of IFN-γ

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SUMMARY

Myeloid derived suppressor cells (MDSCs) are a population of immature myeloid cells that suppress adaptive immune function, yet the factors that regulate their suppressive function in patients with infection remain unclear. We studied MDSCs in patients with leprosy, a disease caused by *Mycobacterium leprae*, where clinical manifestations present on a spectrum that correlate with immunity to the pathogen. We found that HLA-DR^{-}CD33^{+}CD15^{+} MDSCs were increased in blood from patients with disseminated/progressive lepromatous leprosy and possessed T cell-suppressive activity as compared with self-limiting tuberculoid leprosy. Mechanistically, we found ER stress played a critical role in regulating the T cell suppressive activity in these MDSCs. Furthermore, ER stress augmented IL-10 production, contributing to MDSC activity, whereas IFN-γ allowed T cells to overcome MDSC suppressive activity. These studies highlight a regulatory mechanism that links ER stress to IL-10 in mediating MDSC suppressive function in human infectious disease.

INTRODUCTION

Under inflammatory conditions, immature myeloid cells expand, leave the bone marrow, and travel to the site of injury where they may accumulate and/or differentiate. Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells representing an alternative differentiation pathway of myeloid cell development during inflammation or stress that have potent immunosuppressive roles including the suppression of antigen-specific and non-antigen-specific T cell function (Nagaraj and Gabrilovich, 2010).

In humans, MDSCs are defined as being HLA-DR$^{-/low}$CD33^{+}CD11b^{+} and can be further subdivided into granulocytic (expressing CD15^{+}, but CD14^{-}) or monocytic (expressing CD14^{+}). In intracellular bacterial infection in humans, MDSCs have been isolated from patients with tuberculosis and are shown to inhibit CD4^{+} T cell responses and CD8^{+} T cell proliferation by reducing cytokine responses (du Plessis et al., 2013).

Leprosy is caused by the intracellular bacterium *Mycobacterium leprae* (mLEP) and presents as a spectrum in which the clinical manifestations correlate with the immune response to the pathogen. On one end of the spectrum, tuberculoid leprosy (T-lep) is a self-limited infection with the presence of few if any bacilli and involves predominantly a Th1 response. Lepromatous leprosy (L-lep) is the progressive form of the disease characterized by a high bacillary load within macrophages and a Th2 type response (Rea and Modlin, 1991). Less is known about the reactional states that often occur in patients with leprosy. Reactional states provide a window into immunopathology of the disease and occur when a patient's immune status rapidly changes resulting in tissue injury, including nerve damage. Type 1 reactions, or reversal reactions (RRs), are associated with an increase in cell-mediated immunity to *M. leprae* with reduction in viable *M. leprae*, representing a switch from a disseminated/progressive form to a self-limited form of disease. In RR, edema within granulomas and nerves is prominent and the myeloid cells include monocytes, macrophages, and multinucleated giant cells (Sharma et al., 2015). Type 2 reactions, also known as erythema nodosum leprosum (ENL), are characterized by the appearance of skin nodules due to immune complex formation and IL-1β release, occurring in patients with L-lep leading to tissue injury without a reduction in viable bacilli (Kamath et al., 2014; Lee et al., 2010; Yamamura et al., 1992). In ENL reactions, neutrophils are prominent and may be

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accompanied by vasculitis, suggesting the presence of immune complex disease (Anthony et al., 1983; Lee et al., 2010; Mabalay et al., 1965; Walker et al., 2015). What role, if any, MDSCs play in leprosy has not been investigated. Since MDSCs are associated with chronic immunosuppressive states and nosocomial infections in sepsis/septic shock (Mathias et al., 2017), we hypothesized that MDSCs may also participate in the pathogenesis seen in patients with leprosy.

RESULTS

Cells with an MDSC Phenotype Are Expanded in the Blood and Skin of Patients with Reactional Leprosy

We investigated the frequency of human granulocytic MDSC in patients with leprosy, defined as HLA-DR$^-$CD33$^+$CD11b$^+$CD15$^+$ (Nagaraj and Gabrilovich, 2010). Using flow cytometry, we identified the expansion of a population of myeloid cells in the blood of patients with leprosy with increased side scatter properties present only at low levels in healthy control patients (Figure S1). These cells expressed cell surface markers typically expressed by MDSCs (HLA-DR$^-$CD33$^+$CD15$^+$) and were enriched in patients with ENL, RR, and L-lep (Figure 1A). The frequency of HLA-DR$^-$CD33$^+$CD15$^+$ cells was greater in patients with active versus inactive RR and ENL reactions. In contrast, patients with T-lep along with healthy controls had the lowest levels of HLA-DR$^-$CD33$^+$CD15$^+$ cells (Figure 1A). Patients were considered to have active disease if they were undergoing an active immune response. Patients were inactive if they have completed treatment or were weaning off treatment. HLA-DR$^-$CD33$^+$CD15$^+$ cells in patients with leprosy were of the granulocytic subpopulation (Condamine et al., 2016), expressing CD15 but not CD14 (Figure S1).

MDSCs are readily identified in circulation, whereas their presence in tissue indicates local immune regulation at the site of infection as a part of disease pathogenesis (Gabrilovich, 2017; Ostrand-Rosenberg and Sinha, 2009). We next investigated whether cells with an MDSC phenotype (HLA-DR$^-$CD33$^+$CD15$^+$) are present in the skin of patients with leprosy. By immunofluorescence of frozen biopsy specimens and flow cytometry of fresh biopsy specimens, we detected HLA-DR$^-$CD33$^+$CD15$^+$ cells in lesions of patients with RR and ENL (Figures 1B and 1C). The presence of MDSC in leprosy lesions suggests the potential immunomodulatory role at the site of disease.

MDSCs from Patients with Leprosy Suppress *M. leprae*-Specific T Cells

A key characteristic of MDSCs is the ability to suppress the function of immune effector cells. We therefore assessed whether the cells possessing the MDSC phenotype suppressed the function of T cells, measuring their ability to inhibit proliferation and IFN-γ response of an *M. leprae*-specific T cell clone. We performed functional experiments using HLA-DR$^-$CD33$^+$ enriched cells isolated from peripheral blood. These cells were also CD15$^+$ but were only selected based on HLA-DR$^-$CD33$^+$. Following incubation of antigen-presenting cells (APCs) with *M. leprae* sonicate for 1 h, an equal number of *M. leprae*-specific T cells are added to assess their antigen-specific proliferation. To measure the impact of MDSCs on T cell activity, a T cell suppression assay was performed. Patient HLA-DR$^-$CD33$^+$ cells were added to T cells primed with antigen-presenting cells pulsed with antigen to assess the effects of HLA-DR$^-$CD33$^+$ on T cell proliferation and production of IFN-γ (Figures 1C–1F). Although every group of patients possessed HLA-DR$^-$CD33$^+$ cells that did not possess significant suppressive activity (>30% suppression), only HLA-DR$^-$CD33$^+$ cells isolated from patients with ENL (active or inactive) and L-lep suppressed T cell proliferation and IFN-γ production from the majority of patients (Figures 1E and 1F). These data indicate that HLA-DR$^-$CD33$^+$ cells from patients with L-lep, with or without ENL, are MDSCs with the capacity to suppress antigen-specific T cell responses. The HLA-DR$^-$CD33$^+$ cells from patients with RR and T-lep are MDSC-like cells, displaying the same phenotype of MDSC by flow cytometry but not suppressing adaptive immune function. Furthermore, these data suggest that leprosy serves as a model to test the functional plasticity of HLA-DR$^-$CD33$^+$ cells since infection with the same organism can elicit differential function of cells expressing the MDSC phenotype.

Endoplasmic Reticulum Stress Is Activated in MDSCs from Patients with ENL

To better understand why cells with the same surface phenotype isolated from patients with different clinical manifestations vary in their suppressive activity, we performed RNA sequencing (RNA-seq) from isolated HLA-DR$^-$CD33$^+$ cells from patients with suppressive activity, ENL, and from patients who did not possess suppressive activity, RR, and performed differential expression gene analysis (ENL n = 4, RR n = 5, HC n = 3). We chose to focus our RNA-seq studies on patients with ENL and RR since these patients displayed the largest expansion of these cells. We found that 46 genes were increased 2-fold or more in ENL

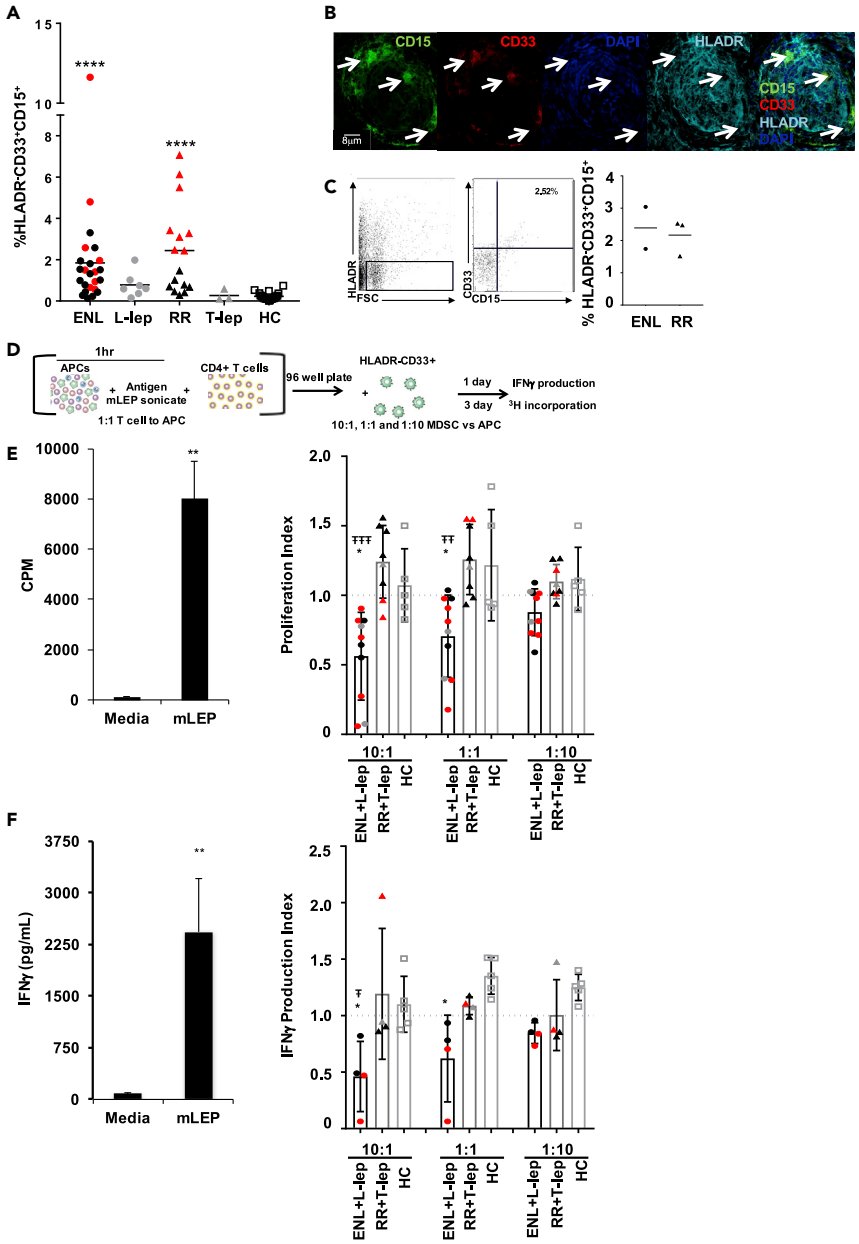

Figure 1. MDSCs Are Increased in Blood and Lesional Skin of Patients with Leprosy

(A) Dot graph depicting the percentage of HLA-DR$^-$CD33$^+$CD15$^+$ cells in the peripheral blood of patients with leprosy (ENL [red circles, active] n = 9, ENL [black circles, inactive] n = 15, RR [red triangles, active] n = 8, RR [black triangles, inactive] n = 8, L-lep n = 7, T-lep n = 3, Healthy Control [HC] n = 24; line represents mean of all samples. Kruskal-Wallis $p < 0.0001$; Dunn's post hoc analysis ****$p < 0.0001$).

(B and C) (B) Confocal microscopy—representative confocal image depicting HLA-DR$^-$CD33$^+$CD15$^+$ cells (pointed out by white arrows) in a representative ENL lesion (63X, one labeled representative section of at least three per group) and (C) representative flow cytometry plot of RR fresh skin lesion biopsy (left two panels) and dot plot from ENL (n = 2) and RR (n = 3) patient biopsies (right panel) reveal the presence of HLA-DR$^-$CD33$^+$CD15$^+$ cells in lesions of patients with reactional leprosy.

(D) Model of suppression assay.

(E and F) (E) ^{3}H incorporation at 72 h. Left panel depicts T cell proliferation in response to mLEP sonicate in the absence of MDSCs or MDSC-like cells (n = 22, average of all samples); right panel depicts Proliferation Index (T cell proliferation [CPM] without MDSC/T cell proliferation [CPM] with MDSC). Addition of ENL but not RR or HC MDSC at a ratio of 10:1 and 1:1 (MDSC to APC) results in suppression of Tcell proliferation (ENL [black circles, inactive] n = 3, ENL [red circles, active]

Figure 1. *Continued*
n = 4, L-lep [gray circles] n = 2, RR [black triangles, inactive] n = 5, RR [red triangles, active] n = 2, T-lep [gray triangle] n = 1, and HC [open squares] n = 5; line represents mean ± SEM of all samples). One-way ANOVA $p < 0.0001$; Tukey's post hoc analysis, *$p < 0.05$, **$p < 0.01$, ŦŦ,**$p < 0.01$, ŦŦŦ,***$p < 0.001$ compared with healthy controls (*); compared with RR (Ŧ). and (F) IFN-γ production at 24 h. Left panel depicts IFN-γ production in response to mLEP sonicate in the absence of MDSCs or MDSC-like cells (n = 13, average of all samples); right panel, IFN-γ production index (T cell IFN-γ production without MDSC/T cell IFN-γ production with MDSC) at 24 h (ENL [active and inactive, circles] n = 4, RR [active and inactive, triangles] n = 4 and HC [open squares] n = 5; line represents mean ± SEM of all samples). One-way ANOVA $p = 0.0011$; Tukey's post hoc analysis *$p < 0.05$ compared with healthy controls (*); compared with RR (Ŧ).

MDSC, with an adjusted p value of <0.05, when compared with RR MDSC, whereas only seven genes were increased 1.8-fold or more in RR MDSC compared with ENL MDSC. Shown in Tables 1 and 2 are the top 10 most differentially expressed genes in MDSC from patients with ENL when compared with patients with RR and the top seven most differentially expressed genes in RR MDSC-like cells compared with ENL MDSC. Of note, one of the most overexpressed genes in ENL MDSC compared with RR MDSC was lectin type oxidized lipid receptor 1 (OLR1), a gene shown to be expressed in granulocytic (PMN)-MDSC with immunosuppressive function when compared with neutrophils of healthy people or patients with cancer who did not possess immunosuppressive function (Condamine et al., 2016).

Since OLR1 was associated with endoplasmic reticulum (ER) stress in MDSCs of patients with cancer, we next performed Gene Ontology (GO) enrichment analysis to determine whether ER stress-related genes (GO: Response to Endoplasmic Stress) are differentially activated in ENL MDSC versus RR MDSC-like cells. Enrichment analysis was performed by investigating the overlap in ER stress-related genes (Table S1, List of Endoplasmic Stress genes related to Figure 2) between the different leprosy datasets using the hypergeometric distribution to control for differences in the overall number of differentially expressed genes (Teles et al., 2013). Indeed, we found a significant >2-fold enrichment of ER stress-related genes in MDSC from patients with ENL over healthy controls, with RR MDSC-like cells displaying an intermediate level of ER stress that was not significant (Figure 2A).

To validate the transcriptomics findings, we performed real-time qPCR on additional MDSCs isolated from patients with ENL or RR reactions. The expression of master regulators of the ER stress response, including the transcription factors X-Box-binding protein 1 (XBP1), activating transcription factor 4 (ATF4), and its

Log_2Fold Change	p Value	p-Adj	Gene Name	
−4.2439	1.17×10^{-4}	0.0374	OSR2	Odd-skipped related 2
−3.7839	1.66×10^{-4}	0.0472	CCL20	Chemokine (C-C motif) ligand 20
−3.6029	7.55×10^{-5}	0.0289	IL24	Interleukin-24
−3.3588	2.3×10^{-6}	0.0020	LIF	Leukemia inhibitory factor
−3.3048	7.28×10^{-6}	0.0047	OLR1	Oxidized low-density lipoprotein (lectin-like) receptor 1
−3.0636	1.06×10^{-6}	0.0010	HES1	Hairy and enhancer of split 1
−3.0529	4.29×10^{-7}	0.0005	PHLDA1	Pleckstrin homology-like domain, family A, member 1
−2.9323	2.01×10^{-7}	0.0003	RGCC	Regulator of cell cycle, response gene to complement 32 protein
−2.0339	1.65×10^{-10}	0.000	IL1B	Interleukin 1 beta
−2.9137	1.06×10^{-5}	0.0062	PGLYRP1	Peptidoglycan recognition protein 1

Table 1. Top 10 Genes Upregulated in ENL MDSC over RR MDSC-like Cells

Log_2Fold Change	p Value	p-Adj	Gene Name	
2.99356	1.70×10^{-4}	0.0472	PRICKLE1	Prickle homolog 1
2.26979	6.47×10^{-8}	0.0001	AIM2	Absent in melanoma 2
1.63421	5.87×10^{-5}	0.0243	GBP4	Guanylate binding protein 4
1.42352	2.38×10^{-5}	0.0115	FCGR2C	Fc fragment of IgG, low affinity IIc, receptor for (CD32)
1.09738	1.10×10^{-4}	0.0356	SRBD1	S1 RNA binding domain 1
0.8841	1.10×10^{-4}	0.0356	APOL3	Adolipoprotein L, 3
0.87008	1.70×10^{-5}	0.008	KIAA0922	Transmembrane protein 131-like (hg19 protein)

Table 2. Top 7 Genes Upregulated in RR MDSC-like Cells over ENL MDSCs

target gene ATF3, along with the cytoplasmic ER to nucleus signaling 1 (ERN1), were expressed 3- to 50-fold higher in HLA-DR$^-$CD33$^+$ cells isolated from patients with ENL compared with patients with RR (Figure 2B) (Rodrigues et al., 2018). These data suggest that the ER stress pathway contributed to suppressor activity of MDSCs.

To confirm whether ER stress may contribute to immunosuppression in lesions, we next examined if an ER stress signature existed in lesional biopsies of patients with leprosy with ENL or RR using the same enrichment analysis as above. This DNA microarray dataset was published previously (Teles et al., 2013). We identified a 3-fold enrichment of ER stress genes in skin lesions from patients with ENL compared with patients with RR (Figure 2C). Taken together, the increased ER stress signature in both blood MDSCs and lesional skin of patients with ENL suggests a role for ER stress in the immune response of these patients.

Endoplasmic Reticulum Stress Regulates MDSC Suppressive Function in Patients with Leprosy

We next tested whether ER stress regulates MDSC suppressor function in patients with leprosy. Similar to MDSCs from patients with cancer, the immunosuppressive activity of MDSCs from patients with ENL can be reversed by pretreatment with the free radical scavenger N-acetyl cysteine (NAC; Figure 2D) (Condamine et al., 2016). Alternatively, the induction of ER stress by thapsigargin (THG) in MDSC-like cells from patients with RR resulted in the ability of these cells to suppress T cell proliferation (Figure 2E). These data highlight the contribution of ER stress to MDSC suppressor function and support a role for ER stress in MDSC-induced immunosuppression in ENL reactions.

Presence of IL-10 Contributes to MDSC Suppressive Effects on T Cell Function in ENL

Since only MDSCs from ENL inhibited T cell proliferation consistently, we wanted to further explore how MDSCs may modulate immune responses. We treated MDSCs from patients across the clinical spectrum of leprosy with TLR2/1L (19-kD triacylated lipopeptide derived from mycobacteria) and measured cytokine production. Although the induction of most cytokines, including IL-6, IL-8, TNFα, and IL-1β, was similar between cells from patients with RR and ENL leprosy (Figure S2), we found that IL-10 was more strongly induced in MDSCs from patients with ENL (Figure 3A). Since MDSCs have also been shown to regulate immunity through IL-10 production (Bunt et al., 2009; Park et al., 2018) and IL-10 participates in the immunopathology of ENL (Sreenivasan et al., 1998), we next tested whether IL-10 produced by MDSCs from patients with ENL contributed to T cell suppressor function. Antibody-mediated neutralization of IL-10 decreased the T cell suppressive function of MDSC isolated from patients with ENL when compared with isotype control antibody (Figure 3B). These data indicate that IL-10 produced by MDSCs from patients with ENL contributes to their suppressive activity.

To examine whether inducing ER stress in RR MDSC-like cells directly regulates IL-10 production we treated MDSC-like cells from patients with RR with thapsigargin or MDSCs from patients with ENL with NAC for 6 h prior to stimulation with 19 kD for 24 h. Although thapsigargin augmented IL-10 production (Figure 3C), the

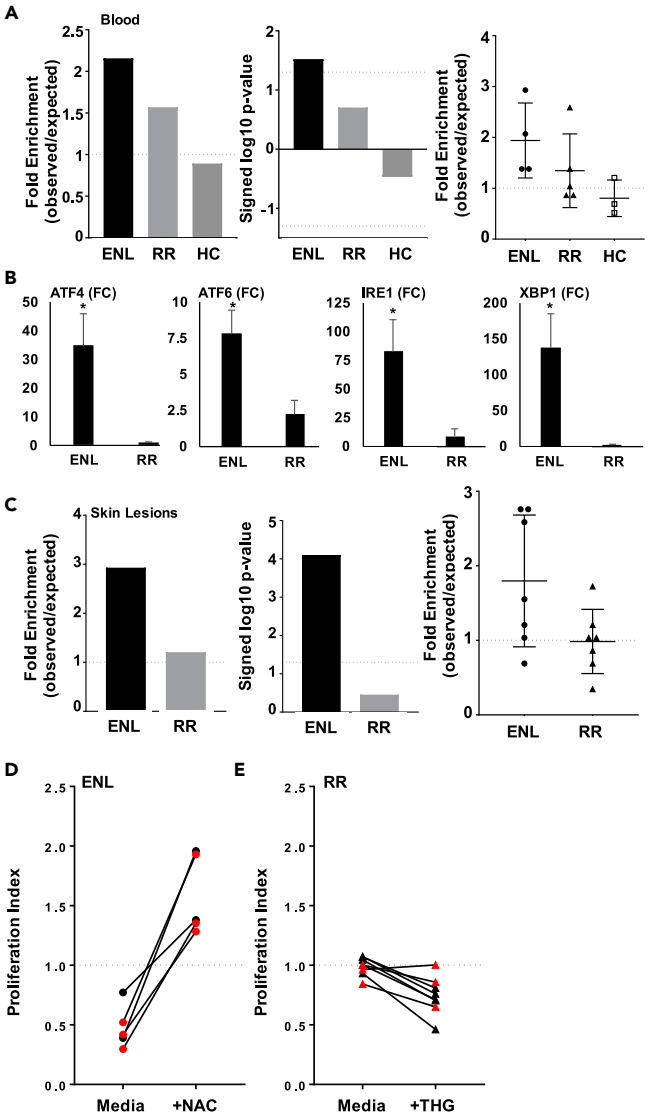

Figure 2. ER Stress in Leprosy

(A) ER stress response enrichment score of MDSC-like cells from patients with reversal reaction (RR) and healthy controls (HC) and MDSCs from patients with ENL was calculated using the hypergeometric distribution to control for differences in the overall number of differentially expressed genes. These tests determine if the degree of observed amount of enrichment is greater than expected. List of genes used is given in Table S1. The left panel displays fold enrichment, the middle panel shows the significance of enrichment (1.3-p = 0.05, negative value represents under-enrichment), and the right panel shows fold enrichment for each patient (ENL n = 4, RR n = 5, HC n = 3, line represents mean ± SEM).

(B and C) (B) Upregulation of ATF4, ATF6, IRE1, and XBP1 in HLA-DR^{-}CD33^{+}CD15^{+} (MDSC) isolated from ENL and RR blood (quantitative RT-PCR) ENL n = 6, RR n = 3, line represents mean ± SEM *p < 0.05 Student's t test. (GSE129033) (C) ER stress response enrichment score of lesions from patients with reversal reaction (RR) and patients with ENL as in (A). The left panel displays fold enrichment, the middle panel shows significance of enrichment (1.3-p = 0.05), and the right panel shows fold enrichment for each patient (ENL n = 7, RR n = 7; line represents mean ± SEM GSE43700).

(D and E) A T cell assay, as in Figure 1D, was performed, except in some cases MDSCs were treated with or without NAC or thapsigargin for 5 h prior to being irradiated and added to the T cell assay. (D) Effect of using 1 μM NAC—lowers ER stress, inhibits suppression (Proliferation Index; ENL n = 5, red are active ENL; line represents mean ± SEM, paired t test: p = 0.0033) or (E) thapsigargin (1 μM)—induces ER stress, augments suppression (Proliferation Index) on MDSCs from patients with RR (n = 7, red are active RR; line represents mean ± SEM, paired t test p = 0.0024).

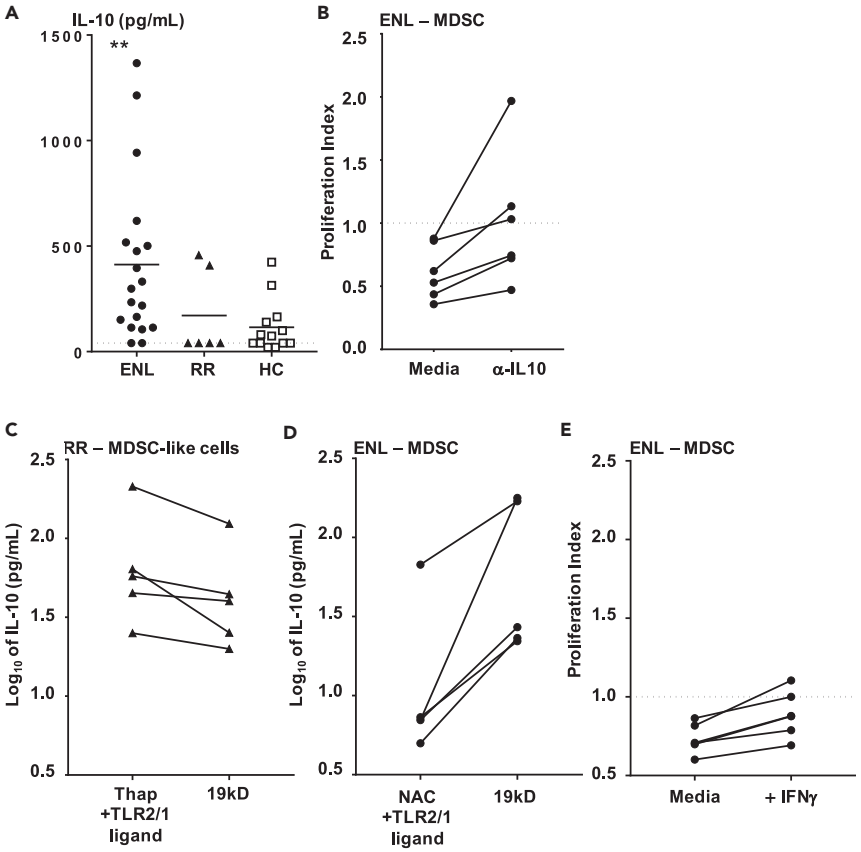

Figure 3. MDSCs from Patients with ENL Produce More IL-10 owing to Increased ER Stress

(A) HLADR^{-}CD33^{+} cells were isolated from ENL, RR, or healthy individuals and stimulated with or without TLR2/1 ligand for 24 h. IL-10 production was measured by ELISA. Dashed line indicates the lowest limit of detection; all unstimulated cells were at this level (ENL n = 17, RR n = 6, HC n = 13; line represents mean of all samples Kruskal-Wallis p = 0.0059; Dunn's post hoc analysis **p < 0.01 compared with healthy controls [HC]).

(B) T cell suppression assay showing inhibition of suppression (Proliferation Index, a PI of 1 indicates no change) by MDSCs from patients with ENL treated with an anti-IL-10 neutralizing antibody (n = 6; paired t test p = 0.044).

(C and D) (C) IL-10 production in MDSC-like cells treated with thapsigargin (to induce ER stress) for 5 h prior to a TLR2/1 ligand stimulation (n = 5; paired t test p = 0.045) (D) or MDSCs from patients with ENL treated with NAC for 5 h prior to stimulation with 19 kD (n = 5; paired t test p = 0.017).

(E) T cell suppression assay showing inhibition of suppression (Proliferation Index) by MDSCs from patients with ENL if recombinant IFN-γ is added to the T cell assay (n = 6; paired t test p = 0.038).

treatment of ENL MDSC with NAC inhibited IL-10 production (Figure 3D), confirming a regulatory role of ER stress in myeloid cell IL-10 production.

Enrichment of an IFN-γ Signature in MDSC-like Cells from Patients with RR

Since both ENL MDSC and RR MDSC-like cells both displayed some ER stress, a factor present in patients undergoing an RR may be able to overcome the ER-stress-dependent function to allow T cell activation. Using RNA-seq, we found only seven genes are increased significantly in RR MDSC-like cells compared with ENL MDSC. Three of these seven genes, AIM2, GBP4, and APOL3, are known IFN-γ inducible genes (Table 2) (Samarajiwa et al., 2009). In addition, monocytes from patients with RR express an IFN-γ signature (Teles et al., 2013). This led us to hypothesize that IFN-γ may be a factor that overcomes the suppressive effect of ER stress by MDSC. To test this hypothesis, we added IFN-γ to T cell suppression assays with MDSC from patients with ENL. Indeed, we found that the addition of recombinant IFN-γ restored T cell proliferation by MDSCs from patients with ENL (Figure 3E).

Classification	Thalidomide Treatment	Prednisone Treatment	Average Age	# Of M	# Of F	Unknown Sex	Total # of Patients
L-lep	(2/11)	(0/11)	54 ± 17.2	9	1	1	11
ENL-inactive	(3/20)	(0/20)	52.6 ± 14.1	17	2	1	20
ENL-active	(19/21)	(0/21)	43.2 ± 9.4	16	1	4	21
RR-inactive	(0/17)	(1/17)	46.5 ± 10.2	15	2	0	17
RR-active	(0/15)	(13/15)	49.1 ± 9.8	13	1	1	15
T-lep	(0/7)	(0/7)	60 ± 13.7	7	0	0	7

Table 3. Leprosy Patient Information

DISCUSSION

A better understanding of host-pathogen interactions is crucial for effectively treating chronic infections in humans. The model of leprosy is powerful in that infection of *M. leprae* leads to a spectrum of disease where some patients can control the bacterial infection and others cannot. Although the infiltration of myeloid cells from leprosy biopsy specimens has served as a key to histopathologic diagnosis of leprosy reactions, the role of these cells or other immune cells has not been evaluated in detail (Eichelmann et al., 2013; Modlin, 2010; Ridley, 1974). Herein, we identify an increase in immature myeloid cells displaying a cell surface phenotype of granulocytic MDSC (HLA-DR$^-$CD33$^+$CD15$^+$) in the blood of patients with L-lep and ENL leprosy, both manifesting disseminated/progressive infection, and also in patients with RR , who are undergoing a cell-mediated immune response associated with the reduction of bacilli in lesions. However, only those MDSCs isolated from patients with L-lep and ENL, i.e., from the patient groups with weak cell-mediated immunity to *M. leprae*, were consistently able to suppress T cell function.

As there are distinct treatments for the different reactional states of leprosy, we considered the role of therapy in affecting MDSC suppressive function. All patients with active RR received systemic glucocorticoids, most commonly prednisone, whereas those with ENL received thalidomide (Table 3). Since glucocorticoids induce the release of myeloid cells into the blood from the bone marrow (Okano et al., 2018; Varga et al., 2008), treatment with prednisone could explain the significant increase in MDSCs observed in patients with RR (Figure 1A). However, glucocorticoid treatment is a potent immunosuppressant that is known to enhance, rather than diminish, the suppressive function of MDSCs (Varga et al., 2008). Since MDSCs from patients with RR demonstrated low suppressive activity, it is likely that glucocorticoid treatment of patients with RR may contribute to their increased numbers in circulation but not their lack of suppressor function. MDSCs from both patients with L-lep and ENL suppressed T cell responses *in vitro*, but only patients with ENL were treated with thalidomide, suggesting that thalidomide did not have a substantial effect on MDSC function.

Our data link ER stress to MDSC immunosuppressive activity across the spectrum of leprosy. An ER stress signature was recently identified as a major signature differentiating PMN-MDSCs from neutrophils in patients with cancer, finding OLR1 as a marker of immunosuppressive MDSCs when compared with neutrophils (Condamine et al., 2014, 2016). Herein, we extend these findings to human infectious disease by demonstrating increased OLR1 expression in immunosuppressive MDSCs from patients with ENL over those from non-suppressive cells from patients with RR by RNA-seq. Additionally, we found a significant enrichment of ER stress-related genes in both skin lesions and circulating MDSCs from patients with ENL. Quantitative real-time PCR from additional patients confirmed that regulators of ER stress were elevated in MDSCs from patients with ENL when compared with those from patients with RR. In summary, our data implicate ER stress in the immunosuppressive function of MDSCs from patients with ENL, which is linked to an ER stress signature at the site of disease. On the other hand, one study found that ER stress proteins are more highly expressed in lesions from patients with T-lep versus patients with L-lep (Hirai et al., 2018), suggesting that the overall pattern of ER stress at the site of infection may not fully correlate with MDSC suppressor activity.

Mechanistically, induction of ER stress, with thapsigargin, in previously non-suppressive MDSC-like cells from patients with RR resulted in increased suppressor function and IL-10 production, whereas blockade

of ER stress or neutralization of IL-10 from previously suppressive MDSCs from patients with ENL resulted in diminished suppressor function and decreased IL-10 production. These findings suggest that clinical states known to be associated with a higher bacterial burden drive ER stress and increased IL-10 production enhancing the suppressive function of MDSCs. In fact, it was previously found that infection with *M. smegmatis* led to increased ER stress in a dose-dependent manner (Kim et al., 2018). Additionally, infection of murine macrophage with Mtb H37Rv or H37Ra was shown to lead to increased ER stress and apoptosis and survival of bacteria, or not (Lim et al., 2011) (Lim et al., 2016). Although ER stress was increased in the groups of patients known to have greater numbers of bacilli in lesions, unfortunately, we do not have the bacterial burden information for all of the patients with leprosy studied to perform a direct correlation.

Alternatively, factors driving enhanced cell-mediated immunity, such as IFN-γ, as occurs in RR with augmentation of host defense resulting in the clinical change from the disseminated/progressive to the self-limited form of leprosy may disable MDSC function. The few genes differentially expressed in MDSC-like cells from patients with RR as compared with MDSCs from patients with ENL are predominantly IFN-γ signature genes, and in the presence of increased IFN-γ, normally suppressive MDSCs from patients with ENL displayed diminished suppressor activity (Figure 3E). Further work is needed to determine the effects of IFN-γ on MDSC function, but the finding that MDSC-like cells from patients with psoriasis also do not suppress T cell function (Soler et al., 2016) and IFN-γ is present at high levels in patients with psoriasis (Lowes et al., 2014) suggest that IFN-γ may provide a signal that can overcome ER stress and disable MDSC function. There are a number of reports of *in vitro* models where IFN-γ has been demonstrated to induce ER stress and lead to decreased suppressive activity (El Jamal et al., 2016; Pirot et al., 2006; Watanabe et al., 2003); however, how tumor cells or cells with a persistent infection are affected by chronic ER stress *in vivo* is not understood. There is evidence that MDSCs from septic patients are not immunosuppressive until after their infection has cleared (Hollen et al., 2019) suggesting that MDSCs may behave differently in the context of cancer versus infection. Here we show that patients with ENL leprosy have MDSCs with an increased ER stress signature, which suppresses both T cell proliferation and IFN-γ production. If recombinant IFN-γ is added back into the assay, the same MDSCs are significantly less suppressive, suggesting a potential target for immunotherapy. IFN-γ is also increased in skin lesions of patients with RR where MDSC-like cells are not suppressive (Teles et al., 2013). Whether IFN-γ directly prevents MDSC suppressive function, induces MDSC apoptosis (Medina-Echeverz et al., 2014), or induces protective changes in the T cells that makes them resistant to suppression by MDSC in patients with RR requires further investigation.

Identifying the factors that disable ER stress in patients with leprosy may represent therapeutic targets to activate cell-mediated immunity to *M. leprae* in these patients. Additionally, treatment with factors that enhance ER stress may serve as an adjunct treatment in inflammatory skin disorders by increasing suppressive function of MDSC. Possible factors contributing to ER stress in patients with progressive leprosy could be increases in circulating IL-1β, IL-6, or type I interferon. All of these cytokines have been shown to increase ER stress in different cell types (O'Neill et al., 2013) and are elevated in progressive forms of leprosy.

MDSCs utilize a variety of pathways to suppress T cell function including arginase, IL-4, iNOS, reactive oxygen species, and induction of other regulatory cell populations such as regulatory T cells (Heim et al., 2015; Kwak et al., 2015; Ostrand-Rosenberg and Fenselau, 2018). IL-10 was shown to be an important mechanism for MDSC suppression of T cell function in a mouse model of ovarian cancer (Hart et al., 2011). Although we have previously shown that IL-10 is elevated in L-lep lesions, we and others have shown that IL-10 is elevated in peripheral blood mononuclear cells (PBMCs) from patients with ENL (Yamamura et al., 1992). Herein, we show that MDSCs from patients with ENL leprosy produce greater amounts of IL-10 in response to TLR2/1 ligand than MDSC-like cells from patients with RR leprosy, and their production of IL-10 contributes to their ability to suppress T cell function. Additionally, we have now linked increased ER stress to increased IL-10 production.

Taken together, our findings highlight the plasticity of immature myeloid cells in patients with leprosy. Suppression of T cell function by these cells may promote chronicity of infection and contribute to poor immunity to *M. leprae*. An ER stress pathway is activated during ENL and likely contributes to the overall immunosuppression seen. Overcoming this ER stress can subvert the suppressive effects of

these cells. Cells of a similar phenotype have been reported in patients with atopic dermatitis, psoriasis, and melanoma. Although those cells from atopic dermatitis and melanoma possessed suppressive function against T cells, those from patients with psoriasis did not demonstrate suppressive function (Poschke et al., 2010; Skabytska et al., 2014; Soler et al., 2016). As opposed to our results showing that ER stress is linked to immunosuppression of T cell responses, ER stress in M1 macrophages has been shown to be required for optimal killing of intracellular pathogens (Lim et al., 2016; Pillich et al., 2012). Further work is needed to understand how the bacterial burden induces ER stress in patients with microbial infection and how it contributes to immune dysfunction. To our knowledge, leprosy is the first model in humans to demonstrate plasticity of MDSC function. Using this model to investigate myeloid cell development can lead to a better understanding of MDSCs, their function, and potentially therapies to prevent or exploit their function.

Limitations of the Study

We acknowledge several limitations to the findings in this study. These include limited demographic and clinical information on each patient owing to samples being de-identified. We also do not know how long each patient has been on or off treatment nor do we have a bacillary index. Our established groups of patients with leprosy are based on a clinical and pathology diagnosis. In addition, because studies are performed on primary human cells, we rely on small molecule inhibitors rather than genetic perturbations for mechanistic studies.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

Raw data files for RNA-seq of MDSCs can be found at NCBI Gene Expression Omnibus Accession number: GEO:GSE129033.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101050.

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AUTHOR CONTRIBUTIONS

K.M.K.-S. – conceived and designed experiments, collected and analyzed data, and wrote the manuscript. A.C. – helped obtain patient samples and contributed data. R.S., H.B., and E.P. – contributed data. P.O.S. – assisted in manuscript preparation. M.T.O. – provided patient samples and clinical information. J.Y., F.M., and M.P. – analyzed RNA-seq data. R.L.M. – helped design experiments and analyze data and assisted in manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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iScience, Volume *23*

Supplemental Information

ER Stress Regulates Immunosuppressive Function of Myeloid Derived Suppressor Cells in Leprosy that Can Be Overcome in the Presence of IFN-γ

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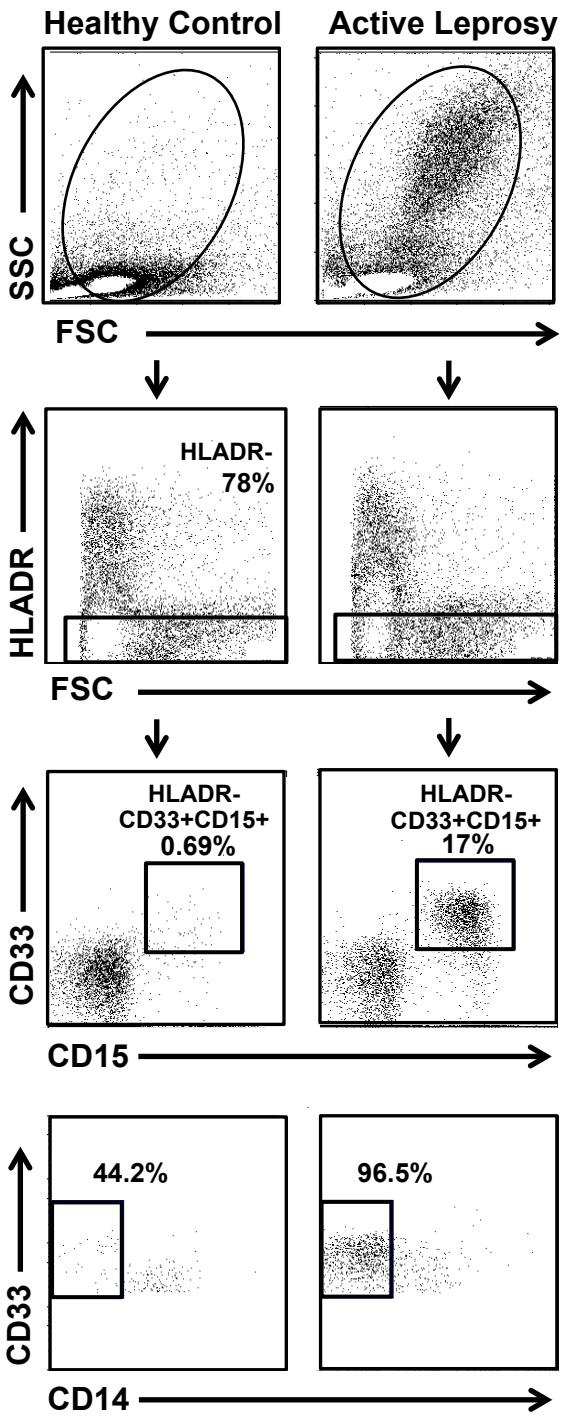

Figure S1. Gating scheme for MDSC ($HLADR^{-}CD33^{+}CD15^{+}CD14^{-}$) related to Figure 1. Flow plots depicting a representative healthy control and active RR leprosy patient. Ficolled cells are larger and more granular as seen by FSC/SSC in leprosy patients than healthy controls. We then gated on the $HLADR^{-}$ cells followed by $CD33^{+}CD15^{+}$ and looked at the expression of CD14.

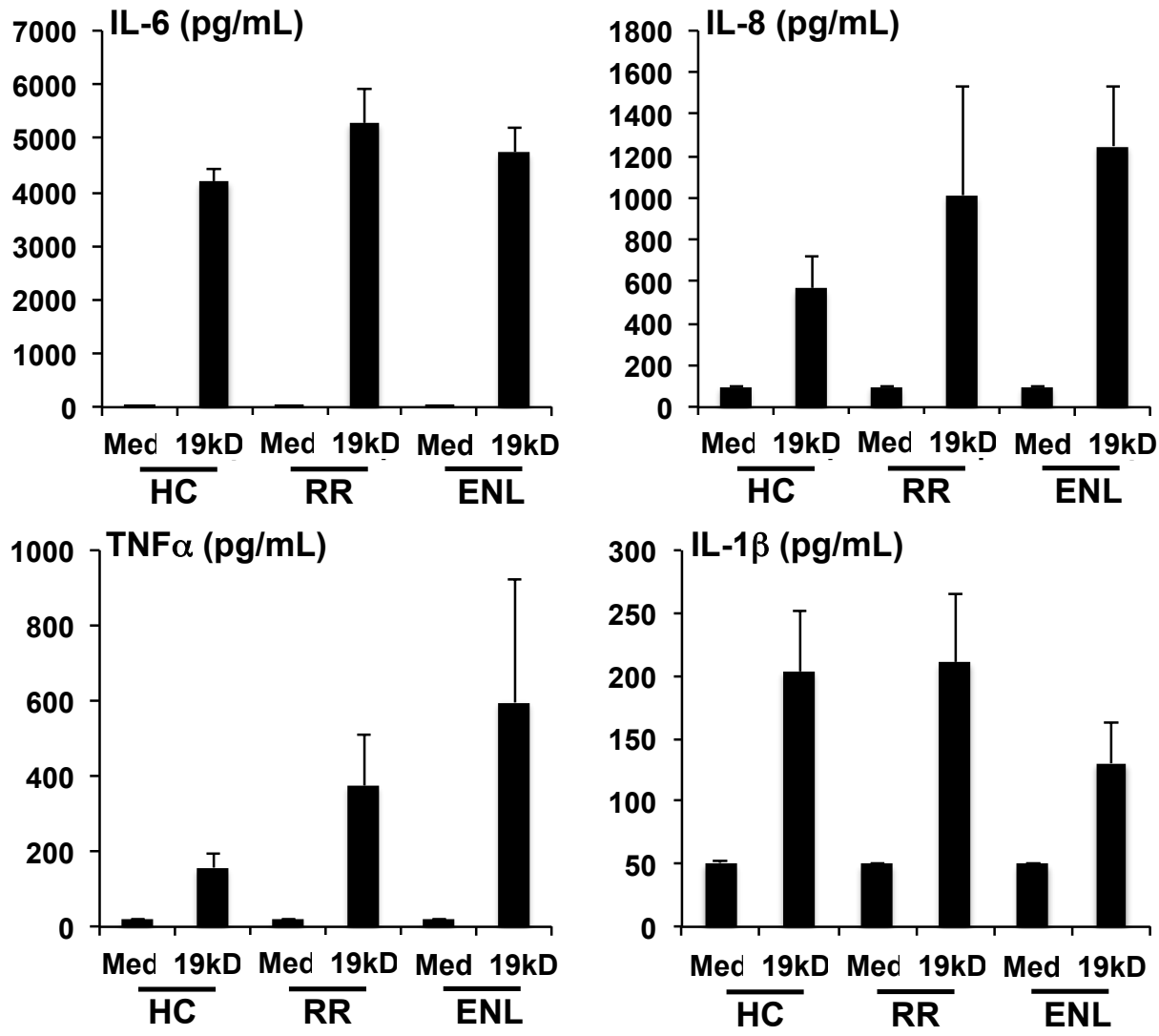

Figure S2. Cytokines produced by MDSC in response to TLR2/1 ligand (19kD). Related to Figure 3. Cytometric bead array results for IL-6, IL-8, TNFα and IL1β from supernatants of MDSC or MDSC-like cells from ENL or RR patients and HC treated with TLR2/1 ligand (19kD) for 24hr. We see no significant difference in the production of these four cytokines by MDSC or MDSC-like cells (ENL n=9, RR n=9, HC n=7). Data are represented at mean+SEM.

Key Resources Table

REAGENT or RESOURCE	RESOURCE	IDENTIFIER
Antibodies		
Anti- human HLADR (Clone: LN3)	eBioscience	11-9956-42
Anti-human CD33 (Clone:P67-6)	BDBioscience	BDB341650
Anti-human CD14, Clone: M5E2	BDBioscience	BDB555399
Anti-human CD15 (Clone: H198)	Invitrogen	12015942
Anti-human IgG1, κ	BDBioscience	BD347202
Anti-human IgG2b, κ	BioLegend	400310
Anti-human IgG2a, κ	BDBioscience	BD551414
Anti-human IgM	eBioscience	50-108-67
Purified NA/LE rat Anti-human and viral IL-10 (JES3-9D7) RUO	Fisher	BDB554495
Purified NA/LE rat IgG1k isotype RUO	Fisher	BDB554682
Anti-human HLADR (Clone:L243)	BioLegend	307602
Anti-human CD15 (Clone: H198)	BioLegend	301902
Anti-human CD33 (Clone: WM53)	BioLegend	303402
Purified Mouse IgG2a, κ Isotype Control	BDBioscience	554126
Purified Mouse IgG1 κ Isotype Control	BDBioscience	556648
Purified Mouse IgM, κ Isotype Control	BDBioscience	553472
Chemicals, Peptides, and Recombinant Proteins		

Thapsigargin	Sigma-Aldrich	T9033
N-acetyl-cysteine (NAC)	Sigma-Aldrich	A8199
mLEP sonicate	ATCC/BEI resources	NR-19336
Pam3Cys-SSNKSTTGSGETTA (19kD)	EMC microcollections GmH	bLP002
Human recombinant IFN-γ	Fisher	BDB554617

Critical Commercial Assays

Human IL-10 Duoset	Fisher	DY217b
Anti-human HLADR microbeads	Miltenyi Biotech	130-046-101
Anti-human CD33 microbeads	Miltenyi Biotech	130-045-501
Mouse anti-human IFNg (Clone: 4S.B3)	Fisher	BDB554550
ELISA plates	Costar / Corning	3690
Anti-Strepavidin	Fisher	PI-21126
Developing solution	SeraCare	506402
CBA human IL-6	Fisher	BDB558276
CBA human IL-8	Fisher	BDB558277
CBA human IL-1b	Fisher	BDB558279
CBA human TNF-a	Fisher	BDB558273
Truseq RNA Library Preparation kit v2	Illumina	RS-122-2001
10X NEB next second strand synthesis reaction buffer	New England Biolabs	B6117S
USER enzyme	New England Biolabs	M5505L
NEBNext mRNA second strand synthesis module	New England Biolabs	E6111S

SuperScriptII	Life Technologies	18080093
dUTP solution	Life Technologies	R0133
Dynabeads M-280 Streptavidin	Life Technologies	11205D
TRIZOL	Invitrogen/Fisher	15-596-018
RNeasy Mini kit	Qaigen	74104

Software and Algorithms

TopHat (version 2.0.6)	Kim and Salzberg, 2011	N/A
DESeq (version 1.5) Bioconductor package	Anders et al., 2010	N/A
Bowtie2 (version 2.0.2)	Langmead , 2010	N/A

Deposited Data

Raw data files for RNA seq of MDSC	NCBI Gene Expression Omnibus	GEO:GSE129033
Raw data for skin lesion microarray	NCBI Gene Expression Omnibus	GEO:GSE43700

Transparent Methods

Patients and clinical specimens

Patients with leprosy were classified according to the criteria of Ridley and Jopling (Ridley and Jopling, 1966). Reversal reaction (RR) skin biopsy specimens were considered upgrading reactions; consistent with activation of cell-mediated immune responses against *M. leprae* towards the tuberculoid pole (usually this reaction occurs

during treatment but can also occur spontaneously before or after treatment). Erythema nodosum leprosum (ENL) reactions also have CD4 T cell infiltration but are associated with the formation of immune complexes that are responsible for panniculitis, arthritis, vasculitis, and nerve injury. A reaction is considered active if the patient is undergoing an active immune response, either RR or ENL. A reaction is inactive if a patient with a previous active reaction is no longer undergoing treatment or are weaning off of treatment. Skin biopsy specimens (6 mm diameter) containing both epidermis and dermis were obtained by standard punch technique following informed consent. Specimens were embedded in OCT medium (Ames, Elkhart, IN), snap frozen in liquid nitrogen and stored at -80°C. Skin biopsy specimens were collected from untreated patients at the Hansen's Disease Clinic at Los Angeles Country and University of Southern California Medical Center as well as the Leprosy Clinic at the Oswaldo Cruz Foundation in Brazil. For flow cytometry, biopsy samples were dissociated mechanically using a surgical scalpel and extruded through a 64mm mesh filter (Bellco Glass Inc.) with a glass rod to obtain a single cell suspension. All leprosy patients were recruited with approval from the Institutional Review Board of University of Southern California School of Medicine (USC Institutional Review Board: for blood HS-08-00220 and for biopsies HS-12-00049). Healthy controls were recruited from the University of California, Los Angeles with informed consent (UCLA Institutional Review Board: 11-001274). Informed consent was attained from all subjects prior to samples being obtained. These studies enrolled both male and female adult subjects (**Table 3**).

Phenotypic Analysis

PBMCs were isolated using Ficoll (GE Healthcare) gradient centrifugation. To purify HLADR$^-$CD33$^+$ cells were first negatively selected on HLA-DR microbeads followed by a positive selection for CD33 (Milteny Biotec microbeads) using LS columns according to manufacturer's protocol (Miltenyi Biotec). Purity of the HLA-DR$^-$CD33$^+$ cells was confirmed to be >75% by flow cytometry.

T-cell assays

CD1a-expressing LCDCs were cultured with the CD1a-restricted T-cell clone derived from a patient with tuberculoid leprosy (1×10^5, LCD4.G) that recognized *M. leprae*, as described previously (Beckman et al., 1996; Niazi et al., 2007; Sieling et al., 1999) in the presence or absence of irradiated (6000 rad, γ irradiation, ^{137}Cs source) HLA-DR$^-$CD33$^+$ MDSC isolated from leprosy patients and healthy controls. Interferon-γ (IFN-γ) was measured by ELISA (BD Pharmingen) and proliferation was measured using [^{3}H] thymidine incorporation as described (Niazi et al., 2007; Sieling et al., 1999). Irradiated MDSC were used as preliminary experiments demonstrated MDSC proliferate during activation and interfere with [^{3}H] thymidine incorporation.

ELISAs

Secreted IFN-γ and IL-10 protein in the supernatant was measured using an IFN-γ or IL-10 Sandwich ELISA kit (BD Pharminigen and Invitrogen).

Flow Cytometry

PBMC were stained as follows. Fc receptor was blocked using human serum. Followed by extracellular staining of cells using anti-HLADR (Clone: LN3, FITC, eBioscience),

CD33 (Clone:P67-6, PerCP-y5.5, BD Biosciences), CD15 (Clone:HI98, PE, eBioscience), and CD14 (Close: MΦP9, APC, BD Bioscience) antibodies or isotype controls. Samples were fixed with 2% paraformaldehyde and processed on an LSRII (BD Biosciences) in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility.

Microbial ligands and cytokines.

The mycobacterial 19-kDa lipopeptide (EMC Microcollections) was used at 10 μg/ml. mLEP sonicate (ATCC/BEI resources) was used at 1ug/mL. Recombinant human IFN-γ. These reagents were all tested for endotoxin by LAL Assay (Limulus amoebocyte lysate, Lonza) to be endotoxin free (detection limit <0.1 EU/ml).

Induction or inhibition of ER stress

ER stress was induced in MDSC from RR patients using thapsigargin (Sigma,1μM) for 5 hours prior to harvest, wash, irradiation and incubation in T-cell assay. N-acetyl-cysteine (NAC, Sigma, 1μM) was used as a free radical scavenger to inhibit ER stress in MDSC from ENL patients 5 hours prior to harvest, wash, irradiation and incubation in T-cell assay or stimulated with 19kD for 24hrs.

Immunofluorescence

Three-color immunofluorescence was performed on cryostat tissue section by incubating with anti-HLADR antibody (Clone: L243; IgG2a), anti-CD33 (Clone: WM53; IgG1) and anti-CD15 (Clone: HI98 ; IgM) (Biolegend, San Diego, CA) followed by secondary isotype-specific, flourochrome (A488, A568, and A647)-labeled goat anti-

mouse antibodies (Invitrogen, Carlsbad CA). Immunofluorescence of skin sections were visualized using Leica SP2 1P-FCS confocal microscope at the Advanced Microscopy/Spectroscopy Laboratory Macro-Scale Imaging Laboratory, California Nanosystems Institute, UCLA.

RNA sequencing

MDSC and MDSC-like cells were isolated from ENL and RR leprosy patients and enriched as above. Total RNA was harvested using TRIZOL (Invitrogen) finishing with from RNAeasy kit (Qiagen) according to manufacturer's instructions, which included the on-column DNAse treatment step. Extracted RNA was quantified with Quant-iT RiboGreen RNA Assay Kit (Invitrogen) and RNA quality was assessed using RNA electrophoresis (Agilent Bioanalyze 2100). Total RNA was subjected to poly-A-selection to purify messenger RNA, then fragmented and converted into double stranded cDNA. Library construction was then done using the TruSeq Sample Preparation Kit (Illumina) according to manufacturer's instructions. This included the ligation of sequencing adapters containing nucleotide indexes for multiplexing. Libraries were quantified using PicoGreen (Invitrogen) and quality was assessed using the Agilent 2200 Tapestation. Library samples were pooled (4-5 samples per lane) at equimolar quantities (10uM each library) and sequenced on a HiSeq 2500 sequencer (Illumina) with 100bp single-end protocol.

Bioinformatics analysis

Sequenced reads were demultiplexed and aligned to the human reference genome hg19 (UCSC) using TopHat (version 2.0.6) and Bowtie2 (version 2.0.2). The HTseq

package was then used to assign uniquely mapped reads to exons and genes using the gene annotation file for build hg19 from Ensembl in order to generate raw count data. Once raw count data was generated, data normalization and differential expression analysis using a negative binomial model was performed in the R statistical programming environment using the DESeq (version 1.5) Bioconductor package. P-values were adjusted for multiple testing using the Benjamini-Hochberg correction. A cutoff of less than 0.05 for adjusted P-values and a fold change greater than 1.8 comparing opposite groups was used to identify significant differentially expressed genes.

Identification of ER stress enrichment signature

Enrichment analysis of the overlap in ER stress related genes (Supplemental Table 1) between the different leprosy datasets was performed using the hypergeometric distribution to control for differences in the overall number of differentially expressed genes (Teles et al., 2013). The hypergeometric distribution (hypergeometric test) is equivalent to the one-tailed version of Fisher's exact test. These tests determine the degree the observed amount of enrichment is greater than expected, and together these are two of the most common enrichment statistics used in bioinformatic analyses (Plaisier et al., 2010).

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