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PRINCIPAL INVESTIGATOR: K.K. Wong, Jr., M.D.

CONTRACTING ORGANIZATION: City of Hope Beckman Research Institute
Duarte, CA 91010

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K.K. Wong, Jr., M.D.

E-Mail: kkwong@coh.org

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14. ABSTRACT
CD4+CD25+FoxP-3+ regulatory T-cells (Tregs) suppress immune responses to "self" antigens, but also have been shown to suppress host anti-tumor responses in several human malignancies, including breast, gastrointestinal, and ovarian cancer. Identification of CML Tregs as suppressors of host anti-CML responses could have a significant impact upon CML treatment strategies. Methods are currently available to selectively suppress Tregs and subsequently boost host anti-CML responses. We have examined the CD4+CD25+FoxP-3+ regulatory T-cell population in the peripheral blood from healthy individuals and those with CML using flow cytometry. Our results demonstrate that subjects with CML who have detectable residual disease have a larger percentage of CD4's that are Tregs (6.89% ± 3.32 vs 1.94% ± 0.99, p 0.003)and a >3.5 fold larger absolute number of circulating CD4+CD25+FoxP3+ T-cells (1,743 ± 1,350 vs 455 ± 234, p 0.02), consistent with our hypothesis. The larger numbers of circulating Treg's appears to correlate with CML disease activity. We are continuing to examine functional correlates of this CML Treg population.

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INTRODUCTION.

Although Imatinib Mesylate (Gleevec®) therapy has clearly made a major impact upon the treatment of chronic myelogenous leukemia (CML), it does not eliminate nonproliferating CML stem cells, and, thus, is unlikely to be curative. Currently, because of age, underlying medical conditions and/or lack of donor availability, only a minority of individuals are eligible for curative treatment with allogeneic hematopoietic stem cell transplantation (HSCT). Therefore strategies that increase the potential for disease control or cure for the majority of individuals with CML are sorely needed. CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) suppress immune responses to "self" antigens, but also have been shown to suppress host anti-tumor responses in several human malignancies, including breast, gastrointestinal, and ovarian cancer. Identification of CML T_{regs} as suppressors of host anti-CML responses could have a significant impact upon CML treatment strategies. Methods are currently available to selectively suppress T_{regs} and subsequently boost host anti-CML responses. Furthermore, reduction of host suppressor CML T_{regs} could act synergistically with administration of CML targeted vaccines to boost host anti-tumor responses. Such immune strategies could aid in the elimination of residual leukemic stem cells that persist after other treatments.

BODY.

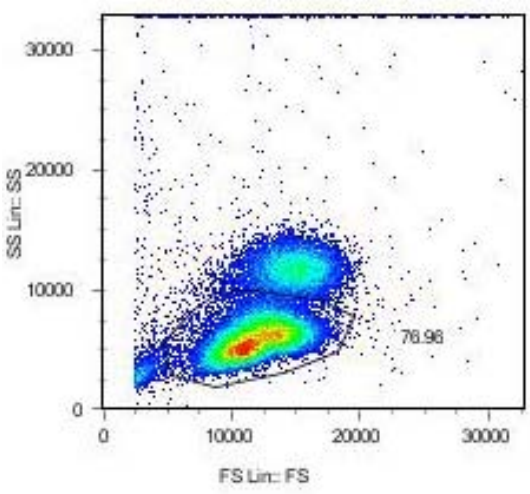
Task 1. **Phenotypic Characterization of CD4+CD25+ T_{regs} in Individuals with Chronic Phase CML:(a) quantification of T_{reg} populations in CML and healthy controls and (b) expression analyses for bcr-abl and FoxP3.** (Months 01-18)

(a) Isolate CD4+ CD25+ T_{regs} from subjects with CML and healthy donor pairs
(b) Test for bcr-abl by PCR and/or fluorescence-in-situ-hybridization (FISH)
(c) Test for FoxP3 by PCR

To assess the potential role(s) of CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) in CML, we first examined the T_{regs} peripheral blood profile of healthy individuals compared to those with CML using a City of Hope Institutional Review Board (IRB) approved protocol. Peripheral blood mononuclear cells were isolated from healthy individuals and those with CML, stained with fluorochrome conjugated antibodies against CD4 (FITC), CD25 (PE), and FoxP3 (APC) antigens, characteristic of the regulatory T-cell population, and analyzed using a CyAn™ ADP 9 Color flow cytometer (Beckman Coulter). Conjugated antibodies were obtained from EBiosciences (San Diego, CA), and staining was performed per manufacturer's recommendations.

Cells are first gated using forward and side scatter parameters, with gating of small cells with low granularity consistent with lymphocytes. Compensation was performed to correct for wavelength overlap of the different fluorochromes. Cells were then gated against CD4-FITC, and CD4+ cells were analyzed against CD25-PE and FoxP3-APC to obtain the CD4, CD25, and FoxP3 triple positive population. We initially compared a standard quadrant compared to a freehand (hand drawn) gate for analysis of the CD4+CD25+FoxP-3+ population, and found the latter to be slightly more consistent. Therefore, freehand gates for CD4+CD25+FoxP-3+ cells are depicted in the Figures, and summarized in Table 1, below. It should be noted that all CML subjects that were analyzed to date were on Nolotinib therapy.

Representative examples of profiles from a healthy individual (**Figure 1**) as well as an individual with CML (**Figure 2**) are provided below and overall results are summarized in **Table 1,** below. It should be noted that the individual with CML depicted in Figure 2 was felt to have poorly controlled disease, with 35.8% cells in the periphery which were positive for the BCR-ABL translocation by cytogenetics (FISH).



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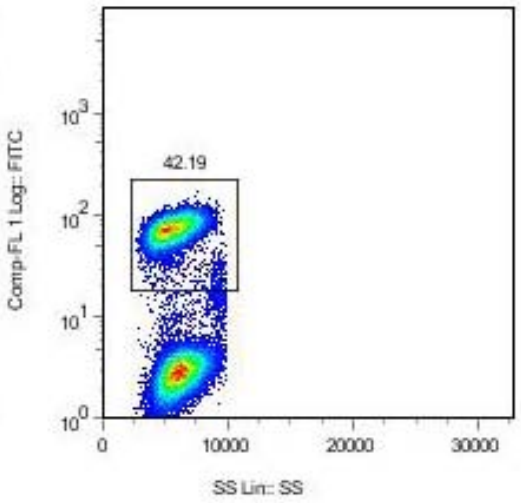

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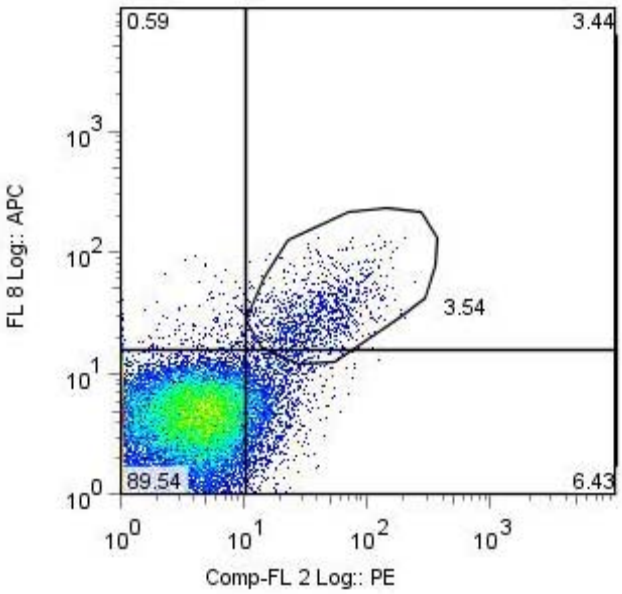

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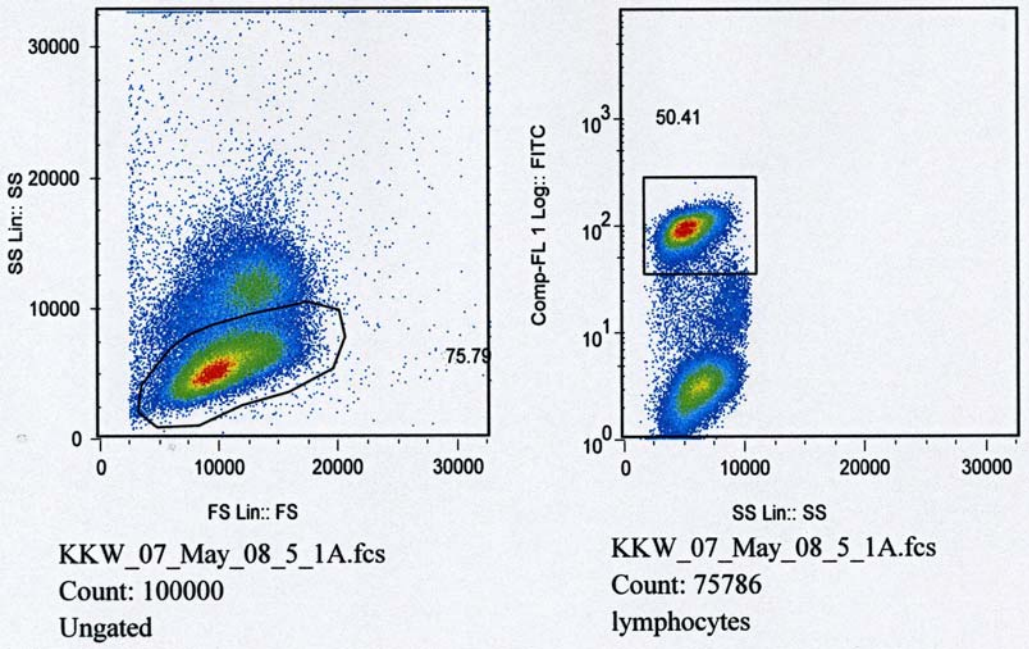
CD4 pos

Figure 1. Flow Analysis of CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) from a healthy individual. Panel Upper Left. Gate of small, low granularity cells consistent with small lymphocytes. Panel Upper Right. The previously gated cells were analyzed for CD4-FITC expression, and gated (square). Lower Panel. The CD4+ cells were analyzed for CD25-PE and FoxP3-APC expression using a freehand gate; **3.54 %** of CD4+ lymphocytes were CD25 and FoxP-3 positive.



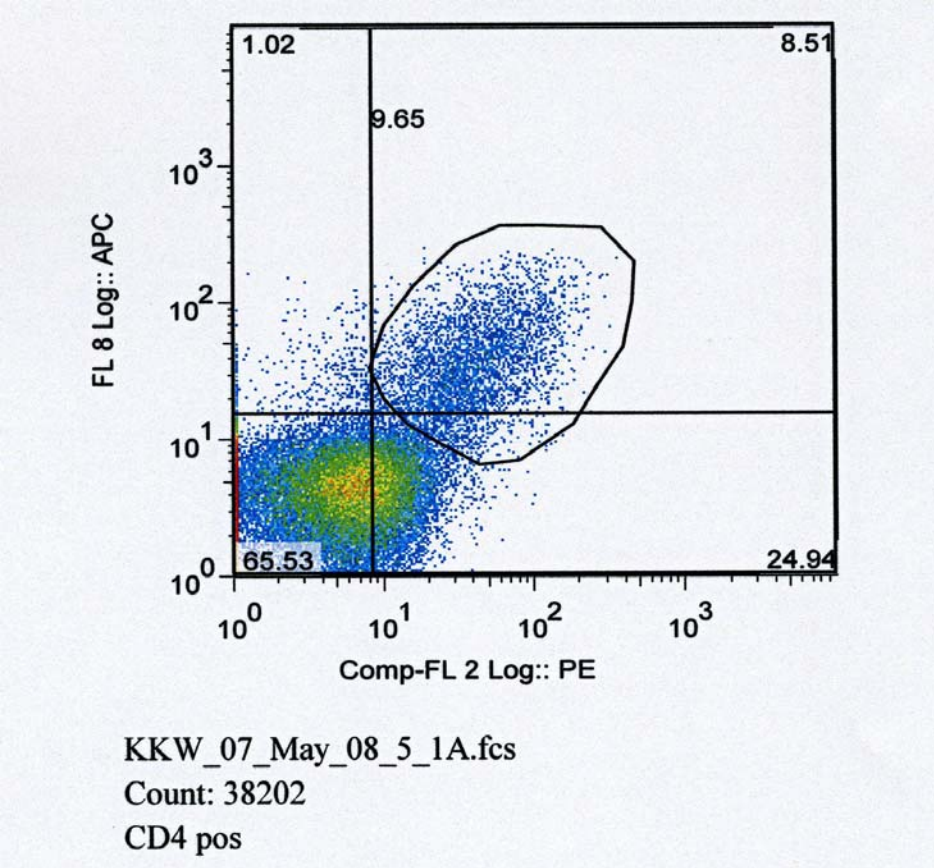


Figure 2. Flow Analysis of CD4+CD25+FoxP-3+ regulatory T-cells (T_{regs}) from an individual with active CML. Panel Upper Left. Gate of small, low granularity cells consistent with small lymphocytes. Panel Upper Right. The previously gated cells were analyzed for CD4-

FITC expression, and gated (square). Lower Panel. The CD4+ cells were analyzed for CD25-PE and FoxP3-APC expression using a freehand gate; **9.65 %** of CD4+ lymphocytes were CD25 and FoxP-3 positive.

To insure that we were analyzing appropriate cell populations in the most reproducible fashion, we examined samples using a number of different parameters, including (1) traditional quadrant vs free hand gates, (2) lymphocyte (low forward and side-scatter) vs total mononuclear gates, and (3) lymphocyte gates vs CD3+ T-cells, but found that, although there was some variance noted with changing the different parameters, using the traditional quadrant lymphocyte gate gave the most reproducible results and that the addition of using a CD3+ gate did not increase the specificity of the analyses.

An updated summary of our analyses of peripheral blood samples from healthy donors (HD) and subjects with CML is depicted in **Table 1**. Samples were run in duplicate with average values and standard deviations (SD) depicted. Clinical characteristics of the CML subjects are also shown; subjects included in **Table 1** under CML had to have detectable residual disease. **Note that the data outlined in Table 1 is slightly different from that provided in the Flow graphs in Figures 1 and 2 (freehand gates depicted in Figures 1 and 2 vs traditional quadrant gates in Table 1) so that the percentages of T_{regs} is slightly different.**

TABLE 1

Sample	Gender	Age	Percent CD4+CD25+FoxP3+ Using Traditional Gate	Absolute # of CD4+ Cells	Absolute # of CD25+ & FoxP3+ Cells	Comments
HD022009	M		1.47	29,901	382	
HD030409	F	37	0.98	18,135	169	
HD030909	M	58	1.91	18,396	343	
HD031109	F	58	3.55	27,650	823	
HD031909	M	35	1.83	33,491	375	
HD041309	F	33	2.06	24,290	498	
HD041509	F	62	1.52	36,078	543	
HD042109	F	47	1.49	23,906	353	
HD042309	M	26	0.84	24,248	201	
HD042709	F	62	3.78	23,091	865	
HD042909	M	53				
MEAN		46	1.94	25,919	455	
SD		13	0.99	5,907	234	
CML-050608	M	53	6.85	41,224	2,822	Chronic Phase
CML-051308	F	58	4.34	36,406	1,577	Chronic Phase Partial Cytogenetic Response
CML-061708-R	F	55	6.48	36,023	2,331	
CML-072808	M	53	4.29	4,774	198	
CML-090108	F	57	8.21	10,963	900	
CML-090308	F	51	13.60	28,745	3,906	
CML-020209-R	F	55	4.49	10,653	468	

CML-022309-R	F	57			
CML-022609-R	F	58			
MEAN		55	6.89	24,113	1,743
SD		2	3.32	14,918	1,350
T TEST			0.0034	0.3848	0.0226
CONFIDENCE			99.7%	61.5%	97.7%

Note that the average number of peripheral blood CD4 T-cells in healthy (25,919 $\pm$ 5,907) vs CML subjects (24,113 $\pm$ 14,918) was pretty comparable, although the CML subjects had a higher variability. However, the average percentage of CD4+ lymphocytes that were CD25 and FoxP-3 triple positive in
Healthy individuals was 1.94% $\pm$ 0.99
CML subjects 6.89% $\pm$ 3.32 (**p 0.003**), suggesting a quantitative difference in the number of circulating T_{regs} between the two populations. If one examines the absolute numbers of CD4+CD25+FoxP-3+ T-lymphocytes in the periphery, the difference is accentuated with healthy individuals having **455 $\pm$ 234 vs 1,743 $\pm$ 1,350** for subjects with CML, over a **3 fold** difference (**p 0.02**). We noted several trends:

- One individual with poorly controlled CML (CML-050608) had the highest numbers of circulating T_{regs} (2,822).
- We found that individuals with CML treated with Gleevec who were in complete remission (no detectable circulating CML cells) usually had a normal T_{reg} population.
- We followed one individual with CML who had detectable disease when first examined (with high T_{regs}) and went into remission with treatment, at which time his T_{regs} were low normal.

ONGOING STUDIES. We continue to examine subjects with CML using the above protocol(s) to increase our CML sample size for greater statistical significance, and are looking for functional characteristics of the CML T_{reg} populations (Task 2, below).

From our previous work, we have isolated human cytotoxic T-lymphocyte (CTL) clones with b3a2 BCR-ABL fusion region specificity that are also HLA DRB5*0101 or B*3501 restricted [1]. If our hypothesis is correct, MHC matched CD4+CD25+FoxP3+ T_{regs} isolated from subjects with CML should suppress BCR-ABL peptide mediated CTL responses from our BCR-ABL-specific CTL clones. After extensive searching, we actually located subjects diagnosed with (1) the b3a2 BCR-ABL fusion region form of CML, and (2) haplotypes that matched our CML clones (e.g. were HLA DRB5*0101 or B*3501), but the subjects had already been treated, were in remission, and sufficient T_{regs} could not be isolated. Alternatively, for those individuals with CML with HLA types different from HLA DRB5*0101 or B*3501, we are currently evaluating the ability of their T_{regs} to inhibit a proliferative and IL-2 secretory response from HLA-matched T-cells stimulated with α-CD3 coated beads which will serve as a measure of their overall T_{reg} suppressor activity.

Task 2. **Immunologic Characterization of CD4+ CD25+ T_{regs} in Individuals with CP CML**

(a) Demonstration and characterization of immune suppression by T_{regs} (Lymphocyte proliferation, gamma IFN ELISPOT, Luminex cytokine secretion assays)

(b) Is suppression specific for CML cells in vitro?
(c) Can suppression be reversed by removing T_{regs} using specific antibodies?

KEY RESEARCH ACCOMPLISHMENTS:

- Identified a quantitative difference in the number of CD4+CD25+FoxP-3+ regulatory T-cells in the CML population compared to healthy controls, supporting our hypothesis that these cells may be regulating host anti-CML responses ($p < 0.003$)
- Followed one individual who had a high number of CD4+CD25+FoxP-3+ regulatory T-cells when CML was active and measurable, and T_{regs} in the normal range when in remission after treatment, supporting our hypothesis.
- Also noted that 3 subjects with CML in remission had CD4+CD25+FoxP-3+ regulatory T-cells populations in the normal range.

PERSONNEL RECEIVING FUNDING FROM GRANT
Ms. Diana Palma, Research Associate

REPORTABLE OUTCOMES:
Regulatory T Cells (T_{regs}) in Chronic Myeloid Leukemia (CML), Diana Palma, Saswati Chatterjee, & K.K. Wong, City of Hope Research Symposium, July 2009.

A manuscript with the above title and authors is in preparation.

CONCLUSION:
We have identified higher numbers of CD4+CD25+FoxP-3+ regulatory T-cells in the CML population compared to healthy controls, supporting our hypothesis that these cells may be regulating host anti-CML responses. The numbers of T_{regs} in 3 subjects with CML in complete remission were similar to our healthy control population. Finally, we were able to monitor one individual with CML serially, and noted that the T_{reg} population started high while the CML was active, and dropped to the normal range following treatment and induction of remission. Our original plan was to assess the ability of T_{regs} from subjects with active CML to suppress anti-b3a2-specific BCR-ABL responses from MHC matched CTL clones that we had derived in the lab. Although we were able to identify MHC-matched subjects with b3a2 BCR-ABL CML, the subjects disease was in remission following treatment. We are continuing to accrue information on CD4+CD25+FoxP-3+ regulatory T-cells in subjects with CML, and are now looking at functional correlates of the T_{reg} population in CML.

Reference List

1. Sun JY, Senitzer D, Forman SJ, Chatterjee S, Wong KK, Jr. Identification of new MHC-restriction elements for presentation of the p210(BCR-ABL) fusion region to human cytotoxic T lymphocytes. Cancer Immunol.Immunother. 2003;52:761-70.

APPENDICES: None

SUPPORTING DATA: See BODY, above.