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Regulatory T cell kinetics in the peripheral blood of patients with Crohn's disease

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Abstract

Estimates of T regulatory cell populations in the periphery of patients with Crohn's disease are confounded by disease activity and concomitant immunotherapeutic agents known to affect T cell proliferation and survival. We performed deuterium pulse/chase experiments in patients with quiescent Crohn's disease on no immunotherapy and healthy control subjects to estimate T regulatory cell kinetics. Quantification of deuterated DNA isolated from T cell subsets over 10 days was determined by mass spectrophotometry. We demonstrate enhanced proliferation within the T regulatory cell population from patients with Crohn's disease when compared to non-T regulatory cells and T regulatory cells from healthy control subjects. We speculate that T regulatory cells isolated from the periphery of patients with Crohn's disease experience persistent antigen stimulation resulting in excess proliferative rates.

1. Introduction

Crohn's disease (CD) is a chronic, relapsing disorder characterized by inflammation of the small and large intestine. Its etiology, a complex interplay between an individual's genetics and the environment, seems to have a common endpoint – dysregulation of the immune system [1,2]. Murine models of colitis have demonstrated a critical role of regulatory T cells (Tregs) in the maintenance of intestinal homeostasis [3–7]. Human Tregs, identified by the cell surface markers CD4, CD25 as well as the transcription factor Forkhead Box P3 (FOXP3), are involved in the pathogenesis of multiple autoimmune diseases through interaction with effector T cell proliferation and function [8–16]. Thus, further characterization of Treg biology and its relation to the non-Treg effector cells is crucial to the understanding of CD pathology.

There is conflicting data as to the number of Tregs in the peripheral blood of patients with CD compared with healthy controls. Some studies have found an increase while others a decrease [17–19]. The effects of concurrent immunosuppressive medication confound some of the results. In addition, almost all the data reflect in vitro, point analysis of percent Tregs in the peripheral blood compartment. The dynamic cell kinetics of Tregs in CD patients is

unknown. Furthermore, it is unclear whether change in peripheral numbers is a result of proliferation, apoptosis or cell migration away from the periphery.

In this study we describe the in vivo kinetics of Tregs in the peripheral blood of patients with inactive Crohn's disease on no immunosuppressive medication. In 1998 a technique was described to measure in vivo cell proliferation using stable isotope mass spectrometry in patients with HIV [20]. Since then, the technique and mathematical modeling has been extrapolated to study the kinetics of subpopulations of human T cells [21–23]. Most recently, cell proliferation and disappearance rates for Tregs from healthy adult subjects were described. The authors demonstrated that, compared to memory T cells, Tregs were hyperproliferative (a doubling time of 8 days compared to 24 days). The authors suggest that Tregs, compared to effector T cells, are more susceptible to apoptosis in that they had shorter telomeres and lower expression of Bcl-2, an anti-apoptotic molecule [24]. We aimed to optimize this technique in patients with Crohn's disease.

We report that compared to healthy controls, CD4+CD45RO+CD25++ T cells from the peripheral blood of patients with CD have the same apoptotic rate as healthy controls but are hyperproliferative compared to effector T cells. This suggests that in patients with CD, there may be a constitutive higher Treg cell turnover independent of disease activity and medication use.

2. Materials and methods

2.1. Human studies

All studies were reviewed and approved by the Institutional Review Board of the Mayo Clinic and informed consent was obtained from all patients included in the study.

2.2. Subjects

For studying the in vivo kinetics of regulatory T cells, three patients with CD (mean age 48.7 ± 3 ; three women) and three healthy controls (mean age 46.7 ± 23.7 ; one woman, two men) were recruited. Subjects were required to be in clinical remission and off any immunosuppressive (steroids, immunomodulators, biologic agents) medication for at least 4 months.

2.3. Treg in vivo kinetics study

Methods have been recently developed to study the turnover of cells in vivo using incorporation of the non-radioactive isotope, deuterium, into the DNA of dividing cells [25]. In the presence of deuterium-labeled glucose, dividing cells, upon deoxynucleoside synthesis of daughter DNA strands incorporate deuterium into the DNA. The quantification of the deuterium enrichment in the DNA by mass spectrometry is directly proportional to the number of cell divisions and the level and duration of precursor (glucose) labeling.

2.3.1. Glucose labeling—As previously described, subjects received deuterium-labeled glucose (6,6 D_2 -glucose, 0.6 g/Kg bodyweight, Cambridge Isotope Laboratories, Inc.) as an oral solution in half hourly aliquots over 10 h during which small, low energy meals (200

kcal per meal, including 15 g carbohydrate and 8 g fat) at 2.5 hourly intervals were also given [22]. Blood samples were taken every 2 h to ascertain plasma deuterated glucose enrichment. Blood samples (50 ml) were taken on day 1 and then days 3, 4, 10 and 21 after labeling in order to ascertain deuterium enrichment in cellular DNA.

2.3.2. Cell isolation, measurement of apoptosis and DNA extraction—CD4⁺ T cells were sorted by magnetic bead separation (CD4⁺ T Cell Isolation Kit 130-091-155, Miltenyi Biotec). An aliquot was removed and then further stained with a mixture of antibodies consisting of anti-CD4 APC (eBioscience), anti-CD25 PE-Cy5 (BD Pharmingen), then stained for caspases, markers of apoptosis, using the CaspaTag kit (green fluorescence, Chemicon). Lastly, cells were stained with anti-FoxP3 PE (BioLegend). Percent apoptosis assessed by flow cytometry in two cell subsets: Tregs (CD4⁺, FOXP3⁺) and non-Tregs (CD4⁺, FOXP3⁻). Flow cytometry was performed using the FACSCalibur and CellQuest software (Becton Dickinson). For analysis, live cells were gated on based upon their forward and side scatter characteristics. Percent FOXP3⁺ per CD4⁺ T cells was ascertained by gating on the FL1 bright cells.

CD45RO⁺ memory cells were further isolated from the rest of the CD4⁺ T cells by negative selection using the CD45RA⁺ T cells isolation kit (Miltenyi Biotec). The CD45RO⁺ memory T cells were further sorted into CD45RO⁺CD25⁺⁺ and CD45RO⁺CD25⁻⁻⁻ T Cells using CD25 microbeads (Miltenyi Biotec). The CD45RO⁺CD25⁻⁻⁻ fraction was then re-exposed to the CD25⁺ microbeads and run over a fresh column in order for a more stringent CD25⁺⁺ extraction. DNA was extracted using Easy-DNA kit (Invitrogen) and digested enzymatically to deoxynucleosides from the two cell groups (CD45RO⁺CD25⁺⁺, CD45RO⁺CD25⁻⁻⁻) and sent for mass spectrometry. FOXP3 enrichment was found to be >75% by flow cytometry (representative dot plot, Fig. 1).

2.3.3. Mass spectrometry—Plasma glucose enrichment was measured on an agilent gas chromatograph/mass spectrometer detector 5973 system (Agilent Technologies Inc., Wilmington, DE) as previously described [26]. To measure the incorporation of deuterium to cells the enrichment of 2H₂-deoxyadenosine (dA) via deoxyribose (dR) moiety was followed as previously described [27]. Analyses on the mass spectrometer were modified as follows: selective reaction monitoring (SRM) was performed on Thermo Electron TSQ7000 gas chromatograph/mass spectrometer system (Thermo Electron, Madison, WI) under negative chemical ionization with isobutane as reagent gas. Ion transitions at m/z 392 > 272, 394 > 274 were monitored for dR and 2H₂ dR, respectively. Isotopic enrichment was calculated against an enrichment standard curve. Results were expressed as a fraction of labeled cells (F). As previously described $F = E/b$ [25], where E represents enrichment of deuterium in DNA, and b stands for the mean plasma glucose multiplied by a dilution factor of 0.65.

2.3.4. Cell turnover analysis—To analyze the data, we used the simple mathematical model published in [19,22]. Briefly, the rate of change of labeled deoxyadenosine in a pool of cells during the labeling period (from $t = 0$ to $t = \tau$) is described by

$$dA/dt = bpA_0 - d^*A, t \leq \tau, A(0) = 0 \quad (1)$$

Here $A(t)$ represents the amount of labeled deoxyadenosine and A_0 is the total amount of deoxyadenosine, assumed to be constant and proportional to the number of cells. The cells are assumed to proliferate with rate constant p and b is the probability that an incorporated deoxyadenosine molecule will be labeled. d^* represents the rate constant for disappearance of labeled deoxyadenosine by cell death, cell migration out of the peripheral blood, or change of cell phenotype.

After the labeling period τ , the labeled deoxyadenosine disappears with the rate constant d^* . There is no further increase of labeled cells because cell division does not result in the loss of labeled deoxyadenosine from the pool. The corresponding equation is

$$dA/dt = -d^*A, t > \tau \quad (2)$$

The solution of Eqs. (1) and (2) is given by

$$A(t) = \begin{cases} (bpA_0/d^*)(1 - e^{-d^*t}) & t \leq \tau \\ (bpA_0/d^*)(e^{-d^*\tau} - 1)e^{-d^*t} & t > \tau \end{cases} \quad (3)$$

Data were fitted by this model equation using the non-linear least squares method. There are only two free parameters: d^* and $P = dpA_0$.

2.4. Statistical analysis

For parametric data results were expressed as mean $\pm$ SD and analyzed by the student t test (GraphPad Prism statistical software). Confidence intervals were set at 95% and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Treg cells from patients with Crohn's disease in remission have the same apoptotic rates in vivo as Treg cells from healthy controls

Tregs have been shown to be extremely susceptible to apoptosis in both mice and humans [28–30]. Peripheral CD4⁺ T cells were isolated from three healthy control subjects and three patients with Crohn's disease in clinical remission without anti-Crohn's therapy that might confound the results. The mean absolute lymphocyte counts in patients with Crohn's disease was normal and not significantly different from the counts in healthy controls (1.68 ± 0.25 vs. $1.2 \pm 0.12 \times 10^9/L$, respectively). The cells were then subsequently stained with anti-CD25, anti-FOXP3 and assessed for active caspase activity. The percentage of apoptotic cells was constant at an average of $2 \pm 0.5\%$ and did not differ between patients with CD and healthy controls (data not shown). Therefore, differences in peripheral Treg frequencies in inactive CD and healthy control subjects or patients do not appear to be explained by apoptotic cell death.

3.2. Treg cells from patients with Crohn's disease in remission are hyperproliferative in vivo compared to non-Treg cells

The in vivo kinetics of Treg cells using deuterium labeling of dividing cells/Tregs has been previously described [21–23,25]. Based upon this technique we studied the in vivo kinetics of two cell populations (Treg, CD4+CD45RO+CD25++ and non-Treg, CD4+CD45RO+CD25—) in three healthy control subjects and three patients with Crohn's disease in clinical remission on no immunosuppressive therapy. While we recognize the existence of naïve Treg population (CD45 RA CD25++), the frequency was too low to study using this methodology. Therefore, all subsequent data reported reflects kinetics of the memory Treg population.

On day 1 of deuterium labeling, blood was sampled from the subjects at 2-hourly intervals in order to determine the enrichment of plasma glucose. A steady state was achieved in both patients and healthy subjects by 10 h (Fig. 2). The mean glucose enrichment in the healthy control subjects was 32.4 ± 4.7 molar percent excess and mean 37 ± 7.9 molar percent excess in the patients with CD. These results are similar to published average plasma glucose enrichment [23].

Blood was sampled in all subjects at days 1, 3, 4, 10 and 21 in order to determine the enrichment of DNA in the Treg, CD4+ CD45RO+CD25++ and non-Treg, CD4+CD45RO+CD25— cells. Maximum measured enrichment occurred at day 4 for CD4+ CD45RO+CD25++ cells and slightly earlier on day 3 for the CD4+CD45RO+CD25— cells (Table 1). This delay might represent a lag in emergence of lymphocytes into the periphery from lymphoid tissue where initial cell division occurs [22]. The assumption is that peak labeling post deuterium pulse occurs in the lymphoid tissue at day 1 prior to subsequent redistribution of cells to the periphery. The measured peak in the periphery may then be used to extrapolate the peak in the lymphoid compartment. Based on the raw data, Treg cells appear to enter the peripheral pool later than non-Treg cells. The higher peak peripheral value in Treg cells reflects greater incorporation of deuterated glucose. In Crohn's disease, peak values were significantly higher in Treg cells compared to non-Treg cells, indicative of enhanced proliferation ($2.1 \pm 0.6\%$ vs. $0.8 \pm 0.3\%$, p -value = 0.030; Table 1). Indeed, peak values in Treg cells isolated from Crohn's disease patients trended to be higher than Treg cells isolated from healthy control subjects ($2.1 \pm 0.6\%$ vs. $1.2 \pm 0.1\%$ p = 0.0699).

Kinetics of DNA labeling from the peak enrichments of deuterated DNA were then calculated. A higher rate of rise to the peak deuterium signal reflects a greater proportion of dividing cells at the time of deuterium pulse and thus an increased rate of proliferation (P) (Fig. 3, Table 2). From the proliferation rate, a doubling time ($i_2 = (\ln 2)/P$) was calculated. The doubling time of Treg cells from patients with Crohn's disease was faster than Treg cells from healthy control subjects (30 days vs. 47 days) and significantly faster than non-Treg cells in CD (30 ± 14 days vs. 63 ± 10 days, p -value = 0.032; Table 2). Thus these data indicate a heightened proliferative rate and shortened doubling time of Treg cells in patients with Crohn's disease compared with healthy control subjects (Fig. 3). As opposed to published data, our analysis did not demonstrate a significantly different proliferative rate between Treg cells and non-Treg cells isolated from healthy control subjects.

3.3. Treg cells from patients with Crohn's disease in remission are not disappearing from the periphery at a faster rate when compared to healthy control subjects

The rate of decrease of labeled DNA in each cell pool reflects disappearance of that cell type from the periphery either by death, migration or phenotypic conversion [22]. The disappearance rates were not significantly different between Treg cells from clinically well Crohn's disease patients and healthy control subjects ($10.9 \pm 9.4\%$ vs. $11.4 \pm 6.7\%$, Table 2). Overall, in remission, Treg cells from patients with Crohn's disease do not appear to be disappearing from the periphery at a faster rate than Treg cells from healthy control subjects.

4. Discussion

Our study of Treg cell kinetics in Crohn's disease patients has three key findings. First, it optimizes the in vivo study of T cell kinetics. Secondly, in patients with CD in remission on no immunosuppressive medication Treg cells appear to divide more rapidly than non-Treg cells, and finally, this difference in proliferative rate was not seen in cell populations isolated from healthy control subjects.

The sample size was small in this study and the chance of type II error exists; thus the results should not be overstated. We did find it interesting that the deuterated Treg cells seem to emerge into the peripheral pool, presumably from the lymph nodes [22], later than non-Treg cells. This difference in emergence of the Treg cell population into the periphery may suggest important insight into the imprinting of adaptive Treg cells with altered integrin repertoire as most certainly exists with Treg cells educated within the mesenteric lymphatics [31]. The Treg cells from patients with Crohn's disease had the highest peak deuterated DNA levels. We speculate that perhaps Treg cells from patients with Crohn's disease, even in remission, are responding to self (intestinal) antigenic stimuli as compared to their healthy counterparts but one needs a larger sample size to draw stronger conclusions.

As has been described previously, there did not seem to be a significant difference in the disappearance rates between the cell populations, perhaps reflecting, as previously reported, a high susceptibility to apoptosis in rapidly dividing memory cells [25]. In our study there was an observed rate of apoptosis of around 2% in Treg cells from both healthy control subjects and patients with Crohn's disease. The Crohn's patients were not on immunosuppressive medication and were in clinical remission. This leads us to conclude that the in patients with inactive Crohn's disease, Treg cells do not appear to leave the peripheral pool at a faster rate than in healthy control subjects. What remains to be seen is if there is a difference in the in vivo disappearance rates once the disease is active or in the presence of immunomodulators or biologic therapy.

There have been several attempts to characterize Treg cells from patients with inflammatory bowel disease. The results have been somewhat contradictory regarding the relationship between the number of these cells in the peripheral blood of patients and disease activity. In healthy adult individuals, CD25⁺⁺ Treg cells are thought to account for approximately 2–10% of the peripheral CD4⁺ T cell population [9,32,33]. Maul et al. using flow cytometry for cell sorting, found CD4⁺CD25⁺ high Treg cells accounted for about 1.6% of the peripheral blood lymphocyte (PBL) compartment in healthy adults [17]. In patients with

Crohn's disease the mean CD4+CD25^{high} Treg count dropped to 0.68% in active disease compared to approximately 2% in patients with inactive disease. This pattern was replicated when they performed an analysis for FOXP3 on cytopins from healthy controls vs. patients with IBD [17]. In contrast to these results, Saruta et al. reported a mean of 5.9% FOXP3+CD4⁺ Treg cells in the peripheral blood of healthy controls vs. 8.2% in active Crohn's disease and 10.2% in inactive disease [18]. Using CD4+CD45RO+CD25⁺ as markers for Treg cells Takahashi et al describe the frequency of Treg cells as 2.9% of PBLs in healthy individuals, which increased to 3.1% in inactive CD and further to 4.4% in active CD although the results were not statistically significant [19]. The issue of the effects of immunosuppressive medication confounds these results. Furthermore, it has been recently described that administration of antigen-specific (ovalbumin) Treg cells to patients with refractory Crohn's disease is a well-tolerated treatment modality and a dose-related efficacy was seen [34]. The ovalbumin-specific immune response correlated with clinical response, which supporting immune-suppressive mechanisms of ovalbumin specific Treg cells.

In conclusion, this report, the first of Treg cell kinetics among patients with inactive Crohn's disease without immunotherapy, demonstrates a heightened proliferative rate within the Treg cell subset when compared to non-Treg cells and Treg cells from healthy control subjects. This may reflect persistent antigenic stimulation in Crohn's patients compared to control subjects, and the pathophysiologic relevance of this finding requires further investigation.

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Abbreviations

CD	Crohn's disease
Tregs	regulatory T cells
FOXP3	Forkhead box P3

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Fig. 1.
Representative flow cytometric dot plot for peripheral blood CD4⁺ lymphocytes isolated from human subjects. The CD25 high expressing cell population (FL3) is enriched in FOXP3⁺ expressing T regulatory cells (FL2).

Fig. 2.

Plasma glucose enrichment was seen to reach a steady state in both the patients with CD (mean 37 ± 7.9 molar percent excess) and the healthy controls (NL) (mean 32.4 ± 4.7 molar percent excess) by 10 h with no significant difference between the two rates. The fit is given by the empirical model $MPE(t) = a(1 - e^{-bt})$ where a and b are positive parameters determined by the least squared method.

Fig. 3.

In vivo kinetics of T cells in Crohn's disease and healthy controls. Proportion of labeled cells for the two cell populations CD4+CD45RO+CD2++ Tregs (solid lines) and CD4+CD45RO+CD25-- non-Tregs (dashed lines) was graphed over time in days for three patients with Crohn's disease in remission (left column) and three healthy controls (right column). The graphs represent the proliferation and disappearance rates for the different cell populations using the mathematical modeling previously described. The thin lines illustrate the coordinates related to half-times of the decays curves accordingly. Data of

labeled cells from individual patients in figure A–C is pooled for healthy control patients (left) and patients with Crohn’s disease (right) and refit by the model Eq. (3) using the non-linear weighted least squares method. CD4+CD45RO+CD25++ Tregs (solid) in patients with Crohn’s disease in remission are hyperproliferative compared to CD4+CD45RO+CD25++ Tregs in healthy controls and significantly more hyperproliferative compared to CD4+CD45RO+CD25— non-Tregs (dashed).

Table 1

Measured peak label of memory Treg and non-Treg cells in Crohn's disease and healthy controls.

Subjects	CD4+CD45RO+CD25++		CD4+CD45RO+CD25--	
	MPE	Peak (%)	MPE	Peak (%)
HC 1	0.27	1.20	0.25	1.10
HC 2	0.29	1.30	0.30	1.30
HC 3	0.24	1.10	0.15	0.70
<i>Mean</i>	0.27	1.20	0.23	1.03
<i>SD</i>	0.03	0.10	0.08	0.31
CD 1	0.45	2.00	0.09	0.40
CD 2	0.61	2.70	0.21	0.90
CD 3	0.34	1.50	0.23	1.00
<i>Mean</i>	0.47	2.10*	0.18	0.80*
<i>SD</i>	0.14	0.60	0.08	0.33

Peak levels of deuterated DNA were observed in peripheral blood samples on day 3 for CD4+RO+CD25-- cells except in HC 3 where it occurred on day 4 and on day 4 for CD4+RO+CD25++ cells except for HC 2 and CD 3 where it occurred on day 3. The peak percent of labeling was higher in the Treg cells from patients with Crohn's disease compared to healthy controls but significantly higher compared to non-Treg cells.

MPE (molar percent excess), HC healthy control, CD Crohn's disease.

*
p-Value = 0.030.

Table 2

In vivo calculated proliferation rates, doubling times, disappearance rates and half-lives of Memory Treg and non-Treg cells in Crohn's disease and healthy controls.

CD4+CD45RO+CD25++				CD4+CD45RO+CD25--			
Peak (%)	P (%/day)	T ₂ (days)	d* (%/day)	Peak (%)	P (%/day)	T ₂ (days)	d* (%/day)
HC1	1.53	1.68	41.31	18.95	1.11	1.20	57.83
HC2	1.37	1.41	49.02	6.21	2.15	2.36	29.31
HC3	1.32	1.38	50.15	8.98	2.00	2.87	24.18
Mean	1.41	1.49	46.83	11.38	1.75	2.14	37.11
SD	0.11	0.16	4.81	6.70	0.56	0.86	18.13
CD1	3.37	3.75	18.50	21.70	0.99	1.33	52.13
CD2	2.67	2.73	25.36	4.67	1.00	1.07	64.77
CD3	1.45	1.50	46.29	6.15	0.94	0.96	72.35
Mean	2.50	2.66	30.05 ⁺	10.84	0.97	1.12	63.08 ⁺
SD	0.97	1.13	14.48	9.44	0.03	0.19	10.21
							31.89

The peak enrichment represents the proportion of cells labeled relative to the mean precursor (glucose) enrichment. *P*, proliferation rate and *d**, disappearance rate are calculated values from the mathematical model. *T*₂, the doubling time, is calculated as (ln 2)/*P*.

⁺ *p*-Value = 0.032.