

Letter to the Editor

Anti-IgE therapy results in decreased myeloid dendritic cells in asthmatic airways

To the Editor:

Immunotherapy targeting IgE reduces aeroallergic hypersensitivity and is a US Food and Drug Administration–approved treatment option for moderate to severe allergic asthma.¹ Treatment with anti-IgE (omalizumab) results in a marked reduction in the airway inflammation of patients with asthma with a significant decrease in bronchial eosinophils and both CD4⁺ and CD8⁺ T cells.² The primary antigen-presenting cells in the lung, dendritic cells (DCs), play a critical role in airway inflammation in asthma because they constantly sample inhaled allergens/antigens and stimulate antigen-specific T cells.^{3,4} It is known that pulmonary inflammation results in an increase in the number of lung DCs.⁵ Accordingly, steroid therapy of patients with asthma that diminished the airway inflammation resulted in a reduction in airway DC numbers.⁶ Thus, we hypothesized that anti-IgE treatment in asthma volunteers might result in reduced airway DC numbers and decrease their maturation phenotype.

The study included 7 patients with intermittent to mild-persistent asthma undergoing anti-IgE treatment. Omalizumab (anti-IgE) was administered subcutaneously over 12 weeks based on body weight and serum IgE levels (for details, see this article's Methods section in the Online Repository at www.jacionline.org). All 7 subjects (2 males and 5 females; mean age, 30 years) had positive methacholine challenges and positive allergen skin tests described in detail elsewhere.⁷ Allergen PC₂₀ values were determined both before and after 12 weeks of anti-IgE treatment. All subjects showed a significant increase in PC₂₀ values from before to after anti-IgE treatment ($P = .03$) except 1 subject who failed to respond to the tested allergen even before anti-IgE treatment (Fig 1). Thus, there was an improvement in allergen-specific airway hyperreactivity in 6 of 7 subjects after anti-IgE treatment, a finding similar to that in a previous report.⁸

Bronchoalveolar lavage fluid and bronchial biopsy samples obtained at 2 weeks before therapy, immediately after 12 weeks of therapy, and 2 months posttherapy were evaluated for inflammatory cell numbers. Subjects were lavaged using 300 mL saline with a mean bronchoalveolar lavage fluid recovery of 197.5 mL (range, 72–256 mL). There was no significant change in the bronchoalveolar lavage fluid cells. The biopsy samples were also analyzed semiquantitatively for intraepithelial and subepithelial inflammatory cell and mucus cell numbers by a pathologist blind to the timing of the samples. Similar to bronchoalveolar lavage fluid cellularity, there was no significant change in mucus cells, intraepithelial or subepithelial mononuclear cells, eosinophils, or neutrophils.

We next examined DCs in bronchial biopsies after multicolor immunostaining and multispectral confocal imaging techniques (for details, see this article's Methods section in the Online Repository at www.jacionline.org). Airway myeloid DCs (mDCs or conventional DCs) and plasmacytoid DCs (pDCs) were identified, localized, and enumerated in these biopsies. For enumeration of DC subsets, the lineage-negative cells (cells that did not express specific T-cell, B-cell, monocyte, neutrophil, or natural killer markers) that expressed the β_2 -integrin CD11c and HLA-DR were identified as mDCs, whereas lineage-negative cells

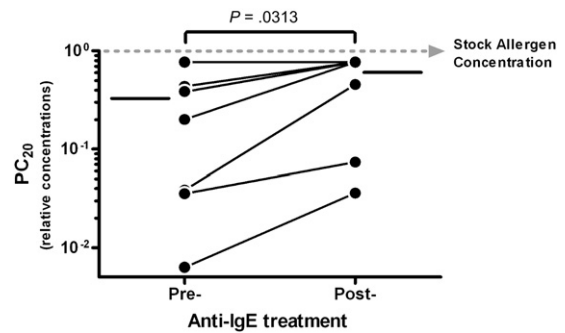


FIG 1. Effect of 12 weeks of anti-IgE treatment on the allergen-specific PC₂₀ values of asthma volunteers. PC₂₀ values or relative allergen concentrations at which the FEV₁ values decreased by at least 20% from baseline were determined both before and after 12 weeks of anti-IgE treatment. The mean PC₂₀ values are indicated on either side of time-points by solid horizontal lines.

expressing the IL-3 receptor α -subunit CD123 and HLA-DR were identified as pDCs.^{3,4} mDCs were observed in both intraepithelial and subepithelial mucosa, whereas all of the pDCs observed were in the subepithelial region (see this article's Fig E1 in the Online Repository at www.jacionline.org). As depicted in Fig 2, A, there was a highly significant ($P = .0046$) decrease in the number of mDCs immediately after anti-IgE treatment that persisted for 2 months posttreatment ($P = .0205$). The average number of mDCs in pretreatment biopsies was 126/mm² (range, 28–246), compared with 38/mm² (range, 10–66) immediately after 12 weeks of treatment and 49/mm² (range, 10–151) 2 months posttreatment. However, the number of pDCs was not significantly changed at either the end of therapy or at 2 months posttherapy (Fig 2, B). The average number of pDCs/mm² in the biopsies was 12/mm² (range, 0–38) pretreatment compared with 31/mm² (range, 0–104) immediately after treatment and 43/mm² (range, 10–85) 2 months posttreatment. It is possible that the trend toward an increase in pDCs could have become significant with additional numbers of study subjects.

The surface expression of selected function-associated markers on DCs was also analyzed, but there were no significant changes in the percentages of mDCs and pDCs that expressed CD80, CD83, and CD1a, and CD80, CD83 and BDCA2 (Blood Dendritic Cell Antigen 2), respectively (see this article's Fig E2 in the Online Repository at www.jacionline.org). The biopsies were also analyzed for changes in CD4⁺, CD8⁺, T, and regulatory T cells, but unlike a previous report,² we found no effect on the bronchial T cells (see this article's Fig E3 in the Online Repository at www.jacionline.org) after anti-IgE therapy. We believe that this inconsistency could be explained by differences in the severity of asthma in the study subjects. However, we observed a trend toward reduction in the CD4⁺ T cells, and it is also possible that samples from additional volunteers would have shown a significant decrease.

In summary, in this study of 7 volunteers, we demonstrate that anti-IgE therapy in intermittent to mild persistent asthma resulted in a significant decrease in airway mDCs associated with improved PC₂₀ values. Asthma-associated airway hyperreactivity likely results from chronic inflammation, and allergen-specific T cells play an important role in the maintenance of

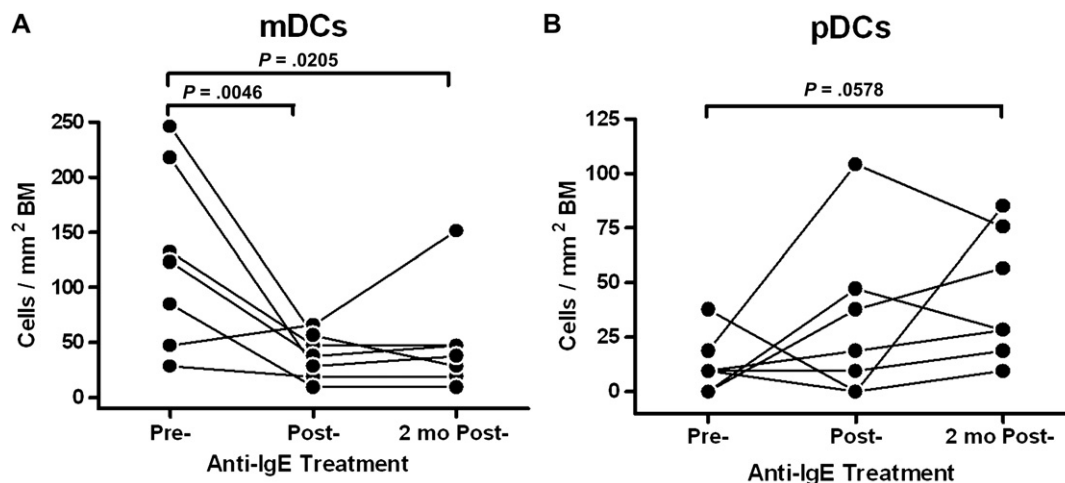


FIG 2. Effect of anti-IgE treatment on the numbers of mDCs and pDCs in asthmatic airways (N = 7). Numbers of mDCs (**A**) and pDCs (**B**) were determined by multispectral image analysis of airway biopsies by counting lineage-negative CD11c⁺HLA-DR⁺ cells and lineage-negative CD123⁺HLA-DR⁺ cells per square millimeter, respectively, from separate serial sections. The data from individual subjects are plotted as an average of 4 sections per time-point. BM, Basement membrane.

chronic inflammation.⁹ DCs are essential for the initiation of primary T-cell responses, and they are also very efficient in restimulating memory T cells. Thus, we speculate that the decrease in airway mDCs after anti-IgE treatment might contribute in the long term to reduced T-cell activity and airway inflammation, and consequently to decreased airway hyperreactivity. We do not believe that the anti-IgE therapy directly reduced mDCs, but rather reduced activation of airway mast cells (because of a decrease in IgE binding to these important effector cells) is most likely the primary effect of therapy. Consistent with this idea, a separate study showed that the same subjects enrolled in this report had a marked reduction in the FcεRI-mediated release of T_H2 cytokines and chemokines by peripheral blood basophils after anti-IgE treatment.⁷ Thus, the diminished release of DC chemotactic chemokines resulting either directly or indirectly from less mast cell activation and secretion could be responsible for the decrease in airway mDC numbers. Strikingly, a decrease in the number of mDCs was detected, although the number of other inflammatory cells did not change significantly, which suggests, in view of the clinical response in these patients, that mDC numbers might be a very sensitive indicator of modulation in tissue inflammation.

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METHODS

Study design, methacholine challenges, and allergen PC₂₀

This study was approved by the institutional Human Research Review Committee and details are described elsewhere.^{E1} Briefly, patients with intermittent to mild-persistent asthma were recruited, consented, and checked for sensitivity to allergens, and bronchoscopy was performed for obtaining the biopsy and the bronchoalveolar lavage fluid (BALF) samples. The airway responsiveness was tested by using a standard methacholine challenge as described previously.^{E2} The allergen PC₂₀ was determined. The starting allergen concentration was 1:100 dilution of the concentration that caused a 3-mm wheal on serial skin testing. If there was <20% decrement in FEV₁, the concentration of allergen was increased sequentially starting at 1:10⁷ dilution to stock. After 2 weeks of recruitment, omalizumab (anti-IgE) was administered subcutaneously 150 to 375 mg, based on weight and serum IgE levels, by giving 4 to 7 injections, each 2 to 4 weeks apart for a total of 12 weeks. Bronchoscopy was done right after anti-IgE treatment and 2 months later. In all cases in which FEV₁ crossed from above to below 80% with increasing allergen concentration, PC₂₀ was determined by linear interpolation between the 2 doses (in the log scale) both before and 12 weeks after anti-IgE treatment.

BALF and bronchial biopsy collection

BALF and bronchial biopsies collected at 3 time-points: one at 2 weeks before therapy, another immediately after therapy, and one at 2 months posttherapy were analyzed. For BALF, the right middle lobe of each subject was lavaged using prewarmed saline. BALF cytopspins were analyzed with 4 slides per time-point, and cells were classified as monocytes, lymphocytes, neutrophils, or eosinophils. The data were analyzed as the percent positive of the total cells. The bronchoscopies were performed by using a fiber optic bronchoscope directed to the carina between the bronchus intermediatus and the right middle lobe bronchus, and 6 to 8 mucosal biopsies samples were obtained using a forceps. The bronchial biopsies collected were either freshly frozen into Tissue-Tek OCT compound (Sakura Finetek, Torrance, Calif) or formalin-fixed.

Histochemical staining and multicolor immunostaining

The cryosections (6 μ) cut at Tricore Laboratories, Albuquerque, NM, were stained with hematoxylin and eosin for the histochemical analysis. For morphologic demonstration of mucus cells, Alcian blue (pH 2.5)/periodic acid-Schiff staining was performed to visualize acidic and neutral mucosubstances as described.^{E3} Tissue sections were also stained with Alcian blue and hematoxylin and eosin essentially as described previously.^{E4} All stained sections were coded and analyzed in a blind fashion by a board-certified anatomic pathologist using a $\times 40$ objective. The biopsy sections from each time-point during therapy were evaluated for eosinophils, lymphocytes, neutrophils, and intraepithelial mucous cells.

For a multicolor fluorescent immunostaining, as the primary antibodies used were all mouse mAbs (Table E1); hence, a step-wise sequential staining protocol was followed (Chand et al, manuscript in preparation). Briefly, cryosections were washed in PBS(+) (Gibco BRL), quenched in 50 mmol/L NH₄Cl/PBS(+), and nonspecific binding was blocked by using 1% gelatin/2% BSA (IgG-free) in PBS(+). After staining with primary antibodies, the sections were probed with goat antimouse F(ab)₂ Alexa-conjugated (Alexa-680, Alexa-633, or Alexa-546) antibodies (Invitrogen Inc, Carlsbad, Calif). This procedure was repeated 2 more times by using specific primary antibodies each time with blocking in between. Slides were mounted by using

ProLong Gold mounting medium with DAPI, 4'-6-diamidino-2-phenylindole, dihydrochloride (Invitrogen).

Confocal microscopy and multispectral image analysis

Confocal microscopic analysis of multicolor stained sections was performed by using a Zeiss LSM 510 Meta (Carl Zeiss MicroImaging, Inc, Thornwood, NY) mounted on an Axiovert 100 scope (Carl Zeiss Microimaging Inc, Thornwood, NY) (for details see Chand et al, manuscript in preparation). For each set of staining, the data from at least 4 optical sections were obtained and represented as means $\pm$ SD per square millimeter. For enumeration of DC subsets, the lineage-negative (Lin⁻) cells (meaning that the cells did not express specific T-cell, B-cell, monocyte, neutrophil, or natural killer markers) that expressed the β_2 -integrin CD11c and HLA-DR were identified as mDCs, whereas Lin⁻ cells expressing the IL-3 receptor α -subunit CD123 and HLA-DR were identified as pDCs to differentiate them from mast cells and basophils.^{E5,E6} For colocalization in a given section, mDCs were identified as Lin⁻/CD11c⁺/CD123⁻, and pDCs were identified as Lin⁻/CD11c⁻/CD123⁺ (Fig E1). The expression of cell-surface markers, CD80, CD83, and CD1a on airway mDCs, was assessed by staining Lin⁻/CD11c⁺ cells. Similarly for airway tissue pDCs, the expression of CD80, CD83, and BDCA2 was assessed by staining Lin⁻/CD123⁺ cells. The data were expressed as the percent positive of total mDCs or pDCs (Fig E2). In separate sections, CD4⁺ T cells were identified as CD3⁺CD4⁺CD8⁻, CD8⁺ T cells as CD3⁺CD4⁺CD8⁺, and regulatory T cells as CD4⁺CD25⁺forkhead box protein 3⁺ cells by using CD4, CD25, and forkhead box protein 3 staining (Fig E3).

Statistical analysis

The data were repeated-measures data with values of all outcome variables measured for each subject at all time-points. Analysis of each outcome variable was a linear mixed model in PROC MIXED of SAS version 9.2 (SAS Inst. Inc, Cary, NC). The covariance structure was assumed to be AR(1), an Autoregressive Time Model, which allows a greater correlation for adjacent times than more distant times. If there was a significant F test for treatment, then 1 degree of freedom pairwise contrasts were examined. Results with $P < .05$ were considered statistically significant.

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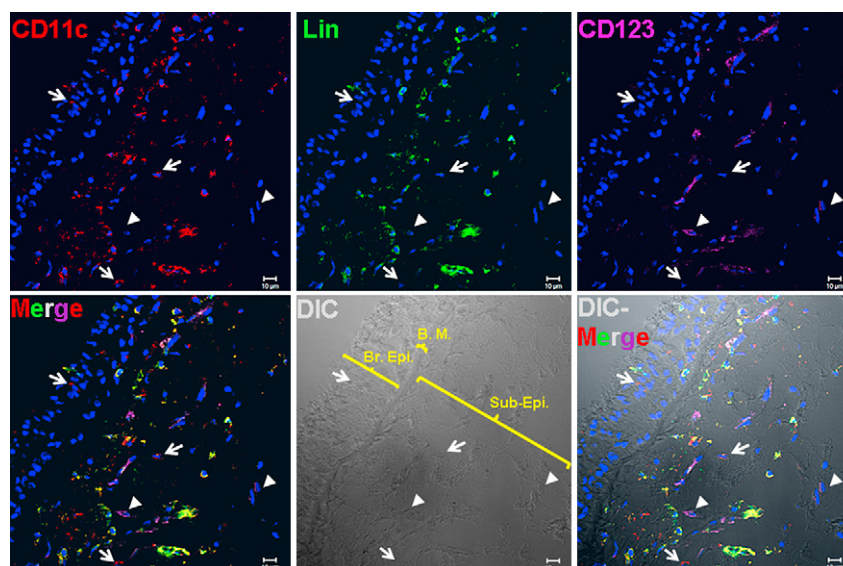


FIG E1. Identification and colocalization of mDCs and pDCs in asthmatic airways. Biopsy sections were sequentially immunostained for CD11c (red), lineage (green), and CD123 (magenta) to identify mDCs (arrows, CD11c⁺CD123⁻Lin⁻) and pDCs (arrowheads, CD11c⁺CD123⁺Lin⁻) and mounted with 4'-6-diamidino-2-phenylindole, dihydrochloride, DAPI (blue). Upper panels show single stains, and the merged images are provided in the lower panels along with a differential interference contrast (DIC) image showing tissue morphology. B.M., Basement membrane; Br. Epi., bronchial epithelium; Sub-Epi., sub-epithelial region.

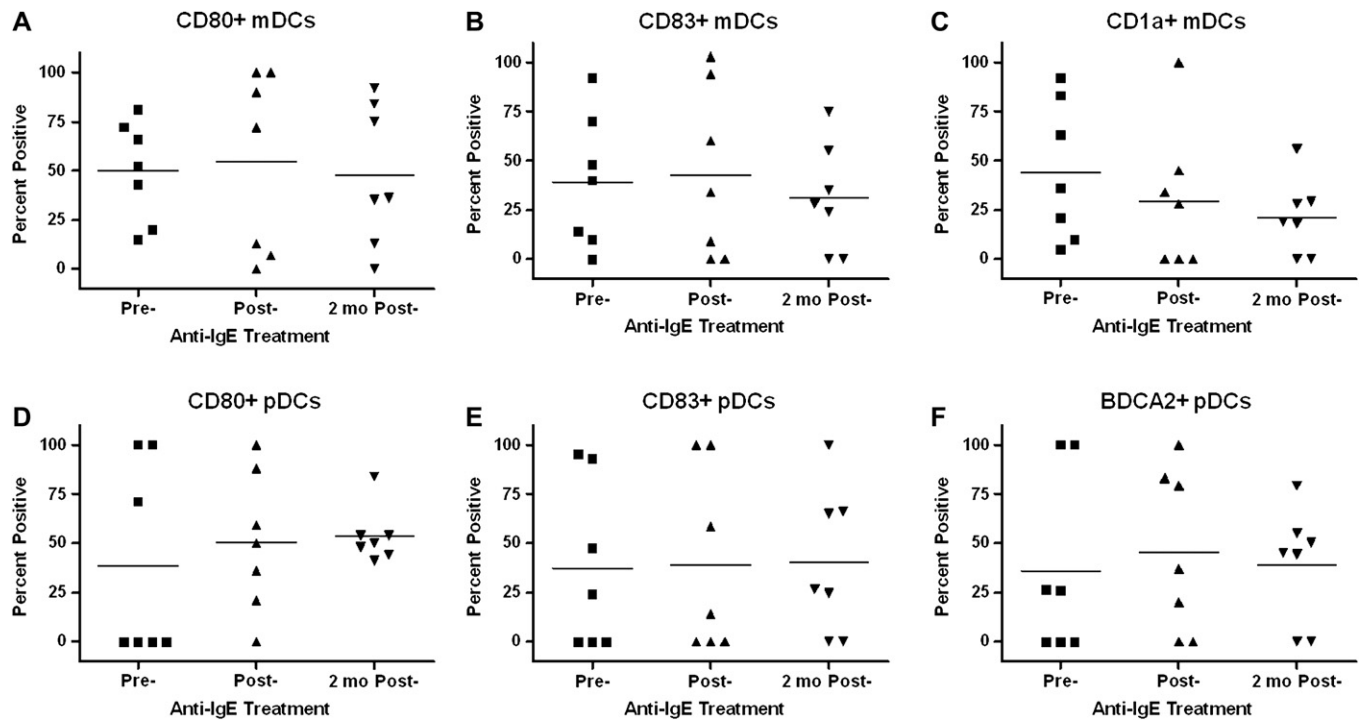


FIG E2. Effect of anti-IgE therapy on the percent of mDCs and pDCs expressing selected function-associated cell surface markers. mDCs were immunostained for the expression of CD80 (A), CD83 (B), and CD1a (C) on Lin⁻CD11c⁺ cells, and pDCs were immunostained for the expression of CD80 (D), CD83 (E), and BDCA2 (F) on Lin⁻CD123⁺ cells. The data plotted are average from 2 to 4 sections evaluated per time-point per subject. There were no changes in the proportion of mDCs and pDCs expressing these cell surface markers. However, there was a noteworthy trend toward decrease in the percent of CD1a⁺ (44.5% to 21.3%) mDCs.

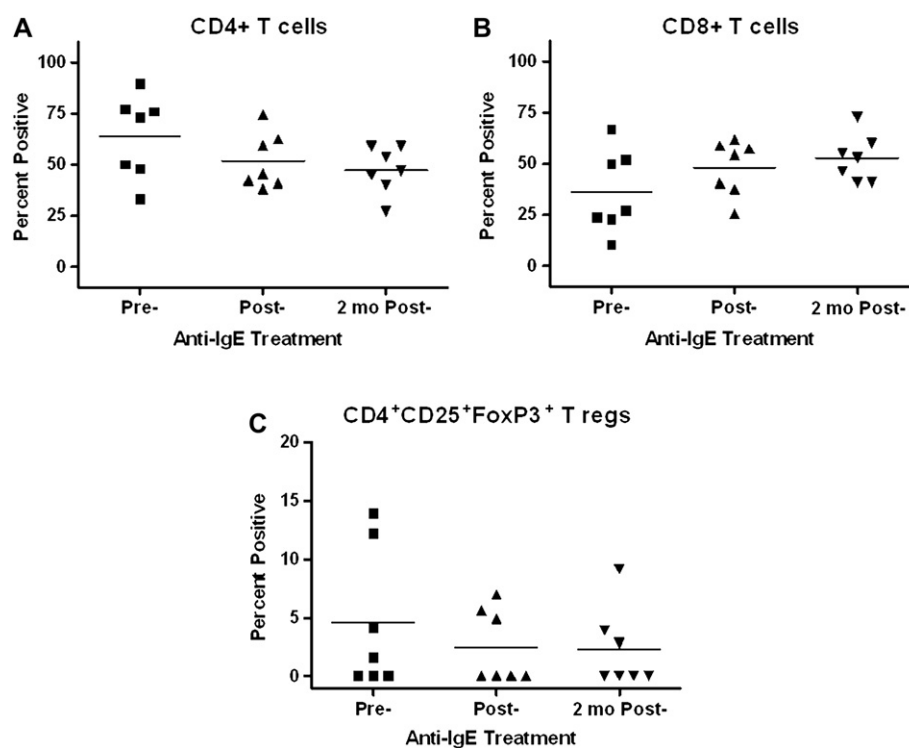


FIG E3. Percentage of CD4⁺, CD8⁺, and regulatory T cells in airways of subjects with asthma after anti-IgE therapy. CD4⁺ T cells (**A**) were counted in each biopsy section as CD3⁺CD4⁺CD8⁻ cells and CD8⁺ T cells (**B**) were counted as CD3⁺CD4⁺CD8⁺ cells by using multispectral image analysis. In addition, the regulatory T cells (*Tregs*; **C**) were enumerated as CD4⁺CD25⁺forkhead box protein 3⁺ (*Foxp3*⁺) cells. The data plotted are average from 2 to 4 sections evaluated per time-point per subject, and data are represented as a percentage of total T cells. There were no significant changes in the number of T-cell subsets during this study. Note the trend toward reduction in the percentages of CD4⁺ T cells after anti-IgE treatment.

TABLE E1. Antibodies used for immunohistochemical identification and characterization of human airway immune cells using confocal microscopy

	Antigen	Mouse isotype	Clone	Company*
Lineage cocktail	CD3	IgG ₁	UCHT1	BioLegend
	CD14	IgG _{2a}	M5E2	BioLegend
	CD16	IgG ₁	3G8	BioLegend
	CD19	IgG ₁	HIB19	BioLegend
	CD20	IgG _{2b}	2H7	BioLegend
	CD56	IgG _{2a}	MEM-188	BioLegend
Other antibodies	CD11c	IgG ₁	3.9	BioLegend
	CD123	IgG ₁	6H6	BioLegend
	HLA-DR	IgG _{2a}	L243	BioLegend
	CD11b	IgG ₁	ICRF44	BioLegend
	CD80	IgG ₁	2D10	BioLegend
	CD83	IgG ₁	HB15e	BioLegend
	CD86	IgG _{2b}	IT2.2	BioLegend
	CD1a	IgG ₁	HI149	BioLegend
	CD1c	IgG _{2a}	AD5-8E7	Miltenyi Biotec
	BDCA2	IgG ₁	AC144	Miltenyi Biotec
	BDCA4	IgG ₁	AD5-17F6	Miltenyi Biotec
	CD4	IgG _{2b}	OKT4	BioLegend
	CD8	IgG ₁	RPA-T8	BioLegend
	CD25	IgG ₁	BC96	BioLegend
	FoxP3	IgG ₁	206D	BioLegend
	IgG ₁	IgG ₁	MOPC-21	BioLegend
	IgG _{2a}	IgG _{2a}	MG2a-53	BioLegend
	IgG _{2b}	IgG _{2b}	25-31	BioLegend

BDCA, Blood dendritic cell antigen; FoxP3, forkhead box protein 3.

*BioLegend, San Diego, Calif; Miltenyi Biotec Inc, Auburn, Calif.