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Allospecific Regulatory Effects of Sirolimus and Tacrolimus in the Human Mixed Lymphocyte Reaction

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Abstract

Background—Tacrolimus (TAC) and sirolimus (SRL), two commonly used immunosuppressive agents, have demonstrated contrasting immunoregulatory effects. We recently described factors affecting the generation of allospecific CD4⁺CD25^{High} forkhead/winged helix transcription factor P3 (FOXP3⁺) T-regulatory (Treg) cells in mixed lymphocyte reaction (Treg MLR) and now report additional findings on the effects of TAC and SRL.

Methods—TAC, SRL, or media without agents were added separately to MLRs using human leukocyte antigen two DR-matched and -mismatched healthy volunteers and prekidney transplant donor/recipient pairs. Concentrations correlated with subtherapeutic and therapeutic blood levels. Stimulation indices of ³H-TDR uptake, cell proliferation, and the generation of carboxy-fluorescein diacetate succinimidyl ester (CFSE) labeled CD4⁺CD25^{High}FOXP3⁺ cells by flow cytometry were initially compared. Each group of (non-CFSE labeled) MLR-generated cells were then added as third components to CFSE-labeled responding cells in freshly prepared primary MLRs, to determine allospecific and nonspecific inhibitory and Treg recruitment effects.

Results—TAC inhibited stimulation indices and CD4⁺CD25^{High} FOXP3⁺ cell generation in both human leukocyte antigen DR-matched and -mismatched pairs, particularly at therapeutic levels (5 ng/mL). SRL had an equivalent effect in matched pairs but was associated with a significantly higher % generation of CD4⁺CD25^{High}FOXP3⁺ cells than TAC. SRL-MLR-generated Tregs

added as third components allospecifically inhibited MLR proliferation and recruited additional CFSE-labeled autologous Tregs compared with addition of TAC- or media-MLR-generated Tregs.

Conclusions—Calcineurin and mammalian target of rapamycin inhibitors have disparate effects on allospecific Treg generation using the Treg MLR. This assay can thereby be helpful in assessing allospecific regulatory effects of diverse immunosuppressive agents.

Keywords

Mixed lymphocyte reaction; Regulatory T cells; FOXP3; Calcineurin inhibitor agents; mTOR inhibition

In clinical organ transplantation, determining immunobiologically appropriate dosing of lifelong immunosuppression (IS) may allow the ability to taper IS to low levels and thereby lower the incidence of long-term IS complications, whereas optimally preventing graft rejection. The current standard IS agents are the calcineurin inhibitors (CNIs), tacrolimus (TAC) and cyclosporine, and alternative drugs such as mycophenolate mofetil and sirolimus (SRL) in combination with CNIs or in select patients as monotherapy. Related to successful IS minimization or withdrawal might be the development of biomarkers, such as peripheral blood mononuclear cell (PBMC) immunophenotyping and functional assays, that signify immunoregulatory effects of these agents singly or in combination.

Previous studies have demonstrated differences favoring individual IS agents in the generation of regulatory T cells (Tregs) (1–4). TAC suppresses alloimmune responses by interrupting TCR signaling pathways and inhibiting production of amplifying cytokines (IL-2) involved in the generation of forkhead/winged helix transcription factor P3 (FOXP3), a transcription factor important for the function of Tregs. TAC has also been proposed to inhibit the development of regulatory dendritic cells (5, 6). However, we also have reported a long-term favorable effect of CNIs on the regulatory properties of bone marrow cells in vitro in patients infused with deceased donor bone marrow at the time of renal transplantation (7, 8). In contrast to TAC, SRL seems to allow for the generation of alloantigen-specific regulatory T cells in murine models (9), although with the exception of a report by Baan et al. (4) that did not examine allospecific Treg generation, clear in vitro and in vivo human data are limited (3, 4, 10–12).

We recently reported a higher percentage of PBMC immunophenotypic Tregs in liver transplant recipients on stable SRL as opposed to TAC monotherapy (13), as well as an increase in these cells in PBMC and bone marrow during and after TAC to SRL conversion (14), but functional donor-specific regulatory effects were not tested. To this end, we have recently described an in vitro assay, the Treg mixed lymphocyte reaction (Treg MLR), to examine the variables affecting the development of allospecific regulatory T cells (15). In addition to allospecific lymphoproliferative inhibition, allospecific new Treg recruitment was observed (a hallmark of this assay) in readouts of carboxy-fluorescein diacetate succinimidyl ester (CFSE) labeled responding cells in third component assays. Therefore, using this assay, the potential to detect ex vivo allo(donor)specific IS regulatory effects of TAC versus SRL associated with CD4⁺CD25^{High} FOXP3⁺ T-cell generation, not previously reported, are now described.

RESULTS

TAC in Primary Treg MLR

Increasing concentrations of TAC (0, 0.05–0.1, 0.3–3, and 5–20 ng/mL) were tested (n=44), using PBMC of healthy volunteers (n=38) and living-related donor/recipient pretransplant pairs (n=6). Figure 1(A and B) demonstrate a stepwise decline in stimulation indices (SI) and % Treg (%CD4⁺CD25^{High}FOXP3⁺ cells) with increasing TAC concentrations, the highest of which was consistent with clinically “therapeutic” ranges (5 ng/mL, $P<0.05$). As a more relevant measure, to avoid variability between the magnitude of responses of individual experiments, the % change from baseline (no TAC added or 100% of full reactivity – positive control) is additionally portrayed (Fig. 1C) showing the dose-dependent reduction in both SI and % Treg with higher TAC concentrations ($P<0.0001$). The percentage change is therefore used in all subsequent calculations. This inhibition occurred similarly in both the DR-matched and -mismatched experiments (see Figure, Supplemental Digital Content 1, <http://links.lww.com/TP/A303>) reflecting that inhibition of both proliferation and Treg generation occurred in parallel.

TAC Versus SRL in Primary Treg MLR

We then compared the SI and % Tregs generated when TAC versus SRL were added to Treg MLRs of five healthy volunteer responder/stimulator pairs and six living-related donor/recipient end-stage renal disease pretransplant pairs (Fig. 2A). The SI decreased with increasing concentrations of both TAC and SRL. In the volunteer normal pairs (columns 1 and 2), SI was more inhibited by TAC than SRL (0.3–3 and 5 ng/mL) with two DR mismatches ($P=0.02$) but inhibition was equivalent to SRL with two DR matches. However, the % Treg was higher with SRL both at subtherapeutic and at therapeutic levels (0.3–3 and 5 ng/mL, $P<0.05$) for both the two DR-matched and two DR-mismatched combinations (again using the same responding cells for each matched vs. mismatched comparison). Therefore, the overall putative “regulatory” effect (MLR inhibition+Treg generation) of SRL was most pronounced in the two DR-matched experiments. Figure 2 (B) displays representative fluorescence-activated cell sorting plots demonstrating that the proliferating CD4⁺ population (column 4) had significantly higher CD25^{High}FOXP3⁺ percentages with SRL (row C) compared with TAC (row B) and media (row A), at higher “therapeutic” concentrations in two DR-matched pairs. The nonproliferating fraction had significantly fewer of these cells in all three groups (column 5), demonstrating that the majority of the CD4⁺CD25^{High}FOXP3⁺ cells are seen in the proliferating fractions (column 4).

The SI and % Treg effects of both agents in the pretransplant donor/recipient end-stage renal disease pairs (all one DR matched) were somewhat similar to the healthy two DR-matched volunteers. At the highest concentrations of both agents (Fig. 2A, column 3), or clinically therapeutic trough levels, TAC caused similar SI inhibition but significantly lower % Tregs generated than SRL ($P=0.03$).

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Treg MLR Inhibition and Allospecific Recruitment by TAC Versus SRL-Generated Tregs

In these experiments, the immunoregulatory allospecific and nonspecific effects of Tregs generated in MLR in the absence or presence of TAC or SRL were assessed by third component Treg MLR inhibition (inhibition by cell count—representative experiment in Fig. 3A and B, both column 4; SI inhibition—three experiments together in Figure 2, Supplemental Digital Content 2, <http://links.lww.com/TP/A304>) and by recruitment of new autologous CFSE positive CD4⁺CD25^{High} FOXP3⁺ cells, as described in our initial Treg MLR report (15). CD4⁺ cells that were CD127⁻ (resulting in >50% CD25⁺ and FOXP3⁺ cells, Table 1, Supplemental Digital Content 2, <http://links.lww.com/TP/A305>) were immunoselected after MLR incubation for 7 days with media alone, with TAC, or with SRL. When added as third component modulators under the prior influence of SRL, there was more marked inhibition of proliferation than when generated in the presence of media alone or of TAC (cell counts in red, Fig. 3A, columns 2 and 4, row D vs. B or C; SI inhibition in Figure 2, Supplemental Digital Content 2, <http://links.lww.com/TP/A304>). The inhibition was antigen specific in that it was less marked when these cells were added back to a two DR-mismatched MLR (cell counts in red, Fig. 3B, columns 2 and 4, row D vs. B or C; Figure 2, Supplemental Digital Content 2, <http://links.lww.com/TP/A304>) than MLRs involving the original two DR-matched stimulator (Fig. 3A; Figure 2, Supplemental Digital Content 2, <http://links.lww.com/TP/A304>).

It was also observed in responses to the original two DR-matched (specific) stimulator that the modulator SRL Tregs induced the development of higher levels of new CD4⁺CD25^{High} FOXP3⁺ cells in the CFSE-labeled responding cells, that is, they recruited additional autologous responding cells to become this phenotype (Fig. 3C; three experiments together). Most notable was that the greatest recruitment was observed with the original MLR-generated Tregs in the presence of SRL in the specific two DR-matched MLR combinations, compared with Tregs generated with media alone, with TAC or with nonspecific reactions. This could be clearly seen when comparing the allospecific recruitment induced by SRL Tregs versus the weaker (or lack of) specific recruitment of Tregs generated with TAC or media alone (SRL vs. TAC and media, $P=0.01$). Because of variability of MLR responses between pairs, the magnitude of the differences in recruitment effects could only be internally compared in each of the three experiments and also contrasted in terms of specific versus nonspecific recruitment.

DISCUSSION

The recently reported Treg MLR was designed to analyze variables affecting donor-specific immunoregulation in solid organ transplant recipients (15). The present report establishes inherent differences between two commonly used IS agents, TAC and SRL, in Treg generation and lymphoproliferation inhibition, particularly with respect to the effect of human leukocyte antigen (HLA) matching. In addition, the third component experiments demonstrated that SRL-generated Tregs also allospecifically recruited additional Tregs compared with TAC or media-generated Tregs, when added to the MLR in which they were specifically generated—a unique characteristic of the Treg MLR assay.

In both the healthy volunteer and prekidney transplant donor/recipient pairs, SRL had a more profound effect in favoring the % Tregs generated and, in DR-matched pairs, had similar MLR inhibition as TAC in “clinically therapeutic” concentrations. We suggest that the higher % Tregs generated in DR-matched experiments, in combination with MLR inhibition (Fig. 2), represents a true “regulatory” effect of SRL, perhaps in the absence of high frequency memory cells capable of alloactivation as recently described (16). Conversely, in the HLA two DR-mismatched MLRs, SRL seemed to have a weaker inhibitory effect on proliferation perhaps due to inherently stronger alloreactivity with greater mismatching (17). These results (seeing high percentages of Tregs in the proliferating fraction) are consistent with an activation process required for their generation. These data extend our previous observations of Treg MLR disparate effects dependent on HLA disparity, with higher % of inhibitory Tregs associated with DR matching and indirect presentation (15). We propose that additional regulatory effects enhanced by self-recognition may be even more enhanced in an SRL environment. This does not imply that SRL is a more effective immunosuppressant than TAC. SRL was less inhibitory of proliferation than TAC in the mismatched MLR experiments and clinically has been less effective in preventing rejection early after transplantation. However, with greater self (class II) major histocompatibility complex recognition by DR matching, Treg generation seems to be more profound with similar inhibition of proliferation. This may translate into clinical applications when considering use of SRL in transplant recipients and, in particular, when designing IS minimization or withdrawal protocols.

An important question posed by this study is whether the phenotypic Tregs generated in the presence of SRL versus TAC in MLR would then differentially cause enhanced regulatory effects that were allospecific. This observation was described in our previous report in the absence of IS agent, that is that Tregs isolated on day 7 of MLR specifically inhibited newly prepared MLR assays and concurrently recruited more autologous responder Tregs (15). While activated T cells are known to express FOXP3⁺, the allospecific modulating effects of the CD4⁺CD127⁻CD25⁺FOXP3⁺ cells isolated in our experiments support that these cells were “regulatory” and not alloimmunized effectors. In the present experiments, this third component allospecific regulatory effect was greatest in SRL-generated Tregs compared with those generated with TAC or no drug influence. While differential Treg effects are consistent with prior studies (4, 12), MLR-generated Tregs, under the influence of different IS agents, added as third components to the primary MLRs in the context allo- versus nonspecific immunoregulation has not previously been demonstrated. This may be analogous to clinical use of the agent, although there was no attempt to generate Tregs in vitro for in vivo use as recently described in mice by Lim et al. (9). Our experiments consequently can help extend current thinking of mammalian target of rapamycin (mTOR) inhibitor immunoregulatory mechanisms (i.e., in vitro demonstration of MLR inhibition and Treg recruitment ability, augmented by allospecificity) and support this method of immune monitoring for donor-specific immunoregulation and “infectious” Treg functions.

A variety of mechanisms have been suggested to explain the differences between CNI and mTOR inhibitors on Treg generation and function (12). As non-CNI agents such as SRL do not effect IL-2 transcription, they may lead to mechanisms that allow Treg generation (2, 18–21). SRL may also inhibit postactivation signaling (i.e., PI3K/mTOR pathway) but have

less inhibitory effect on pathways (i.e., STAT5) involved in T-regulatory cell generation, or SRL may even be antiapoptotic (12, 22). As such, Tregs are likely to be more resistant to the immunosuppressive effects of SRL. Finally, SRL can induce FOXP3 generation in alloantigen-specific T cells perhaps in the context of regulatory cytokines, such as TGF- β and IL-10. Testing proinflammatory versus regulatory cytokines concomitantly in MLRs might provide clues as to the cytokine milieu required for IS immunoregulation (4, 12).

In summary, the previously described Treg MLR (15) now demonstrates a high percentage of Tregs generated in SRL cultures with DR matching. When isolated and added as third components, these cells inhibit MLRs but recruit additional Tregs allospecifically. It is proposed that these third component inhibition/recruitment experiments can be used to more specifically analyze Treg function in clinically applicable transplant settings and provide insights into drug regulatory mechanisms (23).

MATERIALS AND METHODS

Human Subjects and HLA Typing

PBMCs were obtained from healthy volunteers that were HLA-typed by the Northwestern HLA laboratory using molecular methods (reverse SSO) and selected as being two DR matched or two DR mismatched. Also tested were PBMC of donor/recipient prerenal transplant pairs that were one HLA DR mismatched with each other. The research was conducted on human subjects with the approval of our Institutional Review Board.

The Treg MLR

Material, Media, and Culture Conditions—As previously described (15), after Ficoll-Hypaque gradient centrifugation, PBMC were labeled with CFSE following the manufacturer's instructions (labeling efficiency of >99%). They were stimulated with x-irradiated (3000 rads) PBMC from two DR-matched versus two DR-mismatched volunteer donors in sets of three, that is, responding cells versus two DR-matched and -mismatched stimulating cells, in complete media consisting of RPMI 1640 with 15% normal human AB serum, 2 mM L-glutamine, 10 mM Hepes, and 1X pen/strep/glutamine solution (GIBCO-BRL, Gaithersburg, MD). The cell concentrations used were (1) 1×10^5 stimulating and 1×10^5 responding cells, that is 0.2 mL/well in 96-well U-bottom plates for tritiated thymidine (^3H -TdR) uptake proliferation assays; (2) 5×10^5 stimulating and 5×10^5 responding cells, that is 1 mL/well in 48-well plates for four-color flow cytometry analysis and cell counts for quantifying inhibition of proliferation. The cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere for 5, 7 $\pm$ 9 days before processing. The prerenal transplant donor/recipient pairs (one DR mismatched) were similarly tested.

IS Agent Additives—The liquid formulation of TAC and SRL were diluted in complete media from the initial concentration of 1 and 5 mg/mL, respectively, to descending ng/mL concentrations, with at least 5- or 10-fold descending dilutions in each experiment. Equivalent doses of TAC or SRL were added at the culture outset from subtherapeutic to therapeutic trough level concentrations, ranging from 0.05 to 0.1 ng/mL versus 0.3 to 3 ng/mL versus 5 to 20 ng/mL per well. These were also compared with cultures in which the

same volume of complete media (no drug) was added, as we did not previously find differences in using media versus a drug vehicle for adequate control experiments (24).

Flow Cytometric Analysis—The CFSE-labeled responder cells in the 48-well MLR assays were tested at 5, 7±9 days for the surface markers CD4 and CD25 and for intracellular FOXP3 as previously described (25) and in the Supplemental Methods section (see Supplemental Digital Content 4, <http://links.lww.com/TP/A306>).

Assessment of Proliferation—This was measured using both CFSE labeling (as above) and by standard ³H-TdR incorporation assays (15, 26, 27). For the latter, on days 5, 7±9, 1 uCi ³H-TdR was added to each well of triplicate cultures and, after 18 hr, these were processed using a Tomtec cell harvester (Hamden, CT). Radioactive incorporation was measured using a Packard-Beta counter (Meriden, CT). The SI was calculated using the formula: counts per minute in experimental combinations/counts per minute in autologous control combinations. As a control for proliferation, 5 µg of phytohemagglutinin (Invitrogen, Carlsbad, CA) was added to 1×10⁵ responder cells, 3 days before ³H-TdR incorporation.

Assessment of Regulatory Functions (Inhibition/Recruitment) by TAC Versus SRL-Modified Tregs

This was performed as described (15) with slight modifications. First, Tregs were generated in bulk MLR of 40×10⁶ responder cells (A) stimulated with 40×10⁶ irradiated two DR-matched stimulator cells (Bx) in multiple T-75 flasks at 1×10⁶ cells/mL in presence of 5 ng/mL TAC, SRL, or no drugs. After 7 or 9 days in culture, the CD4⁺CD25^{High}CD127⁻ cells were enriched using the regulatory T cell Isolation Kit and AutoMACS (Miltenyi Biotech), after negatively selecting out CD8, CD19, and CD127 by AutoMACS. In general, more than 50% of these cells were CD25⁺ and FOXP3⁺ (see Table, Supplemental Digital Content 3, <http://links.lww.com/TP/A305>). The isolated cells (no drug vs. TAC vs. SRL Tregs vs. nonirradiated autologous PBMC as negative modulator controls) were then tested as third component modulator cells in MLR inhibition and “infectious” Treg-MLR recruitment assays. Briefly, 5×10⁴ modulators were added as third component cells to MLR cultures containing 5×10⁵ freshly prepared CFSE-labeled cells from the original responder (A) stimulated with unlabeled irradiated cells from the original stimulator (Bx; specific) or HLA-mismatched indifferent stimulator (Ix; nonspecific). Equal numbers of responder and stimulator cells were used.

To calculate inhibition of proliferation, 1×10⁵ responding and x-irradiated stimulator cells were cultured for 7 days in 96-well plates as described earlier. One uCi ³H-TdR was added to each well and 18 hr later the cultures were harvested and the radioactive incorporation was measured, and the percentage inhibition (SI) by the third component Tregs was calculated as described earlier. In addition, cells were counted to determine the presence of inhibition in the proliferating (CFSE dim) fraction of the CD4⁺ responders.

In the Treg-MLR recruitment assays, the percentages of newly generated CD4⁺CD25^{High} FOXP3⁺ cells in the CFSE-labeled (read out) responding cells, after the addition of unlabeled third component Treg modulators generated in the presence of media, TAC, or SRL, were measured by flow cytometry after 5 and 7 days. Given that all proliferating cells

and non- CFSE-labeled cells were CFSE low and might have been difficult to distinguish from proliferating (CFSE dim) responders, the stimulator and modulator cells were labeled with PKH26 and gated out before analyzing the cell subsets that arose specifically from the CFSE-labeled responders.

Statistical Methods

Descriptive statistics (mean, standard deviation) and graphical methods were used to record the SI and % Tregs in each MLR. Because our sample size was small, we conducted both parametric and nonparametric statistical methods. Comparisons between the peak SI and %Treg, including the % difference between the MLR with and without the various IS agent dilutions, were performed using student's *t* test and Wilcoxon signed rank test for parametric and nonparametric calculations, respectively. *P* less than 0.05 was considered statistically significant. All statistical analyses were performed using SAS version 9.2 statistical software (SAS Inc., Cary, NC).

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FIGURE 1.

Effect of tacrolimus (TAC) on lymphoproliferation and the generation of T-regulatory cells in mixed lymphocyte reaction (Treg-MLR). (A) 1×10^5 responding peripheral blood mononuclear cell (PBMC) from each of the laboratory volunteers, regardless of DR match, and each prerenal transplant donor/recipient pair were cultured with 1×10^5 autologous or allogeneic irradiated stimulator PBMC in presence of indicated doses of TAC. Standard ^3H -TdR assays were performed on days 3, 5, 7, and 9. The peak stimulation index (SI), usually on day 7, is shown as median $\pm$ SE. (B) 5×10^5 PBMC each of carboxy-fluorescein diacetate succinimidyl ester-labeled responders and unlabeled irradiated stimulators from the same individuals as in (A) were cultured in the presence of indicated doses of TAC, and four-color flow cytometric analysis was performed on days 1 (baseline), 5, 7, and 9 as previously reported (15). The peak percentage levels (usually day 7) of developing CD4⁺ CD25^{High} FOXP3⁺ responder cells are shown. (C) The median declining percentages from the (no drug) controls for indicated TAC doses were calculated both with the respective SIs and CD4⁺ CD25^{High} FOXP3⁺ percentages. Note that TAC inhibited the proliferation and immunophenotypic Treg generation in a dose-dependent manner ($P < 0.0002$ for 0.3–3 and 5 ng/mL vs. 0).

FIGURE 2.

(A) The differential effects of tacrolimus (TAC) and sirolimus (SRL) on the proliferation and generation of CD4⁺CD25^{High}FOXP3⁺ cells in T-regulatory cells in mixed lymphocyte reactions (Treg-MLRs) of DR-matched and -mismatched laboratory volunteers and pretransplant renal donor/recipient pairs. (B) A representative experiment showing the four-color flow analysis scheme and the differential effects of TAC and SRL on Treg-MLR (day 7 shown here). (A) Treg-MLRs were performed in healthy laboratory volunteers (columns 1 and 2). The percentage inhibition from the no drug control for each drug dose was

calculated. Note that both TAC and SRL inhibited stimulation indices (SIs) equally in DR-matched combinations (*column 1*), but inhibition was greater with TAC in DR mismatches (*column 2*) at higher concentrations ($P<0.05$). However, SRL was significantly less inhibitory than TAC at therapeutic (≥ 5 ng/mL) levels in the generation of CD4⁺ CD25^{High} FOXP3⁺ Tregs in two DR-matched (*column 1*) and -mismatched (*column 2*) pairs ($P=0.02$). Drug Treg-MLRs were similarly performed with one DR-matched responder-stimulator combinations of patients/donors before renal transplantation (*column 3*). Note that SRL was significantly less inhibitory than TAC at both the therapeutic (≥ 5 ng/mL) and subtherapeutic (0.3–3 ng/mL) levels in the generation of CD4⁺ CD25^{High}FOXP3⁺ Tregs ($P=0.02$), whereas at highest doses (therapeutic), the effects on SI were similar. (B) A total of 0.5×10^6 carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled responder peripheral blood mononuclear cell (PBMC) were cultured with 0.5×10^6 irradiated PBMC from an human leukocyte antigen DR-matched volunteer in the presence of no immunosuppressive drugs as control, with 5 ng/mL TAC, or 5 ng/mL SRL. The viable CD4⁺ cells that were nonproliferating (CFSE high cells, labeled as Non-Prolif.) or proliferating (CFSE low cells, labeled as Prolif) were gated and analyzed. CFSE-negative (x-irradiated) stimulator cells were gated out (on and before day 5) or were found to be absent on days 7 and 9 in the viable CD4 gate (see Figure, Supplemental Digital Content 5, <http://links.lww.com/TP/A307>). The *dot plots* on the *right* are gated on the proliferating fractions of the CD4⁺ viable responder cells (*red arrows*). The percentages outside the plots represent gated fractions (*right column*). The CD25^{High} FOXP3⁺ cells were found to be CD127 negative in a separate set of experiments (data not shown). Note that although there appears to be a slightly higher proliferation peak (in *column 3*) with SRL, this did not occur when SIs for the entire group were calculated, that is there was no statistical difference between SIs with SRL versus TAC. Finally, the percentage of Tregs in the nonproliferating fraction was found to be significantly lower than the proliferating fraction (*column 5*).

FIGURE 3. Assessment of allospecific (A) versus nonspecific (B) inhibitory functions (inhibition by cell counts) and CD4⁺ CD25^{High} FOXP3⁺ recruitment (C) by T-regulatory cells (Tregs) generated in mixed lymphocyte reaction (MLR) in the presence of media, tacrolimus (TAC), or sirolimus (SRL). Bulk cultures of volunteer responder cells were stimulated with two DR-matched x-irradiated cells in the presence of no drugs, 5 ng/mL TAC, or SRL for 7 days and putative Tregs enriched as described in the methods and reported in Supplemental Table 1 (see Supplemental Digital Content 3, <http://links.lww.com/TP/A305>). These enriched Tregs

compared with fresh autologous (responder) cells were added in equal numbers (see Methods, Supplemental Digital Content 4, <http://links.lww.com/TP/A306>) as modulators to 1×10^5 autologous fresh responding peripheral blood mononuclear cell (PBMC) and x-irradiated stimulator PBMC, the latter from the original two DR-matched (A – Specific) or an human leukocyte antigen two DR-mismatched (B – Non-specific) volunteer donor. Inhibition by cell counts was greatest with SRL-cultured modulators. This was allospecific against the original stimulator and did not occur as significantly against an indifferent two DR-mismatched stimulator (A vs. B: CD4⁺ responder cell counts in *red, columns 2 and 4, rows D* compared with C and B). Inhibition by stimulation index for this experiment is shown with other experiments in Figure 2, Supplemental Digital Content 2, <http://links.lww.com/TP/A304>. As in Figure 2B), the percentage of Tregs in the nonproliferating fraction was found to be significantly lower than the proliferating fraction (*column 6 in A and B*). (C) In three separate experiments, 5×10^4 enriched (non-carboxy-fluorescein diacetate succinimidyl ester [CFSE] labeled) Tregs or fresh autologous control PBMC were added as modulators to 5×10^5 autologous fresh CFSE-labeled responding PBMC and x-irradiated (unlabeled) specific and nonspecific stimulator PBMC. The modulator cells and x-irradiated stimulator cells were all labeled with PKH26 to differentiate these from the readout of CFSE-labeled responding cells. After another 7 days, four-color flow cytometric assays were performed to estimate CD4⁺CD25^{High} FOXP3⁺ cells in the CFSE-labeled responder PBMC. Recruitment data are calculated as percentage increase in CD4⁺CD25^{High} FOXP3⁺ cell generation with the Tregs over that observed with the control fresh PBMC modulators (*dotted line*). Note that a more marked and allospecific effect of SRL-cultured modulators occurred (green diamond bars, SRL vs. TAC and media, $P=0.01$). A value below the *dotted line* or a change of $\pm 10\%$ from control is considered to indicate the absence of recruitment.