Title: Measuring CTLA-4-dependent suppressive function in regulatory T cells

Running Head: Treg suppression

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Summary
Regulatory T cells (Treg) have a central role in controlling the activation of self-reactive T cells and maintaining peripheral tolerance in our body. Many effector mechanisms for Treg function have been described including a role for the protein CTLA-4 which is constitutively expressed by these cells. Despite its importance, there is currently little consensus in the methods and protocols for studying CTLA-4 function, which is partially due to debate over CTLA-4 function itself. In this chapter, we outline protocols used in our lab to study CTLA-4 function, which have been generated based on the observation that CTLA-4 acts to physically remove and degrade its ligands expressed by antigen presenting cells. Accordingly, we provide protocols for isolation of human monocytes and their differentiation into dendritic cells (DC), purification of conventional and regulatory T cell populations and the assembly of CTLA-4-dependent Treg suppression assays. We hope that this will offer a reliable platform for dissecting the biology of CTLA-4 on Treg and for testing reagents aimed at modulating CTLA-4 function. Such assays are increasingly vital for the study of immune function in both healthy individuals and patients with a variety of autoimmune and immune dysregulation syndromes.

Keywords: conventional T cells, regulatory T cells, CTLA-4, CD80, CD86, CD28, transendocytosis, dendritic cell, costimulation, suppression.

1. Introduction
Regulatory T cells (Treg) are key regulators in the immune system and play an essential role in maintaining immune homeostasis and tolerance (1). Loss of Treg due to mutation in the key transcription factor Foxp3 results in profound autoimmunity (2) (3). This has focused attention on the effector mechanisms whereby Treg suppress self-reactivity and multiple mechanisms have been identified. These include cell-contact dependent molecules and soluble factors that are expressed on and secreted by Treg, respectively (4-8). However, amongst these mechanisms, is a role for the protein CTLA-4 which is increasingly recognised as imparting a dominant suppressive function to Treg in vivo (9-12). Accordingly, animals lacking CTLA-4 die from fatal autoimmune disease characterised by multiple tissue infiltration and organ failure caused by self reactive T cells (13-15). Interestingly, it is also evident that autoimmunity due to the lack of CTLA-4 is dependent on the presence of the immune stimulatory protein CD28 and its ligands CD80 and CD86 for which CTLA-4 is a shared receptor. The importance of the CD28/CTLA-4 pathway has therefore led to the development of numerous therapeutic interventions in recent years including the generation of multiple soluble CTLA-4 variants (16), the use of Treg for adoptive therapies (17) and the use of anti-CTLA-4 for tumour therapy (18). While CTLA-4 is accepted as a crucial negative regulator of T cell responses, there is still controversy over its fundamental behaviour at a molecular level (19). This debate appears to have impeded the development of robust assays for CTLA-4 function and as a result, there are no widely accepted protocols available.

Accepting the on-going mechanistic controversies, it is nonetheless our view that there is compelling evidence that CTLA-4 works in a cell-extrinsic manner: that is CTLA-4 expressed on Treg works via influencing the antigen presenting cells (APC) (9). A number of in vivo studies point to the fact that CTLA-4 expressing cells can control CTLA-4 deficient cells indicating a cell-extrinsic function. More importantly from an assay perspective we recently observed a novel - molecular mechanism (transendocytosis) where CTLA-4 acts to capture and destroy its ligands CD80/CD86 from cells in contact. This cell-extrinsic function deprives the APC of the capacity to costimulate T cells via CD28 (20). The core of this new idea is that T cells expressing CTLA-4 control costimulatory signals derived from CD28 interactions with its ligands. It is well established that CD28 costimulation is generally required for full T cell activation and CTLA-4
therefore acts to limit this function (21). A more detailed consideration of this idea reveals that the function of CTLA-4 is likely not ubiquitous, but rather limited to specific contexts. Perhaps most germane to this argument, is the fact that CTLA-4 function cannot be measured if the T cell response is not specifically dependent on the presence of CD28 ligands. Accordingly, widely used protocols using beads coated with CD3 and CD28 antibodies cannot be used to measure CTLA-4 functions. In addition, the balance between the total number of ligands (CD80/CD86) and the amount of CTLA-4 available will also determine the functional efficacy of CTLA-4 behaviour. Accordingly, low amounts of ligand will generally be susceptible to CTLA-4 suppression. In contrast, high levels of ligand and/or relatively low levels of CTLA-4 would permit CD28 costimulation. We have shown that such predictions hold true in experimental models and act to predict CTLA-4-dependent Treg function (22). Based on this concept, we have developed quantitative assays to examine Treg suppressive function mediated by CTLA-4 which are capable of identifying defects in CTLA-4 expression (23). Here, we describe the details of isolation and differentiation of relevant cells and the assembly of in vitro assays capable of measuring CTLA-4-dependent regulatory T cell function in humans.

2. Materials

2.1 Reagents and Monoclonal Antibodies

2.1.1 For reagents see Table 1

2.1.2 For monoclonal antibodies see Table 2

2.2 Isolation Buffer and Culture Media

2.2.1 Isolation buffer: PBS supplemented with 2mM EDTA and 0.5% bovine serum albumin.

2.2.2 Culture media: RPMI 1640 supplemented with 10% FBS, 1% Penicillin (10,000U/ml), 1% streptomycin (10mg/ml) and 1% L-glutamine (2mM).

2.2.3 Culture conditions: cells are cultured at 37°C, 95% humidity and 5% CO₂ in an incubator.

2.3 Cellular Components

2.3.1 Isolation of human peripheral blood mononucleated cells

1. Peripheral blood mononucleated cells (PBMC) isolations are performed by density gradient centrifugation (1000g for 25 minutes) following overlay of 25-30ml diluted blood onto 15ml Ficoll-Paque PLUS.

2. Yield is approximately 50-100 x10⁶ depending on donors.

3. PBMC are resuspended at 100x10⁶/ml in isolation buffer for further separation and phenotyping by flow cytometry.

2.3.2 Isolation of human CD14⁺ monocytes by magnetic particle separation.

1. Monocytes are purified from PBMC by negative selection using human monocyte enrichment kit as follows:

2. PBMC are incubated with bispecific tetrameric antibody complexes containing monoclonal antibodies against CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A and dextran at 50µl/ml of PBMC at 4°C for 10 minutes in a 5ml tube.

3. Dextran-coated D magnetic particles are vortexed thoroughly and added to the cells at 50µl/ml and incubated at 4°C for 5 minutes.

4. Isolation buffer is added to a total volume of 2.5ml and the tube is placed into the magnet for 5 minutes.

5. Whilst still attached to the magnet, cells (containing negative fraction- enriched CD14⁺ monocytes) are poured into a new 5ml tube.

6. The magnetised tube is left inverted for 2-3 seconds and magnetically labelled (positive fraction-linage/cocktail⁺ cells) cells remain.
7. The magnetic removal step is repeated to improve yield and negatively selected cells are collected into a 20ml universal.
8. Yield is approximately 10-15% of PBMC depending on donors.
9. Monocytes are re-suspended in culture media at a density of 2x10^6/ml for further differentiation into DC in culture.
10. Monocytes are phenotyped by flow cytometry and are typically CD3^-CD4lowCD14^-CD11c+ cells (Figure 1A).

2.3.3 Differentiation of immature DC derived from human CD14^+ monocytes
1. Monocyte are cultured in flat-bottom 24-well plates in 0.5ml (1x10^6 cells)/well in culture media supplemented with human GM-CSF at 20ng/ml and human IL-4 at 100ng/ml.
2. At day 2, cultures are further supplemented with 0.5ml fresh culture media containing human GM-CSF and IL-4 at final volume of 1ml.
3. At day 7, immature DC (iDC) are harvested for use in T cell stimulations. When collecting DC, the culture plate is placed on ice for 20 minutes to reduce adherence. Wells are washed with ice-cold PBS and cells are suspended by gentle pipetting.
4. Yield is approximately 25-40% or 0.25-0.4x10^6/well depending on donors.
5. iDC are re-suspended in culture media at a density of 0.36x10^6/ml and used as APC in T cell proliferation and suppression assays.
6. iDC are phenotyped by flow cytometry and typically CD11c^-HLA-DR^-CD80^-CD86^- cells (Figure 1B).

2.3.4 Enrichment of human total CD4^+ T cells by magnetic particle separation
1. Total CD4^+ T cells are purified from PBMC by negative selection using human CD4^+ T cell enrichment kit as follows:
2. PBMC are incubated with bispecific tetrameric antibody complexes containing monoclonal antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCRγδ, glycophorin A and dextran at 25µl/ml at room temperature for 10 minutes in a 5ml tube.
3. Dextran-coated D magnetic particles are vortexed thoroughly and added to the cells at 50µl/ml and incubated at room temperature for 5 minutes.
4. Isolation buffer is added to a total volume of 2.5ml and the tube is placed into the magnet for 5 minutes.
5. Whilst still attached to the magnet, cells (containing negative fraction- CD4^+ T cells) are poured into a new 5ml tube.
6. The magnetised tube is left inverted for 2-3 seconds and magnetically labelled (positive fraction-lineage/cocktail^-cells) cells remain.
7. The magnetic removal step is repeated to improve yield and negatively selected cells are collected into a 20ml universal.
8. Yield is approximately 20-30% of PBMC depending on donors.
9. CD4^+ T cells are re-suspended in culture media at a density of 100x10^6/ml for further separations.

2.3.5 Isolation of human CD4^+CD25^- naïve conventional T cells by magnetic particle separation
1. CD4^+CD25^- naïve conventional T cells (nTcon) are further purified from total CD4^+ T cells (following step 2.3.4) by negative selection using human naïve CD4^+ T cell enrichment kit as follows:
2. Cells are incubated with biotinylated anti-CD45RO antibody at 50µl/ml at room temperature for 15 minutes in a 5ml tube.
3. To obtain purified CD25− conventional T cells by depleting CD25+ T reg, anti-CD25 tetrameric antibody complexes at 50µl/ml are also added in combination with the above step.

4. Add the bispecific tetrameric antibody complexes containing monoclonal antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγδ, glycophorin A and dextran at 50µl/ml at room temperature for 10 minutes.

5. Dextran-coated magnetic nanoparticles are mixed thoroughly and added to the cells at 100µl/ml and incubated at room temperature for 10 minutes.

6. Isolation buffer is added to a total volume of 2.5ml and the tube is placed into the magnet for 10 minutes.

7. Whilst still attached to the magnet, cells (containing negative fraction- nTcon) are poured into a new 5ml tube.

8. The magnetised tube is left inverted for 2-3 seconds and magnetically labelled (positive fraction-lineage/cocktail’ cells) cells remain.

9. The magnetic removal step is repeated to improve yield and negatively selected cells are collected into a 20ml universal.

10. Yield is approximately 10-50% of total CD4+ T cells depending on donors.

11. nTcon are phenotyped by flow cytometry and are typically CD3+CD4+CD45RA−CD45RO+CD25− cells (Figure 2A).

12. Cells are then labelled with 2.5μM CellTrace Violet Stain reagent as follows:

13. Cells were washed twice with PBS and resuspended at a density of 5-10 x 10⁶/ml in a 20ml universal.

14. CellTrace Violet Stain is added at 2.5μM and cells are incubated at 37°C for 20 minutes.

15. To quench the labelling reaction, the universal is filled with 25ml culture media and cell are incubated at 37°C for a further 10 minutes followed by two washes with culture media.

16. CellTrace Violet labelled nTcon are re-suspended in culture media at a density of 1.8 x 10⁶/ml (or 90,000/50µl) and used as responder cells in T cell proliferation and suppression assays.

2.3.6 Isolation of human CD4+CD127lowCD49d− Treg by magnetic particle separation

1. Treg are further purified from total CD4+ T cells (following step 2.3.4) by negative selection using human CD4+CD127lowCD49d− Treg enrichment kit as follows:
2. Cells are incubated with human CD4⁺CD127lowCD49d⁻ Treg enrichment cocktail at 50µl/ml at room temperature for 10 minutes in a 5ml tube.
3. D2 magnetic particles are vortexed thoroughly and added to the cells at 100µl/ml and incubated at room temperature for 5 minutes.
4. Isolation buffer is added to a total volume of 2.5ml and the tube is placed into the magnet for 5 minutes.
5. The magnet holding the tube is picked up and desired fraction are poured into a new 5ml tube. The tube is left inverted for 2-3 seconds.
6. The tube containing unwanted cells from the magnet is removed and discarded.
7. 50µl of dextran selection cocktail is added to the cell suspension in the new tube and incubated at room temperature for 10 minutes.
8. 50µl of D2 magnetic particles are added to the cells and incubated at room temperature for 5 minutes.
9. The magnet holding the tube is picked up and cells (Negative fraction- Treg) are poured into a new 5ml tube. The tube is left inverted for 2-3 seconds.
10. The magnetic removal step is repeated to improve yield and negatively selected cells are collected into a 20ml universal.
11. Yield is approximately 2-5% of total CD4⁺ T cells depending on donors.
12. Treg are re-suspended in culture media at a density of 1.8x10⁶/ml (or 90,000/50µl) and used as suppressors in T cell suppression assays (see Note 1).
13. Treg are phenotyped by flow cytometry and typically CD3⁺CD4⁺FoxP3⁺ CD25⁺CD127lowCD49d⁻ cells (Figure 2B).

2.4 Flow Cytometric Analysis
2.4.1 FACS staining buffer: RPMI 1640 supplemented with 2% FBS.
2.4.2 PBMC, isolated monocytes and differentiated iDC, isolated nTcon and Treg are FACS stained using monoclonal Abs (see Table 2) at 4°C for 30 minutes for flow cytometry (Figure 1 and 2).
2.4.3 Single cell suspension are acquired by FACS-LSRII (Becton Dickinson) and analysed by FlowJo (TreeStar).

3. Methods
3.1 CTLA-4 dependent Treg suppression assay
3.1.1. To establish Treg suppression assay, iDC, nTcon and Treg are co-cultured in 250µl culture media in the presence of anti-CD3. The ratio of iDC:Treg are the key variable for CTLA-4 function. Accordingly we routinely titrate the iDC:Treg ratio (see Note 2).
3.1.2. In a typical setting, 90,000 of CellTrace Violet labelled nTcon are added into culture as responder T cells, typically along with 9,000 of iDC and 9,000 of Treg (Figure 3B).
3.1.3. To test the effect of CTLA-4 blockade on the proliferative response of nTcon, anti-CTLA-4 blocking Ab is added into culture condition without Treg. We routinely find blocking CTLA-4 alone has little or no effect on responder T cell proliferation in the absence of Treg (Figure 3b).
3.1.4. To test the CD80/CD86 dependency of T cell responses, CTLA-4-Ig which blocks CD80/CD86 is added into culture at 10µg/ml final concentration (Figure 3c). The purpose of CTLA-4-Ig is to serve as a control to establish that the response is CD28-CD80/CD86 dependent. We routinely find high number of iDC is less CD80/CD86 dependent [Figure 3A(c) and C(c)] (see Note 3).
3.1.5. To stimulate T cell receptor (TCR), soluble agonistic anti-CD3 Ab is added into culture at typically at 0.5µg/ml final concentration. This needs to be determined for each antibody. To test the stimulatory strength of the Ab, soluble agonistic anti-
CD3 Ab alone (ie. without iDC) to simulate T cells is included as negative control (see **Note 4**).

3.1.6. To test the effect of Treg numbers on responder T cell proliferation, different number of Treg are added into culture (see **Note 5**). We find the suppressive effect of high number (relative to iDC) of Treg is more pronounced than that of low numbers **[Figure 3(d), A versus B]**.

3.1.7. To test the CTLA-4 dependency of Treg suppression, anti-CTLA-4 blocking Ab is added together with Treg into cultured at 40µg/ml final concentration (see **Note 6**). We find consistently that CTLA-4 blockade is able to largely if not completely reverse Treg suppression in CD80/CD86 dependent T cell responses **[Figure 3B, (d) and (e)]**.

3.1.8. The proliferation of responder nTcon is measured by the dilution of CellTrace Violet. The absolute number of proliferating T cells is calculated using counting beads (see **Note 7**).

### 3.2 Non detectable Treg suppression in CD3/CD28 bead driven T cell proliferation

3.2.1 To test the effect of Treg on nTcon proliferation in a non-CD80/CD86 dependent model, we include a control using T cells stimulated by CD3/CD28 beads in which T cells are still stimulated in TCR and CD28 driven signals but no ligand (CD80/CD86) exists in this system.

3.2.2 To test the impact of the number of CD3/CD28 beads on Treg suppression. The ratio between the number of CD3/CD28 bead and Treg is titrated.

3.2.3 The remaining settings are same as in step 3.1.2-4 and 3.1.6-8.

3.2.4 We find that Treg cannot suppress T cell proliferation driven by CD3/CD28 beads regardless of the bead dose **[Figure 4(d), A versus B]**. This is consistent with that CTLA-4-Ig has no impact on this type of T cell proliferation **[Figure 4(c)]**. This suggests that the presence of ligand (CD80/CD86) is required for CTLA-4 mediated Treg suppression.

### 4. Notes

1. A mixed leukocyte reaction is not detected even though nTcon and Treg are from the same donors whereas iDC are originated from different donors. This is because in the presence of soluble anti-CD3 Ab, responder nTcon proliferate more rapidly and in greater numbers, such that any alloresponse is not observed.

2. The number of responder nTcon cells and concentration of soluble anti-CD3 Ab are the same in all culture conditions, whereas the number of iDC and Treg are titrated to vary the ratio between iDC and Treg (or CD80/CD86 and CTLA-4).

3. We find a strong correlation between inhibitory effect of CTLA-4-Ig blockade and Treg suppression mediated by CTLA-4 (cell bound). This indicates that CTLA-4 on Treg only suppress T cell responses that are driven by CD80/CD86 costimulation.

4. The soluble form and low concentration of anti-CD3 used here provides a relatively weak TCR signal. This signal alone without costimulation provided by CD80/CD86 on iDC is insufficient to drive T cell proliferation.

5. The proliferation of unlabelled Treg is not taken into account. The readout is determined by the proliferation of CellTrace Violet labelled responder nTcon as even after several (typically 1-6) rounds of division responder T cells can still be distinguished from unlabelled (violet negative) Treg by FACS analysis.

6. The reasons why we use relatively high concentration (40µg/ml) of anti-CTLA-4 blocking antibody are as follows. Firstly, although anti-CTLA-4 is very effective (almost 100%) at blocking, accessibility of the antibody to the intracellular and recycling CTLA-4 at immune synapses is likely limiting. Secondly, we only give one dose of anti-CTLA-4 at the beginning of the 5-day assays and the antibody bound to CTLA-4 is internalised and degraded. Blocking at 20µg/ml is less effective in our hands.
7. The quantitative nature of this assay is sufficient to reveal functional defects (ie. impairment of Treg suppression) in patients with point mutations in their CTLA-4 (23).

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References

Figure Legends

Figure 1. Generation of human iDC
A. Purification of human monocytes. Monocytes are sorted from PBMC by negative selection using human monocyte enrichment kit. Pre-sort PBMC populations are FACS stained with CD3 and CD4 and gated to show the expression of CD11c and CD14 (upper panels) in CD3 low compartments. Purified monocytes are re-analyzed to show the sorting efficacy (lower panels). B. Phenotype of differentiated human iDC from sorted monocytes in A. Human monocytes are cultured with GM-CSF and IL-4 for 7 days and stained for CD11c, HLA-DR, CD80 and CD86 expression.

Figure 2. Purification of human CD4\(^+\) nTcon and Treg
A. Purity of human CD4\(^+\)CD25\(^-\) nTcon. Purification of nTcon is performed in two successive steps: (1) total CD4\(^+\) T cell enrichment by negative selection using human CD4\(^+\) T enrichment kit and (2) nTcon isolation by negative selection using human naïve CD4\(^+\) T enrichment kit combined with Treg depletion by negative selection using anti-CD25. PBMC are stained and CD3\(^-\)CD4\(^+\) T cells were gated to show the expression of CD25, CD45RA and CD45RO (upper panels). Freshly purified nTcon were re-analyzed to show the sorting efficacy (lower panels). B. Purity human CD4\(^+\) Treg. Purification of Treg is performed in two successive steps: (1) total CD4\(^+\) T cell enrichment by negative selection using human CD4\(^+\) T enrichment kit and (2) Treg isolation by negative selection using human CD4\(^+\)CD127\(^{low}\)CD49d\(^-\) Treg enrichment kit. PBMC are stained and CD3\(^-\)CD4\(^+\) T cells are gated to show the expression of CD25, FoxP3, CD127 and CD49d (upper panels). Freshly sorted Treg are re-analyzed to show the sorting efficacy (lower panels).

Figure 3. CTLA-4 mediated Treg suppression is associated with low iDC and high Treg frequency
Violet labeled responder human CD4\(^-\)CD25\(^-\) nTcon are activated with human monocyte derived iDC and soluble anti-CD3 Ab. Culture was either untreated (a, red) or treated with anti-CTLA-4 Ab (b, black), CTLA-4-Ig (c, orange), Treg (d, blue) or Treg plus anti-CTLA-4 Ab (e, green). The proliferation of responder T cells is measured by the division of violet dye shown in histogram. The number of proliferating T cells (x10\(^3\)) is calculated using counting beads and are plotted in bar chart. A and B. Treg:nTcon ratio is 1:10. C and D. Treg:nTcon ratio is 1:20. Proliferation was measured at d5.

Figure 4. Treg suppression is not observed in the absence of CD80 and CD86
Violet labeled responder human CD4\(^-\)CD25\(^-\) nTcon are activated with CD3/CD28 beads. Culture is either untreated (a, red) or treated with anti-CTLA-4 Ab (b, black), CTLA-4-Ig (c, orange), Treg (d, blue) or Treg plus anti-CTLA-4 Ab (e, green). The proliferation of responder T cells is measured by the division of violet dye shown in histogram. The number of proliferating T cells is calculated using counting beads and are plotted in bar chart. A and B. Treg:nTcon ratio is 1:5.
Reference:

Figure 1

A

PBMC (Pre-sort)

CD3
CD11c
CD4
CD14

Monocyte (Post-sort)

B

CD11c
CD11c
HLA-DR

CD11c
CD80
CD86

iDC
Figure 2

A

PBMC (Pre-sort)

nTcon (Post-sort)

B

PBMC (Pre-sort)

Treg (Post-sort)
Figure 3

(a) Control
(b) +anti-CTLA-4 Ab
(c) +CTLA-4-Ig
(d) +Tr
(e) +Tr + anti-CTLA-4 Ab

iDC : Tr
A
4: 2

B
2:2

C
4: 1

D
2:1

Cell no.

CellTrace Violet
Figure 4

(a) Control  
(b) +anti-CTLA-4 Ab  
(c) +CTLA-4-Ig  
(d) +Tr  
(e) +Tr + anti-CTLA-4 Ab

Bead:Tr

A
10:1

B
1.25:1

CellTrace Violet

Cell no.