Identification of Novel Human and Mouse Regulatory T cell Surface Markers via Flow Cytometry Screening

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ABSTRACT

Regulatory T cells (Tregs) are crucial components of the immune response. These cells mediate self-tolerance, suppress auto-immunity, and therefore, play a central role in the maintenance of immune homeostasis. There has been an increased interest in identifying Treg cell surface markers that would be useful not only for facilitating the identification of these cells in a variety of samples, but also for developing new tools to characterize Treg subpopulations, improve Treg enrichment protocols (used in Treg-based transplantation immunotherapy), and activate or neutralize Tregs. The identification of blocking antibodies for Tregs is of special interest, given the potential for the development of drugs that can improve anti-tumor immune responses. We have screened approximately 1,800 antibodies, via flow cytometry, using human PBMCs and mouse splenocytes (cultured with anti-CD3/anti-CD28/TGFβ and IL-2), to identify surface markers in $CD4^+/CD25^+$ and $FoxP3^+$ populations. About 170 antibodies were positively identified, of which approximately 100 recognize proteins that have already been reported in the literature to be expressed by Tregs. Approximately 30 constitute a group of potentially new Treg markers, which will be further tested for their significance.

INTRODUCTION

Regulatory T cells (Tregs) are important in several aspects of the immune system, including dominant self-tolerance (suppression of autoimmune responses and allergies), homeostasis (monitoring the stable and constant condition of the system), *in vivo* suppression of the activation, proliferation, and effector functions of several immune cell types (CD4/CD8 T cells, NK, NKT, B cells, and APCs), allograft tolerance, and fetal-maternal tolerance in pregnancy. However, in unbalanced conditions, Tregs can also suppress anti-tumor immune responses, which favors tumor progression, and limit sterilizing immunity to certain pathogens. Tregs act via multiple mechanisms: secretion of inhibitory cytokines (IL-35, IL-10, TGF-β) and cytolytic factors (Granzyme A and B), promotion of metabolic disruption (*i.e.* IL-2 deprivation), and inhibition of the maturation and function of dendritic cells (by attaching to them via CTLA-4 and LAG-3, for example).

The main Treg subset is $CD4^+/CD25^+/FoxP3^+$. FoxP3 is the standard Treg marker utilized thus far, but its intracellular/nuclear localization makes it a difficult target in a number of applications, such as systematic identification of Treg subpopulations by flow cytometry, antibody-based purification/enrichment of Tregs, and activation or neutralization of Tregs. The identification of blocking/neutralizing antibodies for Tregs is of special interest, given the potential for the development of drugs that can improve anti-tumor responses. Although Treg surface markers may be useful for all of these applications, relatively few have been identified. To systematically identify novel potential surface markers for Tregs, we screened approximately 1,800 antibodies by flow cytometry, using both human and mouse cells.

METHODS

1. Samples: human PBMCs and mouse splenocytes were cultured for one week in media containing anti-CD3/anti-CD28/TGF-β/IL-2, to promote Treg proliferation (3 independent experiments).
2. R&D Systems antibodies for cell surface markers were screened in $CD4^+/CD25^+$ populations by flow cytometry.
3. Selected antibodies not found in the literature as Treg markers were tested in non-cultured PBMCs and splenocytes in $CD4^+/CD25^+$ populations (data not shown).
4. Selected antibodies were co-stained with FoxP3 antibodies.

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RESULTS

FIGURE 1 Examples of Negative Results using Human Treg Cells

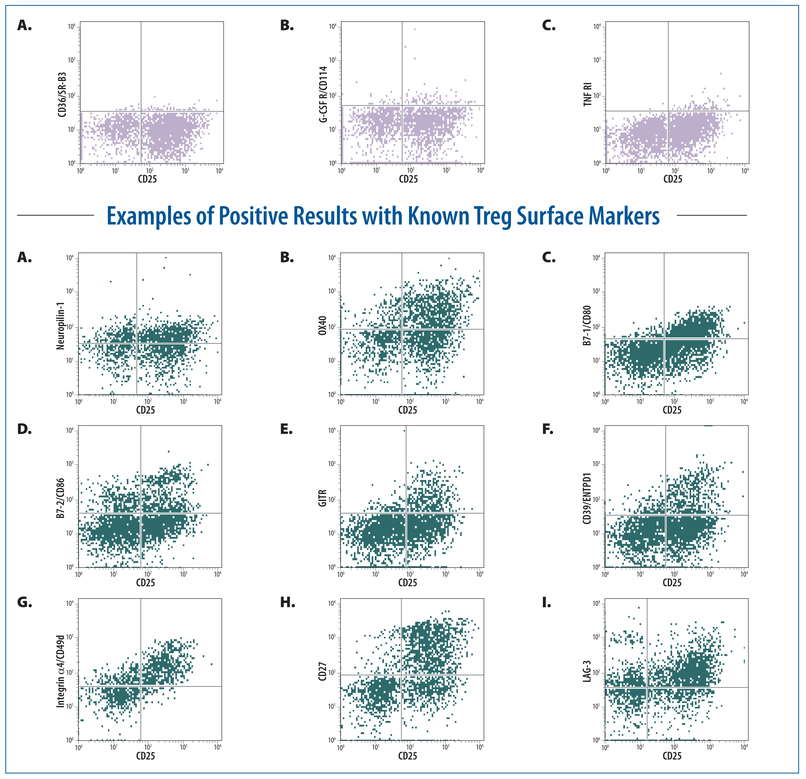

FIGURE 1 Human peripheral blood mononuclear cells (PBMCs), cultured for one week in media containing anti-CD3/anti-CD28/TGF-β/IL-2, were stained with an APC-conjugated Mouse Anti-Human CD25/IL-2 Rα Monoclonal Antibody (Catalog # FAB1020A) and one of several antibodies recognizing either a protein not expressed on Treg cells or a known Treg cell surface marker. The graphs shown were gated on $CD4^+$ cells.

FIGURE 2 Examples of Potential Novel Markers on Human Treg Cells

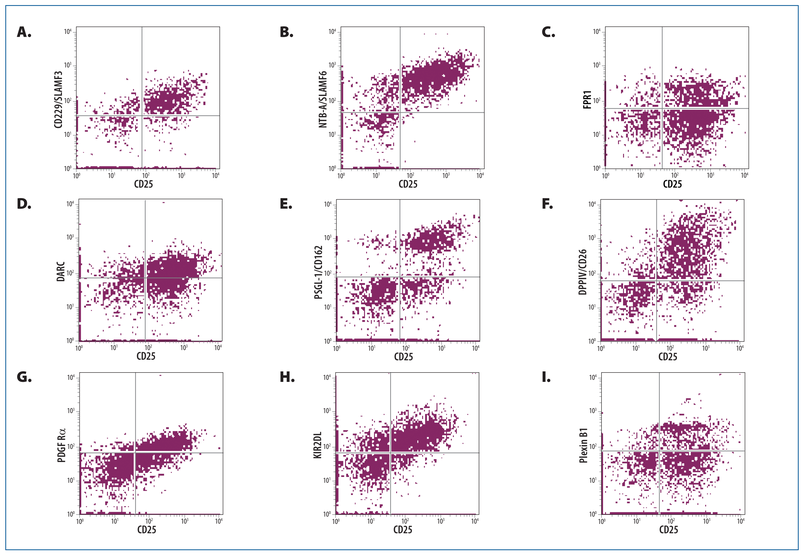

FIGURE 2 Human PBMCs, cultured for one week in media containing anti-CD3/anti-CD28/TGF-β/IL-2, were stained with APC-conjugated Mouse Anti-Human CD25/IL-2 Rα Monoclonal Antibody (Catalog # FAB1020A) and one of several antibodies recognizing potential novel Treg cell surface markers. Specific expression of these proteins on Treg cells was confirmed using non-cultured PBMCs. The graphs shown were gated on $CD4^+$ cells.

FIGURE 3 Examples of Potential Novel Markers on Human FoxP3+ Treg Cells

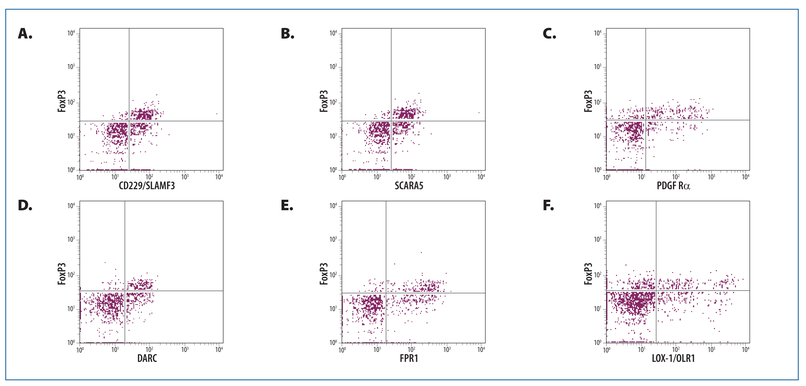

FIGURE 3 Human PBMCs, cultured for one week in media containing anti-CD3/anti-CD28/TGF-β/IL-2, were stained with APC-conjugated Goat Anti-Human/Mouse/Rat FoxP3 Antigen Affinity-purified Polyclonal Antibody (Catalog # IC3240A) and one of several antibodies recognizing potential novel Treg cell surface markers. The graphs shown were gated on $CD4^+$ cells.

FIGURE 4 Examples of Positive Results with Known Treg Surface Markers using Mouse Treg Cells

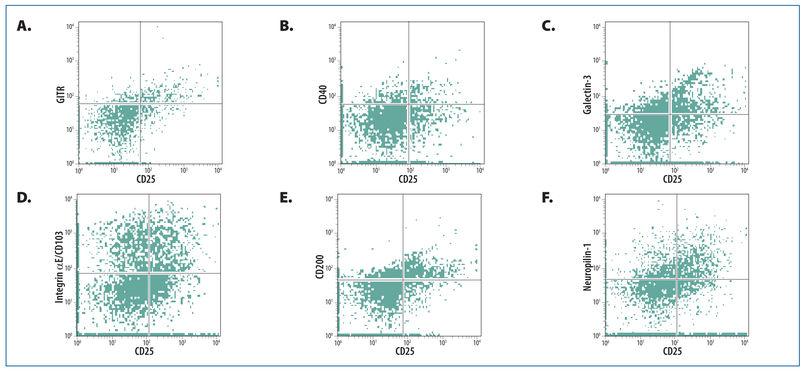

FIGURE 4 Mouse splenocytes, cultured for one week in media containing anti-CD3/anti-CD28/TGF-β/IL-2, were stained with an APC-conjugated Rat Anti-Mouse CD25/IL-2 Rα Monoclonal Antibody (Catalog # FAB2438A) and one of several antibodies recognizing known Treg cell surface markers. The graphs shown were gated on $CD4^+$ cells.

FIGURE 5 Examples of Potential Novel Markers on Mouse Treg Cells

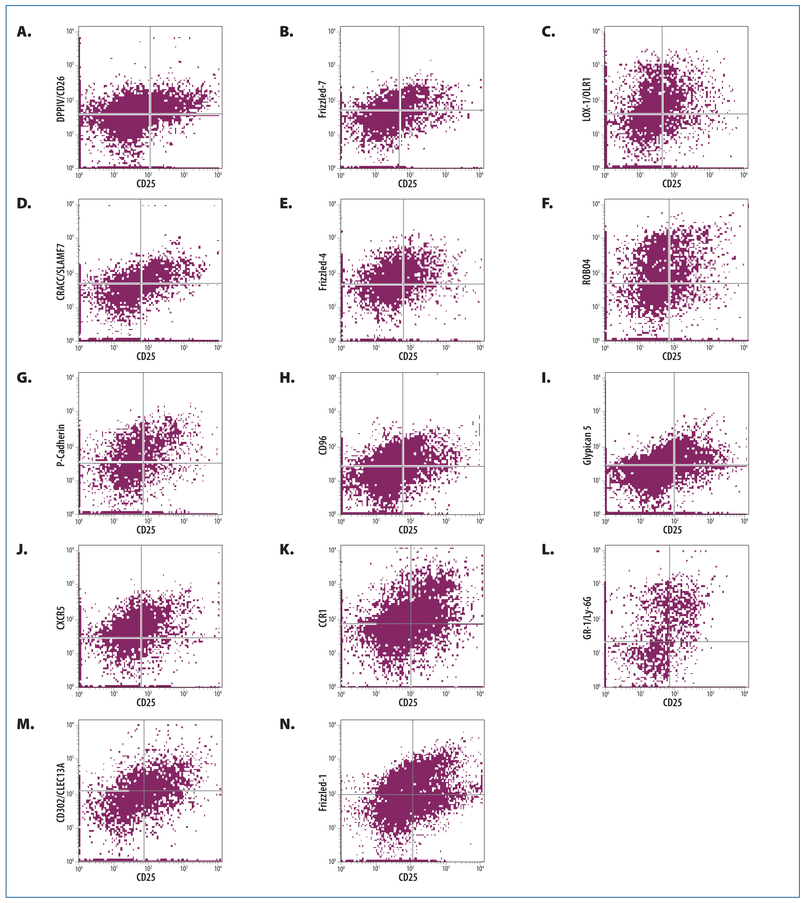

FIGURE 5 Mouse splenocytes, cultured for one week in media containing anti-CD3/anti-CD28/TGF-β/IL-2, were stained with APC-conjugated Rat Anti-Mouse CD25/IL-2 Rα Monoclonal Antibody (Catalog # FAB2438A) and one of several antibodies recognizing potential novel Treg cell surface markers. Specific expression of these proteins on Treg cells was confirmed using non-cultured splenocytes. The graphs shown were gated on $CD4^+$ cells.

CONCLUSIONS

Approximately 170 antibodies were positively identified as recognizing Treg cell surface markers in human and mouse cells. Of these, approximately 100 have already been reported in the literature to recognize proteins expressed by Tregs, and about 30 constitute a group of potentially new Treg cell surface markers. These proteins are being further tested to confirm their biological relevance, including testing their expression by induced and natural Tregs, and determining if their expression correlates with other Treg markers, and with immune suppression.

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