CD6: New low/negative surface marker for human FOXP3+ naturally-occurring regulatory T-cells

Carlos A. Garcia Santana M.D., Ph.D.[1], James W. Tung Ph.D.[2], and Sergei Gulnik Ph.D.[1]

[1]Life Science Research; [2]Life Science Development, Beckman Coulter, Inc., Miami, FL., USA

SUMMARY

Natural T-regulatory cells, nTreg, are responsible for the maintenance of dominant self tolerance. nTreg cells play an important role in the prevention of autoimmune disorders, allergy, and in maintenance of fetal maternal tolerance and organ graft tolerance. For these reasons nTreg cells have been the subject of extensive research in the past decade[1]. While the nuclear transcription factor, FOXP3, uniquely defines nTreg cells, there is a need for more convenient surface markers that can be used for nTreg isolation. Several surface markers have been already identified. Among them, CD25 and CD127 are the most commonly used. We report here the identification of CD6 as a low/negative surface marker for natural occurring regulatory T-cell (nTreg). CD6 is an important costimulatory molecule in T cell response. Lack of CD6 expression on nTreg may have significant biological consequences as it could explain anergy and peripheral antigen-induced tolerance, a central characteristic of nTreg suppressor cells. The high level of FOXP3+ cells afforded by $CD4^+CD25^+CD6^{lo/-}CD127^{lo/-}$ marker combination provides a new approach for identification, enrichment and isolation of nTreg by surface staining. In addition, $CD4^+CD25^+CD6^{lo/-}CD127^{lo/-}$ nTreg functional differentiation stages can be assessed based on the expression of CD45RA and CCR4 markers, where $CD45RA^-CCR4^{hi}HLA\text{-}DR^+$ cells could represent a mature effector stage of the nTreg population. This allows analysis of nTreg differentiation using only surface markers. Lack of or low CD6 expression on nTreg could contribute to a better understanding of the biology of nTreg immune regulation.

MATERIAL & METHOD

Subjects. Heparinized blood samples were obtained with informed consent with Institutional Review Board approval at Blood Service, Beckman Coulter,

Multicolor Flow Cytometry. Conjugated monoclonal antibodies used are summarized in Table 1. Staining was performed according Grant et al. protocol[2]. Multicolor flow cytometry was used to evaluate Treg markers using the panels show in Table 2.

Table 1. Conjugated monoclonal antibodies

Antibody-Conjugate	Clone	Function	Source
CD4-PC7	SFCI12T4D11	MHC- II R	BCI
CD4-Krome Orange	13B8.2	MHC- II R	BCI
CD6-APC	6D3	TCR Costimulation	BCI
CD6-FITC	6D3	TCR Costimulation	BCI
CD8-APC	B9.11	MHC-I R	BCI
CD25-PE	B1.49.9	IL-2R, activation	BCI
CD25-APC	B1.49.9	IL-2R, activation	BCI
CD25-PC7	B1.49.9	IL-2R, activation	BCI
CD45RA-ECD	2H4	Naïve/memory	BCI
CD45RA-AF750	2H4	Naïve/memory	BCI
CD62L-ECD	DREG56	LN Homing R	BCI
CD127-PE	R34.34	IL-7^{α} R	BCI
CD127-APC-AF700	R34.34	IL-7^{α} R	BCI
HLA-Dr-Pacific Blue	Immu-357	Activation	BCI
CD45-FITC	J.33	Pan Leukocyte	BCI
CD45-PE	J.33	Pan Leukocyte	BCI
CD45-ECD	J.33	Pan Leukocyte	BCI
CD45-PC5	J.33	Pan Leukocyte	BCI
CD45-PC7	J.33	Pan Leukocyte	BCI
CD45-APC	J.33	Pan Leukocyte	BCI
CTLA-4-PCy5 (CD152)	BNI3	Inhibitory signal	BD PharMingen
GARP-PE	7B11	Activation	BD PharMingen
CCR4-PE (CD194)	205410	Homing R	R&D Systems
CD39-APC	A1	Ecto-nucleotidase	BioLegend
FOXP3-Pacific Blue	206D	Treg transcript	BioLegend

Flow cytometry analysis was performed using a Gallios* flow cytometer (Beckman Coulter) equipped with 405nm, 488nm, and 633nm lasers. Autofluorescence, isotype and FMO controls were used to establish negative and positive fluorescent events (Figure1A, B), Compensation controls were performed using single tube staining with antibodies conjugated with the fluorochromes used in our staining protocols.

The results were expressed as the percentage of cells above the negative region for each mAb; alternatively, results were expressed as the mean of fluorescence intensity (MFI) of discrete populations.

Table 2. Multicolor flow cytometry panels

Panels	Pacific Blue	Krome Orange	FITC	PE	ECD	PC5/ 7AAD	PC7	APC/ AF647	APC-AF700	APC-AF750 APC-PC7
10 color Panel 1	HLA-Dr	CD4	CD6	CCR4	CD62L	7AAD	CD25	CD39	CD127	CD45RA
10 color Panel 2	HLA-Dr	CD4	CD6	GARP	CD62L	7AAD	CD25	CD39	CD127	CD45RA
8 color panel	FOXP3		CD6	CD127	CD45RA	CTLA-4	CD4	CD25		HLA-Dr
6 color panel	FOXP3		CD6	CD127			CD4	CD25		HLA-Dr
CD6-Treg sorting				CD25			CD4	CD6		
CD127-Treg sorting			CD127				CD4	CD25		

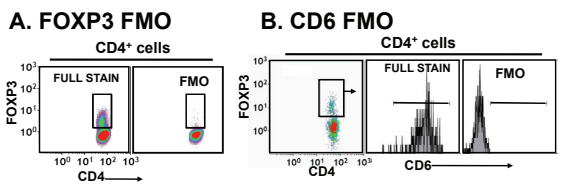


Figure 1. Fluorescense minus one for FOXP3 and CD6 staining

Lymphocytes were gated based on forward and side light scatter, viable cells were selected as 7AAD negative cells, side and forward scatter doublets were excluded, and $CD4^+$ population was analyzed (Figure 2)

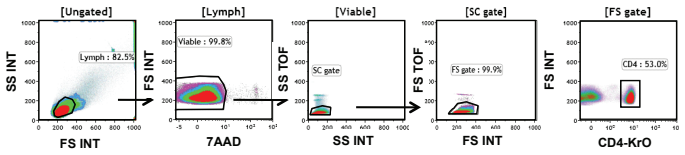
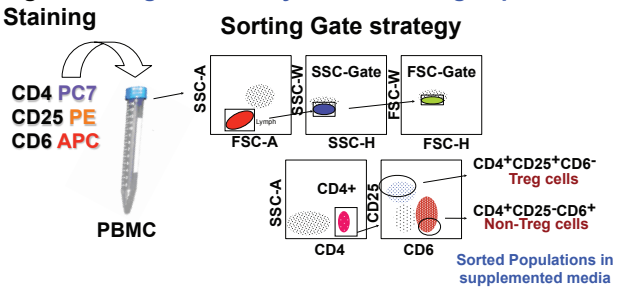


Figure 2. Gating strategy for 10-color nTreg panel analysis.

In vitro suppression assay. Sorting regulatory T-suppressor cells. After PBMC staining, cells were pelleted and resuspended at 10×10^6 per mL cell culture media. Stained cells were isolated using a MoFlo* XDP (Beckman Coulter, Inc.) high speed cell sorter.

Figure 3. Treg isolation by MoFlo XDP high speed cell sorting



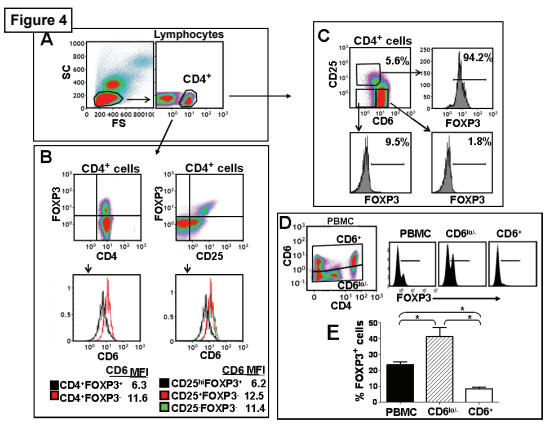
In vitro suppression assay was performed based on the capacity of added nTreg to suppress the proliferation of allogeneic $CD8^+$ responder T-cells stimulated with CD3/CD28 soluble mAbs[3]. A carboxyfluorescein diacetate succinimidyl ester (CFSE)-based proliferation assay was used[4]. A CFSE histogram of unstimulated responder cells defined the parent population, and the proliferation of activated responders was determined by calculation of precursor frequency (pf) using ModFit LT software (Verity Software House, v3.0; Topsham ME). Results are expressed as % suppression of pf for each nTreg:Tresp ratio sample.

Methods of analysis. Flow cytometric analysis was performed using Kaluza* software (v1.2; Beckman Coulter, Inc., USA). Statistical analyses included mean, standard deviation and regression testing using Excel (Microsoft Office 2003) and U-Mann Whitney non parametric tests (GraphPad Software, Inc., USA). Proliferation of activated responders was determined by calculation of precursor frequency (pf) using ModFit LT software (Verity Software House, v3.0; Topsham ME).

RESULTS

Low/ negative expression of CD6 in nTreg.

Figure 4



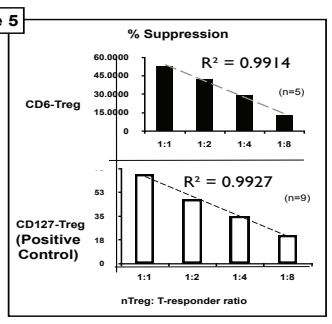
B. MFI analysis reveals low CD6 expression on $CD4^+FOXP3^+$ and $CD4^+CD25^+FOXP3^+$ cells

C. $CD4^+CD25^{hi}CD6^{low/-}$ lymphocytes have significantly higher % of FOXP3 positive cells compared to $CD4^+CD25^-CD6^{lo/-}$ and $CD4^+CD25^-CD6^+$.

E. FOXP3 expression in $CD6^+$ and $CD6^{lo/-}$PBMCs. Bars represent mean ± SD from four healthy donors; * p<0.03, U-Mann Whitney test.

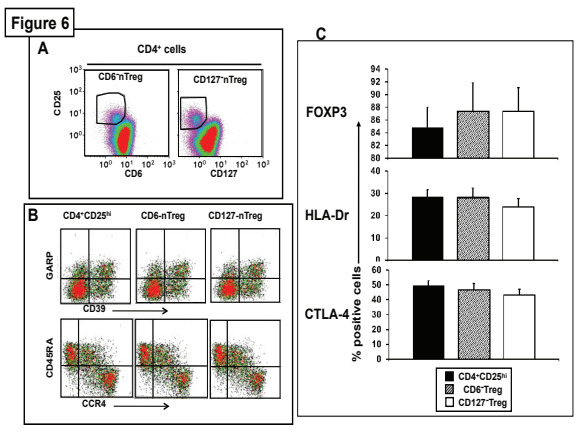
$CD4^+CD25^{hi}CD6^{lo/-}$ cells show suppressive function Treg-suppression assay

Figure 5



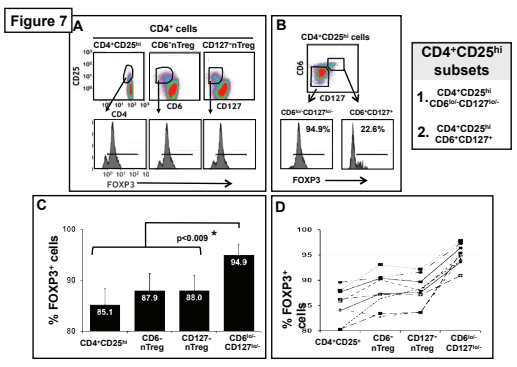
No differences in the expression of nTreg-associated markers in CD6-Treg and CD127-Treg populations

Figure 6



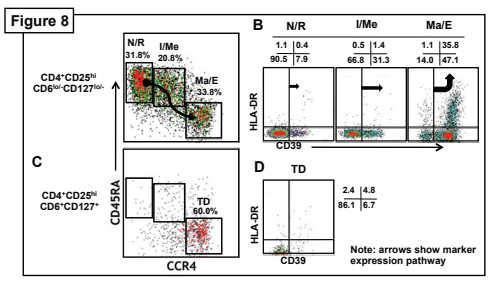
Highly enriched FOXP3+ population identified by surface nTreg markers.

Figure 7



nTreg functional maturation compartments defined by CD45RA/ CCR4 bivariate analysis

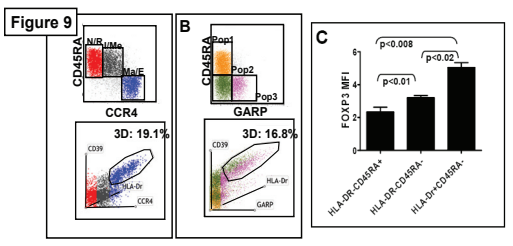
Figure 8



-$CCR4^{hi}HLA\text{-}DR^+$ cells could

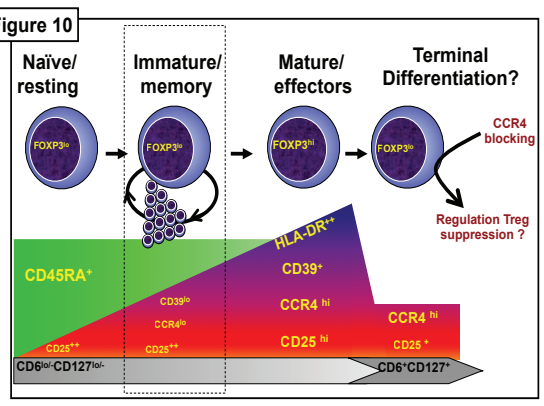
$CD45RA^-CCR4^{hi}HLA\text{-}DR^+$ cells could represent a mature effector stage of the nTreg population

Figure 9



Postulated nTreg functional maturation pathway model

Figure 10



DISCUSSION & CONCLUSION

$CD6^{lo/-}$: New insights into nTreg cell biology

Explains anergy in nTreg cells

Thymus Treg selection

Mechanism to induce tolerance

$CD6^{lo/-}$ nTreg

Depletion of bone marrow with anti-CD6 will enrich the cell preparation with untouched nTreg cells without loosing lymphoid progenitors and could deplet inflammatory/ cytotoxic T-cells

Identification and isolation of high pure $FOXP3^+$ population

↓

Accurate evaluation of nTreg subset

ACKNOWLEDGEMENTS

We wish to acknowledge Dr Tewfik Miloud and David Bloodgood for preparation of anti-CD6 conjugates, and Dianne Prendergast and Joe Gao for MoFlo* XDP assistance. We thank Dr Vincent T. Shankey for his valuable discussions and suggestions, and Dr. William Godfrey, Dr Li Yang and Dr. Mike Reed for their useful comments. The authors are employees of Beckman Coulter, Inc., and presented research was undertaken under context of their employment.

REFERENCES

1. Sakaguchi et al. (2004) Annu Rev Immunol 22, 531-562.
2. Grant, J. et. al.Cytometry B Clin Cytom. (2009) Mar;76(2):69-78).
3. Putnam, A. et al. (2009) Diabetes 58, 652-662.
4. Lyons and Parish, (1994) J Immunol Methods 171, 131-137.

**Gallios, MoFlo XDP and Kaluza are for research use only. Not for use in diagnostic procedures.*