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Accessory cells precondition naïve T cells and regulatory T cells for cytokine-mediated proliferation

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Naïve T cells and regulatory T cells, when purified, do not proliferate to the γ_c-cytokines IL-2, IL-7, or IL-15, despite their expression of cognate cytokine receptors. Dendritic cells (DCs) enabled the T cell proliferation to these cytokines, through cell-to-cell contact, but independent of T cell receptor stimulation. This effect lasted after separation of T cells from DCs, enabling enhanced proliferation of the T cells in DC-depleted hosts. We propose calling this a "preconditioning effect". Interestingly, IL-2 alone was sufficient to induce phosphorylation and nuclear translocation of STAT5 in T cells, but could not activate MAPK and AKT pathways and failed to induce transcription of IL-2 target genes. "Preconditioning" was necessary to activate these two pathways and induced weak Ca^{2+} mobilization independent of calcium release-activated channels. When preconditioning was combined with IL-2, full activation of downstream mTOR, 4E-BP1 hyperphosphorylation, and prolonged S6 phosphorylation occurred. Collectively, accessory cells provide T cell preconditioning, a unique activation mechanism, controlling cytokine-mediated proliferation of T cells.

naïve T cells | regulatory T cells | dendritic cells | gamma chain cytokine | proliferation

Significance

This study addresses the requirements for naïve T cells and $CD4^+CD25^+$ T cells (Tregs) to undergo proliferative responses to cytokine stimulation, segregated from T cell receptor signals. These T cells, when purified, do not proliferate to γ_c-cytokines IL-2, IL-7, or IL-15, despite their expression of the cognate receptors for the cytokines and phosphorylation and nuclear translocation of STAT5. The study demonstrates that accessory cells convert naïve T cells and Tregs from a resting status to a "preconditioned" status, through cell-to-cell contact, without T cell receptor stimulation. "Preconditioning" enables full transduction of cytokine signals and cytokine-mediated proliferation of T cells. It also enhances cytokine-induced proliferation of other T cell subsets and natural killer cells, further suggesting the pivotal role of accessory cells.

Cytokines, especially those that share the common gamma (γ_c) receptor chain, including interleukin (IL)-2, IL-7, and IL-15, play important roles in the survival, maintenance, activation, and differentiation of T cells (1–4). Naïve T cells in lymphopenic conditions undergo homeostatic proliferation to maintain cell populations in vivo, which are driven by recognition of self-antigen in the context of major histocompatibility complex (MHC) molecules and IL-7 (5–8).

Upon encountering foreign antigens, naïve T cells become activated, integrate signals from cytokines such as IL-2, undergo robust proliferation, and differentiate into effector T cells. Several mechanisms that control the induction of naive T cell proliferation upon antigen recognition have been proposed. In quiescence, the condensin II complex in naïve T cells maintains the condensation of chromatin. When naïve T cells were stimulated with IL-2 in the absence of T cell receptor (TCR) signals, chromatin remained inaccessible to phosphorylated signal transducers and activators of transcription 5 (STAT5), preventing gene induction and subsequent cellular proliferation (9). Therefore, chromatin condensation acts as a regulator of naïve T cell proliferation. However, upon TCR stimulation, chromatin became accessible to STAT5, histone modifications occurred, and cytokine-driven proliferation took place (9). Tumor suppressor p53 was reported to be another regulator of naive T cell proliferation (10). In the absence of antigen-induced TCR signals, p53 proteins were reported to show a sustained increase in naive CD4 T cells stimulated by IL-2, inhibiting the proliferative responses of the cells. TCR signals decreased p53 mRNA expression and strongly induced p53-specific E3 ubiquitin ligase mouse double minute 2 homolog (MDM2), resulting in early termination of p53 expression that allowed the cells to proliferate (10).

In certain pathological conditions in humans, such as smoldering/chronic adult T cell leukemia (ATL) or human T cell lymphotropic virus I (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP), both caused by lymphocytes infected with HTLV-1, spontaneous proliferation of infected CD4 T cells in peripheral blood mononuclear cells (PBMC) has been observed (11, 12). In HAM/TSP, IL-2 and IL-15 autocrine loop seems to be the mechanism underlying the proliferation; however, the presence of antigen-presenting, HTLV-I infected dendritic cells (DCs) and monocytes appears essential for T cell proliferation as well. Notably, HAM/TSP spontaneous proliferation of T cells could be inhibited by removal of DCs or addition of antibodies to MHC class-II (MHC-II) and HTLV-I antigens (13). Similar to HAM/TSP, IL-2 and IL-15 autocrine loop may be involved in the early stages in ATL (14, 15), when HTLV-1 Tax protein plays an important role transactivating various growth-promoting genes, including those of IL-2 and IL-2 receptor α (IL-2Rα) (16). Previous studies from our group showed that

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The authors declare no competing interest.

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smoldering/chronic ATL cells produced IL-2, IL-9, and IL-15 in ex vivo cultures and that IL-9 expression was dependent on IL-2 signaling; however, similar to HAM/TSP, proliferation of the cells in response to these cytokines required presence of monocytes and involved interactions with MHC-II molecules (17).

In the present study, we addressed the requirements for $CD4^+CD25^+$ T cells (Tregs) and naïve T cells to undergo proliferative responses to cytokine stimulation, segregated from TCR signals. In steady state, there exist long-lived quiescent Tregs and chronically TCR-activated proliferating Tregs (18–20). We found that naïve T cells and Tregs, when purified, failed to proliferate to such γ_c-cytokines as IL-2 and IL-15, despite the T cell expression of the cognate receptors for the cytokines and phosphorylation and nuclear translocation of STAT5. These T cells required contact with accessory cells, independent of antigens, for their proliferative responses. Accessory cells seemed to convert naïve T cells and Tregs from a resting status to a preconditioned status that is distinct from activation induced by TCR signaling. "Preconditioning" allowed cytokine signals to fully transduce and enable naïve T cells to proliferate in response to the cytokines. Furthermore, preconditioning also acted on other subsets of T cells and on natural killer (NK) cells to enhance their responses to cytokines.

Results

Naïve T Cells Require Accessory Cells In Order To Proliferate to Cytokines. Naïve T cells are considered to be maintained in vivo by γ_c cytokines. We first examined whether mouse CD8 T cells proliferated in response to IL-2 when they were purified. CD8 T cells purified from the spleen were labeled with 5-chloromethylfluorescein diacetate (CMFDA) and cultured with IL-2. These T cells expressed IL-2 receptor β-chain (IL-2Rβ, CD122) and γ_c (CD132) that should be able to bind IL-2 and activate downstream signaling pathways (*SI Appendix*, Fig. S1*A*). However, proliferation, indicated by dilution of CMFDA, was observed only with the $CD8CD44^{hi}$ T cell population (Fig. 1*A*), an observation that contradicted the notion that naïve T cells proliferate in vivo when cytokines such as IL-2, IL-15, and IL-7 are available (1–4). Thus, we next examined the proliferation of these CD8 T cells to IL-2 in whole splenocytes, a situation which is more similar to the in vivo environment. Interestingly, this enabled proliferation of $CD8CD44^{lo}$ naïve T cells, although not robustly, to IL-2 (Fig. 1*B*). $CD8CD44^{hi}$ cells in the splenocyte culture divided even further in response to IL-2 as compared to their proliferation after purification and stimulation with IL-2. This led us to hypothesize that these T cells required additional cells for their proliferation to γ_c-cytokines that were present in the splenocyte cultures.

To test this hypothesis, we stimulated purified CD8 and CD4 T cells with IL-2 in the presence of splenic purified B cells, the most abundant cell type residing in the spleen. However, B cells failed to enhance proliferation of CD8 and CD4 T cells as shown by ^{3}H-thymidine incorporation assays (Fig. 1*C*). We then asked whether DCs could enhance the T cell responses. The presence of DCs enabled purified CD8 and CD4 T cells to proliferate to IL-2 (Fig. 1*C*). DCs, even at small numbers relative to T cells, significantly enhanced the T cell proliferation, exhibiting a T:DC ratio dependency that peaked between 2:1 and 1:2 T:DC ratios (Fig. 1*D*). To this end, we isolated $CD8CD44^{lo}$ naïve T cells (*SI Appendix*, Fig. S1*B*) and stimulated them with IL-2 with and without an addition of irradiated DCs. $CD8CD44^{lo}$ naïve T cells alone failed to proliferate to IL-2 but adding DCs to the cultures enabled the cells to respond and proliferate to the cytokine (Fig. 2*A*). Of note, without IL-2 (shown as 0 nM), DCs alone did not induce proliferation of $CD8CD44^{lo}$ T cells, and the irradiated DCs did not incorporate ^{3}H-thymidine (*SI Appendix*, Fig. S1*C*). A similar effect of DCs was observed with IL-15 and IL-7, enabling $CD8CD44^{lo}$ naïve T cells to proliferate (Fig. 2*A*). Consistent with the CMFDA dilution assay above, $CD8CD44^{hi}$ memory phenotype T cells were able to proliferate to IL-2 alone, showing 50-times higher ^{3}H-thymidine uptake with 10 nM IL-2 than the $CD44^{lo}$ cell counterpart (Fig. 2*B*). However, the addition of DCs further enhanced $CD8CD44^{hi}$ T cell proliferation. This augmentation effect of DCs on $CD8CD44^{hi}$ T cell

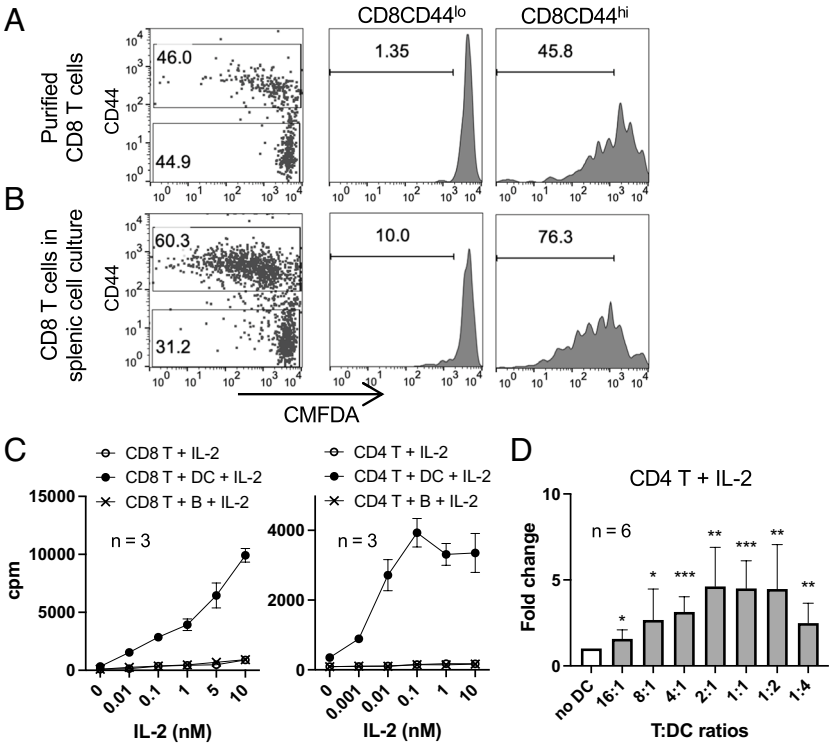

Fig. 1. Purified naïve T cells fail to proliferate to IL-2. (*A*) Flow cytometry analysis of CMFDA-labeled purified CD8 T cells cultured with IL-2 (8 nM) for 5 d indicated that $CD44^{lo}$ cells did not proliferate, whereas $CD44^{hi}$ cells did (representative data of two independent experiments). (*B*) Proliferation of CD8 T cells within CMFDA-labeled splenocytes cultured with IL-2 (8 nM) was analyzed by flow cytometry on day 5. $CD8CD44^{lo}$ cells in splenocyte culture proliferated and $CD8CD44^{hi}$ cells proliferated more than purified $CD8CD44^{hi}$ cells (representative data of 3 independent experiments). (*C*) ^{3}H-thymidine incorporation studies indicated that addition of B cells (nonirradiated) to the culture failed to enhance proliferation of purified CD8 and CD4 T cells to IL-2, but addition of irradiated DCs enhanced their proliferation in a 3 d culture. B cells or DCs were added at twice the T cell numbers (n = 3). (*D*) Addition of irradiated DCs, even at low numbers, significantly enhanced proliferation of purified CD4 T cells to 10 nM IL-2 as indicated by the fold increase of ^{3}H-thymidine incorporation over IL-2 alone on day 3. The effect peaked when T cell:DC ratios were between 2:1 and 1:2 (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: $P > 0.05$ by two-tailed ratio paired *t* tests.

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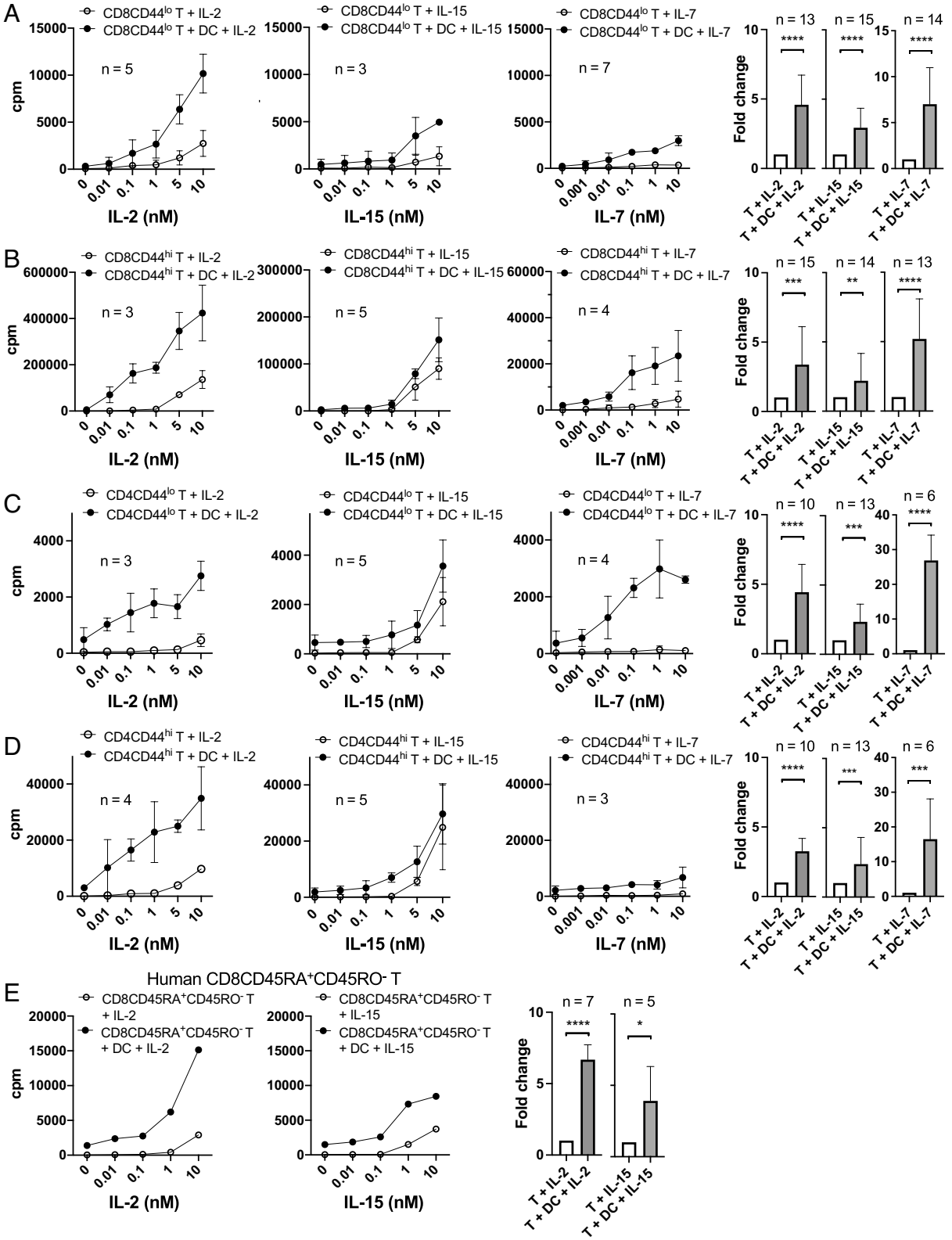

Fig. 2. Accessory cells augment proliferation of naïve T cells in response to cytokines. (*A–D*) ^{3}H-thymidine incorporation of purified mouse CD8CD44^{lo} T cells (*A*), CD8CD44^{hi} T cells (*B*), CD4CD44^{lo} T cells (*C*), and CD4CD44^{hi} T cells (*D*), cultured with IL-2, IL-15, and IL-7, was enhanced in the presence of irradiated DCs (day 3). Fold increase of ^{3}H-thymidine incorporation by the presence of DCs at 10 nM of each cytokine over cytokine alone is indicated at the right end for each cell population. (*E*) Proliferative responses of human CD8CD45RA^{+}CD45RO^{-} T cells that include naïve T cells cultured with IL-2 and IL-15, with or without irradiated human monocyte-derived DCs (representative data of four independent experiments). Fold increase of incorporated ^{3}H-thymidine activity significantly increased by the presence of DCs at 10 nM of each cytokine (right end). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by two-tailed ratio paired t tests, n: number of replicates.

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proliferation was also observed with IL-15 and IL-7 (Fig. 2*B*). Similarly, purified CD4CD44^{lo} T cells required DCs to proliferate to IL-2, IL-15, and IL-7 (Fig. 2*C*), and the presence of DCs augmented proliferation of CD4CD44^{hi} T cells to the cytokines (Fig. 2*D*). Moreover, human CD8CD45RA^{+}CD45RO^{-} T cells that include naïve T cells also depended on the presence of DCs for their proliferative responses to the cytokines (Fig. 2*E*). Accessory cells seemed to condition T cells, enabling T cells to respond to the cytokines, and induce proliferation. In fact, monocytes, similar to DCs, augmented the naïve T cell proliferation to the cytokines (*SI Appendix*, Fig. S1*D*). We call this effect "preconditioning". Interestingly, although these T cells proliferated less in response to IL-7 alone compared to IL-2 or IL-15, the fold-change increase of proliferation by preconditioning seemed greater with IL-7, when examined by ^{3}H-thymidine incorporation at 10 nM of each cytokine (Fig. 2 *A*–*D*). To determine how other cytokines might be influenced by preconditioning, granulocyte macrophage-colony stimulation factor (GM-CSF), IL-12, IL-9, IL-21, and IL-5 were examined. In all cases, these factors did not induce T cell proliferation even in the presence of DCs (*SI Appendix*, Fig. S1*E*). These results suggest that the effect of accessory cells to induce naïve T cell proliferation to cytokines may be limited to certain γ_c-cytokines, especially IL-2, IL-15, and IL-7.

We further tested whether IL-6 or its family cytokines were involved in preconditioning by examining T cell proliferation to IL-2, with or without DCs, in the following conditions: addition of a combination of IL-6 and IL-6Rα (*SI Appendix*, Fig. S1*F*), using IL-6 deficient T cells and DCs (*SI Appendix*, Fig. S1*G*), addition of leukemia inhibitory factor (LIF) or neutralizing anti-LIF antibody (*SI Appendix*, Fig. S1 *H* and *I*), and neutralization of gp130 by an antibody (*SI Appendix*, Fig. S1*J*). None of these conditions altered the proliferative responses of CD8CD44^{lo} or CD4 T cells to IL-2 nor the "preconditioning effect" by DCs, indicating that IL-6, LIF, and gp130 were not involved in preconditioning.

Expression of Receptors for Cytokines Is Necessary But Not Sufficient for Resting T Cells to Proliferate to the Cytokines. Resting T cells required DCs for their proliferative responses to IL-2, IL-15, and IL-7, despite their expression of the receptors, IL-2Rβ (CD122) and γ_c (CD132) for IL-2 and IL-15, or CD127 and γ_c for IL-7 (Fig. 2 and *SI Appendix*, Fig. S1*A*). To further examine whether the lack of cytokine proliferation of purified naïve T cells was unrelated to the expression of the nominal receptors for the cytokine, we examined IL-2 responses of purified CD4CD25^{+} Tregs, which express all the receptor components for IL-2, namely IL-2Rα (CD25), IL-2Rβ and γ_c. Furthermore, CD8CD44^{lo} T cells purified from human IL-15Rα transgenic mice were examined for their responses to IL-15. These cells expressed all the three IL-15 receptor components, IL-15Rα, IL-2Rβ and γ_c, mirroring the Treg expression of IL-2Rα, IL-2Rβ and γ_c for IL-2 (*SI Appendix*, Fig. S2*A*). As a control, we confirmed that CD4CD25^{-} non-Tregs that do not express IL-2Rα do not proliferate in responses to IL-2, and also IL-15, without the presence of DCs (*SI Appendix*, Fig. S2*B*). CD4CD25^{+} Tregs and IL-15Rα^{+}CD8CD44^{lo} cells alone also failed to proliferate to either IL-2 or IL-15 (*SI Appendix*, Fig. S2 *C*–*E*), despite their expression of the high-affinity receptor complexes for the cytokines, and addition of DCs augmented their proliferation. With DCs, robust proliferation was observed for Tregs stimulated with IL-2 and IL-15Rα^{+}CD8CD44^{lo} cells stimulated with IL-15, both responding to low doses of cytokines consistent with their expression of high-affinity tri-receptors (*SI Appendix*, Fig. S2 *C* and *E*). These results support the view that although receptor expression is necessary for the cytokines to bind to T cells, it is not sufficient for the proliferative responses. Importantly, IL-15Rα on DCs that could transpresent endogenous or exogenous IL-15 to T cells, and DC-derived IL-15, was dispensable for preconditioning T cells, as DCs deficient for IL-15Rα or IL-15 enhanced T cell proliferation to IL-2 or IL-15 as well as wild-type (WT) DCs (*SI Appendix*, Fig. S2*F*). DC-augmented proliferation of bulk CD4 T cells tended to be higher than that of CD44^{lo} and CD44^{hi} T cell proliferation (Fig. 2 *C* and *D*). This might reflect the shorter purification time required for CD4 T cells compared to that of the subpopulations, therefore better maintaining their cellular function.

Of note, CD25, CD122, or CD132 was not induced in the CD8CD44^{lo} T cells stimulated with IL-2 and/or DCs, in contrast with CD25 upregulation observed in CD8CD44^{hi} T cells (*SI Appendix*, Fig. S3) or in the TCR-stimulated T cells that is commonly observed. Interestingly, we observed CD69 upregulation in preconditioned T cells, which could be a marker for preconditioned cells (*SI Appendix*, Fig. S3), although it is not specific for these cells.

Preconditioning Is Distinct from TCR Activation. As DCs enabled naïve T cells and Tregs to respond to IL-2, IL-15, and IL-7, we asked whether DC–T cell interaction through the TCR is involved. To address this, we used transporter associated with antigen processing 1 (Tap1)-deficient (Tap1KO) DCs to stimulate CD8CD44^{lo} T cells and MHC class-II-deficient (MHC-II KO) DCs for CD4 T cells. Tap1KO DCs that were unable to present peptide antigens to CD8 T cells were capable of preconditioning CD8CD44^{lo} T cells to proliferate in response to IL-2 and IL-15 as well as WT DCs, as indicated by ^{3}H-thymidine incorporation (Fig. 3*A*) and CMFDA dilution (Fig. 3*B*) assays. Similarly, MHC-II KO DCs were as effective as WT DC to precondition CD4CD25^{+} Tregs and CD4CD25^{-} non-Treg CD4 T cells (Fig. 3*C*). These findings suggest that neither foreign- nor self-antigen recognition was required for preconditioning.

To confirm this conclusion that TCR signaling is dispensable in T cell preconditioning by DCs, we used cyclosporin A (CsA), a well-known inhibitor of TCR signaling. T cells were pretreated with CsA (5 μM) before culture with IL-2 with and without DCs, or T cells were cultured in the presence of CsA (150 nM). These doses of CsA were carefully selected as those that inhibited T cell proliferation induced by the combination of anti-CD3 and anti-CD28 antibodies but those that did not inhibit proliferation of antigen experienced T cells to IL-2 (Fig. 3 *D*–*F*). We found that the preconditioning effect was not blocked by the CsA (Fig. 3 *D* and *E*), further supporting the view that preconditioning does not involve TCR stimulation.

These findings prompted us to examine whether NK cells that lack TCRs also proliferated better to IL-2, IL-15, and IL-7 in the presence of DCs. Indeed, purified NK cells proliferated robustly to IL-2 and IL-7 in the culture with DCs (Fig. 3*G*), suggesting that the preconditioning effect extends to NK cells and that preconditioning does not require TCR–MHC molecule interactions.

We previously observed that the PBMCs of chronic/smoldering ATL patients proliferated spontaneously in the presence of monocytes (17). Thus, we next asked whether proliferation of ATL cells depended on the TCR signaling using CsA. CsA treatment strongly inhibited the spontaneous proliferation of ATL cells (*SI Appendix*, Fig. S4*A*), indicating the requirement of TCR signaling. We further examined the effect of rapamycin, an inhibitor of mammalian target of rapamycin (mTOR). As expected from the dependency of ATL cells on IL-2, IL-9, and IL-15, the

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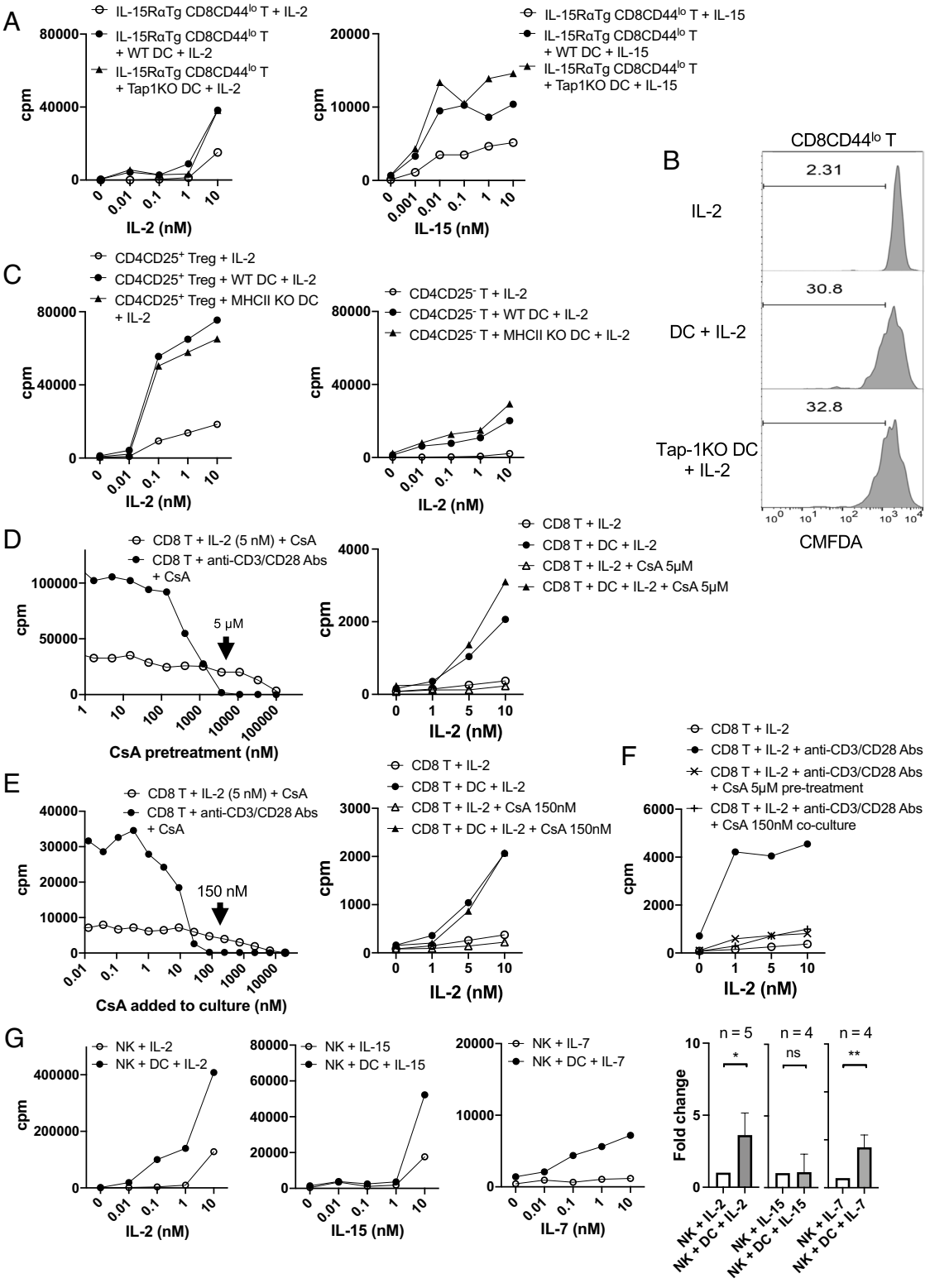

Fig. 3. TCR activation is not required for preconditioning. (*A*) Tap1KO DCs were as effective as WT DCs in preconditioning IL-15Rα transgenic CD8CD44^{lo} T cells when examined by ^{3}H-thymidine uptake assays on day 3 (representative data of five and four independent experiments for IL-2 and IL-5, respectively). (*B*) Tap1KO DCs induced proliferation of CMFDA-labeled CD8CD44^{lo} naïve T cells as well as WT DCs as indicated by the dilution of CMFDA analyzed by flow cytometry on day 5 (representative data of two independent experiments). (*C*) MHC-II KO DCs were as effective as WT DCs in preconditioning CD4CD25^{+} Tregs (*Left*) and CD4CD25^{-} T cells (*Right*) as indicated by the ^{3}H-thymidine incorporation on day 3 (representative data of two independent experiments). (*D* and *E*). Cyclosporin A (CsA) pretreatment (*D*) and coculture (*E*) doses that inhibited TCR activation of CD8 T cells, but not IL-2 responses of antigen-experienced CD8 T cells, were first selected to be 5 μM and 150 nM, respectively (*Left*). A pretreatment of CD8 T cells with 5 nM CsA 30 min, followed by washing, (*D*), or presence of 150 nM CsA in culture (*E*), did not affect the preconditioning effect on the T cells for IL-2 responses (*Right*). TCR was stimulated by plate-coated anti-CD3 antibody (Ab, 3 μg/mL) and 1.5 μg/mL anti-CD28 Ab (^{3}H-thymidine uptake assays on day 3, representative data of two independent experiments). (*F*) Both a pretreatment of CD8 T cells with 5 μM CsA and presence of 150 nM CsA in the culture inhibited the proliferation of the CD8 T cells to a combination of IL-2 and TCR stimulation (representative data of two independent experiments). (*G*) Purified NK cells proliferated to IL-2 and IL-15, but not to IL-7 (representative data of five IL-2, four IL-15, and four IL-7 experiments). Fold increase of ^{3}H-thymidine incorporation by the presence of DCs over cytokine alone, at 10 nM of each cytokine, is summarized at the right end. Presence of DCs significantly increased proliferation of NK cells to IL-2 and IL-7 (*P < 0.05, **P < 0.01, ns: P > 0.05 by two-tailed ratio paired t-tests).

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proliferation of the cells was strongly inhibited (*SI Appendix*, Fig. S4*A*). Although ATL cells are reported to have a phenotype similar to that of Tregs, such as expression of Foxp3 and C-C chemokine receptor type 4 (CCR4) (21–23), the preconditioning effect on purified human CD4CD25^{+} Tregs was not inhibited by CsA (*SI Appendix*, Fig. S4*B*).

B7, CD28, and CD2 Costimulatory Molecules Are Not Involved in Preconditioning. We further examined whether B7 molecules, major costimulatory molecules involved in TCR stimulation of T cells, are required for preconditioning. DCs generated from the bone marrow of B7 double-knockout (CD80$^{-/-}$CD86$^{-/-}$) mice enabled naïve T cell responses to the cytokines as well as WT DCs, indicating that B7 molecules are dispensable in preconditioning (*SI Appendix*, Fig. S5 *A* and *B*). To support this finding, CD28 stimulation alone using an agonistic antibody was not sufficient to induce proliferation of naïve T cells to IL-2 either in soluble form or in plate-coated form (*SI Appendix*, Fig. S5*C*). Moreover, the preconditioning effect was observed with CD8CD44^{lo} naïve T cells purified from CD28-deficient mouse spleens as well as with WT naïve T cells (*SI Appendix*, Fig. S5*D*).

Similarly, OX40, CD47, inducible T cell costimulator (ICOS), 4-1BB, "homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for herpesvirus entry mediator, a receptor expressed on T cells" (LIGHT), and lymphocyte function-associated antigen 1 (LFA-1) did not seem to be involved in preconditioning, based on the examination of proliferation of CD8CD44^{lo} T cells or CD4 T cells to IL-2 in the presence of DCs with an inhibitory or activating antibody against each costimulatory molecule or by using T cells and DCs deficient for the costimulatory molecule (*SI Appendix*, Fig. S6 *A–D*).

It has been reported that human naïve T cells proliferate when stimulated by a combination of anti-CD2 and anti-CD2R antibodies (24–26). Therefore, we examined whether CD2 stimulation could be the mechanism of preconditioning. As reported, the anti-CD2/CD2R antibody combination induced proliferation of human naïve CD8 T cells to increasing doses of IL-2 (*SI Appendix*, Fig. S6*E*). However, when T cells were cultured in the presence of CsA, the cells lost their proliferation capabilities to IL-2, similar to the CsA effect in TCR-stimulated cells (*SI Appendix*, Fig. S6 *E*, *Bottom*), in contrast to the effect of DCs to naïve T cells in preconditioning that is insensitive to CsA (*SI Appendix*, Fig. S6 *E*, *Top*). The difference in the sensitivity to CsA between preconditioning and CD2 stimulation was observed with human CD4 T cells as well (*SI Appendix*, Fig. S6*F*), further indicating CD2 stimulation is not a mechanism of preconditioning.

P53 Does Not Cause Nonresponsiveness of Naïve T Cells to Cytokines. It has been demonstrated that antigen-specific proliferative responses of CD4 T cells require the downmodulation of the tumor suppressor p53 molecule (10). In the absence of TCR signaling, IL-2 induced a sustained increase in p53, which prevented proliferation. With TCR signaling, there was an early termination of p53 protein expression, leading to antigen-specific proliferation of T cells (10). Therefore, we examined an inhibition of p53 transactivation by pifithrin-α (PFT-α). By contrast, with the responses of antigen-specific proliferation of T cells reported (10), inhibition of p53 did not augment proliferation of CD4 T cells to IL-2 alone (*SI Appendix*, Fig. S7*A*). As p53 protein expression is also regulated posttranscriptionally by MDM2-mediated ubiquitination and proteasomal degradation (27), we also examined activation of p53 by pretreating CD4 T cells with MDM2 inhibitor Nutlin-3, or with inhibitor of the p53–MDM2 interaction RITA or RO5963. These treatments did not affect the preconditioning effect (*SI Appendix*, Fig. S7 *B–D*), suggesting that nonresponsiveness of naïve T cells to cytokines is not mediated by p53.

Preconditioning Requires Cell-to-Cell Contact. The preconditioning effect we observed in DC-naïve T cell cocultures could, theoretically, be mediated by some soluble factor(s) or through physiological contact between DCs and T cells. To examine these possibilities, we used a transwell system to separate DCs and T cells in culture. When CD8CD44^{lo} T cells or CD4 T cells were separated from DCs, the preconditioning effect was abrogated, suggesting that the preconditioning effect was not mediated by soluble factors, but rather by cell-to-cell contact between DCs and T cells (Fig. 4*A*). Addition of supernatants from DC cultures or supernatants from DC/T cell cocultures did not enable naïve T cell proliferation to IL-2 (Fig. 4*B*). Hence, bidirectional crosstalk was not producing a soluble factor that led to T cell proliferation. We further found that, once fixed, DCs lost preconditioning effect (*SI Appendix*, Fig. S8).

Preconditioning Has a Lingering Effect and Enhances T Cell Proliferation In Vivo. We then examined whether the contact between DCs and naïve T cells provided naïve T cells some signals that allowed these T cells to proliferate to the cytokines later. To this end, naïve T cells were first cocultured with adherent DCs overnight and then the two cell types were separated by magnetic beads against DCs. CD8CD44^{lo} T cells or CD4 T cells preincubated with DCs were capable of proliferation to subsequent stimulation with IL-2 or IL-15 (Fig. 4 *C* and *D*). The results indicate that preconditioning had a lingering effect on the T cells.

IL-7 is a factor that drives homeostatic proliferation of T cells in vivo (2, 3, 8). We hypothesized that preconditioning enhanced cytokine-derived naïve T cell homeostatic proliferation in vivo. To test this, we first created an acute lymphopenic condition by irradiating mice expressing diphtheria toxin (DT) receptor transgene under a CD11c promotor (CD11c-DTR Tg) at 5 Gy. CMFDA-labeled CD8CD44^{lo} or CD8CD44^{hi} T cells transferred to these mice underwent homeostatic proliferation. Depletion of endogenous DCs by DT injection abrogated proliferation of the CD8CD44^{lo} T cells, but not that of CD8CD44^{hi} T cells (Fig. 4*E*, day 5), indicating a greater need for DCs in homeostatic proliferation with naïve T cells than memory phenotype T cells. To examine the role of preconditioning in homeostatic proliferation of naïve T cells, CD8CD44^{lo} T cells were preconditioned ex vivo overnight with Tap1KO DCs before they were labeled with CMFDA and transferred to CD11c-DTR Tg mice on a recombination activating gene 1 (Rag1)-deficient background (CD11c-DTR Tg-Rag1KO mice). Preconditioned CD8CD44^{lo} T cells underwent greater proliferation than nontreated cells and, furthermore, underwent homeostatic proliferation in DC-depleted hosts (Fig. 4*F*, day 7), demonstrating that preconditioning and its lingering effect enhanced in vivo proliferation of naïve T cells on a lymphopenic, cytokine-rich environment, independent of self-antigen recognition. These data suggest a potentially critical, previously undescribed role for DC-T cell interaction independent of antigens in maintenance of naïve T cell homeostasis.

Preconditioning Induces Gradual Ca^{2+} Mobilization Distinct from That Induced by TCR Stimulation. We next asked whether Ca^{2+} flux was induced in naïve T cells upon contact with accessory cells, a phenomenon known with T cells undergoing TCR stimulation (28). Interestingly, contact with DCs in the absence of antigen or IL-2 induced an Indo-1 ratio increase in CD8CD44^{lo} naïve T cells, indicating an induction of Ca^{2+} flux in the T cells. However, Indo-1 ratios declined quickly as T cells

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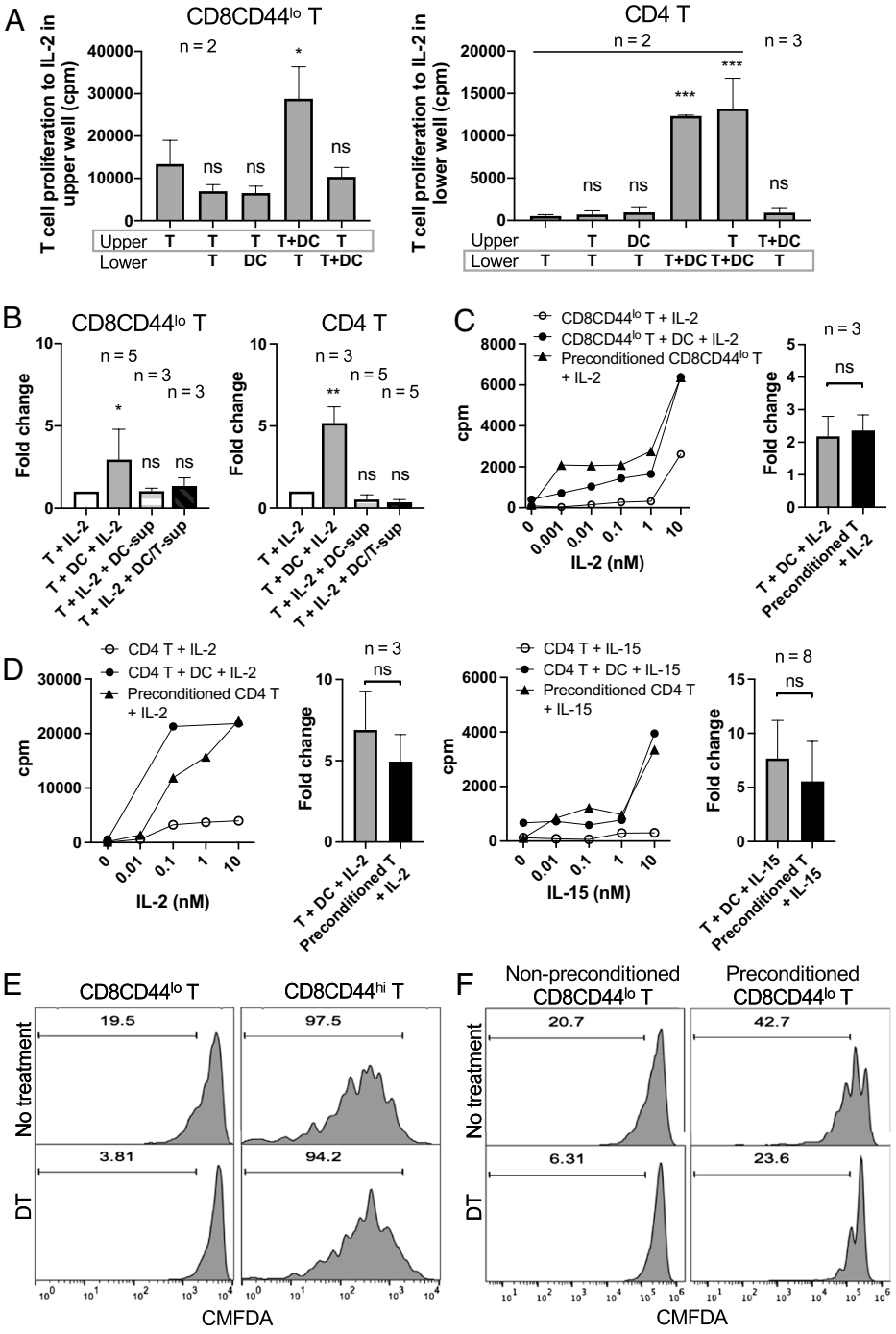

Fig. 4. Preconditioning requires cell-to-cell contact between DCs and resting T cells, has a lingering effect, and enhances T cell proliferation in vivo. (*A*) CD8CD44^{lo} T cells (*Left*) or CD4 T cells (*Right*) were cultured with 8 nM IL-2 in a transwell system separated from or in contact with irradiated DCs as indicated. Proliferation of CD8CD44^{lo} T cells in upper wells and that of CD4 T cells in lower wells was examined by ^{3}H-thymidine incorporation (day 3). T cells proliferated only when DCs were present in the same side of the well (representative data of three independent experiments, each performed with n = 2 or 3. *P < 0.05, ***P < 0.001, ns: P > 0.05 against CD8CD44^{lo} T cells alone in the upper wells or CD4 T cells alone in the lower wells analyzed by one-way ANOVA). (*B*) Fold increase of ^{3}H-thymidine uptake to CD8CD44^{lo} (*Left*) or CD4 (*Right*) T cells either by the presence of irradiated DCs or by an addition of supernatant of DC culture (DC-sup) or DC and T cell coculture (DC/T-sup) over IL-2 alone, at 10 nM IL-2, is shown (day 3). The supernatants failed to enhance T cell proliferation (n: number of replicates, *P < 0.05, ****P < 0.0001, ns: P > 0.05 by two-tailed ratio paired t tests). (*C* and *D*) Proliferation of CD8CD44^{lo} T cells (*C*) and CD4 T cells (*D*) contacted with Tap1KO (*C*) and WT (*D*) DCs overnight followed by purification and stimulation with IL-2 or IL-15 for 3 d was compared with that of CD8CD44^{lo} (*C*) and CD4 (*D*) T cells stimulated with the cytokine for 3 d, with or without DCs, respectively (line graphs, representative data of three experiments). Fold increase of T cell ^{3}H-thymidine uptake by the overnight pretreatment with DCs and that by the coculture with DCs compared to IL-2 or IL-15 alone, at 10 nM cytokine, was comparable (bar graphs, n: number of replicates, ns: P > 0.05 by unpaired two-tailed t test). (*E*) CMFDA-labeled Ly5.1^{+}CD8CD44^{lo} T cells or Ly5.1^{+}CD8CD44^{hi} T cells were transferred to Ly5.2^{+}CD11c-DTR Tg mice irradiated at 5 Gy on day (−1). The mice received no treatment or diphtheria toxin (DT) i.p. injections on days (−1), 1, and 3 (6 ng/g body weight). Five days later, spleens of host mice were harvested, and CMFDA dilution of Ly5.1^{+}CD8CD44^{lo} or Ly5.1^{+}CD8CD44^{hi} cells was analyzed by flow cytometry. Proliferation of transferred CD8CD44^{lo} T cells was suppressed by the depletion of DCs but that of CD8CD44^{hi} T cells was unaffected (representative data of two experiments). (*F*) Ly5.1^{+}CD8CD44^{lo} T cells without or with overnight preconditioning with Tap1KO DCs were labeled with CMFDA and transferred to Ly5.2^{+}CD11c-DTR-Rag1KO mice. The mice received no treatment or DT i.p. injections on days (−1), 1, 3, and 5. Seven days later, spleens of the host mice were harvested, and CMFDA dilution of Ly5.1^{+}CD8CD44^{lo} cells was analyzed by flow cytometry. Preconditioning of CD8CD44^{lo} T cells by DCs before transfer enhanced in vivo proliferation of the T cells. Moreover, the preconditioned T cells were capable to proliferate in a DC-depleted environment (representative data of two experiments).

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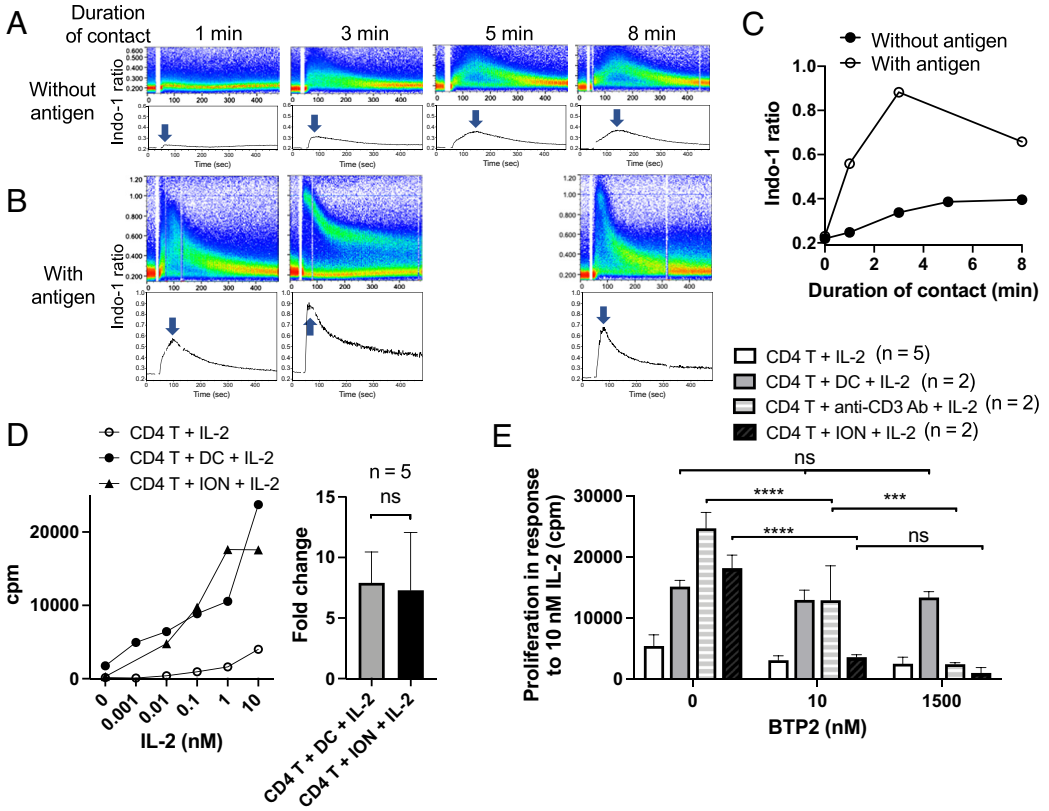

Fig. 5. Preconditioning induced gradual Ca^{2+} mobilization distinct from that induced by TCR stimulation. (*A*) Indo-1-loaded OT-1 Tg CD8CD44^{lo} T cells (1.8 × 10^6 cells) were spun down with DCs without antigen (3.6 × 10^6 cells) and left in contact for the indicated times before applying to a flow cytometer for Ca^{2+} mobilization analyses. T cells showed increased concentrations of intracellular Ca^{2+}, indicated as increased Indo-1 ratios, depending on the time of contact with DCs. Graphs of median of the top 60% Indo-1 ratios are shown below the corresponding Indo-1 profiles of all T cells. (*B*) As control, DCs preloaded with ovalbumin peptide were used to stimulate CD8CD44^{lo} T cells through TCR and Indo-1 ratios are indicated as above. (*C*) The peak of the graph of median of the top 60% Indo-1 ratios, indicated by the arrows in *A* and *B*, was plotted against the duration of DC-T cell contact. Preconditioning (without antigen) showed gradual and low Ca^{2+} flux. (*D*) Proliferation of CD4 T cells was examined in the presence of DCs or a low-dose ionomycin (ION, 0.8 μM) by ^{3}H-thymidine uptake assays. Ionomycin exhibited a similar effect as preconditioning (representative data of four experiments). Fold increase of ^{3}H-thymidine uptake at 10 nM IL-2 in the presence of DCs or ionomycin over IL-2 alone showed no significant difference (day 3, n = 5, unpaired two-tailed *t* test). (*E*) Effect of BTP2, a CRAC inhibitor, on proliferation of CD4 T cells stimulated with IL-2 alone, IL-2 with DCs, plate-coated anti-CD3 antibody (Ab, 1 μg/mL), or ionomycin (0.8 μM) was examined. Fold increase of ^{3}H-thymidine uptake at 10 nM IL-2 with 0, 10, or 1,500 nM BTP2 on day 3 indicated that preconditioning effect was not affected by BTP-2 (IL-2 alone: n = 5, others: n = 3, ***$P < 0.001$, ****$P < 0.0001$ and ns: $P > 0.05$ by two-way ANOVA).

and DCs were no longer in contact during the flow cytometry data acquisition (Fig. 5*A*). By contrast, antigen-loaded DCs induced higher Ca^{2+} flux that lasted longer than that induced by DCs without antigen (Fig. 5*B*). The peak of median of the top 60% Indo-1 ratios for each of the samples incubated for different times was plotted against the duration of DC-T cell contact to obtain Ca^{2+} mobilization data induced by the contact with DCs (Fig. 5*C*). These studies showed a gradual Ca^{2+} mobilization in CD8CD44^{lo} T cells, which was distinct from the rapid and high Ca^{2+} flux induced by TCR stimulation.

As Ca^{2+} influx is critical for full T cell responses to TCR signaling, and that a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin can mimic TCR signaling and induce T cell proliferation, we examined whether Ca^{2+} influx induced by an ionophore could mimic the preconditioning effect and enable naïve T cells to respond to cytokines. CD4 T cells cultured in the presence of a low dose of ionomycin (0.8 μM) were responsive to IL-2 and proliferated in similar fashion to preconditioned cells (Fig. 5*D*).

To examine whether the Ca^{2+} mobilization observed in preconditioned T cells was mediated by the calcium release-activated Ca^{2+} channels (CRAC), BTP2 was used to block the CRAC channels. Unlike TCR or ionomycine stimulation-induced T cell proliferation to IL-2, the CRAC channel inhibitor did not block preconditioning of CD4 T cells (Fig. 5*E*), further supporting the notion that the Ca^{2+} mobilization in preconditioned T cells is distinct from that induced by TCR simulation or ionomycin, although they exhibit similar consequences (i.e., cellular proliferation) on resting T cells.

STAT5 Is Phosphorylated and Translocates to the Nucleus in IL-2 Stimulated Naïve T Cells, but Transcriptional Activation Requires Preconditioning. The data obtained so far indicated that signaling from IL-2, IL-15, or IL-7 alone did not lead to cellular proliferation of resting T cells. To address whether the cytokines induce the signaling events downstream of the cytokine receptors, phosphorylation of STAT5 was first examined. We found that STAT5 phosphorylation occurred in nonproliferating CD8CD44^{lo} naïve T cells upon cytokine stimulation, similar to that observed in CD8CD44^{hi} T cells that can readily proliferate to the cytokines by western blotting and flow cytometry (Fig. 6 *A* and *B*). We then examined whether the phosphorylated STAT5 was translocated to the nucleus. Western blotting of subfractionated cellular samples indicated the presence of phosphorylated STAT5 in the nucleus of CD8CD44^{lo} T cells stimulated with IL-2, as well as in CD8CD44^{hi} T cells (Fig. 6*C*). This led us to examine further downstream events, including transcription of *cish* (cytokine-inducible SH2-containing protein gene), a STAT5 target gene, in CD8CD44^{lo} naïve T cells with and without preconditioning. Despite the phosphorylation and nuclear translocation of STAT5, naïve T cells stimulated with IL-2 failed to induce

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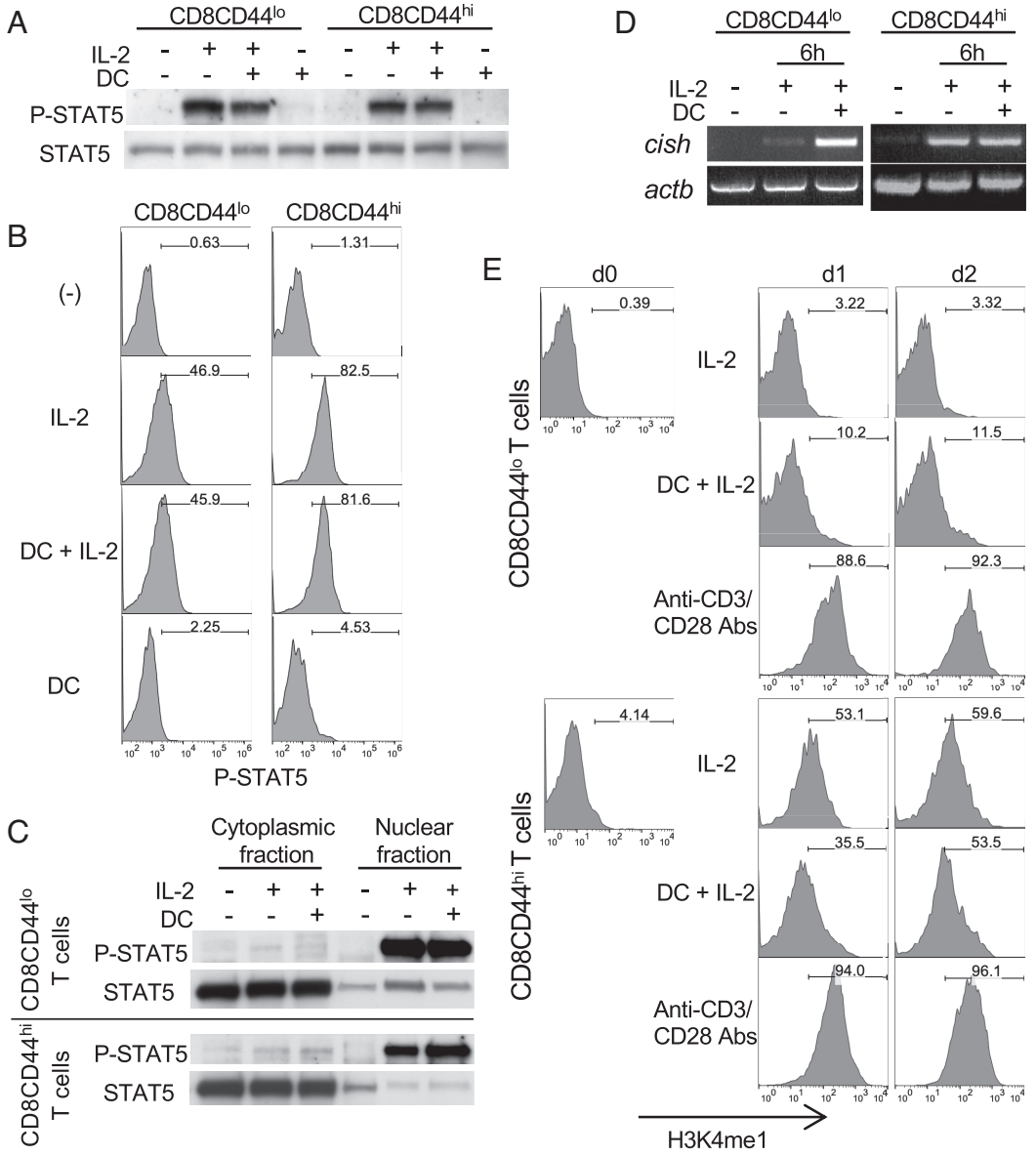

Fig. 6. STAT5 is phosphorylated and translocated to the nucleus in IL-2-stimulated naïve T cells, but transcriptional activation required preconditioning. (*A*) Western blotting of CD8D44^{lo} and CD44^{hi} T cells stimulated with 8 nM IL-2 and/or Tap1KO DCs for 20 min indicated that STAT5 was phosphorylated in CD8CD44^{lo} T cells as well as in CD44^{hi} cells in the presence of IL-2 (representative data of two experiments). (*B*) Flow cytometry analysis of CD8CD44^{lo} and CD44^{hi} T cells stimulated with 8 nM IL-2 and/or Tap1KO DCs for 30 min demonstrated phosphorylation of STAT5 in the presence of IL-2 in both cell types (representative data of two experiments). (*C*) Western blotting of CD8D44^{lo} and CD44^{hi} T cells stimulated with 8 nM IL-2 with or without Tap1KO DCs for 6 h and subfractionated into cytoplasmic and nuclear fractions indicated that the majority of phosphorylated STAT5 induced by IL-2 translocated to the nucleus in CD8CD44^{lo} T cells as well as in CD44^{hi} cells (representative data of two experiments). (*D*) Induction of *cish*, an early IL-2 target gene, was examined by PCR using *actb* as control in CD8D44^{lo} and CD44^{hi} T cells stimulated with IL-2 (8 nM) and/or DCs for 6 h. IL-2 alone failed to induce *cish* in CD8CD44^{lo} cells, but the combination of IL-2 and DCs enabled this. In CD8CD44^{hi} T cells, IL-2 alone was sufficient to induce *cish* (representative data of two experiments). (*E*) Levels of H3K4me1 in CD8CD44^{lo} and CD44^{hi} T cells, stimulated by 8 nM IL-2 alone, IL-2 and Tap1KO DCs, or anti-CD3 (3 μg/mL)/anti-CD28 (1.5 μg/mL) antibodies, were examined by flow cytometry over the course of 2 d. In CD8CD44^{lo} T cells, IL-2 alone did not induce H3K4me1 and DCs were required to induce this chromatin modification. By contrast, CD8CD44^{hi} T cells did not require DCs to induce H3K4me1 (representative data of two experiments).

cish transcription, contrasting to the CD44^{hi} T cells in which *cish* transcription occurred (Fig. 6*D*). IL-2 signaling and the downstream transcriptional events seemed to be disconnected in resting CD44^{lo} naïve T cells. We examined the status of histone H3 mono-methylation at lysine 4 (H3K4me1), as a whole, to address the general transcriptional activation status in naïve T cells. H3K4me1 did not clearly increase even after stimulation with IL-2 and an addition of DCs was required to induce H3K4me1 in CD8CD44^{lo} T cells (Fig. 6*E*), in line with the findings of *cish* induction. By contrast, CD8CD44^{hi} T cells showed higher levels of H3K4me1 immediately after purification, compared to CD44^{lo} naïve T cells, and IL-2 stimulation alone was sufficient to increase H3K4me1 as well as IL-2 with DCs (Fig. 6*E*). It seems that contact with accessory cells provides an additional signal to the resting naïve T cells, which allows the cytokine signal to be transcribed and enables cellular proliferation in response to the cytokine.

Preconditioning Activates the MAPK and PI3K-mTOR Pathways, Likely Leading to Proliferation upon Cytokine Stimulation. To dissect the signaling events preconditioning provides, CD8CD44^{lo} naïve T cells were stimulated with IL-2 and/or Tap1KO DCs and activation of various signaling pathways were analyzed. As shown in Fig. 7*A*, in contrast to the IL-2 situation alone,

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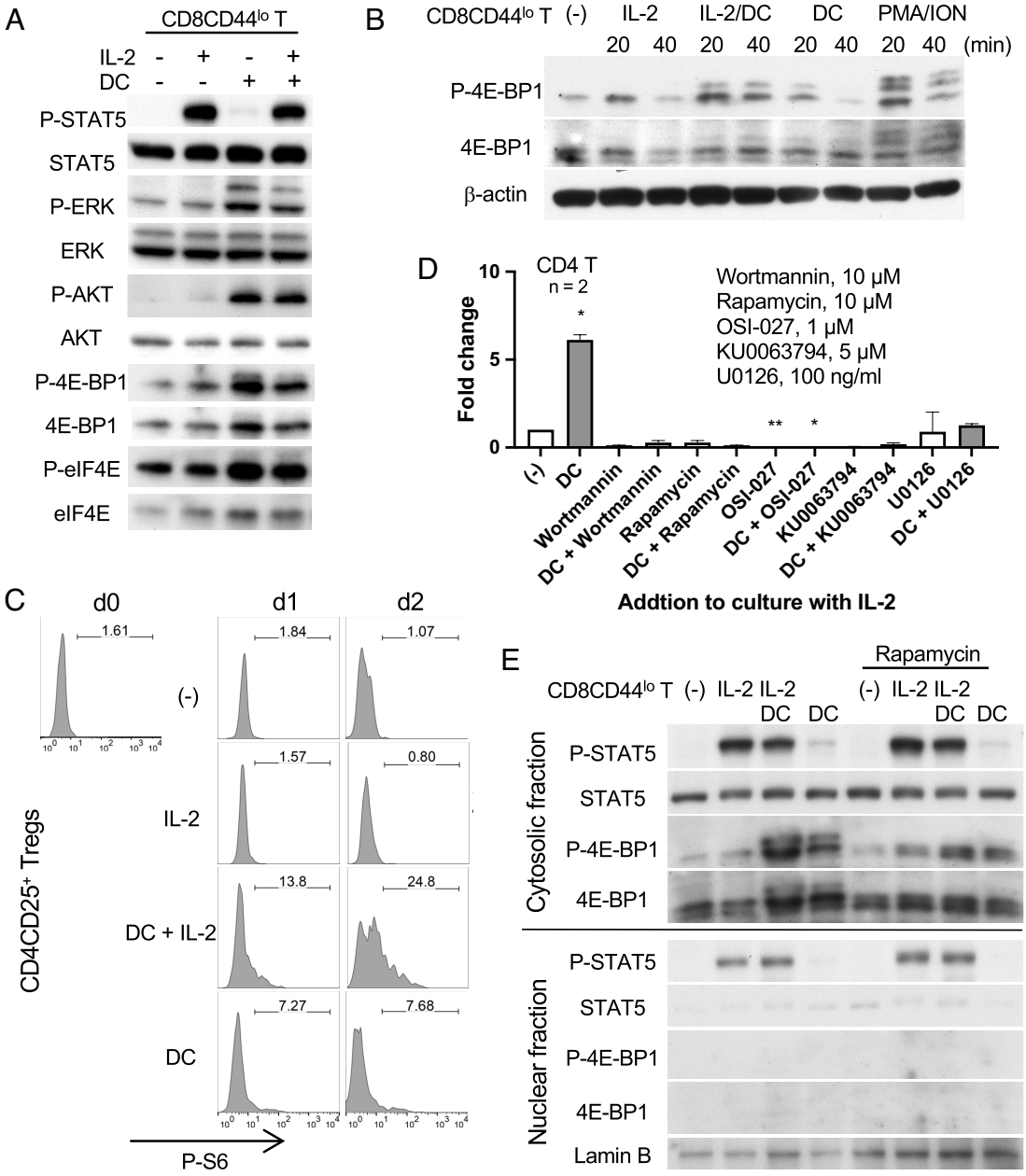

Fig. 7. Preconditioning allows PI3K-mTOR pathway activation likely leading to proliferation upon cytokine stimulation. (*A*) Western blotting of CD8CD44^{lo} T cells stimulated with 8 nM IL-2 and/or Tap1KO DCs for 30 min indicated that STAT5 phosphorylation occurred in the presence of IL-2, but the MAPK pathway and the PI3K-mTOR pathway known to be activated by the cytokine stimulation in cell lines were not activated. Presence of DCs induced ERK phosphorylation on p44, AKT S^{473} phosphorylation, and hyperphosphorylation of 4E-BP1. (*B*) Hyperphosphorylation of 4E-BP1 in CD8CD44^{lo} T cells was sustained when stimulated with 8 nM IL-2 and DCs but decreased by 40 min when stimulated with DCs alone, suggesting that 4E-BP1 is downstream of cytokine and preconditioning pathway convergence. PMA (2.5 ng/mL) and ionomycine (0.8 μM) were used to mimic a weak TCR stimulation as a control. (*C*) Phosphorylation of S6, another signaling molecule downstream of mTOR, was examined by flow cytometry analysis. S6 phosphorylation was induced in CD4CD25^{+} Tregs in the presence of DCs and the combination of DCs and IL-2 (8 nM) induced stronger phosphorylation of S6 than IL-2 alone over 2 d. (*D*) Fold increase of ^{3}H-thymidine uptake to CD4 T cells cultured with various PI3K or mTOR inhibitors at 10 nM IL-2 over IL-2 alone. The inhibitors abrogated preconditioning (n = 2, *$P < 0.05$, **$P < 0.01$ by two-tailed ratio paired *t* tests). (*E*) Western blotting indicated that rapamycin (10 μM) did not inhibit phosphorylation of STAT5 nor its nuclear translocation, induced by the presence of IL-2 (8 nM), in CD8CD44^{lo} T cells, but blocked hyperphosphorylation of 4EBP1 (30 min stimulation, representative data of two experiments).

preconditioning induced phosphorylation of extracellular signal-regulated kinase (ERK) p44, AKT S^{473}, and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) in naïve T cells. Multiphosphorylation of 4E-BP1, a downstream target of mTOR signaling, occurred in the presence of DCs (Fig. 7*B*), which could lead to translational activation in naïve T cells, leading to cellular proliferation. Another molecule downstream of mTOR is S6. We observed the basal S6 phosphorylation in purified Tregs, which gradually decreased over time. Presence of DCs prolonged S6 phosphorylation and its combination with IL-2 further retained S6 phosphorylation (Fig. 7*C*), which seemed to correlate well with cellular proliferation.

As the mTOR pathway seems to be important in preconditioning, we treated CD4 T cells with a phosphoinositide 3-kinase (PI3K) inhibitor, a mitogen-activated protein kinase kinase (MEK) 1/MEK2 inhibitor, and mTOR inhibitors. These additions abrogated the preconditioning effect (Fig. 7*D*). Importantly, rapamycin did not inhibit phosphorylation of STAT5 nor its nuclear translocation, but blocked phosphorylation of 4E-BP1 in CD8CD44^{lo} T cells stimulated in the presence of DCs (Fig. 7*E*).

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Thus, incubation of naïve T cells with IL-2 alone was associated with STAT5 phosphorylation, whereas that with DCs and cytokines was associated with cellular proliferation and with activation of AKT-mTOR-S6 and MEK-ERK mitogen-activated protein kinase (MAPK) pathways that are not activated when naïve T cells are cultured with cytokines alone.

Discussion

Naïve CD8 T cells, CD4CD25$^-$ T cells, as well as Tregs, when purified, do not proliferate to γ_c-cytokines, such as IL-2, IL-7, or IL-15, despite the fact of their expression of receptors for these cytokines. IL-2 alone was sufficient to induce phosphorylation and nuclear translocation of STAT5 in T cells, however failed to induce transcription of IL-2 target genes and did not activate MAPK and AKT pathways. This observation contradicts the common perception that IL-2 activates MAPK and AKT pathways through the recruitment of the Shc adaptor protein to IL-2Rβ upon binding of IL-2 to its receptor (29–31). Interestingly, we found that DCs enabled naïve T cells to activate MAPK and AKT pathways and to proliferate in response to the cytokines. Such preconditioning by DCs is distinct from TCR-mediated T cell activation, although it depends on cell-to-cell contact with DCs, in that it is not inhibited by CsA, an inhibitor of calcineurin that is well known to inhibit TCR-mediated T cell activation (32). In line with this, preconditioning induced a weak and gradual Ca^{2+} mobilization in naïve T cells that is distinct from a rapid Ca^{2+} flux induced by TCR stimulation, similar to that previously reported for CD4 T cells forming an antigen-independent synapse with DCs (33). Moreover, preconditioning seemed to act on NK cells that lack TCR. In line with this observation, MHC-I and -II molecules and B7 costimulatory molecule were not involved in preconditioning, along with other costimulatory molecules such as OX40, 4-1BB, ICOS, and CD2.

The activation pathway of T cells requiring cell-to-cell contact between accessory cells and naïve T cells observed in the preconditioning appears to be distinct from major pathways that have been previously described to activate naïve T cells (9–11). For example, an IL-2 transpresentation by mature DCs expressing CD25 has been reported to occur in an antigen-dependent manner, supporting antigen-specific T cell activation (34). However, our data indicate that preconditioning is an antigen-independent process, and it can enhance T cell responses to IL-7 and IL-15, in addition to IL-2, via nonactivated (nonirradiated) accessory cells. Therefore, it seems unlikely that IL-2 transpresentation is fundamental to the preconditioning mechanism. Tumor suppressor p53 has been reported to prevent naïve CD4 T cell proliferation to IL-2 alone, and a downmodulation of p53 induced upon TCR stimulation enables antigen-specific proliferation of the T cells (10). However, the preconditioning we described did not involve p53 as evidenced by our results showing that neither inhibiting nor activating p53 had effects on T cell proliferation to IL-2 by the preconditioning effect. In a separate system, leukemic cells and PBMCs of smoldering/chronic ATL patients proliferate spontaneously ex vivo (11, 12), which required Tax-transactivated IL-2, IL-9, and IL-15 and the presence of monocytes (17), similar to the preconditioning phenomenon. However, with HTLV-1, the proliferation involved interactions with the MHC-II molecules and, as shown in the present study, was inhibited by CsA, in contrast to the preconditioning pathway.

We have observed a gradual proliferation of transferred naïve CD8 T cells in hosts with DCs, which was abrogated in hosts depleted of DCs. In line with this, homeostatic proliferation of CD4 T cells in the absence of an MHC-II-driven signal has been studied previously by examining the proliferation of CD4 T cells transferred to irradiated H-2m^0 mice or Ab^0 mice (35). In that experimental system, T cells underwent homeostatic proliferation in MHC-II-deficient hosts. These T cells showed no increase in CD25 and CD69, sustained high expression of CD62L and CD45RB, and exhibited a gradual increase of CD44 that shifted toward an intermediate level in 2 d, all of which are quite different from the observation with CD4 T cells undergoing antigen-induced proliferation. As the authors only analyzed T cell proliferation in vivo, whether this naïve T cell proliferation in MHC-II-deficient mice is mediated by cell-to-cell interactions between CD4 T cells and accessory cells remains unanswered. Of note, in our in vitro naive T cell cultures with DCs, CD25 expression levels did not change but CD69 was up-regulated in the T cells. This difference in CD69 expression levels may stem from the difference between in vivo and in vitro systems, but remains to be elucidated.

The effect of preconditioning, observed by fold increase of T cell proliferation, seemed to be greater with IL-7 than IL-15. This might favor naïve T cells for their homeostatic proliferation over resting memory T cells in the steady state. Naïve T cells are known to patrol the lymphoid organs/tissues, such as spleen, lymph nodes, and mucosa-associated lymphoid tissues, repeatedly surveying accessory cells for presented nominal antigens (36). Through these random contacts, naïve T cells could be preconditioned for cytokine-mediated proliferation. Our results indicated that even a small number of DCs relative to naïve T cells was sufficient to precondition the T cells. In addition, monocytes also showed preconditioning effects on naïve T cells, increasing the chances for the T cells to be preconditioned in the body. With its lingering effect, the preconditioning provides naïve T cells some window to receive cytokines and allow proliferation, as we demonstrated both in vitro and in vivo. Added to this, a T cell recognizing IL-15 transpresented by an accessory cell (37) could always receive such a preconditioning through the cell-to-cell interaction. In other words, recognition of transpresented IL-15 could intrinsically encompass activation of two signaling pathways, IL-15 cytokine signaling and preconditioning signaling pathways, that could merge downstream and effectively induce naïve T cell proliferation. With aging, naïve T cell pools and the diversity of the TCR repertoire are known to shrink (38, 39) as thymic function declines. Low-level proliferative responses of naïve T cells to γ_c-cytokines independent of antigens enabled by preconditioning, which is not occurring otherwise, could help maintain a naïve T cell pool in the steady state without disturbing the homeostasis. This ultimately could help maintain the TCR repertoire of T cells as a whole. In line with this, preconditioning enabled proliferation of T cells to low concentrations of IL-7, for instance 0.1 nM. As for naïve Tregs, which reside in lymphoid organs in the proximity of CD4CD25$^-$ T cells producing IL-2 (40), TCR stimulation (41) and costimulatory stimulations via CD28 (42) and ICOS (43), besides IL-2, are considered important for their maintenance. However, preconditioning without these stimulations was sufficient to induce a robust proliferation of Tregs in response to IL-2, and thus could be a critical mechanism for Treg maintenance in vivo.

On the other aspect, the preconditioning mechanism could act as a regulator for immune reaction. Upon pathogen invasion, cytokines produced by activated immune cells could be delivered to neighboring T cells that are not specific for the pathogen. The requirement for a contact with accessory cells would regulate cytokine-induced proliferation of nonantigen-specific T cells, minimizing undesired competition for resources, such as nutrients, with the critical pathogen-specific T cells. Signals induced by contact with accessory cells, segregated from the TCR and cytokine signals, might also modulate functionality of the pathogen-specific T cells, which remains to be explored.

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A requirement for accessory cell interaction with resting T cells segregated from TCR or MHC molecules is of interest. In this context, the requirement for accessory cell interaction in the absence of TCR is also observed for optimal NK cell activation in its role in antibody-dependent cell-mediated cytotoxicity (44) or in tumor killing (45, 46). In the former system, as in the present study, cell-to-cell contact between NK cells and macrophages was required for an optimal response. In the latter system, Toll-like receptor ligand-activated DCs or macrophages augmented cytotoxic activity of NK cells (45, 46) and interferon-γ production by NK cells, in turn, induced maturation of DCs, showing bidirectional crosstalk between NK cells and DCs (46). Such interaction of immunological cells with DCs may be required for the host to maintain regulation of an immediate immune response to invading pathogens rather than solely to increased cytokine levels.

In summary, we have defined a unique pathway involving accessory cells preconditioning naïve cells and Tregs for select γ_c-cytokine-mediated proliferation.

Materials and Methods

Animals. All animal experiments were performed in accordance with a protocol approved by the Animal Care and Use Committee of the National Cancer Institute. C57BL/6 WT (Ly5.2$^+$) and Ly5.1$^+$ congenic mice; mice expressing diphtheria toxin receptor transgene under a CD11c promotor (CD11c-DTR Tg); mice expressing MHC class I-restricted TCR specific for ovalbumin (OT-1 Tg); and mice deficient for Tap1, MHC class II, both CD80 and CD86 B7 molecules, CD28, IL-6, OX40, CD47, and IL-15 receptor alpha (IL-15RαKO) were purchased from Jackson Laboratories. IL-15-deficient (IL-15KO) mice were purchased from Taconic Biosciences. CD11c-DTR Tg mice were crossed with Rag1-deficient mice (Jackson Laboratories) to generate CD11c-DTR-Rag1KO mice. IL-15RαKO and IL-15KO mice were backcrossed to the C57BL/6 strain for 10 additional generations in our facility. IL-15Rα transgenic mice were generated by our group (47).

Patient Materials. All patient samples were obtained in accordance with the Declaration of Helsinki. All patients signed a written informed consent for participation in the clinical studies approved by the Intramural Review Board of the National Cancer Institute.

T, NK, and B Cell Purification and Generation of DCs and Monocytes. Unless indicated as human cells, experiments were performed using mouse cells. Mouse T cell subsets, NK cells, and B cells were purified from splenocytes using magnetic beads or an Isolation Kit (Miltenyi). The purification procedure was repeated as necessary to achieve a purity of >96% for CD8 T, CD4 T, CD8CD44^{lo} T, CD4CD25$^-$ T, and B cells, and >85% for CD8CD44^{hi} T, CD4CD25$^+$ T, and NK cells. Mouse DCs and monocytes were derived from the bone marrow flushed from femurs and tibias of mice using 20 ng/mL murine GM-CSF and M-CFS (PeproTech), respectively. Human CD45RA$^+$CD45RO$^-$ CD8 T cells and CD4CD25$^+$ Tregs were purified from elutriated peripheral lymphocytes of healthy donors using Isolation Kits (Miltenyi), with purities of >90% and >85%, respectively. Human DCs were derived from elutriated peripheral monocytes using 50 ng/mL human GM-CSF and 50 ng/mL human IL-4 (PeproTech). See *SI Appendix* for details.

Proliferation Assays. To analyze cell proliferation in vitro by ^{3}H-thymidine incorporation, T cells (2.5×10^5 cells) were cultured with or without B cells, DCs, or monocytes (5×10^5 or indicated cell number) and different doses of cytokines specified. DCs and monocytes were irradiated at 36 Gy using a cesium-137 irradiator and used in experiments, with GM-CSF (5 ng/mL) and M-CFS (5 ng/mL) added to all samples, respectively. In some experiments, an additional antibody, cytokine, or reagent (*SI Appendix*) was included in the culture as indicated. ^{3}H-thymidine was pulsed on day 3 of culture, or on day 6 in experiments involving patient cells, and after 6 h, culture plates were frozen at −20 °C and later thawed and harvested for counting the incorporated radioactivity by a β-counter (MicroBeta TriLux, PerkinElmer). In experiments using transwells (0.2 μm Anopore Membrane 8-Well Strip inserts, Nunc, Cat. No. 136730), T cells (2.5×10^5 cells) and DCs (2.5×10^5 cells) were cultured in the indicated combinations. On day 3, the cells in the upper wells were transferred to a new 96-well plate and volumes of all samples were adjusted to 150 μL by medium containing IL-2 before adding ^{3}H-thymidine. In some assays, T cells cocultured with DCs overnight underwent purification using Pan DC MicroBeads (Miltenyi, Cat. No. 130-092-45) to remove DCs for further assays.

To analyze proliferation by flow cytometry, T cells were labeled with CMFDA (ThermoFisher Scientific, Cat. No. C7025) at 2.5 μM in PBS for 15 min at 37 °C, washed twice using the culture medium, and then cultured with or without non-irradiated DCs and cytokines. On day 5, dilution of CMFDA was analyzed by flow cytometry (FACSCalibur, BD Biosciences, or CytoFLEX LX, Beckman Courter).

Proliferation of T cells in vivo was examined by transferring CMFDA-labeled Ly5.1$^+$ T cells to Ly5.2$^+$CD11c-DTR Tg hosts that were lymphodepleted by 5 Gy irradiation using a cesium-137 irradiator. The hosts received either no treatment or intraperitoneal injection (i.p.) of DT (Millipore-Sigma) at 6 ng/g body weight on days (−1), 1, and 3. The splenocytes collected from the host mice on day 5 were analyzed by flow cytometry for donor T cell proliferation. Ly5.2$^+$CD11c-DTR Tg-Rag1KO hosts were also used and received DT i.p. on days (−1), 1, 3, and 5. The donor Ly5.1$^+$ T cell proliferation was analyzed on day 7.

Flow Cytometry Analysis. Cell surface staining was performed in 0.1% fetal calf serum in PBS (FCM buffer) for 30 min on ice, and the cells were washed in the buffer. For intracellular staining, cells were fixed in 0.5% paraformaldehyde in PBS for 15 min at 37 °C, washed twice in PBS, and permeabilized by incubating in −20 °C 90% methanol for 15 min. The cells were then washed in PBS, blocked in 0.5% bovine serum albumin (BSA) in PBS for 10 min at room temperature, and labeled with an antibody for 30 min at room temperature. The cells were then washed in 0.5% BSA in PBS. Flow cytometry data were acquired using FACSCalibur or CytoFLEX LX, except for Ca^{2+} flux analyses described below, and analyzed using FlowJo software (BD Biosciences). See *SI Appendix* for antibody information.

Ca^{2+} Flux Assays. CD8CD44^{lo} OT-1 T cells (5×10^6 cells) were loaded with 1.8 μM Indo-1 (ThermoFisher Scientific, Cat. No. 1223) in Indo buffer (HBSS with calcium and 0.5% BSA) at 37 °C for 30 min. The cells were washed twice with the buffer and let stand for 40 min at 4 °C with a gentle shaking at 20 min, followed by washing twice in Indo buffer. The cells resuspended in the buffer (0.5 mL) were heated to 37 °C for 2 min, added with DCs (5×10^6 cells in 0.5 mL), and spun using a minifuge for 20 s to have the T cells contact with DCs, without IL-2. Various times later (1 to 8 min), the cell mixture was resuspended by a short vortexing and applied to a flow cytometer (BD FACSVantage, BD Bioscience). The 405/510 nm emission signal ratio for Indo-1 (Indo-1 ratio) was used to analyze the intracellular Ca^{2+} flux. The peak of median of the top 60% Indo-1 ratios for each of the samples incubated for different times was plotted against the duration of DC-T cell contact to obtain intracellular Ca^{2+} concentration changes induced by the contact with DCs. In some experiments, DCs loaded with ovalbumin peptide (SIINFEKL, American Peptide Company/Bachem) were used to induce TCR stimulation-mediated Ca^{2+} flux as a control.

Western Blotting. Tap1KO DCs of day 8 or 9 of differentiation were plated in a CellBIND 12-well plate (Corning, Cat. No. 3337) overnight to allow the cells to adhere to the plate. On the following day, floating and loosely attached DCs were carefully removed by washing with 37 °C culture medium. T cells were added to adhered DCs or plated by themselves, with or without IL-2 (8 nM). The plates were spun at 1,500 rpm for 3 min to allow T cells to contact the adhered DCs. After culture at 37 °C for various times, the T cells were gently collected and washed in ice-cold PBS. Of note, the purity of collected T cells was comparable to that of the original cells (>96% for CD8CD44^{lo} T cells and >85% for CD8CD44^{hi} T cells). Whole-cell lysate was generated in RIPA buffer (Cell Signaling Technologies) or M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Cat. No. 78501) at 2×10^6 cells in 30 μL. Nuclear Extraction Kit (CHEMICON International/Millipore, Cat. No. 2900) was used for subcellular fractionation to collect cytoplasmic and nuclear lysates. Western blotting was performed using the lysates in the amount that derived from 0.67×10^6 cells per sample loaded to 10 or 12 % tris-glycine gels (ThermoFisher Scientific) and transferred to Immobilon-P PVDF membranes (Millipore, Cat. No. IPVH15150). The membranes were then blotted using antibodies against indicated proteins (*SI Appendix*) and underwent chemiluminescence signal detection using SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, Cat. No. 34075) and exposure to films.

PCR. RNA was extracted from T cells using a Total RNA Purification Micro Kit (Norgen, Cat. No. 35300), and complementary DNA (cDNA) was synthesized

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using a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche, Cat. No. 11483188001, discontinued), following the manufacturers' instructions. PCR was performed using the cDNA, Taq DNA polymerase (Takara, Cat. No. R001B), and primer pairs as follows; *cish*: 5′-TCCTTTGCGTACAGG GATCTT -3′(forward) and 5′-CACAGGAGGCCACATAGTGCT-3′ (reverse). *actb*: 5′-TTCTTTGCAGCTCCTTCGTTG-3′ (forward) and 5′-AACACCCCAGCCATGTACGTA-3′ (reverse).

Statistical Analyses. Statistical comparisons concerning sample means in two independent groups of subjects were performed by two-tailed unpaired *t* tests and that concerning sample means within the same group of subjects over two different conditions were performed by two-tailed paired *t* tests or by two-tailed ratio paired *t* tests. Statistical comparisons concerning sample means over more than two different conditions in one factor were performed by one-way (ANOVA) and those in more than two factors by two-way ANOVA. Sample means and SDs are presented.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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