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Long-term effects of Alemtuzumab on regulatory and memory T cell subsets in kidney transplantation

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Abstract

Background—Induction with lymphocyte-depleting antibodies is routinely employed to prevent rejection but often skews T cells towards memory. It is not fully understood which memory and regulatory T cell subsets are most affected and how they relate to clinical outcomes.

Methods—We analyzed T cells from 57 living-donor renal transplant recipients (12 reactive and 45 quiescent) 2.8±1.4 years after Alemtuzumab induction. 34 healthy subjects and 9 patients with acute cellular rejection (ACR) were also studied.

Results—We found that Alemtuzumab caused protracted CD4>CD8 T lymphocyte deficiency, increased proportion of $CD4^+$ memory T cells (T_M), and decreased proportion of $CD4^+$ regulatory T cells (T_{REG}). Reactive patients exhibited higher proportions of $CD4^+$ effector memory (T_{EM}) and $CD8^+$ terminally differentiated effector memory ($T_{EM}RA$), with greater $CD4^+$ T_{EM} and $CD8^+$

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T_{EM}RA to T_{REG} ratios, than quiescent patients or healthy controls. Patients with ongoing ACR had profound reduction in circulating $CD8^+$ T_{EM}RA. Mixed lymphocyte assays showed significantly lower T cell proliferation to donor than third party antigens in the quiescent group, while reactive and ACR patients exhibited increased effector molecules in $CD8^+$ T cells.

Conclusions—Our findings provide evidence that T cell skewing towards effector memory may be associated with anti-graft reactivity long after lymphodepletion. Further testing of T_{EM} and T_{EM}RA subsets as rejection predictors is warranted.

Keywords

kidney transplantation; memory T cells; regulatory T cells; alemtuzumab

Introduction

Approximately 80% of kidney transplant recipients currently receive antibody induction therapy at the time of transplantation to prevent acute rejection and decrease the burden of chronic immunosuppression (1–3). Although several clinical studies have shown that transient T cell depletion could afford patients steroid-free maintenance immunosuppression without jeopardizing graft survival in the short-term (4–7), some patients still develop acute or chronic rejection despite aggressive T cell depletion at the time of transplantation, and immunosuppression withdrawal is seldom achieved without significant risk of graft rejection (8, 9).

In an effort to better understand the effects of lymphodepleting induction therapy, several groups have performed immunological measurements on transplant recipients after Thymoglobulin or Alemtuzumab administration (10–18). The consensus from these studies is that transient T cell depletion leads to skewing of T cells that repopulate the host towards memory, but that donor-specific hyporesponsiveness could occur in some recipients (12, 18). Most memory T cells (T_M) arise from either undepleted naïve T cells (T_N) or T_M undergoing homeostatic expansion, a phenomenon known as lymphopenia-induced proliferation (LIP) that is driven by IL-7 and IL-15 present in excess amounts in lymphopenic hosts (19, 20). Evidence in mice and humans indicates that T_M, including those generated by LIP, carry alloreactive specificities, cause rejection, and prevent tolerance (21–24). In addition to alterations in T_M and T_N proportions, lymphodepletion influences regulatory T cell (T_{REG}) populations. Some studies reported an increase in percentage of T_{REG}, defined as $CD4^+CD25^{high}Foxp3^+$ cells, after either induction therapy (11, 25, 26). These findings, however, are inconclusive, as Foxp3 expression in humans is not restricted to T_{REG} but is also present in recently activated T cells (27–29).

Despite these important insights, several questions remain: does skewing of the T cell pool towards memory after transient lymphocyte depletion persist long-term; which T_M subsets are most affected; what changes are observed in the T_{REG} population (identified by stringent phenotypic criteria in addition to Foxp3); and are there immunological markers that could identify patients at risk of rejection or, conversely, those who are quiescent? In a prior study, we found that alloreactive T_M cells in healthy human subjects consist of heterogeneous subpopulations characterized by differential proliferative capacity and variable expression of effector molecules (23). $CD4^+$ and $CD8^+$ central memory (T_{CM}) cells proliferated robustly in response to alloantigen, while effector memory (T_{EM}) and terminally differentiated effector memory (T_{EM}RA) cells proliferated less but expressed higher levels of perforin and granzyme B. This led us to hypothesize that T_{EM} and T_{EM}RA present a threat to transplanted organs. To test this hypothesis and to further define the long-term immunological effects of transient lymphocyte-depletion, we performed a cross-sectional

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phenotypic and functional analysis of peripheral blood T cells from renal transplant recipients up to six years after receiving Alemtuzumab induction.

Results

Long-term effects of Alemtuzumab induction on $CD4^+$ and $CD8^+$ memory T cell subsets and their relationship to clinical status

The number and proportion of lymphocyte subpopulations were measured in the peripheral blood of the patient groups outlined in Table 1 and compared to those of healthy controls (HC). As shown in Fig. 1A–C, a significant reduction in proportion and absolute number of total $CD3^+$ and $CD3^+CD4^+$ T cells, and absolute number but not proportion (%) of $CD3^+CD8^+$ T cells was observed in transplant recipients compared to HC. The $CD4^+$ T cell population contracted more than the $CD8^+$ T cell population in all patient groups, leading to reduced CD4/CD8 ratio (data not shown). Neither B nor NK cells were diminished in absolute number (data not shown). These data indicate that Alemtuzumab induction is associated with protracted deficiency of CD4>CD8 T cells but not B or NK cells.

We next investigated the relative distribution of T_N and T_M subsets among $CD4^+$ and $CD8^+$ T cells (Fig. 1D–F). The gating strategy used to identify T_N ($CD45RO^-CD62L^+$), T_{CM} ($CD45RO^+CD62L^+$), T_{EM} ($CD45RO^+CD62L^-$), and $T_{EM}RA$ ($CD45RO^-CD62L^-$) is shown in Fig. 1D (23). Among $CD4^+$ T cells, we found a significant decrease in % T_N accompanied by a reciprocal increase in % T_{EM} in all patient groups (except ACR) compared to HC (Fig. 1E–F). Importantly, % T_{EM} was significantly higher in the reactive than the quiescent patient group (Fig. 1F). Receiver operating characteristic (ROC) curves to assess accuracy of % $CD4^+$ T_{EM} in discriminating between reactive and either quiescent or HC yielded an area under ROC (AUROC) of 0.79 and 0.92, respectively (Supplemental Fig. 1A). CD4 T_{CM} and $T_{EM}RA$ subsets were similar among patient groups and between patients and HC (data not shown). Among $CD8^+$ T cells, we observed a significant increase in % $T_{EM}RA$ in the reactive group compared to quiescent patients (AUROC = 0.68) and HC (AUROC = 0.70) (Fig. 1F & Supplemental Fig. 1B). A striking decline in $CD8^+$ $T_{EM}RA$, however, was observed in ACR patients compared to all other groups (Fig. 1F), most significantly when compared to reactive or quiescent patients (AUROC 0.88 and 0.79, respectively) (Supplemental Fig.1C), suggesting that this cell population may have exited the circulation or differentiated into a different phenotype during rejection. These results indicate that T cells are skewed towards memory (T_{EM} and $T_{EM}RA$) even several years after Alemtuzumab induction and that increased proportions of $CD4^+$ T_{EM} and $CD8^+$ $T_{EM}RA$ may be associated with clinical reactivity.

Long-term effects of Alemtuzumab on regulatory and effector T cell subsets among $CD4^+CD25^{high}$ T cells

Previous studies provided evidence that T_{REG}, defined as $CD4^+CD25^{high}$ or $CD4^+CD25^{high}Foxp3^+$ cells, increase after Alemtuzumab induction (11, 25, 26). This evidence is inconclusive because in humans the $CD4^+CD25^{high}Foxp3^+$ phenotype also encompass recently activated or effector T cells (T_{EFF}) (27–29). We therefore quantified % and absolute number of T_{REG} based on low IL-7Rα (CD127) expression (T_{REG} = $CD3^+CD4^+CD25^{high}Foxp3^+CD127^{low}$) (30–33) (Fig. 2A). We found that while absolute counts of Tregs were significantly reduced in all patient groups compared to HC, % T_{REG} of all $CD4^+$ T cells was not altered (data not shown). However, the % T_{REG} among $CD4^+CD25^{high}$ T cells was significantly decreased in all patient groups compared to HC (Fig. 2B), with AUROC values of 0.88, 0.88, and 0.75 for the ACR, reactive, and quiescent patients, respectively (Supplemental Fig. 2A). Importantly, we observed a reciprocal increase in % $CD4^+CD25^{high}Foxp3^-CD127^{high}$ (Fig. 2B), which in humans represents T

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effector (T_{EFF}) that are precursors of T_M (33). This T_{EFF} population was increased in all patients compared to HC and over-represented in the ACR group compared to quiescent patients. The data therefore suggest that Alemtuzumab induction does not favor long-term immune regulation but instead effector and memory generation.

Increased ratios of $CD4^+$ T_{EM}/T_{REG} and $CD8^+$ $T_{EM}RA/T_{REG}$ are associated with reactive status

Kreijveld *et al*. reported that increased T_M/T_{REG} ratio is associated with acute rejection upon tacrolimus reduction in kidney transplant recipients who had not received induction therapy (34). Given that we observed skewing of T cells towards higher T_M and lower T_{REG} proportions after Alemtuzumab, we asked whether the T_M/T_{REG} ratio correlates with clinical status in our patient cohort. We found that all patient groups had higher $CD4^+$ T_{EM}/T_{REG} ratio than HC (Fig. 2C) and this ratio was predictive for reactive patients when compared to either quiescent patients or HC (AUROC = 0.76 and 0.88, respectively) (Supplemental Fig. 2B). A similar pattern was observed for the $CD8^+$ $T_{EM}RA/T_{REG}$ ratio except that it was significantly diminished in the ACR group (Fig. 2C & supplemental Fig. 2C). These results suggest that the ratio of effector memory to regulatory T cells after Alemtuzumab induction could potentially distinguish reactive patients at risk of rejection from those who are quiescent.

Long-term effects of Alemtuzumab induction on $CD4^+$ and $CD8^+$ T cell function

We next investigated whether alterations in naïve, memory, and regulatory T cell proportions after Alemtuzumab induction translate to differences in T cell alloreactivity. $CD4^+$ and $CD8^+$ T cells from quiescent patients proliferated significantly less in response to donor than third party cells, and less than HC cells (Fig. 3A). On the other hand, $CD8^+$ T cells from reactive and ACR patients expressed more IFNγ than those from HC (Fig. 3B). T cells from ACR patients showed heightened $CD8^+$ T cell perforin/granzyme B compared to reactive patients, and of both perforin/granzyme B and IFNγ compared to quiescent patients (Fig. 3B,C). These studies suggest that increased effector molecule expression may mark $CD8^+$ T cells involved in ACR and identify reactive patients.

Identification of T cell subsets in renal allograft biopsies

We analyzed kidney biopsies from patients undergoing ACR (n=4), borderline rejection (n=3), or drug toxicity (n=3) (Fig. 4). Enumeration of T cells in areas of tubulitis identified 9.3±5.8 T ($CD3^+$) cells/mm^2 in ACR patients versus 2.7±1.3 and 2.6±1.7 in borderline rejection and drug toxicity patients, respectively (p=0.1). Similar numbers of $CD3^+CD8^+$ and $CD3^+CD8^-$ cells were identified in all groups, but infiltrating $CD3^+CD8^+$ T cells were significantly greater in ACR (4.7±3.0 *vs.* 1.4±0.8 and 0.7±0.2 cells/mm^2, respectively, p=0.03). Among $CD8^+$ T cells, 93% had a $CD45RO^+CD62L^-$ phenotype, consistent with effector or effector memory T cells. $CD8^-$ T cells were evenly divided between $CD45RO^+CD62L^-$ (T_{EM}) and $CD45RO^-CD62L^-$ ($T_{EM}RA$). These results support the possibility that effector memory T cell subsets are preferentially recruited to the graft during acute rejection.

Discussion

We investigated the phenotype of regulatory and memory T cell subsets after induction in a cohort of patients with diverse clinical outcomes and at a later time point after transplantation than previous studies (10–18). First, we found that Alemtuzumab-induced patients have long-term skewing of $CD4^+$ T cells towards T_{EM} and a reduction in T_{REG} with reciprocal increase in T_{EFF} among $CD4^+CD25^{high}$ T cells. Since serial blood samples were not obtained in our study, the mechanisms by which this skewing occurred (differential

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depletion of T cell subsets *vs* differential repopulation) were not determined. Second, we observed a correlation between increased $CD4^+$ T_{EM}/T_{REG} and $CD8^+$ $T_{EM}RA/T_{REG}$ ratios and clinical reactivity. Third, the $CD8^+$ $T_{EM}RA$ population, which is characterized by high cytolytic activity (23), was consistently and profoundly diminished in the circulation of patients with ongoing ACR. Fourth, despite skewing towards memory and increased CD8 effector molecule expression in some recipients, other patients achieved clinical quiescence associated with lower donor-specific T cell proliferation.

Our finding that T cell repopulation after Alemtuzumab does not favor T_{REG} but that they are reduced as a proportion of $CD4^+CD25^{high}$ T cells is at odds with previous studies (11, 25). Gurkan *et al* found that administration of Thymoglobulin in adult renal transplant recipients induced peripheral expansion and new thymic emigration of $CD4^+Foxp3^+$ Treg in the first 6 months post-depletion (17). Ciancio *et al* found that Foxp3 mRNA expression and the proportion of $CD4^+CD25^+$ T cells among circulating T lymphocytes were significantly higher between two and eight months after transplantation in patients that received Alemtuzumab than those induced with either Thymoglobulin or Daclizumab (11). We believe that the discrepancy can be explained by the fact that we defined T_{REG} as $CD4^+CD25^{high}Foxp3^+CD127^{low}$ T cells. In humans, Foxp3 is expressed on recently activated $CD4^+$ T cells in addition to T_{REG} (27–29), and among $CD4^+CD25^{high}$ lymphocytes only the $CD127^{low}$ are suppressive (27, 30–32). Another potential reason for the discrepancy is that we analyzed peripheral blood T cells at late time points (average 2.8 years) after Alemtuzumab induction. It is possible that increased % T_{REG} observed early after lymphodepletion does not persist long-term. In addition, certain immunosuppressive drugs used in other studies, such as rapamycin, could favor T_{REG} expansion (25).

Previous studies have found preferential expansion of $CD4^+$ T_{EM} after lymphodepletion in kidney and pancreatic islet transplant recipients (10, 15), but no clear correlation with clinical outcomes or ACR was established. Our results showed that Alemtuzumab is associated with long-term increase in the proportion of $CD4^+$ T_{EM} and identified reactive patients as having the highest proportion of this memory subpopulation. These data indicate a correlation between $CD4^+$ T_{EM} and risk of immune reactivity or rejection after T cell repopulation, but other factors likely contribute, as not all patients with increased $CD4^+$ T_{EM} proportion develop rejection and *vice versa*. Two other cell populations that participate in determining patient risk are $CD4^+$ T_{REG} and $CD8^+$ $T_{EM}RA$. This is borne out by our finding that a high proportion of $CD8^+$ $T_{EM}RA$ and a high ratio of $CD4^+$ T_{EM} or $CD8^+$ $T_{EM}RA$ to T_{REG} correlate with reactive status. Finally, in the absence of a comparison group receiving a different depleting or non-depleting induction agent, we could not definitively determine that alterations in T cell subsets were indeed due to Alemtuzumab. Reports in which such groups were studied suggest that skewing towards memory is a consequence of lymphopenia, irrespective of depleting agent used (15, 17, 18).

A striking finding in our study is the profound and consistent decline in proportion of circulating $CD8^+$ $T_{EM}RA$ in patients with ongoing ACR. It is possible that $CD8^+$ $T_{EM}RA$ migrate to the graft and differentiate into effectors or enter other non-lymphoid tissues during rejection. These cells are rich in perforin/granzyme B and could be pathogenic (35). We found in biopsy analyses that the majority of tubule-infiltrating T cells during ACR have an effector phenotype. An alternative explanation is that $CD8^+$ $T_{EM}RA$ are regulatory lymphocytes whose decline heralds acute rejection. Trzonkowski *et al*. have shown that most repopulating $CD8^+$ T cells after Alemtuzumab induction display a senescent ($CD28^-$) phenotype (36), and others suggested that $CD8^+CD28^-$ T cells may be suppressive (37, 38). $CD8^+CD28^-$ suppressor T cells however had a T_{CM} and not $T_{EM}RA$ phenotype (38). While it is recognized that $CD8^+$ $T_{EM}RA$ are mostly $CD28^-$ (39), our results and those of others demonstrate that this subpopulation is rich in perforin/granzyme B and IFNγ (23, 40, 41),

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indicating that it has effector rather than regulatory functions. Finally, it is possible that higher tacrolimus levels at the time of blood draw in the ACR group (Table 1) could alter memory T cell distribution, preferentially reducing number of $CD8^+$ $T_{EM}RA$ cells.

In conclusion, our results show that Alemtuzumab induction in kidney transplant recipients is associated with long-term phenotypic and functional T cell alterations. We found significant differences in relative proportion of memory and regulatory T cell subsets among quiescent, reactive, and ACR patients. Although promising, the prognostic value of peripheral blood T cell profiling in our patient cohort should be interpreted with caution because of the cross-sectional nature of the study, heterogeneity and small size of the reactive group, and lack of control patients not receiving lymphodepleting induction therapy. Therefore, future prospective randomized studies comparing larger cohorts of patients receiving different induction therapies and employing serial monitoring, similar to that recently performed by Cherkassky *et al* (18), are needed to determine whether T cell profiling would accurately identify patients experiencing ongoing acute rejection, patients at risk of rejection, or patients who are quiescent. Such studies would also address whether observations made after Alemtuzumab induction can be generalized to kidney transplant recipients managed by different immunosuppression protocols.

Materials and Methods

Human subjects

Sixty-three first time living-donor renal transplant recipients (66 samples), 34 healthy control (HC) volunteers, and 30 living kidney donors at the University of Pittsburgh Medical Center (UPMC) were consented over a period of three years (2007–2010) under IRB-approved protocols to participate in this cross-sectional, observational study. Demographic and clinical characteristics of recipients and HC are shown in Table 1. Patients were induced with Alemtuzumab (30mg) plus methyl-prednisolone (2×1000mg) i.v. at the time of transplantation. Maintenance immunosuppression consisted of tacrolimus ± mycophenolate mofetil (MMF) (Table 1). Recipients were classified as reactive (n=12) or quiescent (n=45) based on clinical status ±3 months from the time of sample collection. Specifically, reactive patients were those who within this 6 month window (i) developed *de novo* donor specific antibodies (DSA) (n = 2); (ii) had biopsy-proven acute cellular or humoral rejection within the 6 month window but *not* on the day of blood sampling (n=2), or (iii) had a significant change in serum creatinine (> 20% of baseline without evidence of non-immunological causes of acute renal failure) and/or borderline ACR on biopsy, triggering pulse corticosteroids and/or increase in maintenance tacrolimus ± MMF based on the clinician's judgment (n= 10, including 2 patients who also had either de novo DSA or biopsy-proven ACR). Quiescent patients were those who had none of the above. ACR (n=9) patients were those who had biopsy-proven ACR on the day of blood sample collection before any anti-rejection therapy was administered.

Isolation of peripheral blood mononuclear cells (PBMC)

One ml heparinized blood was used directly for lymphocyte phenotyping and the remainder to isolate PBMC by density gradient centrifugation. PBMC were frozen for functional assays.

Phenotyping of T cell subsets

Cells were surface stained with mAbs which included anti-CD62L-FITC (Beckman Coulter, Fullerton, CA), anti-CD45RO-PE, anti-CD8 or CD4-PerCpCy5.5, CD25-PECy7, anti-CD3-V450 (BD, San Jose, CA), anti-CD127-APC-eFluor-780, and intracellularly stained with anti-FoxP3-APC (eBioscience, San Diego, CA) prior to RBC lysis (23). Events were

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collected using a LSRII flow cytometer (BD) and analyzed with Diva (BD) or FlowJo software (Tree Star, Ashland, OR).

One-way CFSE-MLR

Responder PBMC were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Eugene, OR) and incubated with γ-irradiated allogeneic donor or third party PKH-26 (Sigma, St. Louis, MO)-labeled PBMC (1:1 ratio) for 5 days. Proliferation of $CD3^+CD8^-$ ($CD4^+$) or $CD3^+CD8^+$ T cells was measured by CFSE dilution by flow cytometry (23). Surface-stained, fixed cells were further permeabilized and incubated with perforin-APC (Biolegend, San Diego, CA) and granzyme B-Alexa700 (BD). For IFNγ intracellular staining, cultured cells were re-stimulated *in vitro* for 4hrs with PKH26-labeled, γ-irradiated allogeneic CD3-depleted (ratio 1:1) PBMC in the presence of Golgi-Plug (BD) prior to staining with anti-IFNγ-Alexa700 mAb (BD).

HLA typing and measurement of DSA

HLA typing was performed in the Tissue Typing Laboratory at the UPMC. Screening for anti-HLA antibodies was performed by ELISA (LAT-M kit, One Lambda, Canoga Park, CA) followed by Luminex single-antigen bead analysis (One Lambda). MFI values ≥1000 were considered positive.

Multiplex quantum dot staining and whole slide image

Formalin-fixed, paraffin-embedded renal allograft tissue was immunostained using multiplex quantum dot staining as reported (42). Antibodies used were anti-CD45RO (NeoMarkers, Fremont, CA), anti-CD62L (Novocastra, Newcastle Upon Tyne, UK), anti-CD8 and anti-CD3 (DAKOCytomation, Carpinteria, CA).

Statistical analysis

Data were reported as mean±standard deviation (SD). Significance was measured by one-way ANOVA and two-tail Student *t* tests for normally distributed values or Wilcoxon's rank test for non-normally distributed data. Fisher's test was used on qualitative data. Statistically significance was set at $p \leq 0.05$. ROC curves were plotted to assess sensitivity for ≥ 80% specificity over the entire range of cut-offs, and calculated the AUROC to measure how well the markers discriminated between groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACR	Acute cellular rejection
AUROC	Area under ROC curve
DSA	Donor specific antibodies
HC	Healthy controls

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ROC	Reporter operator characteristic
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
$T_{EM}RA$	Terminally differentiated effector memory T cell
T_N	Naïve T cell
T_{REG}	Regulatory T cell

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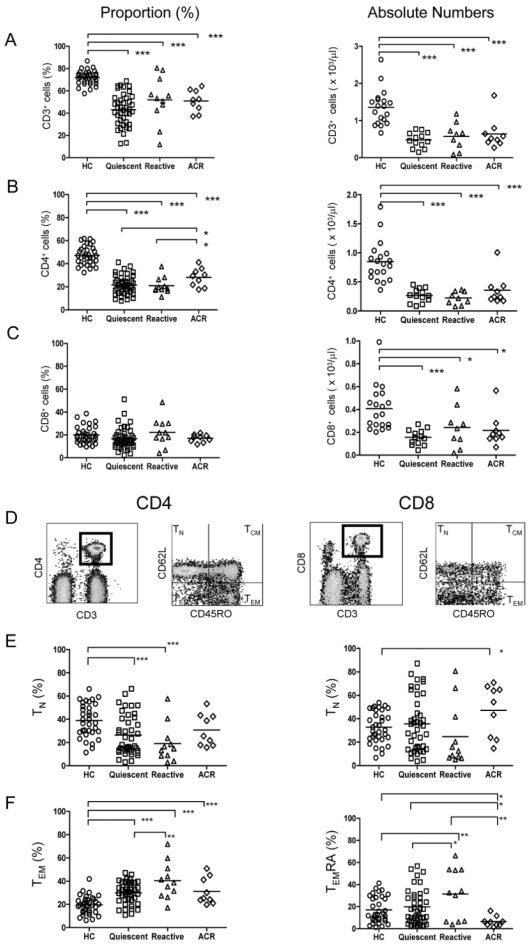

Figure 1. Identification and quantitation of peripheral blood T cell subsets

Proportion (%) and absolute numbers of $CD3^+$ (A), $CD3^+CD4^+$ (B), and $CD3^+CD8^+$ (C) T cells in each study group. (D) Gating strategy for identifying T_N ($CD45RO^-CD62L^+$), T_{CM} ($CD45RO^+CD62L^+$), T_{EM} ($CD45RO^+CD62L^-$), and $T_{EM}RA$ ($CD45RO^-CD62L^-$) CD4 and CD8 T cells. (E–F) Proportion (%) of $CD4^+$ T_N, T_{EM}, and $CD8^+$ T_N, $T_{EM}RA$ cells for each study group. Each symbol represents a single subject, while the horizontal line reprsents the mean value. *p<0.05; ** p<0.01; *** p<0.005.

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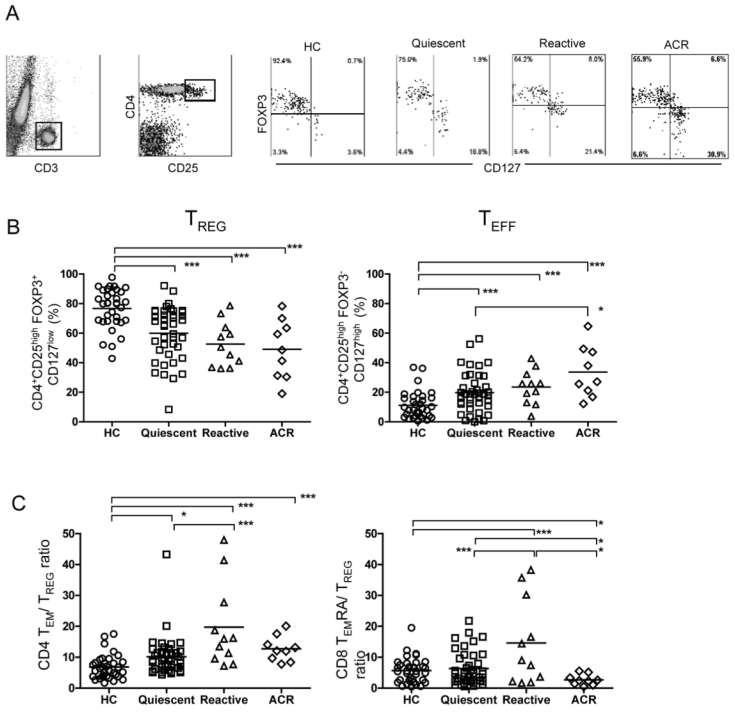

Figure 2. Identification and quantitation of peripheral blood CD4 T_{REG} and T_{EFF} cells

(A) Gating strategy and representative flow cytometry of T_{REG} ($CD3^+CD4^+CD25^{high}FOXP3^+CD127^{low}$) and T_{EFF} ($CD3^+CD4^+CD25^{high}FOXP3^-CD127^{high}$) in the $CD3^+CD4^+CD25^{high}$ T cell gate in each of the study groups. (B) Proportion (%) of T_{REG} and of T_{EFF} within the $CD3^+CD4^+CD25^{high}$ T cell gate. (C) CD4 T_{EM}/T_{REG} and CD8 $T_{EM}RA/T_{REG}$ ratios in each of the study groups. Each symbol represents a single individual, and the horizontal line represents the mean value. *p<0.05; ** p<0.01; *** p<0.005.

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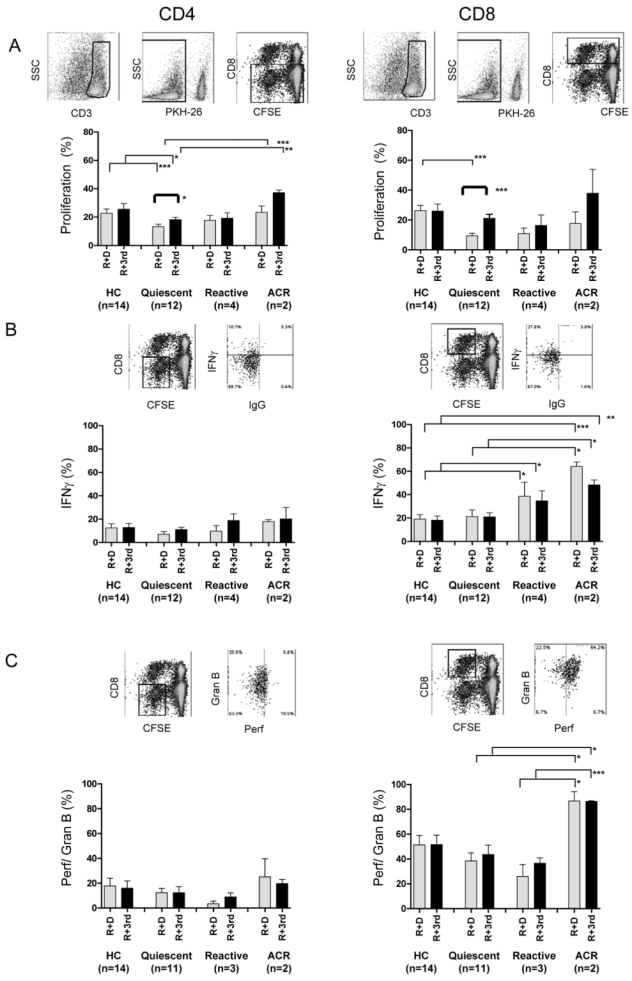

Figure 3. T cell proliferation and IFNγ and peforin/granzyme B expression in one-way CFSE-MLR

Analyses were performed after excluding PKH-26^{+} stimulator cells and gating on CD3^{+}CD8^{-} (CD4^{+}) and CD3^{+}CD8^{+} T cells at the end of the 5-day MLR. (A) Proliferation of responder CD4^{+} and CD8^{+} T cells, presented as proportion (%) of cells that diluted CFSE, after stimulation with allogeneic donor (R+D) or third party PBMC (R+3rd). (B) IFNγ production by proliferated CD4^{+} and CD8^{+} T cells 4 hrs after re-stimulation with donor or third party CD3-depleted allogeneic PBMC. (C) Perforin/granzyme B expression by proliferated responder CD4^{+} and CD8^{+} T cells. HC (n=14), quiescent (n=12), reactive (n=4), and ACR (n=2). *p<0.05; ** p<0.01; ***p<0.005.

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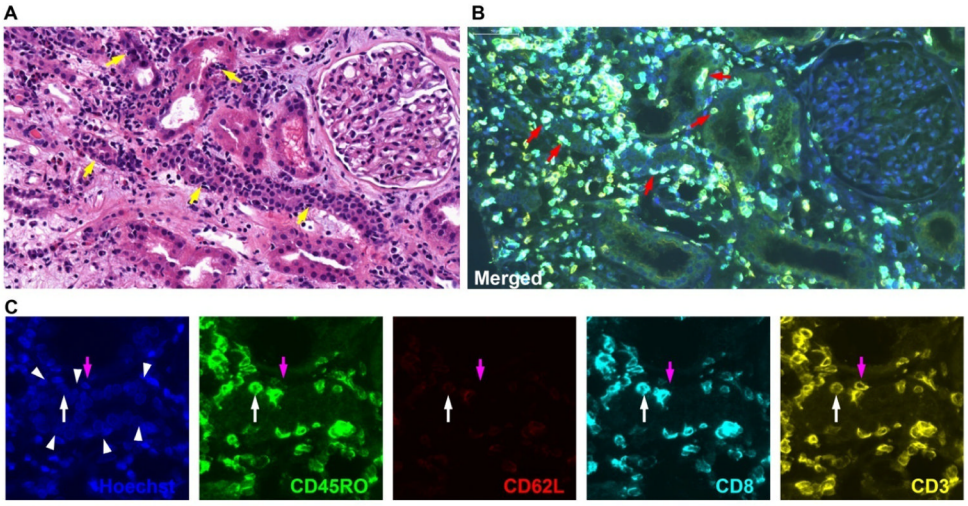

Figure 4. Identification of T cell subsets in renal allograft tissue of a patient undergoing ACR

(A) H&E staining shows irregularly shaped tubules and mononuclear cell infiltrates (tubulitis) (yellow arrows). (B and C) Multiplex quantum dot staining of CD45RO (green), CD62L (red), CD8 (cyan), and CD3 (yellow) in the same tissue section as A. Panel B depicts merged image, red arrows indicate infiltrating cells which are mostly $CD8^{+}CD3^{+}CD45RO^{+}CD62L^{-}$ T cells (T_E or T_{EM}). Panel C depicts cropped images from Panel B. White arrow heads indicate tubules. Purple arrows identify $CD3^{+}CD8^{-}CD45RO^{-}CD62L^{-}$ ($T_{EM}RA$) while white arrows identify $CD3^{+}CD8^{+}CD45RO^{+}CD62L^{-}$ (T_{EM}) infiltrating T cells.

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TABLE 1

Demographics of patients and healthy controls

	Quiescent n=45	Reactive n=12	ACR n=9	HC n=34	ANOVA p-value
Time (yrs) from transplant to blood draw: mean ± SD (range)	2.8 ± 1.2 (1.0–5.7)	2.9 ± 1.6 (1.0–5.9)	2.8 ± 0.6 (0.9–3.8)	N/A	NS
Age: mean ± SD (range)	53.3 ± 15.4 (21.7–86.5)	58.6 ± 18.1 (20.6–76.6)	38.8 ± 16.7 (20.7–60.6)	49.4 ± 11.3 (29.1–70.9)	0.009[1]
Female/Male gender*	17/28	4/8	6/3	16/18	N/A
African American race*	4/45	1/12	1/9	1/34	N/A
De novo DSA*	8 (17%)	4 (33%)	3 (33%)	N/A	N/A
DSA during follow-up*	2 (4%)	2 (17%)	2 (22%)	N/A	N/A
HLA mismatches class I: mean ± SD	2.3 ± 1.0	2.3 ± 0.6	2.4 ± 0.7	N/A	NS
HLA mismatches class II: mean ± SD	1.1 ± 0.5	1.2 ± 0.4	1.1 ± 0.4	N/A	NS
Estimated GFR at blood draw: mean ± SD (ml/min)	50.9 ± 7.9	42.9 ± 8.9	35.6 ± 6.6	N/A	<0.001[2]
Tacrolimus level at blood draw: mean ± SD (ng/ml)	4.6 ± 2.3	5.7 ± 1.8	8.5 ± 2.3	N/A	0.001[3]
Daily MMF usage	7/45	2/12	5/9	NA	N/A
Daily steroid usage	1/45	0/12	0/9	N/A	N/A
Living donor	45/45	12/12	5/9	N/A	N/A

NS= non-significant; N/A= not applicable

*Not significant by Fisher's exact test, pairwise comparisons.

†Any DSA occurring post-transplant.

‡Any DSA present with ±3 months of blood sampling.

[1]Student *t* Test: HC vs. Reactive: p=0.02; HC vs. ACR: p=0.02; ACR vs. Reactive: p=0.02; ACR vs. Quiescent: p=0.01

[2]Wilcoxon's rank test: ACR vs. Quiescent: p=0.02; Reactive vs. Quiescent: p=0.03

[3]Student *t* Test: ACR vs. Quiescent: p<0.001; ACR vs. Reactive: p=0.03

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