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CD28–CD80 Interactions Control Regulatory T Cell Motility and Immunological Synapse Formation

Timothy J. Thauland,* Yoshinobu Koguchi,*-1 Michael L. Dustin,†‡ and David C. Parker*

Regulatory T cells (Tregs) are essential for tolerance to self and environmental Ags, acting in part by downmodulating costimulatory molecules on the surface of dendritic cells (DCs) and altering naive CD4 T cell–DC interactions. In this study, we show that Tregs form stable conjugates with DCs before, but not after, they decrease surface expression of the costimulatory molecule CD80 on the DCs. We use supported planar bilayers to show that Tregs dramatically slow down but maintain a highly polarized and motile phenotype after recognizing Ag in the absence of costimulation. These motile cells are characterized by distinct accumulations of LFA-1–ICAM-1 in the lamella and TCR-MHC in the uropod, consistent with a motile immunological synapse or “kinapse.” However, in the presence of high, but not low, concentrations of CD80, Tregs form stationary, symmetrical synapses. Using blocking Abs, we show that, whereas CTLA-4 is required for CD80 downmodulation, CD28–CD80 interactions are critical for modulating Treg motility in the presence of Ag. Taken together, these results support the hypothesis that Tregs are tuned to alter their motility depending on costimulatory signals. The Journal of Immunology, 2014, 193: 000–000.
We suggest a model wherein highly motile Tregs are constantly scanning the surface of APCs, only stopping when the APC is activated and displaying high levels of costimulatory activity. This behavior may allow Tregs specific for self and environmental Ags to efficiently downmodulate costimulatory molecules on inappropriately activated cells while ignoring resting cells displaying those Ags.

### Materials and Methods

#### Animals

Heterozygous AD10 TCR transgenic mice on a B10.BR background, specific for pigeon cytochrome c (MCC) 88–103 (22), were provided by S. Hedrick (University of California at San Diego, La Jolla, CA) by way of P Marrack (National Jewish Medical Center, Denver, CO). B6.Cg-Foxp3^GFP^/J (Foxp3-GFP) mice were obtained from The Jackson Laboratory. Male AD10^+^ mice were bred to homozygous female Foxp3-GFP mice and the AD10^+^ progeny were used as a source of TCR transgenic GFP^+^ Tregs. All mice were housed in specific pathogen-free conditions and used in accordance with National Institutes of Health guidelines under an animal protocol approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

#### Abs

The Abs used for flow cytometry were as follows: anti-CD4 Alexa Fluor 488 (GK1.5; eBioscience), anti-CD4 PerCP (RM4-5; BioLegend), anti-CD152 PE (UC10-4B9; BioLegend), anti-Foxp3 Alexa Fluor 647 (150D; BioLegend), anti-CD11c FITC and PE (HL3; BD Biosciences), anti-CD25 PerCP (PC61; BioLegend), and anti-CD80 FITC and PerCP-Cy5.5 (16-10A1; BD Biosciences).

#### DC culture

Bone marrow–derived DCs (BMDCs) were cultured as described (23). Briefly, bone marrow cells were cultured for 9 d in bacteriologic media plates in complete RPMI 1406 media supplemented with GM-CSF superantigen (final concentration, 20 ng/ml). On day 9 of culture, immature DCs were plated in LabTek II eight-well chambers (no. 1.5; Nunc) or six-well plates in fresh media with 20 ng/ml GM-CSF and 1 μg/ml LPS.

#### In vitro Treg polarization

CD4^+^ cells and B cells were purified from AD10 and B10.BR spleen cell suspensions, respectively, using EasySep immunomagnetic negative selection (StemCell Technologies). B cells (5 × 10^5) and CD4^+^ cells (2.5 × 10^6) were cultured in 1 ml complete RPMI 1640 media in six-well plates with 2.5 μM MCC peptide, 20 ng/ml TGF-β, 100 U/ml IL-2, and 10 nM all-trans retinoic acid. On days 2 and 3, 1 ml media supplemented with 100 U/ml IL-2 was added to the cultures. To confirm that T cells were polarized to a Treg phenotype, cells were fixed, permeabilized, and stained for CD4, CTLA-4, and Foxp3. Fixation and permeabilization reagents were from BioLegend. Cells were used on day 4.

#### CD80 downmodulation assay

Day 9 BMDCs (1.6 × 10^5) were seeded onto 12-well plates and treated with LPS as described above. One day later, the indicated numbers of day 4 Tregs were added per well in the presence or absence of 2.5 μM MCC. In some experiments, Fab fragments of anti-CD80 (E18; gift from Thomas Hünig) (24) or anti–CTLA-4 (UC10-4F10-11; Bio X Cell) were added at 100 μg/ml. The Fab fragments were generated with a Pierce Fab preparation kit (Thermo Scientific). As a control, Tregs were not added to some wells. Forty-eight hours later, the cells were harvested and stained for 30 min on ice for CD4, CD11c, and CD80. All Abs were used at 1:200. EDTA (1 μM) was included in the FACS buffer to discourage continued interactions. Samples were collected on a BD FACSCalibur with CellQuest software and analyzed with FlowJo (Tree Star).

#### Imaging Treg–DC interactions

Day 9 BMDCs (3 × 10^4) were seeded onto coverslips in eight-well chambers and treated with LPS as described above. One day later, 1.5 × 10^4 CFSE-loaded, day 4 Tregs were added to the wells. Imaging commenced as soon as Tregs were added to the wells. Differential interference contrast (DIC) and fluorescent images were obtained every minute for 2 h. All imaging was conducted at 37°C with 5% CO_2_. Wide-field imaging was performed with an Applied Precision DeltaVision system using an Olympus ×200/0.75 numerical aperture Plan Apo objective. This system included an Applied Precision chassis with a motorized XY stage, WeatherStation environmental chamber, Olympus IX71 inverted fluorescent microscope, xenon lamp, and CoolSnap HQ2 camera. The DeltaVision SoftWorx software package was used for image acquisition.

#### Supported planar bilayer experiments

GPI-linked forms of Oregon Green 488 labeled 1-E^+^ (200 molecules/μm^2) and Cy5-labeled ICAM-1 (300 molecules/μm^2) were incorporated into dioleoylphosphatidylcholine bilayers exactly as described (25, 26). For some experiments, GPI–linked CD80 was incorporated into biotinylated PLD as described previously (27). These bilayers were supported on a coverslip in a Bioptechs flow cell and were loaded with 100 μM MCC or hemoglobin peptide (GKKVITAFNEGLK) in a PBS/citrate buffer (pH 4.5) for 24 h at 37°C (25). T cells (10^4) in 1 ml HBS buffer with 1% BSA were injected onto bilayers at 37°C. In some experiments, anti-CD28 and/or anti–CTLA-4 Fab fragments were added to the T cells at 50 or 100 μg/ml 10 min prior to injection onto the bilayers. Images were acquired every minute for 30–60 min on the DeltaVision system using an Olympus ×60/1.42 numerical aperture Plan Apo objective.

Imaris 6.3 (Bitplane) was used to track cells interacting with the bilayer. To ensure that only cells productively interacting with the bilayer were analyzed, Cy5 fluorescence (ICAM-1 accumulation) was tracked automatically with the Spot tool in Surpass mode. In situations where the ICAM-1 signal was ambiguous, DIC images were examined to determine whether the cell was flattened against the bilayer. DIC images were used to track cells interacting with bilayers loaded with irrelevant peptide, as there was only sparse ICAM-1 accumulation under these conditions.

#### Scoring T cell–bilayer interactions

Cells were scored as nonmotile when they moved one cell diameter or less during the entire imaging session. Cells were considered to have reformed an IS when they stopped forward progress, lost their uropod and ICAM-1 arc, and remained nonmotile for at least 10 min. Cells were considered to have broken symmetry when they were nonmotile for 10 min or more before gaining motility and moving more than one cell diameter. The vast majority of cells were symmetrical when first contacting the bilayer, but this state was transient (much less than 10 min) and cells that subsequently became motile were not considered to have broken symmetry. Cells were scored as having a uropod anchor when the uropod of a motile cell was attached to the same place for at least 10 min, causing the cell to pivot.

#### Results

**Tregs transition from stable to motile contacts with DCs, concomitant with downmodulation of costimulatory molecules**

We set out to study the IS formed between Tregs and APCs. To generate a population of Ag-specific cells for use in imaging experiments, Tregs were induced in vitro by culturing purified CD4 T cells from AD10 TCR transgenic mice with naïve B cells in the presence of MCC peptide, TGF-β, IL-2, and all-trans retinoic acid, as previously described (28). After 4 d of culture under Treg conditions, the cells were almost completely polarized to a Treg phenotype with high levels of Foxp3 and CTLA-4 (Fig. 1A). Day 4 Tregs were loaded with CFSE, introduced to peptide-loaded BMDCs, and imaged over time.

The initial interactions between Tregs and BMDCs were very stable, with the vast majority of Tregs flattening against BMDCs and maintaining a rounded, unpolarized shape for the duration of the experiment (Fig. 1B, Supplemental Video 1). However, after 24 h of coculture, the Tregs became highly motile and formed large, swarming clusters around the BMDCs (Fig. 1C). To further investigate this change in motility, we added fresh Tregs labeled with CFSE to the Treg-BMDC cocultures and observed their behavior. Surprisingly, the freshly added Tregs also displayed a motile, swarming phenotype. As shown in Fig. 1D and Supplemental Video 2, the freshly added Tregs adopted a polarized shape and crawled along the surface of the BMDCs. The tendency of freshly added cells to form motile, rather than stable, contacts indicates a change in the BMDCs rather than a change in the Tregs following 24 h of culture together.
nTregs have been shown to cluster around DCs after a 12-h incubation, and this phenotype was associated with downmodulation of CD80 and CD86 on the DC surface (15). To determine whether changes in the surface phenotype of the DCs could be responsible for the altered motility of Tregs after a 24-h coculture, we conducted assays to measure downmodulation of CD80. Incubating Tregs with BMDCs resulted in Ag-specific downmodulation of CD80 at both 1:1 and 5:1 Treg/BMDC ratios (Fig. 1E, 1F). In agreement with published work (15), Fab fragments of an anti–CTLA-4 Ab completely inhibited CD80 downmodulation, even at a 10:1 Treg/BMDC ratio (Fig. 1G). Treatment with Fab fragments of an anti-CD28 Ab known to efficiently block CD28–CD80 interactions (24) resulted in a decrease in the efficiency of CD80 downmodulation, but the effect was relatively minor compared with anti–CTLA-4 treatment (Fig. 1H).

**CD80 modulates IS formation in Tregs**

Although qualitatively informative, the Treg–DC interactions were not conducive to precise measurements of Treg motility because the DCs in these experiments were often highly motile. To carefully measure the interactions between Tregs and APCs in two dimensions, we used supported planar bilayers containing GPI-linked, fluorescently labeled ICAM-1 and MHC class II (I-Ek) loaded with MCC peptide (25).

Day 4 Tregs were injected onto the supported planar bilayers and examined for the distribution of peptide-MHC (pMHC)–TCR and ICAM-1–LFA-1 interactions at the Treg–APC interface. Although the cells fluxed calcium immediately upon touching the bilayers (data not shown), we did not observe symmetrical IS with well-defined supramolecular activation clusters, in agreement with a recent report (Fig. 2A) (29). Instead, the Tregs interacting with the bilayer had the polarized morphology of motile cells, with a well-defined uropod. An arc of ICAM-1 was located in the mid-cell region (Fig. 2B), as previously described for motile T cells (30). The strongest accumulation of pMHC-TCR was in the uropod of the crawling cells (Fig. 2B), consistent with a phenotype seen for CD8 T cell blasts (31). As shown in Fig. 2C, the Tregs broke radial symmetry and formed motile synapses almost im-
mediately upon interacting with the bilayers. Motile IS, or “kinapses,” have been previously reported in naïve T cells in vitro (32) and in vivo (33). Our examination of Treg–DC interactions suggested that the level of costimulatory molecules on the APC surface could modulate Treg behavior. Therefore, we examined IS and kinapse formation in the presence of 0, 40, and 200 molecules/μm² GPI-linked CD80 in the bilayers. In the presence of low levels of CD80 (40 molecules/μm²), the Tregs predominantly formed motile kinapses, similar to the phenotype observed in the absence of CD80 (Fig. 3A, 3B, Supplemental Videos 3, 4). However, when Tregs were introduced to bilayers containing 200 molecules/μm² CD80, their movement was drastically reduced, with some of the Tregs forming stable IS (Fig. 3C, Supplemental Video 5). Analysis of the motility of Tregs on bilayers containing varying amounts of CD80 showed that high levels of CD80 significantly decreased the mean square displacement (MSD) of Tregs over time (Fig. 3D, 3E). As shown in Fig. 3F, the average speed of Tregs was significantly reduced in the presence of high levels of CD80. Because changes in the centroid of relatively nonmotile cells can inflate speed measurements, we also measured the total displacement of each cell and divided by track duration. This measurement yielded values close to 0 for nonmotile cells and showed that Tregs are dramatically less motile when introduced to bilayers with high levels of CD80 (Fig. 3G). We also noted a decrease in the straightness of Treg tracks in the presence of high levels of CD80 (Fig. 3H).

Although the fraction of cells that maintained symmetrical, nonmotile interactions with the planar bilayers for the entirety of a 45- to 60-min experiment was 3-fold higher in the presence of 200 molecules/μm² CD80, this result did not quite meet the standard for statistical significance (Fig. 4A). However, significantly fewer cells were motile for the entire experiment in the presence of high levels of CD80 (Fig. 4B). These results indicate that in addition to slowing the motility of Tregs, high levels of CD80 also induced stable IS formation. As shown in Fig. 2C, Tregs that formed kinapses broke radial symmetry almost immediately upon contacting the bilayers. However, we observed that a fraction of the cells regained symmetry and formed a stable IS during the course of the experiment (Fig. 4C). The percentage of Tregs displaying this behavior was dramatically increased in the presence of high levels of CD80 (Fig. 4D).

In the absence of CD80, the vast majority of Tregs moved freely across the bilayer, but the addition of CD80 caused some motile cells to become anchored to the substrate via their uropods, frustrating the forward motion of the cells and causing them to turn in circles (Fig. 4E). This behavior was seen at both concentrations of CD80, but was especially prominent when high levels were used (Fig. 4F).

It has been shown that TCR transgenic mice contain a population of nTregs that are most likely derived from cells expressing endogenous TCR α-chains in addition to transgenic α- and β-chains (34). Indeed, we found that a fraction of naïve CD4⁺ cells from AD10 mice expressed Foxp3 (Fig. 5A). To confirm that our findings were generalizable to nTregs, we crossed Foxp3-GFP mice to our AD10 TCR transgenic mice and introduced purified C4 cells to bilayers (Fig. 5B, 5C). Unlike activated Tregs, a significant fraction of the nTregs was sessile regardless of CD80 concentration (Fig. 5E), possibly because these cells are relatively metabolically inactive compared with in vitro–generated Tregs. Nevertheless, the presence on CD80 in the bilayers resulted in a significant decrease in motility (Fig. 5D–F). Interestingly, naïve non-Tregs were much less motile than Tregs in the absence of CD80. These results demonstrate that nTregs behave similarly to in vitro–induced Tregs in the presence and absence of CD80.

**Treg motility decreases dramatically in the presence of Ag**

Tregs interacting with bilayers accumulated pMHC-TCR in the contact zone (Fig. 2B) and fluxed intracellular calcium (data not shown), but they were almost always continuously motile over the entire imaging experiment (Fig. 4B). This led us to ask whether there were any changes in Treg motility upon Ag recognition, or whether motility was only altered in the presence of high levels of CD80. Therefore, we introduced AD10⁺ Tregs to bilayers containing ICAM-1 and MHC loaded with either cognate peptide (MCC) or an irrelevant peptide (hemoglobin). As shown in Fig. 6A, in the absence of cognate Ag, Tregs moved rapidly across the bilayers with only transient accumulations of ICAM-1, and at no time were pMHC-TCR clusters apparent. Analysis of the tracks made by Tregs in the absence of Ag showed that Tregs scanning the surface of an APC lacking cognate Ag move much faster and straighter than did Tregs that have recognized Ag (Fig. 6B–F). Thus, Tregs are highly motile when scanning the
surface with ICAM and MHC without specific Ag, slow considerably in the presence of Ag, and slow even more, often to the point of stopping, in the presence of Ag and high levels of co-stimulation.

**CD28–CD80 interactions control Treg motility**

To dissect the mechanism by which CD80 modulates Treg motility, we incubated cells with Fab Abs against CD28 or CTLA-4 and introduced them to bilayers containing pMHC, ICAM-1, and high levels of CD80. As shown in Fig. 7A, blockade of CD28 resulted in highly motile Tregs in the presence of CD80. In contrast, many of the Tregs treated with anti–CTLA-4 formed relatively nonmotile contacts with the bilayer or had their uropods anchored (Fig. 7B). We analyzed the tracks of Tregs loaded onto bilayers without CD80 or with CD80 and either anti-CD28, anti–CTLA-4, or no Ab. Tregs treated with anti-CD28 were much more motile than cells in the no Ab control condition and were almost as motile as cells loaded onto bilayers lacking CD80 (Fig. 7C–G). Treatment with anti-CD80 did not have an appreciable effect on motility by most measures, as 10 min MSD displacement/duration and speed were not different from the no Ab control (Fig. 7C–E). Treatment with anti–CTLA-4 did result in somewhat straighter tracks, although the significance of this finding is unclear (Fig. 7G).

Taken together, these data show that CD28–CD80 interactions control the motility of Tregs.

**Discussion**

In this study, we propose a model wherein Treg–DC interactions are modulated by relative levels of costimulatory molecules. In the absence of Ag recognition, Tregs rapidly migrate over the surface of APCs, scanning for their cognate Ag. Upon Ag recognition, Tregs form either relatively slow-moving kinapses or nonmotile IS depending on the levels of costimulatory molecules. In the presence of high levels of costimulation, symmetrical ISs are favored. After levels of costimulatory molecules are reduced, kinapse formation is favored and the Treg starts migrating again. Thus, by alternating between kinapses and stable ISs, Tregs could efficiently contact and modulate levels of costimulatory molecules or otherwise suppress inappropriately activated DCs.

Our results are consistent with a recent study showing that nTregs interacting with supported planar bilayers containing 90 molecules/μm² CD80 form unstable ISs, but because CD80 was not tested at higher concentrations in that study, the profound effect that CD80 has on Treg motility was not discovered (29). In contrast to our study, human Tregs were observed to form hyperstable ISs compared with T effector cells (35). This dis-
crepancy could be due to a species difference, but is probably best explained by differences in the TCR stimulus provided to the Tregs. Zanin-Zhorov et al. (35) introduced T cells to supported planar bilayers containing anti-CD3ε. It is likely that the presence of such a strong and qualitatively different TCR stimulus obviates the need for CD80 to induce stopping of Tregs, masking the differences in motility we noted when using saturating concentrations of a physiological TCR ligand. However, note that CD25 expression of non-Tregs displayed rapid symmetry breaking and motility in the same conditions, demonstrating that anti-CD3 does not generate a durable stop signal for all human T cells.

How does signaling through CD28 alter Treg motility? Crosslinking CD28 results in Lck-mediated phosphorylation of the YMNM motif and recruitment of PI3K and Grb2 (36). The activity of PI3K leads to activation of Akt, whereas Grb2 recruits the guanine nucleotide exchange factor Vav1, resulting in activation of the small GTPases Rac1 and Cdc42, which in turn activate WAVE2 and WASp, respectively, ultimately leading to Arp2/3-mediated actin remodeling (37). Additionally, a proline-rich domain of CD28 is known to recruit several kinases downstream of the TCR, including Lck and PKC-θ (36). We hypothesize that Tregs have a relatively higher threshold for activation than do conventional T cells, which allows them to monitor their environment without being inappropriately activated, even in the face of ubiquitously expressed, low-affinity cognate Ags. Under this scenario, Tregs would require a robust signal through CD28 before transitioning from motile kinapses to stable synapses. In support of a model where CD28 signaling through PI3K is critical for Treg function, it has been shown that Tregs expressing an inactive form of the PI3K catalytic subunit, p110δ, are poor suppressors and fail to prevent inflammation in a colitis model (38). Strong CD28 crosslinking may simply augment TCR-induced signaling and/or cytoskeletal remodeling, or it could provide a unique signal. A similar phenomenon has recently been observed in thymocytes, in which CD28-dependent actin remodeling was required for maximal activation after TCR triggering (39).

An annular ring of CTLA-4 surrounding the central supramolecular activation cluster characterizes the IS formed by induced Tregs interacting with bilayers containing CD80 (40). This dense accumulation of CTLA-4 may increase the efficiency of costimulatory molecule downmodulation. Thus, the reduced motility and increased IS formation we observed in the presence of high levels of costimulation may serve two purposes: 1) to increase the dwell time of a Treg on a particular activated APC, and 2) to enhance the efficiency of downmodulation. The IS functions as a platform for the polarized secretion of effector molecules toward the APC (41, 42). Therefore, it is possible that additional Treg functions, including Ag-specific delivery of perforin (43, 44) or IL-10 (42), may require the formation of a stable IS.

CTLA-4 ligation has been reported to increase motility of T cells both in vitro and in vivo (45–47). Because CTLA-4 has a much higher affinity for CD80 than CD28 (48), it follows that high levels of CD80 are needed to trigger the CD28-mediated stop signal we see in our experiments. We suggest that the abundant expression of CTLA-4 on Tregs tunes these cells to be highly motile unless they recognize Ag in the context of large amounts of costimulation. Conventional CD4 T cells, unlike self-reactive Tregs that are constantly seeing Ag, are able to efficiently scan APCs without this mechanism. In disagreement with our model, a recent report showed that activated CD4+CD25+ cells from CD28−/− mice did not migrate differently in lymph node slices from CD4+CD25+ cells from CD28+/+ mice (49). The apparent
disparity between these results and ours could be explained by the fact that the absence of CD28 has a profound effect on the development and homeostasis of Tregs (8). In the absence of CD28, the small number of surviving peripheral Tregs may have compensatory mechanisms for activation and motility.

Mice that have CD28 specifically ablated in Foxp3+ cells develop severe multiorgan autoimmunity (50). We suggest that efficient downmodulation of costimulatory molecules on activated APCs presenting self-Ag may be a CD28-dependent function that is missing in these mice. This hypothesis leads to the prediction that blocking CD28 will result in less efficient downmodulation, and we did see a modest decrease in downmodulation in the presence of anti-CD28 (Fig. 1H). Alternatively, CD28-dependent arrest and symmetrical synapse formation may be required for other Treg suppressive functions, as mentioned above.

Mice deficient in the actin regulatory protein WASp develop chronic colitis, and Tregs from these mice are poor suppressors both in vitro and in vivo (51–53). WASp is also required for T cells to maintain IS symmetry (30). Although defective migration to lymphoid tissue likely plays a role in the phenotype described for WASp-deficient Tregs, defective IS formation may also be important. Tregs incapable of IS formation could also be deficient in CD80/86 downmodulation. Thus, constant surveillance and modulation of DCs (via IS-dependent downmodulation of costimulatory molecules) may be required to prevent autoimmune disease. Consistent with this idea, Tang et al. (19) have demonstrated that nTregs from BDC2.5 TCR Tg mice (which have TCRs specific for an Ag associated with pancreatic β cells) are motile in the pancreatic lymph node when transferred into NOD mice. However, when the same nTregs are transferred into NOD, CD28−/− mice lacking Tregs, they are nonmotile (19). Given the results presented in the present study, it is likely that the DCs in the Treg-deficient animals had relatively high levels of CD80/86 compared with animals containing endogenous Tregs, thereby altering the behavior of the transferred Tregs. Higher levels of costimulatory molecules on DCs in the pancreatic lymph node of these already diabetes-prone animals could partially explain why NOD mice lacking Tregs develop diabetes more quickly than do wild-type mice (54).

To maintain tolerance and immune homeostasis, Tregs need to monitor the activation state of all the DCs in the body. Our finding
that Ag can slow Treg migration may help explain how Tregs accumulate where their Ags are displayed. High CD80/CD86 levels then alter Treg behavior further to enable Ag-specific delivery of suppressive signals. The functional consequences of regulation of Treg motility and synapse formation by CD28 will be a fertile field of investigation in future experiments.

FIGURE 6. Tregs migrate rapidly in the absence of cognate Ag. Tregs were introduced to bilayers containing ICAM-1 and I-Ek loaded with either MCC or irrelevant (hemoglobin) peptides. (A) Representative time-coded tracks (blue to white from the beginning to the end of the experiment) of Tregs migrating on bilayers containing MHC loaded with hemoglobin peptide overlaid on an ICAM-1 image are shown. Scale bar, 10 μm. (B) MSD over time is plotted for Tregs interacting with bilayers loaded with MCC or hemoglobin peptide, and the MSD at 10 min is shown (C). The straightness (D), speed (E), and average displacement/duration (F) of Treg tracks on bilayers with MCC or hemoglobin peptide are shown. Results (B–F) are from one representative experiment of two with n = 62 and 84 for MCC and hemoglobin peptide, respectively. Error bars represent SEM. The p values were determined with a two-tailed Student t tests. Hb, hemoglobin peptide.

FIGURE 7. CD28–CD80 interactions are responsible for modulating Treg motility. Tregs were introduced to bilayers containing pMHC, ICAM-1, and 200 molecules/μm² CD80 in the presence of anti–CTLA-4 (A) or anti-CD28 (B) Fab Abs. Time-coded tracks (blue to white from the beginning to the end of the experiment) are overlaid on ICAM-1 images. Scale bars, 10 μm. The tracks shown are representative of two (A) or three (B) independent experiments. (C) MSD over time is plotted for Tregs on bilayers lacking CD80 and on bilayers with 200 molecules/μm² CD80 with or without anti–CTLA-4 and anti-CD28 Fab Abs, and the MSD at 10 min is shown (D). The average speed (E), average displacement/duration (F), and straightness (track displacement/length) (G) of Treg tracks on bilayers with or without CD80 and Fab Abs are shown. Results (C–G) are from one representative experiment of three with n = 135, 92, 113, and 109 for no CD80, CD80, CD80 plus anti-CD28, and CD80 plus anti–CTLA-4, respectively. Error bars represent SEM. One-way ANOVAs followed by Tukey HSD tests were used to determine p values. A p value >0.05 was considered not significant.
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Disclosures

The authors have no financial conflicts of interest.

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