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Reversal of T-cell Tolerance in Myelodysplastic Syndrome through Lenalidomide Immune Modulation

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Letter to the Editor

Myelodysplastic syndromes (MDS) represent a spectrum of senescence-dependent, hematopoietic stem cell disorders[1] with dysplastic cytological features, ineffective hematopoiesis, and a propensity for transformation into acute myeloid leukemia (AML)[2]. Response biomarkers to inform delegation of FDA-approved therapies such as the thalidomide analog lenalidomide (Revlimid ®, Celgene Inc.), are needed to improve outcomes. High rates of erythroid response to lenalidomide occur in del(5q)-MDS patients due to suppression of haplodeficient phosphatases encoded within the proximal Commonly Deleted Region (CDR)[3]. A previous report showing that bone marrow lymphoid aggregates appear in association with hematologic response implicates immune modulation in this process[4]. Thalidomide, lenalidomide, and other structural analogues of this drug class induce potent immune modulation independent of del(5q), with documented activation of T-cells and NK-cells both *in vitro* and *in vivo* in multiple myeloma and chronic lymphocytic leukemia[5-7].

In an effort to understand how lenalidomide's immunomodulatory activity may be linked to hematologic response in MDS, we evaluated T-cell activity before and after lenalidomide treatment *in vitro*, and examined *in vivo* immune correlates related to hematologic response based on International Working Group (IWG) 2000 criteria. For this analysis, one hundred patients with pathologically defined MDS were consented at Moffitt Cancer Center to

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Conflict of Interest

Dr. List is a consultant, honoraria, member of the scientific advisory board, principal investigator for, and receives clinical research support from Celgene Corporation. J. McDaniel, Dr. Zou, Dr. Fulp, Dr. Chen, and Dr. Epling-Burnette declare no potential conflict of interest.

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evaluate immune responses. Thirteen of these were lower-risk, treated with lenalidomide, and had samples collected before-and-after treatment. Blood samples from an additional 5 patients with only lenalidomide pre-treatment samples available were used for *in vitro* experiments, but did not contribute to hematologic response analysis. Clinical characteristics and lenalidomide responses are shown in Supplementary Table 1. There was no difference between responders and non-responders with regard to international prognostic score (IPSS), World Health Organization (WHO) classification, or age (p=0.224).

To evaluate basal T-cell competency in pre-lenaliomide-treated patient samples (n=13) compared to healthy donors (HD, n=28), the T-cell receptor complex was stimulated by anti-CD3 antibody-cross-linking and proliferation was determined. Figure 1a-b shows that the percentage of stimulated T-cells induced to proliferate was significantly less in patient samples compared to controls for both CD4+ and CD8+-T-lymphocyte subsets ($p<0.0001$). This functional difference was age-independent, as shown in Figure 1ai-1bi indicating that MDS T-cells are anergic, or hypo-responsive, to T-cell stimulation.

Defective proliferation in incompletely tolerant T-cells can be rescued by high doses of exogenous interleukin-2 (IL-2)[8]. We therefore examined anti-CD3-induced proliferation in the presence and absence of IL-2 (data not shown). Although a 57% increase in CD4 and CD8 T-cell proliferation was observed, the amount of TCR-stimulated proliferation in the presence of IL-2 in cases was still significantly below that of HD. PBMCs were cultured with lenalidomide *in vitro* during anti-CD3/CD28 antibody stimulation to assess effects of the drug on these tolerant, or hypo-responsive, T-cells in MDS patients. PBMCs from 18 (untreated) MDS patients were stimulated in the presence of 5 μM lenalidomide or vehicle (dimethyl sulfoxide, DMSO), and proliferation was determined by bromodeoxyuridine (BrdU). Data in Figure 1ci-ii shows significantly greater TCR-induced proliferation in CD4+ ($p<0.001$) and CD8+ ($p<0.001$)-treated T-cells after lenalidomide compared to DMSO, and in some cases, proliferation was restored to the level of HD as indicated (shaded region) (Figure 1c). Lack of proliferation to lenalidomide without TCR stimulation (Unstim) indicates the drug has no direct mitogenic activity in T-cells (Figure 1c).

In addition to proliferation, lenalidomide-treated T-cells increased TCR-stimulated cytokine production (Figure 1di-ii). The cytokine response favored T-helper 1 (Th1)-type cytokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-2, which are effectors of anti-tumor immunity and potentially important for the elimination of pre-malignant or dysplastic myeloid clones. As shown in Figure 1di-ii, lenalidomide either decreased (IL-4) or induced no change (IL-10) in T-helper 2 (Th2) cytokines. Since lenalidomide increased the liberation of effector cytokines and augmented proliferation of both CD4+ and CD8+ T-cell subsets, its effect on hematopoiesis may be mediated by eradication of specific abnormal myeloblasts involved in MDS clonal evolution. In support of this idea, Neuber et al. recently reported enhanced antigen-specific T-cell activity *in vitro* and *in vivo* in multiple myeloma patients, indicating that lenalidomide can potentiate tumoricidal activity of effector T-cells[5].

Although lenalidomide is able to increase function in anergic MDS T-cells *in vitro,* the *in vivo* immunological response in MDS patients treated for anemia has not been examined.

First, the change (%post-pre) was calculated for CD4+ and CD8+ T-cells with a naïve (N), central memory (CM), effector memory (EM), and terminal effector memory (TEM) phenotype using multicolor flow cytometry analysis, as detailed in Supplemental Figure 1 in responders (n=7) and non-responders (n=6). Samples for this analysis were collected before (pre) and 16 weeks after lenalidomide therapy (post), as shown in Figure 2a. Memory phenotype skewing has been previously reported in MDS and correlated to chronic immune activation *in vivo*[9]. Cells with the TEM phenotype represent a unique, poorly studied, population of effector cells that are generally senescent, lack the CD28 co-stimulatory molecule, and increase through aging and autoimmunity[10, 11]. Figure 2b shows that the percentage of naïve CD4 and CD8 T-cells are significantly increased (p=0.004 and p=0.003, respectively), while CD4 and CD8 EM (p=0.046 and p=0.02, respectively) and TEM (p=0.0032 and p=0.015, respectively) decreased significantly after lenalidomide therapy. Lenalidomide increased CD8+ CM T-cells (p=0.004, Figure 2b) with a similar trend in CD4+ CM cells (p=0.09). Thus, changes in the composition of the peripheral T-cell compartment were significantly associated with an erythroid response in MDS. Lack of an increase in total lymphocytes (Supplemental Figure 2) suggests that the observed improvement in naïve and CM T-cells may be due to improved homeostatic balance within the T-cell compartment[12].

Next, we calculated the percentage change in BrdU positive cells (%post-pre) in responders and non-responders after *ex vivo* anti-CD3 antibody-stimulation in lenalidomide-treated patient samples collected and analyzed as shown in Figure 2. The percent increase in proliferation was significantly greater in responders compared to non-responders after therapy (Figure 2c, p=0.03 CD4; p=0.004 CD8) without an absolute increase in lymphocytes (Supplemental Figure 2). The ability of T-cells to produce cytokines was also determined by intracellular flow cytometry in responders and non-responders in *ex vivo* TCR-stimulated cells (Figure 2d-e). IL-2 (p=0.05 CD4; p=0.01 CD8), IFN-γ (p=0.03 CD4; p=0.01 CD8), and TNF-α (p=0.004 CD4; p=0.01 CD8) secreting CD4 (**d**) and CD8 (**e**) T-cells were increased significantly more after lenalidomide in responders compared to non-responders. Responders, however, had either no change or a decrease in Th2-type cytokines (IL-4 and IL-10) (**Figure d-e**), indicating that the type of T-cell immune response is similar to lenalidomide treatment *in vitro*.

Based on these data, we conclude that lenalidomide improves functional response in anergic T-cells in MDS, not by acting as a mitogen, but by augmenting T-cell receptor-induced signals. A significant improvement in the ratio of naïve-to-memory T-cells, increased cytokine production, and increased proliferation was evident in MDS patients with erythropoietic activity to lenalidomide (Figure 2b)**.** Preferential augmentation of naïve T-cells suggests that lenalidomide increases proliferation, thymic output, or a combination of both in addition to improving homeostasis within the T-cell compartment[13, 14].

We then determined the association between proliferation, cytokine production, and naïve T-cell changes. The percentage change in naïve T-cells showed a significant positive correlation with the change in proliferative response (CD4, p=0.0003 and CD8, p=0.0220) (Supplemental Figure 3) and IFN-γ production (CD8 T-cells, p=0.0118 and CD4+ T-cells, p=0.0941), although the latter in CD4+ T-cells was not statistically significant

(Supplemental Figure 3). Positive correlations were also identified between IL-2 production and changes in naïve T-cells in both subsets (CD4, p=0.0118 and CD8, p=0.0428, respectively) suggesting that reconstitution of the naïve T-cell compartment is at least partially responsible for improved function that is associated with hematologic response.

Our findings, therefore, implicate homeostatic reconstitution of naive T-cells in hematologic improvement in lower-risk MDS patients. It was shown that treatment with lenalidomide restored differentiation potential accompanied by up-regulation of the natively suppressed erythroid gene signature in a select subgroup of anemic patients with non-del5q MDS[15]. It is possible that lenalidomide stimulates not only erythropoiesis, but also restores naïve T-cells by increasing lymphopoiesis. If so, the production of naïve T-cells may be limited by age-dependent thymic involution. In support of this idea, the change in naïve CD4+ T-cells after lenalidomide was significantly less with age ($r=-0.92$, $p=0.007$) (Figure 2fi) suggesting that an age-related process, such as thymic involution, may limit the increase in naïve T-cells. Changes in naïve CD8+ T-cells, by contrast, was age-independent (Fig. 2fii) in both NR and R ($p=0.224$) indicating that these two T-cell subsets are mechanistically different. To confirm thymic involvement, examination of T-cell receptor excision circles (TRECs) to identify recent thymic emigrants is needed. However, the relationship between lenalidomide and therapeutic response preference in younger MDS patients has been documented in a large cohort suggesting that age-dependent immune modulation may play a role[16].

In summary, establishment of normal T-cell homeostasis, increased effector function, and reduction in T-cell tolerance is associated with hematologic improvement after lenalidomide treatment in MDS. Our data provides a rationale to examine a larger cohort of patients to determine if basal T-cell function is predictive for response to lenalidomide in MDS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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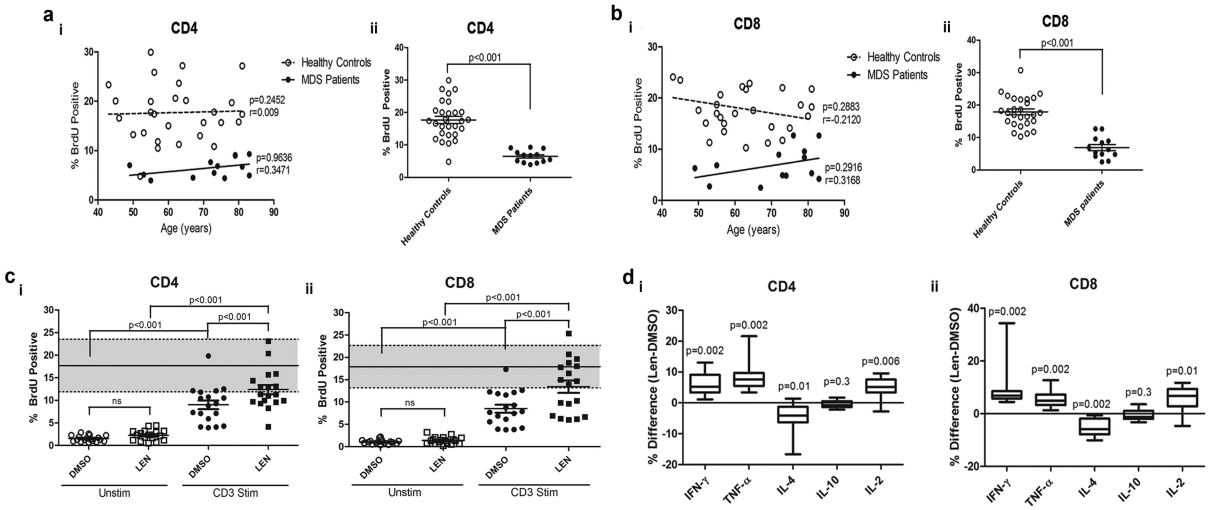

Figure 1. Lenalidomide augments Th1-type cytokine production, proliferation, and overcomes inherent anergy in MDS patient T-cells

Proliferation of T-cells was measured by bromodeoxyuridine (BrdU) incorporation after 2-day culture in the presence of immobilized anti-CD3 antibody (10 μg/ml). The percentage of BrdU positive cells was determined in both CD4+ (**a i-ii**) and CD8+ T-cells (**b i-ii**) from 13 MDS patients (MDS) prior to lenalidomide treatment and 28 healthy donors (Controls) (obtained from the Southwest Florida Blood Services, St. Petersburg, FL). T-cells from healthy donors (HD) were isolated from buffy coats using RosetteSep® Human CD3+ T-cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC Canada) according to the manufacturer's protocol. 10μM BrdU was added during the last 45 minutes of T-cell stimulation. The cells were fixed and permeabilized with BD Cytofix/Cytoperm buffer and incubated with DNase for 1 hour at 37°C. Cells were run on an LSRII flow cytometer (BD Biosciences, San Jose, CA USA) and the percentage of BrdU positive cells from each population were analyzed using Flow-Jo Software (BD Biosciences). A Spearman Correlation was used to determine correlation of age and % BrdU incorporation, with insignificant p values (**ai and bi**). **c and d)** MDS patient PBMCs were treated *in vitro* with either 5μM lenalidomide or vehicle control (DMSO) for 5 days and stimulated with anti-CD3/CD28 antibodies. The drug was weighed and dissolved at the time of use in dimethyl sulfoxide (DMSO) and diluted 1:1000 in culture media to a final concentration of 5 μM because storage of stock solutions at 20°C resulted in variable loss in activity. On day 5, an aliquot of cells was taken and stained for BrdU incorporation in both CD4+ and CD8+ T-cells (**c i-ii**). The solid line at 17.71 (CD4+) and 17.90 (CD8+) represents the mean proliferation of untreated healthy donor T-cells. Gray shading indicates the normal range of one standard deviation above, and one standard deviation below the mean (**c**). Also on day 5, cells were stimulated with PMA (5ng/ml) and Ionomycin (250ng/ml) for 6 hours, with the last 4 hours in the presence of the protein transport inhibitor Brefeldin-A (BFA, 10μg/ml) for intracellular cytokine staining. Cells were collected and incubated in EDTA (2mM) for 15 minutes at room temp, fixed with 2% formaldehyde, and washed with PBS containing bovine serum albumin (BSA). Cells were permeabilized with FACS permeabilization solution (BD Biosciences) and triple-stained with CD3-Pe-Cy5, CD8-FITC and intracellular cytokines (all PE-conjugated antibodies, BD Biosciences, San Jose, CA USA). **d)** Flow

cytometry was used to determine the percentage of CD4+ (**i**) and CD8+ (**ii**) Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), Interleukin (IL) −2, −4, and −10 secreting cells. The difference between Len and DMSO treated samples for each patient is shown. **a-d)** A Wilcoxon rank sum test was used to compare the mean proliferation between Healthy Donor and MDS patient samples (**a and b, ii)** and between stimulated and un-stimulated samples (**c i-ii).** A Wilcoxon rank sum test was used to determine statistical difference between Len and DMSO treated groups, with DMSO treatment used as baseline cytokine secretion (**d i-ii)**; p values are indicated.

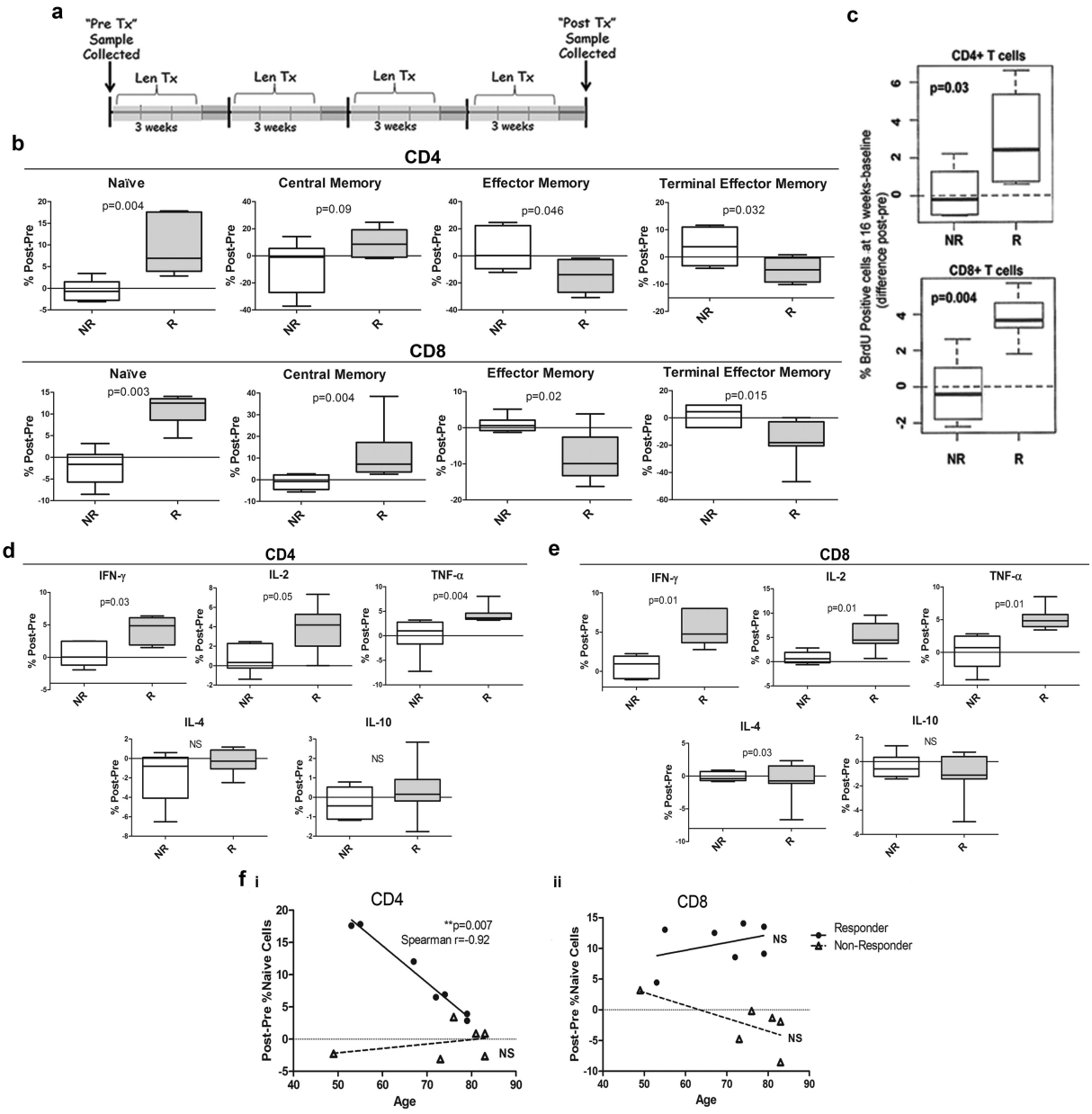

Figure 2. Lenalidomide reverses T-cell tolerance in MDS patients with hematologic response through increased proliferation and cytokine production and increased naïve T-cell reconstitution *in vivo*

Characteristics of the 13 MDS patients treated with lenalidomide *in vivo* are described in Supplemental Table 1. Seven patients exhibited a major erythroid response out of 13 (53.8%), as determined by international working group 2000 criteria. The median age of the group was 74 (range 49 – 83) and the median age of the responders was 72 years (mean 68.3, range 53 – 79 years), which did not differ from the non-responders (median 78.5, mean 73.5, range 49 - 83) (p = 0.224). Two patients with del(5q) were treated, one with del(2)(q11.2), one with complex abnormalities, and the remaining patients had a normal karyotype (n=9). All patients, including the patients with del(5q), were treated for severe anemia and the responsive patients demonstrated a sustained increase in hemoglobin for at least 4 weeks

duration. Patients with del(5q) also had a complete cytogenetic response with elimination of the clonal myeloid cells. The mean hemoglobin for the group was 9.1 g/dL, absolute neutrophil count 3.22×10^9 cells/L, and platelet count was required to be greater than 50,000 cells/L for eligibility on the clinical trial. **a.** Schematic of patient sample collection and lenalidomide treatment during clinical trial. **b-e.** PBMCs were collected from MDS patients (n=13) prior to (pre) and 16 weeks after (post) receiving lenalidomide therapy. Patients were evaluated using the IWG 2000 criteria for response, with 7 responders (R) and 6 non-responders (NR). **b.** Cells were stained for CD3, CD8, CD45RA, CD62L, and DAPI as described in Supplemental Figure 1. The proportion of Naïve, Central Memory, Effector Memory, and Terminal Effector Memory T-cells for both CD4 and CD8 was determined using flow cytometry. Naïve cells are described as $CD3^+CD62L^+CD45RA^+$, Central Memory as $CD3^+CD62L^+CD45RA^-$, Effector Memory as $CD3^+CD62L^-CD45RA^-$, and Terminal Effector Memory are described as $CD3^+CD62L^-CD45RA^+$.The difference Post-Pre of each of the phenotypes within Responding and Non-Responding patients is shown. Statistical analysis was performed using Wilcoxon Rank Sum. P values are indicated. **c.** Cells were cultured in the presence of anti-CD3/CD28 antibodies for 48 hours before measurement of BrdU incorporation. Percentage of proliferating CD4+ (upper) and CD8+ (lower) T-cells was determined via flow cytometry. The difference in proliferation (post-pre) was analyzed using Wilcoxon Rank Sum Test, with p values indicated. **d-e.** *In vivo* cytokine production was determined for both CD4 (**d**) and CD8 (**e**) T-cells**.** PBMCs were stimulated for 48 hours with CD3/CD28 antibodies, and then for the last 6 hours with PMA/ionomycin. Golgi-block and cytokine staining was performed as described in Figure 1d. **f)** MDS patient PBMCs were stimulated with antiCD3/antiCD28 antibodies and the percentage difference (post-treatment - pre-treatment) of naïve CD4+ (**i**) and CD8+ (**ii**) T-cells after treatment with lenalidomide in both Responders and Non-Responders was correlated with age. A trend line was created for both Responders and Non-Responders in both the CD4+ and CD8+ populations. Data was analyzed via Spearman correlation. P values are indicated, as well as correlation of the data to the trend line (Spearman r), with 1 or −1 representing a perfect correlation. Non-Responders are represented by an open triangle symbol; Responders are represented by closed circle.