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# Direct Alloreactivity Is More Susceptible to Regulation by Natural Regulatory T Cells Than Indirect Alloreactivity

**Grégory Noël, Meriam Belghith, Benoit Bélanger, Caroline Leduc, and Claude Daniel**

**The contribution of natural CD4$^+$CD25$^+$ regulatory T cells (nTregs) in controlling graft rejection and the mechanism used remain controversial. Using the duality of the 2.102 TCR Ag recognition, we were able to study, for the first time to our knowledge, the involvement of nTregs in the two pathways of allorecognition in a murine adoptive transfer model in which TCR-transgenic nTregs were or were not depleted before transplantation. We show that nTregs used at a physiological ratio were able to delay graft rejection after direct alloreactivity by controlling proliferation and differentiation of alloreactive CD4$^+$ conventional T cells in draining lymph nodes. In contrast, similar results were found in the indirect alloreactivity pathway only when nTregs were used in high numbers. In the latter pathway, nTregs used at a physiological ratio failed to delay graft rejection and to control proliferation of conventional T cells. These results support recent therapeutic approaches aimed at producing and using in vitro Ag-specific Foxp3$^+$ nTregs to control graft rejection in transplantation. Finally, late inhibition of Th1 differentiation was shown in indirect alloreactivity, but this suppression could also be mediated by Foxp3$^+$-induced Tregs.** ***The Journal of Immunology*****, 2013, 190: 3764–3771.**

During transplantation, alloreactive CD4$^+$ T cells are key elements in graft rejection and can be activated by two classical pathways: direct and indirect (1). In the direct pathway, CD4$^+$ T cells recognize intact allogeneic MHC molecules on donor APCs, whereas in the indirect pathway, they recognize allogeneic peptides presented by MHC molecules of self-APCs after Ag processing. Two broad classes of CD4$^+$ T cells have been described: the conventional T cells (Tconvs), which are responsible for graft rejection after differentiation into effector T cells, and the regulatory T cells (Tregs).

It is now clear that Tregs are a crucial component of the immunoregulation process, in which they generate or maintain peripheral tolerance (2). Natural CD4$^+$CD25$^+$ regulatory T cells (nTregs) play a major role in the maintenance of tolerance to self-antigens and alloantigens (3, 4). Injection of a high number of nTregs provides protection against graft rejection in organ transplantation (5, 6) and against graft-versus-host disease in hematopoietic stem cell transplantation (7, 8). The forkhead-family transcription factor Foxp3, which is important for the development and suppressive activity of these regulatory T cells, currently seems to be the best marker for nTregs (9).

Using MHCII-deficient mice (10) or bone marrow–reconstituted mice (11), or blocking Ag presentation by APCs (12), many studies have shown that CD4$^+$ alloreactive T cells with direct or indirect allospecificity can initiate graft rejection, but the role of nTregs in the regulation of each pathway is still unclear. The nTregs must be Ag specific for an efficient control of graft rejection, particularly in direct alloreactivity, because of the higher frequency of Ag-specific alloreactive T cells in Tconv populations in comparison with nTregs (11). Unfortunately, no model allows the study of the involvement of the same Ag-specific nTreg in each individual pathway.

Our laboratory has established a unique model to investigate alloresponses, based on TCR transgenic (Tg) mice (2.102Tg), wherein both pathways could be independently studied using the same (i.e., 2.102) clonotype, identical recipients, and similar experimental conditions (13, 14). In this model, direct alloreactivity is provided by the recognition of I-E$^p$ on the surface of a donor APC, whereas recognition of the Hb(64–76) peptide after uptake, processing, and presentation by I-E$^k$ on self-APCs represents indirect alloreactivity.

In this article, we show that Ag-specific CD4$^+$CD25$^+$Foxp3$^+$ T cells generated in 2.102Tg mice are phenotypically and functionally identical to nTregs. These cells used at a physiological ratio delay graft rejection by controlling proliferation, differentiation, and migration of alloreactive CD4$^+$ Tconv cells in direct alloreactivity. In contrast, similar results were found in the indirect alloreactivity pathway only when nTregs were used in high numbers.

## Materials and Methods

### *Mice*

C57BL/6J Tcra$^{tm1/Mom}$ mice (15) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred with B6.AKR to introduce the mutation into an H-2$^k$ haplotype (referred to herein as TCRαKOK). The rag1-sufficient 2.102 TCR-Tg (2.102Tg, H-2$^k$, Thy1.1$^+$) and the B6(mHEL-Hb) (H-2$^b$) mice have been previously described (14, 16, 17). B10.P, B6.AKR, 2.102Tg, and B6(mHEL-Hb) mice were kindly provided by Dr. Paul M. Allen (Washington University, St . Louis, MO) and maintained in our pathogen-free animal facilities.

### *Cell purification*

Splenocytes were isolated from 2.102Tg mice and purified on the basis of CD4 and CD25 expression by MACS (Miltenyi Biotec, Auburn, CA), as

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Abbreviations used in this article: Dep, depleted; Dir, direct pathway graft; DLN, draining lymph node; Hb, indirect pathway; Ind, indirect pathway graft; Iso, isogeneic graft; iTreg, induced regulatory T cell; MST, median survival time; nTreg, natural CD4$^+$CD25$^+$ regulatory T cell; Tconv, conventional T cell; Tg, transgenic; Tot, total; Tr mouse, TCRαKOK mouse reconstituted with Tconvs and nTregs at a 1:1 ratio.



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described previously (17). CD25-positive cells were depleted using biotin-labeled anti-CD25 mAb (7D4; BD Biosciences), antibiotin microbeads, and LD columns. The CD25-depleted cells were incubated with L3T4 beads to isolate $CD4^+CD25^-$ T cells. The nTreg cells were purified using the mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit according to the manufacturer's instructions. Purity of sorted cells was > 90%.

### *In vitro cultures for proliferation assays*

Cells were cultured in complete RPMI 1640 supplemented with GlutaMAX, 10% FCS, 0.05 mM β-mercaptoethanol, and antibiotics. After CFSE staining at 2 μM, $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells were seeded, alone or cocultured at 1:1 ratio, in round-bottom 96-well microplates ($2 \times 10^4$ cells per well). Cells were cultured in the presence of irradiated APCs from either B10.P ($6 \times 10^4$ cells per well) or B6.AKR ($1.25 \times 10^5$ cells per well) with Hb(64–76) peptide (0.1 μM). Cells were cultured at 37°C for 4 d in a humidified atmosphere containing 5% $CO_2$, and proliferation was quantified by CFSE dilution.

### *Adoptive cell transfer and skin grafting*

TCRαKOK mice 8–10 wk old were used as recipients. Animals were injected i.v. with $3 \times 10^6$ $CD4^+$ (Tot for total) or $CD4^+CD25^-$ (Dep for depleted) T cells isolated from 2.102Tg mice before grafting. For some experiments, T cells were stained with CFSE before injection. In cotransfer experiments, mice were reconstituted with $3 \times 10^6$ $CD4^+CD25^+$ T cells isolated from 2.102Tg mice and $3 \times 10^6$ $CD4^+CD25^-$ T cells isolated from B6.AKR or 2.102Tg mice. All mice were transplanted with tail skin grafts from male donors onto the dorsum 1 d after reconstitution, as previously described (17). Graft survival was monitored daily and defined as complete loss of viable skin.

### *Flow cytometry*

Splenocytes or draining lymph node (DLN) cells were isolated and stained with anti-CD4 (L3T4) APC, anti-Thy1.1 (OX-7) PerCP-Cy5.5 or APC, anti-CD25 (7D4) PE, anti-CD45RB (H1-2F3) biotin, and streptavidin FITC conjugate. Intracellular IFN-γ staining and intranuclear Foxp3 staining were performed with a detection kit (eBiosciences) according to the manufacturer's instructions, using anti–IFN-γ (XMG1.2) APC or anti-Foxp3 (FJK-16s) FITC or PerCP-Cy5.5. All Abs were purchased from eBioscience. Data acquisition was performed on a FACSCalibur (Becton Dickinson, San Jose, CA) operated with CellQuest software (Becton Dickinson) and analyzed using FCS Express v3 (De Novo Software, Los Angeles, CA).

### *Isolation of skin graft–infiltrating cells*

At the time that mice were killed, skin grafts were retrieved from the recipient's back, minced into small pieces, and then digested for ~3 h at 37°C in RPMI 1640 medium containing 20 U/ml Collagenase D (Sigma-Aldrich, Oakville, ON, Canada). Collagenase-pretreated tissues were then ground, washed, and separated by centrifugation on Lympholyte-M media (Cedarlane, Burlington, ON, Canada). Recovered cells were frozen in RNAlater.

### *RNA isolation and cDNA synthesis*

Total RNA was extracted from cells recovered from skin grafts using the RNeasy Kit (Qiagen, Mississauga, ON, Canada). For cDNA synthesis, total RNA was primed with Hexanucleotide Mix (Roche Diagnostics, Indianapolis, IN) and reverse transcribed with SuperScript III (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions.

### *Quantitative RT-PCR*

Real-time PCR was performed on an Mx3000P QPCR System (Stratagene, La Jolla, CA) using the SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) and continuous fluorescence monitoring, as previously described (18). PCR reactions were cycled 40 times after initial denaturation (95°C, 10 min) with the following parameters: 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Samples were run in duplicate, and relative quantification of mRNA levels was performed by the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous reference and ungrafted donor skin specific for each pathway as a calibrator. Primers were purchased from IDT (Coralville, IA). The following primers were used: GapdhS (5′-CATGTTCCAGTATGACTCCACTC-3′), GapdhR (5′-GGCCTCACCCCATTTGATGT-3′), Tcrb 2.102S (5′-CAACATCTGGGACATAATGC-3′), Tcrb 2.102R (5′-GGCCGCGGCAGCTGAGTCTTCTGGATCC-3′), IfngS (5′-ATGAACGCTACACACTGCATC-3′), IfngR (5′-CCATCCTTTTGCCAGTTTCTC-3′), Foxp3S (5′-CACAATATGCGACCCCCTTTC-3′), and Foxp3R (5′-AACATGCGAGTAAACCAATGGTA-3′).

### *Statistical analyses*

Skin graft survival curve $p$ values were calculated using Kaplan–Meier/log-rank test methods. Flow cytometry and real-time PCR analyses were compared using the ANOVA test. Values are expressed as the mean ± SEM (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$). Data were analyzed with GraphPad Prism Software.

## Results

### *Phenotypic and functional characterization of 2.102Tg $CD4^+$ $CD25^+$ T cells*

Using a unique model of TCR Tg mice (2.102Tg mice), which allows us to study independently the two pathways of allorecognition during skin graft, we have previously shown that the same 2.102Tg alloreactive $CD4^+$ T cells were able to initiate graft rejection by both pathways (19). In this model, the kinetics of rejection were similar, although the initiation of alloreactive responses seems to occur earlier in the direct pathway, suggesting that tolerance mechanisms were operational in this pathway. We first determined whether nTregs were generated in 2.102Tg mice and then investigated their potential involvement in graft tolerance in the two pathways of allorecognition. As shown in Fig. 1A, two populations of 2.102Tg $Thy1.1^+$ splenocytes could be identified: the $CD4^+CD25^-$ and the $CD4^+CD25^+$. The $CD4^+CD25^+$ T cells express high levels of Foxp3 and low levels of CD45RB, a phenotype reminiscent of Tregs, whereas the $CD4^+CD25^-$ T cells have a conventional phenotype with expression of high levels of CD45RB and almost no expression of Foxp3. These two populations were then functionally characterized in vitro. According to our previous studies (13, 17), 2.102Tg $CD4^+CD25^-$ T cells are able to proliferate when activated by self-APCs pulsed with the Hb (64–76) peptide or by B10.P APCs (Fig. 1B, *upper panel*, gray lines), but this proliferation was inhibited when they were cocultured with 2.102Tg $CD4^+CD25^+$ T cells (Fig. 1B, *upper panel*, black lines). $CD4^+CD25^+$ T cells were able to proliferate in vitro under these conditions when cocultured with $CD4^+CD25^-$ T cells (Fig.1B, *lower panel*), but not when cultured alone (data not shown). Thus, the $CD4^+CD25^-$ and the $CD4^+CD25^+$ 2.102Tg T cell populations have functional characteristics of Tconvs and nTregs, respectively. To confirm the Ag specificity of the 2.102Tg nTregs, TCRαKOK mice were adoptively reconstituted with B6.AKR $CD4^+CD25^-$ T cells alone or with 2.102Tg nTregs and grafted with B6(mHEL-Hb) or C57BL/6 skin. As shown in Fig. 1C, 2.102Tg nTregs could specifically delay the graft rejection only when the Hb(64–76) peptide was expressed by the allograft. Altogether, these results confirm the Ag-specific suppressive function of the 2.102 nTregs in vitro and, more importantly, in vivo.

### *nTregs delay graft rejection by alloreactive 2.102Tg $CD4^+$ $CD25^-$ T cells only after priming by direct pathway*

We used the activation duality of the 2.102Tg nTregs to study the two pathways of allorecognition (direct and indirect) during skin graft rejection. For this, TCRαKOK mice were adoptively reconstituted with total $CD4^+$ T cells (Tot) isolated from 2.102Tg mice, in which the Tconv/nTreg ratio can be considered physiological (≈5:1) or with Tconv alone (Dep) and grafted for the two allogeneic pathways [direct pathway graft (Dir); indirect pathway graft (Ind)] (Fig. 2). Isogeneic grafts (Iso), which survived indefinitely, were used as controls (data not shown). Depletion of $CD25^+$ cells significantly accelerated rejection by the direct pathway [median survival time (MST) = 17.58 ± 0.43 versus 11.75 ± 0.49, Tot versus Dep]. In contrast, rejection by the in-

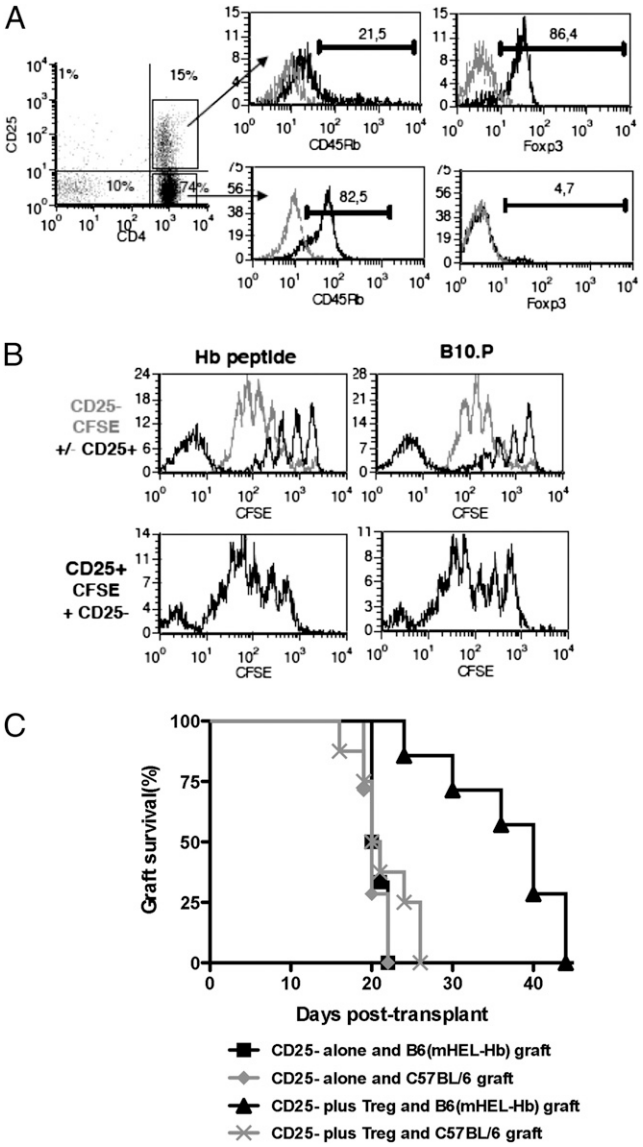


**FIGURE 1.** Phenotypic and functional characterization of 2.102Tg $CD4^+CD25^+$ T cells. (**A**) Surface expression of CD4, CD25, and CD45RB and intracellular expression of Foxp3 were analyzed by flow cytometry on $Thy1.1^+$ splenocytes prepared from 2.102Tg mice. Staining by isotypic controls is represented by gray lines. (**B**) 2.102Tg $CD4^+CD25^-$ T cells (*upper panels*) or $CD4^+CD25^+$ T cells (*lower panels*) were stained with CFSE and cultured alone (gray lines) or cocultured at a 1:1 ratio (black lines). Irradiated B6.AKR splenocytes pulsed with Hb(64–76) peptide (0.1 μM) were used for indirect priming (*left panels*), whereas irradiated B10.P splenocytes were used for direct priming (*right panels*). Results are representative of five independent experiments. (**C**) B6.AKR $CD4^+CD25^-$ T cells ($3 \times 10^6$) were transferred alone or with 2.102Tg nTregs ($2 \times 10^6$) into TCRαKOK mice, and recipients were transplanted with B6(mHEL-Hb) or C57BL/6 skin allografts ($n$ = 5–8). Mice were scored for graft rejection.

direct pathway occurred with similar kinetics, whether $CD25^+$ T cells were depleted (MST = 17 ± 0.85, Ind Dep) or not (18.45 ± 0.53, Ind Tot). In contrast to B6(mHEL-Hb) allografts, C57BL/6 skin allografts survived indefinitely, ruling out a possible contribution of direct alloreactivity from $H\text{-}2^b$ alloantigens after transplant of B6(mHEL-Hb) allografts (Supplemental Fig. 1). This finding is also supported by in vitro proliferation assays showing exquisite specificity of the 2.102Tg T cells for B10.P or B6(mHEL-Hb) alloantigens for direct or indirect Ag presentation, respectively (17). In conclusion, it seems that Tconvs are more efficient at mediating graft rejection in the direct than in the indirect pathway and, more importantly, nTregs at a physiological ratio could delay rejection only in the direct pathway.

### *nTregs inhibit Tconv proliferation in DLNs only in the direct pathway*

To understand why nTregs delay graft rejection only in the direct pathway, we first analyzed the proliferation of Tconvs in DLNs in the absence of $CD25^+$ T cells. Tconvs were stained with CFSE prior to their adoptive transfer, and DLNs were retrieved at days 3, 5, and 7 posttransplant and analyzed by flow cytometry (Fig. 3A). At day 3, all Tconvs ($CD4^+Thy1.1^+Foxp3^-$) were $CFSE^{hi}$ and thus had not yet proliferated, confirming that no homeostatic proliferation occurs in DLNs in our model. At day 5, Tconvs started to dilute their CFSE, and by day 7, most cells had diluted their CFSE extensively, with few undivided ($CFSE^{hi}$) cells remaining. As shown in Fig. 3B, the percentage of undivided cells in Tconvs stimulated by direct alloreactivity was higher at days 5 and 7 posttransplant in mice reconstituted with total $CD4^+$ T cells (Dir Tot), in comparison with CD25-depleted $CD4^+$ T cells (Dir Dep). In contrast, no difference between both groups was found in mice that received indirect pathway allografts (Ind Tot versus Ind Dep). Because proliferation seemed mostly completed by day 7 posttransplant and few 2.102Tg T cells had then infiltrated the allografts (see below), we chose this time point to calculate the precursor frequency of Tconvs that achieved no (Undiv), intermediate (Div 1 to 5), or extensive (Div 6 to 10) cell divisions, as illustrated by the CFSE dilution profile shown in Fig. 3A. The Tconv percentage found for each group was divided by the formula $2^n$, and results were expressed in relative precursor frequency. Our analyses show that precursor frequency that remained undivided after activation by the direct pathway was significantly higher in Dir Tot than in Dir Dep mice (Fig. 3C). This finding correlates with a higher frequency of precursors that achieved one to five cell divisions in Dir Dep mice. Again, no difference was found between mice reconstituted with total or CD25-depleted $CD4^+$ T cells in the indirect pathway. No significant difference was found for the frequency of Tconvs that had divided more than six times. However, the total number of $CD4^+Thy1.1^+Foxp3^-$ 2.102Tg T cells (Tconv) recovered at days 7 and 11 posttransplant was significantly higher in Dir Dep than in Dir Tot mice (Fig. 3D), confirming that more Tconv had achieved extensive proliferation in Dir Dep mice. No significant difference was found between both Ind mice. Overall, these results suggest that nTregs can control Tconv proliferation in DLNs only after direct allorecognition. To rule out the possibility that lack of regulation of the indirect pathway by nTregs was not somehow due to their inability to migrate to DLNs, the frequency of nTregs in $Thy1.1^+$ cells was analyzed at days 3, 5, and 7 (Fig. 3E). No difference was found between Dir Tot and Ind Tot mice at any time point. Of interest, nTregs were barely detectable at day 3 but started to be recruited in the DLNs by day 5. Thus, despite their presence at the same ratio as in the direct pathway, nTregs seem unable to effectively control Tconv proliferation in the indirect pathway.

### *nTregs inhibit Th1 differentiation in DLNs at an early stage in the direct pathway, but only at a later stage in the indirect pathway*

Next, we addressed the issue of Tconv differentiation into Th1 cells, as determined by their production of IFN-γ after activation. Using intracellular flow cytometry, we have found that Dir Dep mice, in comparison with Dir Tot mice, show significantly higher proportion and total number of $IFN\text{-}\gamma^+$ cells in Tconvs of the

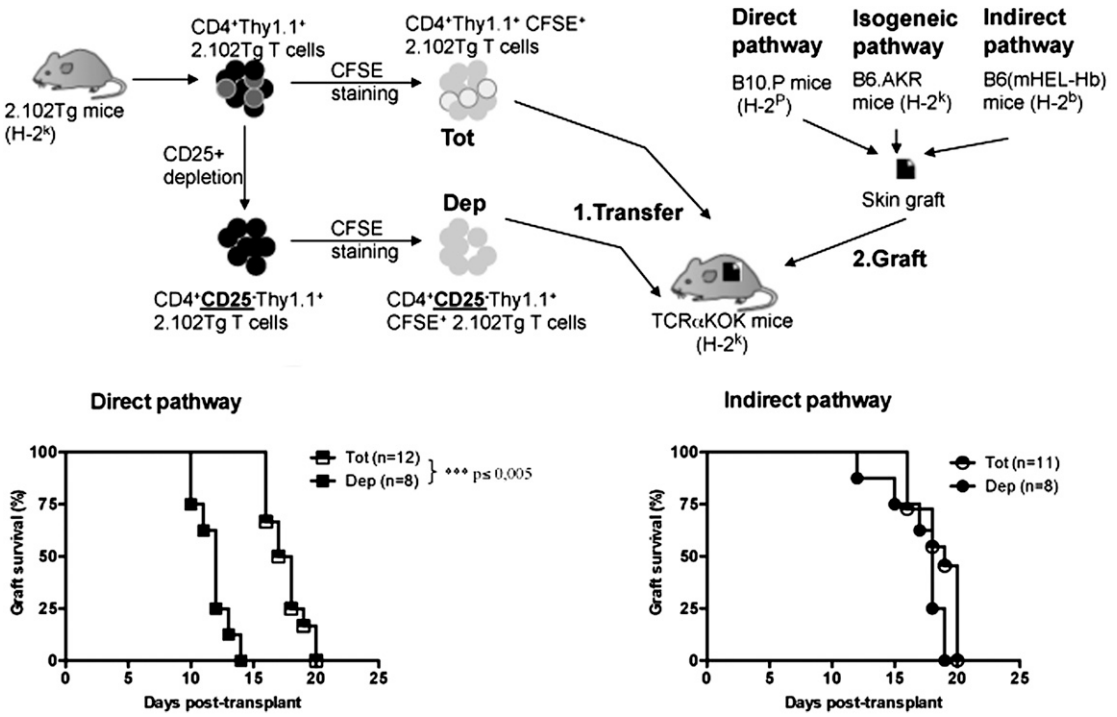


**FIGURE 2.** nTregs delay graft rejection by alloreactive 2.102Tg $CD4^+CD25^-$ T cells only after priming by direct pathway. $CD4^+$ T cells (Tot) and $CD4^+$ $CD25^-$ T cells (Dep) were purified from 2.102Tg mice and transferred ($3 \times 10^6$ cells) into TCRαKOK mice. At 1 d after, mice were grafted with skin from B10.P mice for priming by direct pathway (*bottom left panel*) or from B6(mHEL-Hb) mice for priming by indirect pathway (*bottom right panel*). Mice were grafted with B6.AKR skin as isogeneic controls. Mice were scored for grafted rejection.

DLNs at days 7 and 11, whereas no difference was found at day 7 in both Ind mice (Fig. 4). However, at day 11, the frequency and number of IFN-$\gamma^+$ cells were significantly higher in Ind Dep than in Ind Tot mice. In conclusion, nTregs inhibit Th1 differentiation in DLNs in the direct pathway at day 7 posttransplant, whereas this differentiation is controlled only at day 11 in the indirect pathway.

### *A high number of nTregs is necessary to inhibit Tconv proliferation in the indirect pathway*

As nTregs were unable to control Tconv proliferation at the physiological ratio in the indirect pathway, we wanted to know if this lack of control was intrinsic to this alloreactive pathway or could be attributed to the low number of nTregs present. Indeed, many studies had shown that only high numbers of nTregs were able to delay graft rejection when the indirect pathway was involved (11, 20). Thus, TCRαKOK mice were reconstituted with Tconv and nTreg at a 1:1 ratio (Tr mice) and grafted for the indirect pathway. In these conditions, nTregs could delay graft rejection significantly (MST = 38.0 ± 0.82, Fig. 5A). These results are in agreement with work by Joffre et al. (21), which showed that specific Tregs could control chronic rejection during skin graft, but only when bone marrow chimerism was induced and nTregs persisted in the host. Of note, nTregs used at a 1:1 ratio were able to delay graft rejection even further by the direct pathway (MST = 29.0 ± 3.32, data not shown), compared to the physiological ratio. To seek mechanisms responsible for the delay in graft rejection by the indirect pathway, we estimated the Tconv proliferation in DLNs at day 7. The precursor frequency of undivided Tconvs (Fig. 5B) and the percentage of undivided cells in Tconvs (Fig. 5C, *left panel*) were significantly higher in Ind Tr mice than in Tot and Dep Ind mice. Consistent with these observations, the precursor frequency of Tconvs that had divided one to five times (Fig. 5B) and the total number of Tconvs in DLNs were lower (Fig. 5C, *right panel*). These observations correlated with a lower Tconv/Treg ratio in DLNs at day 7 in Ind Tr mice (1.3 ± 0.43) than in Ind Tot mice (3.4 ± 1.6) (Fig. 5D, $*p \leq 0.05$). Thus, nTregs could control graft rejection and Tconv proliferation in DLNs in the indirect pathway only when injected in numbers higher than physiological.

### *nTregs inhibit Tconv graft infiltration in the direct pathway*

Finally, we evaluated the infiltration of Tconvs and nTregs as well as the IFN-γ production in the graft after transplant. For this purpose, we used quantitative RT-PCR because the very low number of 2.102Tg T cells recovered from the allografts did not allow us to perform conclusive flow cytometry analyses. Hence, grafts were retrieved at different days, total RNA was extracted and reverse transcribed to cDNA, and 2.102 *Tcrb*, *Ifng*, and *Foxp3* expression was analyzed. As shown in Fig. 6, 2.102Tg T cells infiltrated the graft early and strongly in Dir Dep mice, whereas this infiltration was progressive and late in Dir Tot mice and both Ind mice. The *Ifng* production correlated with 2.102Tg T cell infiltration in both Dir mice, but not at day 15 in both Ind mice. Finally, if we consider the *Foxp3* expression, nTregs might infiltrate the graft in Dir Tot and Ind Tot mice, although earlier in Ind Tot mice. In conclusion, nTregs could migrate into the graft via both pathways, but they delay Tconv infiltration into the graft only in the direct pathway.

## Discussion

Using our unique 2.102Tg model, we have been able to study, for the first time to our knowledge, the role of Ag-specific nTregs in direct and indirect pathways of allorecognition during skin graft rejection in identical recipients and under the same experimental conditions. We have demonstrated that nTregs, at a physiological ratio, are able to delay graft rejection in the direct pathway by controlling Tconv proliferation and differentiation in DLNs, and

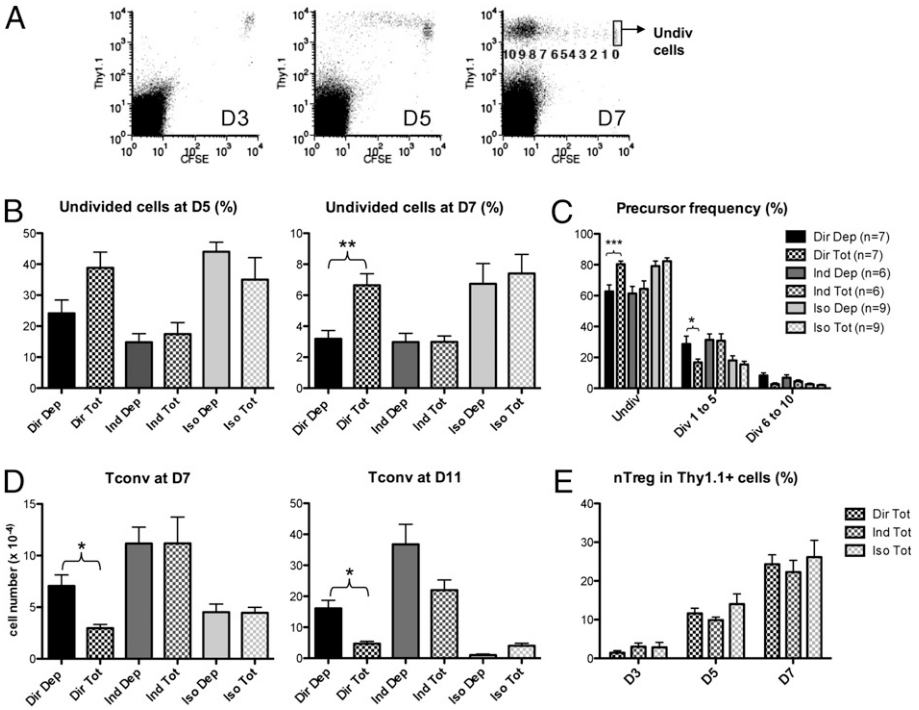


**FIGURE 3.** nTregs inhibit Tconv proliferation in DLNs only in the direct pathway. TCRαKOK mice were adoptively reconstituted and grafted as described in Fig. 2. (**A**) DLNs were collected at days 3, 5, and 7 posttransplant, and cells were isolated and analyzed for the expression of Thy1.1 marker and for CFSE dilution. Data shown are representative of mice reconstituted with CD25-depleted 2.102Tg $CD4^+$ T cells (Dep) and grafted with B10.P skin. (**B**–**E**) Cells recovered from DLNs collected at different days posttransplant were analyzed for the expression of Thy1.1 and CD4 markers and for CFSE dilution (B–D). $Foxp3^+$ T cells were excluded from all analyses of CFSE dilution. The percentages of undivided cells at day 5 (*left panel*) and at day 7 (*right panel*) are shown in (B). The calculated precursor frequency of undivided and divided cells in $CD4^+Thy1.1^+$ T cells is shown in (C). The total number of $CD4^+Thy1.1^+Foxp3^-$ T cells (Tconv) found at day 7 (*left panel*) and day 11 (*right panel*) are shown in (D). DLN cells were analyzed for the expression of Thy1.1, CD25, and Foxp3 markers, and the proportion of nTregs ($Thy1.1^+CD25^+Foxp3^+$) in the $Thy1.1^+$ T cell population at days 3, 5, and 7 is shown in (E). $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.005$.

T cell graft infiltration. Indeed, the extent of proliferation and the number of 2.102Tg Th1 cells found in Dir Tot mice were actually similar to those found in isogeneic controls. Although we found that 20% of Tconvs had proliferated in isogeneic control mice, we consider this proliferation a consequence of the inflammatory response and activation of the skin APCs induced by the graft surgery, because no Tconvs had proliferated before graft vascularization (Day 3 in Fig. 3A) and no proliferation was detected in CFSE dilution or BrdU incorporation analyses of mice adoptively reconstituted but not grafted (Supplemental Fig. 2 and data not shown). However, despite the limited proliferation of Tconvs in Dir Tot mice (similar to control mice), allografts were rejected,

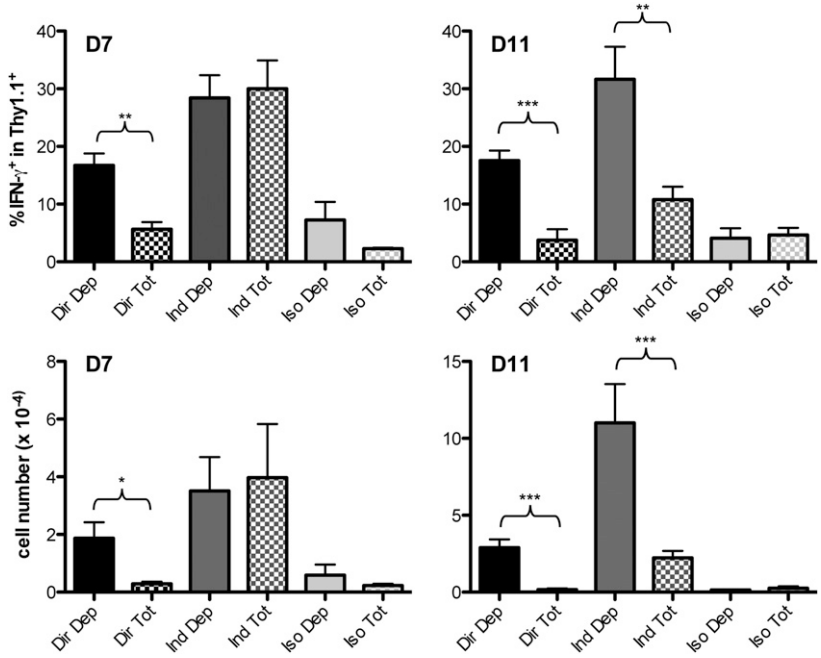


**FIGURE 4.** nTregs inhibit Th1 differentiation of Tconvs in DLNs at an early stage in the direct pathway, but only at a later stage in the indirect pathway. TCRαKOK mice were adoptively reconstituted and grafted as described in Fig. 2. DLNs were collected at days 7 (*left panel*, $n = 6$–9) and 11 (*middle panel*, $n = 6$–8) posttransplant. Cells were isolated and cultured with irradiated B6.AKR splenocytes pulsed with Hb(64–76) peptide (1 μM) and brefeldin A for 4 h. After activation, cells were analyzed for the expression of CD4 and Thy1.1 markers and for IFN-γ production. The frequency of IFN-$\gamma^+$ cells in the $CD4^+Thy1.1^+$ population and the number of $CD4^+Thy1.1^+$IFN-$\gamma^+$ (Th1) T cells are shown by the *upper* and *lower panels*, respectively. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.005$.

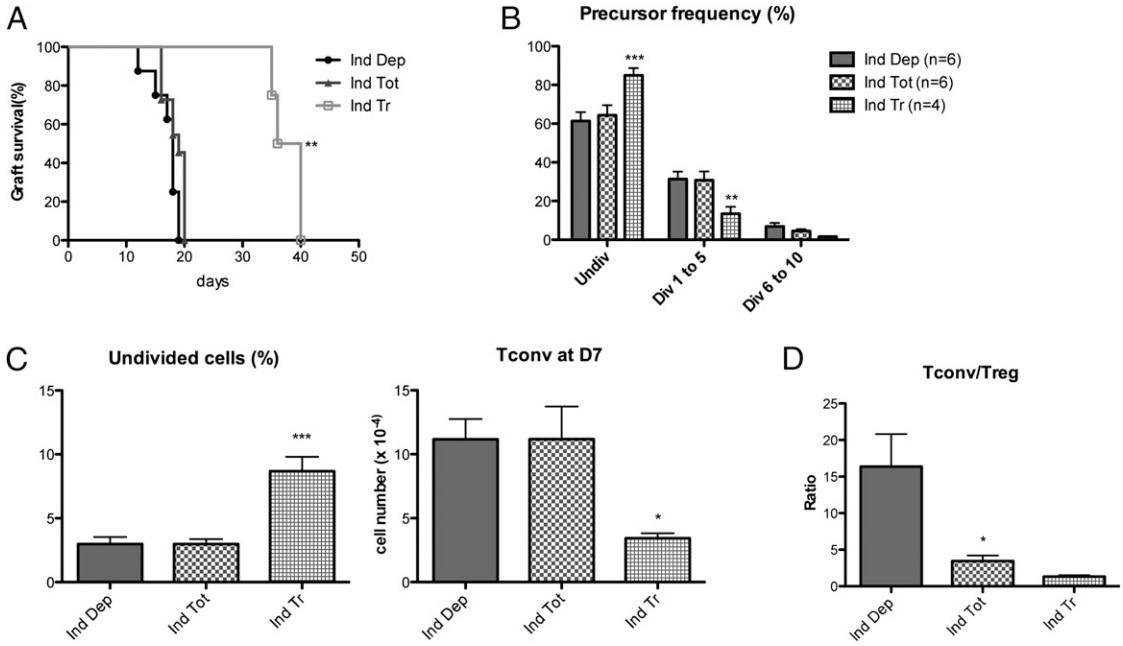


**FIGURE 5.** A high number of nTregs are necessary to inhibit Tconv proliferation in the indirect pathway. TCRαKOK mice were adoptively reconstituted as described in Fig. 2. An additional group (Tr) was reconstituted with $3 \times 10^6$ $CD4^+CD25^-$ T cells coinjected with $3 \times 10^6$ $CD4^+CD25^+$ nTregs. Recipients were grafted for the indirect pathway. (**A**) Mice were scored for grafted rejection. Data from two independent experiments are shown. (**B**–**D**) DLNs were collected at day 7 posttransplant. Cells were analyzed for the expression of Thy1.1 and CD4 markers and for CFSE dilution (B, C). $Foxp3^+$ T cells were excluded from all analyses of CFSE dilution. The calculated precursor frequency of undivided (Undiv) and divided (Div) cells in $CD4^+Thy1.1^+$ T cells is shown in (B). The percentage of undivided cells (*left panel*) and the number of $CD4^+Thy1.1^+Foxp3^-$ T cells (Tconv) found at day 7 (*right panel*) are shown in (C). Cells were analyzed for the expression of Thy1.1, CD25, and Foxp3 markers (D), and the ratios of Tconvs ($Thy1.1^+Foxp3^-$) and nTregs ($Thy1.1^+CD25^+Foxp3^+$) were calculated. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.005$.

suggesting that the highly immunogenic skin graft environment is able to overcome nTreg tolerance and induce rejection. These results are in agreement with those of Sánchez-Fueyo et al. (22), using a similar Tg model (ABM TCR Tg mice) to study the role of nTregs in the direct pathway during skin and heart graft rejection. Moreover, they are also consistent with the study from Carvalho-Gaspar et al. (23) showing that Tregs are able to control alloreactive $CD8^+$ T cells both in lymphoid organs and in the allograft.

In addition, we have shown that in contrast to the direct pathway, nTregs failed to control proliferation of Tconvs in DLNs in the indirect pathway. As the proportion of nTregs was similar at days 3, 5, and 7 between Dir Tot and Ind Tot mice (Fig. 3E), the failure to suppress Tconvs in the indirect pathway could not be attributed to a higher number of Tconvs or to delayed migration of nTregs in DLNs. Thus, we would argue that failure of nTregs to suppress Tconv proliferation can be attributed to a higher number of APCs presenting Hb(64–76) peptide–MHC complexes in DLNs. Indeed, the total number of leukocytes found in DLNs is much higher in Ind Tot mice (11.09 ± 1.66 million) than in Dir Tot mice (4.6 ± 0.82 million), suggesting that the inflammatory response is also more important in the indirect pathway. It is now clear that nTregs can control the maturation and the Ag-presenting functions of APCs (24, 25). Moreover, using two-photon laser-scanning microscopy, recent studies have demonstrated that in autoimmune models, nTregs interact with APCs rather than Tconvs, thus leading to a decreased number of contacts between Tconvs and APCs (26, 27). More specifically, in addition to inhibiting Tconv–APC contacts, nTregs are able to control cytokine production by APCs in DLNs, leading to decreased inflammation and cell recruitment (28, 29). Our study suggests that when present at a physiological ratio, nTregs in DLNs are not present in numbers sufficient to control activation and presentation by APCs in the

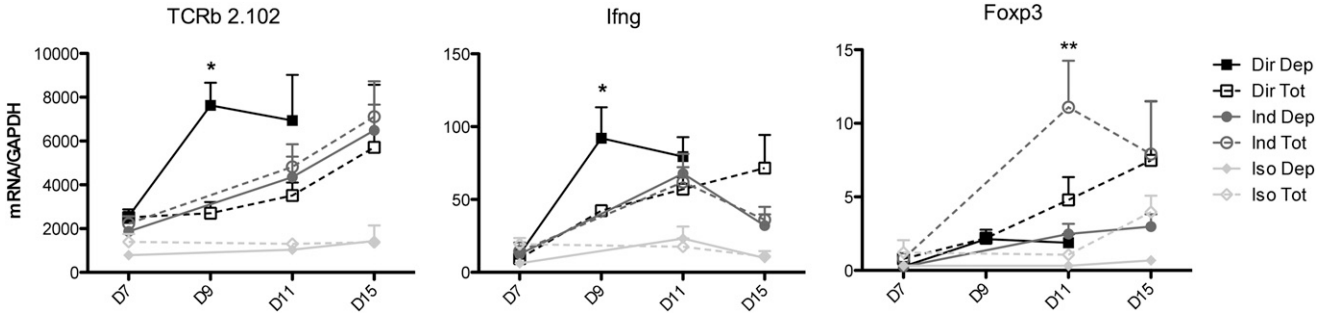


**FIGURE 6.** nTregs inhibit Tconv graft infiltration in the direct pathway. TCRαKOK mice were adoptively reconstituted and grafted as described in Fig. 2. Total RNA was obtained from allogeneic and isogeneic skin grafts retrieved at days 7 ($n$ = 4–6), 9 ($n$ = 4), 11 ($n$ = 4–7), and 15 ($n$ = 4–6) posttransplant and analyzed by qRT-PCR for 2.102 *Tcrb* (*left panel*), *Ifng* (*middle panel*), and *Foxp3* (*right panel*) expression. Fold changes in gene expression levels are relative to level observed in ungrafted skin of each respective pathway and calibrated with the housekeeping gene *Gapdh*. $*p \leq 0.05$, $**p \leq 0.01$.

indirect pathway. One could argue that a higher affinity of the 2.102 TCR for the Hb(64–76) peptide/I-$E^k$ (indirect) ligand may be responsible for the lack of regulation in indirect alloreactivity. However, many arguments seem to contradict this hypothesis. First, our Ag-specific nTregs and Tconvs share the same TCR, so nTregs would be also strongly activated. Next, we demonstrated that Tconvs are more efficient at initiating graft rejection in the direct pathway, although the number of 2.102Tg T cells found in DLNs is higher in the indirect pathway, suggesting that Tconvs are not so strongly activated in the indirect pathway. These results are consistent with numerous studies showing that direct alloresponses play a major role in the early phase of rejection, whereas alloresponses via the indirect pathway are involved later in rejection (1, 6). Moreover, the kinetics of skin graft rejection we observed are similar to those found in other studies using specific T cells (11, 21).

Finally, we cannot ignore a potential contribution of $Foxp3^+$-induced Tregs (iTregs) in our model. A recent study, using color-coded proteins and endoscopic confocal microscopy, has demonstrated that iTregs can be generated in DLNs during islet pancreas transplantation and that these iTregs can infiltrate the graft (30). Furthermore, Ochando et al. (31) have shown that plasmacytoid dendritic cells were required to generate iTregs, suggesting that in regard to transplantation, only the indirect pathway would be involved. In our study, infiltration by iTregs could be responsible for the greater expression of *Foxp3* mRNA in indirect pathway allografts. Thus, although iTregs might be generated in this pathway, they would be induced too late to control Tconv proliferation but could control later differentiation into Th1 cells. Of interest, these iTregs were not found in Ind Dep mice, suggesting that nTregs are necessary for their generation, as described in recent studies (32, 33).

In conclusion, we have shown that in our model of direct pathway alloreactivity, nTregs were able to regulate the proliferation and differentiation of conventional T cells in DLNs and their graft infiltration but could not abrogate graft rejection, probably owing to the high number of allogeneic APCs present in the allograft and thus the high immunogenicity of the skin. In contrast, nTregs failed to regulate Tconv proliferation in DLNs in the indirect pathway at a physiological ratio. The latter pathway is an important player in chronic rejection, which today remains a major hurdle in transplantation. We suggest that the early control of Tconv proliferation in DLNs may be crucial to avoid this rejection. In this article, we show that only a high number of nTregs are able to control this proliferation. Thus, these results support recent therapeutic approaches aimed at producing and using in vitro–generated Ag-specific $Foxp3^+$ nTregs to control graft rejection during transplantation (34, 35).

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## Disclosures

The authors have no financial conflicts of interest.

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