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# Comment on "MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells"

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# LETTERS TO THE EDITOR

## Comment on "MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells"

In the April 15, 2006 issue, Baecher-Allan et al. (1) demonstrated that HLA-DR expression on human $CD4^+CD25^{high}$ cells identifies a mature and highly active regulatory T cell (Treg) population. The authors (1) used an anti-HLA-DR mAb (L243; BD Pharmingen) to sort and to identify highly suppressive $CD4^+CD25^{high}$ regulatory T cells and suggested a specific role of HLA-DR expression in the homeostatic maintenance of Tregs in vivo. Monoclonal L243 Ab, as stated by the manufacturer, reacts with a nonpolymorphic HLA-DR epitope and should not cross-react with other MHC class II molecules. Binding of mAb L243 on the surface of the T cells indicates its reactivity toward N-terminal sequence of HLA, representing type I membrane protein.

In our hands, mAb L243 recognized approximately 20% of $Foxp3^+CD4^+CD25^{high}$ T cells (Fig. 1), thus confirming results of Baecher-Allan et al. With the aim of comparing the specificities of a variety of class II MHC mAbs, we tested binding specificity of the L243 by epitope mapping on the HLA-DP $\beta$-chain (HLA-DP$\beta$) peptide scan. Surprisingly, the peptide scan results revealed that L243 binds to the HLA-DP-specific linear motif (LERYIYN REEFA) in the N terminus of the protein (Fig. 2*A*, *left panel*). In contrast, we could not observe any reactivity of mAb L243 toward HLA-DR peptide of the corresponding N-terminal sequence (Fig. 2*A*, *right panel*). Since HLA-DP and HLA-DR share only 58% sequence identity in the region corresponding to the mapped L243 epitope, a potential cross-reactivity appears very unlikely. These results were confirmed by competition assay using a blocking peptide corresponding to the HLA-DP$\beta$ epitope. Preincubation of L243 with blocking peptide clearly reduced its specificity toward human Tregs (Fig. 2*B*), unambiguously proving its anti-HLA-DP specificity.

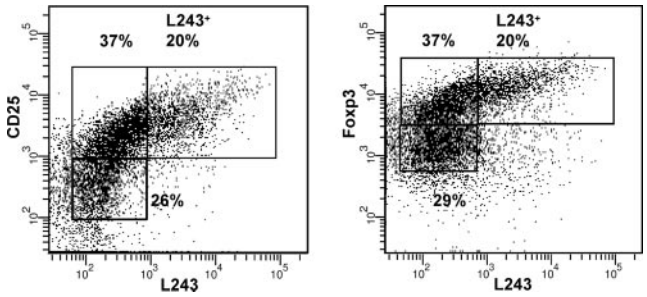


**FIGURE 1.** mAb L243 recognizes a surface Ag expressed on human peripheral blood $CD4^+CD25^{high}Foxp3^+$ Tregs. Human $CD4^+CD25^{high}$ T cells were isolated from peripheral blood and analyzed by flow cytometry for the expression of CD25 (M-A251), Foxp3 (PCH101), and L243. Individual populations expressing L243 Ag and/or CD25 and Foxp3, including their relative ratios in percentages, are shown.

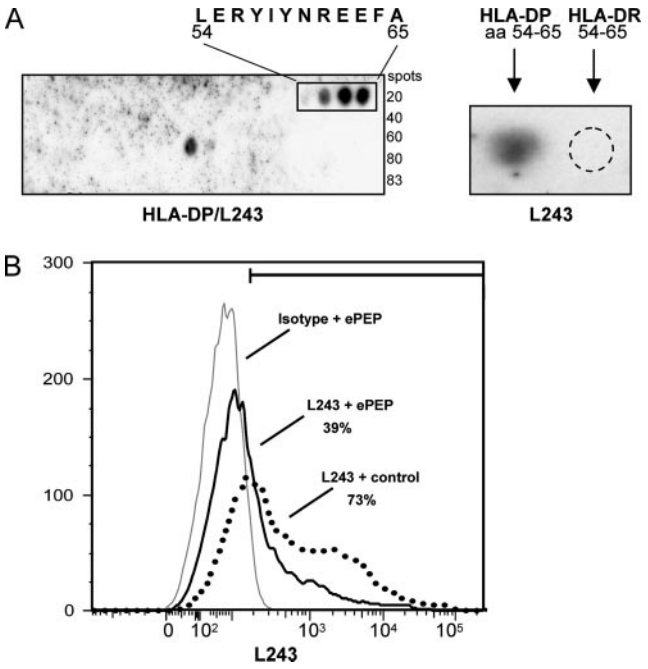


**FIGURE 2.** *A*, *Left panel*, Mapping of L243 epitope. A total of 83 overlapping peptides, covering the entire HLA-DP$\beta$-chain sequence, were synthesized and immobilized on cellulose membrane (PepSpots; JPT Peptide Technologies) and subsequently incubated with mAb L243. The boxes mark mAb-specific epitopes; recognized amino acid residues and their position in HLA-DP$\beta$ sequence are listed. *Right panel*, Specificity of mAb L243 was tested against synthesized peptide corresponding to the N-terminal sequence in the $\beta$-chain of HLA-DP or HLA-DR. Absence of the signal for HLA-DR reactivity is indicated by the circle. *B*, Demonstration of mAb specificity using an epitope-derived blocking peptide. Isotype control and mAb L243 were preincubated with 10× stoichiometric excess of the LERYIYNREEFA epitope peptide (ePEP) before the staining of human $CD4^+CD25^{high}$ Tregs. Positive cells were gated and their percentages were calculated, demonstrating that L243 binding to Tregs was significantly reduced in the presence of HLA-DP-specific epitope peptide. For control experiments, mAb L243 were preincubated with 10× stoichiometric excess of scrambled control peptide (control) before staining.

Taken together, these findings do not essentially change the conclusion of Baecher-Allan et al.; however, they clearly demonstrate that the expression of HLA-DP and not HLA-DR defines a functionally distinct and highly suppressive $CD4^+CD25^{high}$ population of regulatory T cells.

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# Response to Comment on "MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells"

In the current issue of *The Journal of Immunology*, the letter from Swiatek-de Lange et al. (1) presents data confirming our observation that class II expressing $CD25^{high}$ human natural regulatory T cells (nTregs) express the highest levels of FoxP3. We welcome additional studies into this nTreg subset because we believe that the class II-expressing nTregs ($DR^+$ Treg) are an important and functionally distinct subset that functions as end-stage effector nTregs, as they induce a strong and rapid suppression and exhibit low in vitro expansion capabilities (2).

The purpose of the report by Swiatek-de Lange et al., however, is to suggest that the subset of cells that we had referred to as $DR^+$ Treg should be referred to as $DP^+$ Treg. This is based on their contention that the anti-HLA-DR mAb, clone L324, which we had used to FACS sort the $DR^+$ Treg subset, is specific for HLA-DP peptide. We believe their claim, that the L243 Ab does not bind DR but instead binds DP, is based on flawed experimental design and ignores a large body of literature. The simplest explanation for their observation that the L243 mAb binds DP is that they are detecting a low-level cross reactivity of the Ab on denatured linear epitopes spotted at high density, which in other studies also has resulted in the demonstration of Ab polyspecificity (3). However, the biological reality of denatured linear peptides is questionable. Furthermore, in seminal work Gorga et al. (4) demonstrated that the L243 mAb specifically recognized native, highly pure HLA-DR molecules that had been immunoprecipitated and ultimately crystallized and that it did not cross-react with purified native HLA-DP molecules. Thus, there is little doubt that L243 recognizes native DR molecules; that the Ab can recognize linear denatured peptides on a solid medium would appear to have little biologic relevance.

We are pleased that Swiatek-de Lange and coworkers have begun to examine the interesting class II + ($DR^+$ Treg) subpopulation of human nTregs. However, owing to the preponderance of data demonstrating the DR specificity of mAb L234 and because of our ability to amplify HLA-DR$\alpha$ transcripts from $DR^+$ Tregs, we do not agree with their conclusion that the subpopulation should be referred to as $DP^+$ Treg.

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