Immunology and Cell Biology (2014), 1–7

www.nature.com/icb

ORIGINAL ARTICLE

Neuropilin-1$^+$ regulatory T cells promote skin allograft survival and modulate effector CD4$^+$ T cells phenotypic signature

Mauricio Campos-Mora, Rodrigo A Morales, Francisco Pérez, Tania Gajardo, Javier Campos, Diego Catalan, Juan Carlos Aguillón and Karina Pino-Lagos

During allograft rejection, several immune cell types, including dendritic cells, CD4$^+$ and CD8$^+$ T cells among others, recirculate between the graft and the nearest draining lymph node, resulting in immunity against the 'foreign' tissue. Regulatory CD4$^+$ T cells are critical for controlling the magnitude of the immune response and may act to promote or maintain tolerance. They are characterized by the expression of CD25 and Foxp3, and more recently, Neuropilin-1 (Nrp1). The role of these suppressor cells during allograft rejection is not well understood. Our work shows that during graft rejection, there is an increase in the frequency of total CD4$^+$ T cells expressing Nrp1, but the expression of this molecule is downregulated in the regulatory CD4$^+$ T-cell compartment. Interestingly, the expression of the transcription factor Eos, which renders cell function stability, is also reduced. In adoptive transfer experiments, we observed that during allograft rejection: (i) natural regulatory CD4$^+$ T cells maintain high levels of Nrp1 expression, (ii) effector CD4$^+$ T cells (Nrp1$^-$) become Nrp1$^+$Eos$^+$ and (iii) the transfer of regulatory CD4$^+$ T cells (Nrp1$^+$) can promote allograft survival, and also enhance the gain of Nrp1 and Eos on T-effector cells. Together, these data suggest that rejection occurs, at least in part, through the loss of Nrp1 expression on regulatory CD4$^+$ T cells, their stability or both. Additionally, the transfer of regulatory CD4$^+$ T cells (based on Nrp1 expression) permits the acceptance of the allograft, placing Nrp1 as a new target for immune therapy.
Immunology and Cell Biology advance online publication, 23 September 2014; doi:10.1038/icb.2014.77

INTRODUCTION

Transplantation represents a unique opportunity to restore the function of a particular tissue after suffering a disabling injury or failure. However, transplant rejection is still a major clinical problem, despite the use of immunosuppressive drugs to overcome the inflammatory response in the graft for reaching long-term transplant tolerance. Recently, evidence had shed light on the orchestrated cellular processes governing allograft rejection. In a murine model of mismatched skin allograft rejection, the use of intravital immune-imaging techniques has allowed the analysis of the temporal interplay between different immune cells during allograft rejection.[1] After the initial influx of recipient monocytes to the graft and their migration to the draining lymph nodes bringing foreign antigens, the priming of T cells enables their proliferation and subsequent migration to the tissue. This is followed by a massive infiltration of monocytes into the graft, along with both CD4$^+$ and CD8$^+$ effector T cells migrating from adjacent tissues to the graft. In this model, about 10 days post-transplant, CD8$^+$ T cells have destroyed most of the foreign cells, leading to necrosis and total rejection.[2]

Among relevant cell populations during immune responses, strong evidence indicates the essential role of regulatory T cells (Treg).[3,4] Treg cells are a very heterogeneous population with the capacity to modulate the immune system through several mechanisms.[5] They are characterized by the expression of the master regulator Foxp3,[6,7] and they can be mainly found as naturally ocurring thymus-derived Treg cells (nTregs) or as peripheral-induced Treg cells (iTregs).[8,9] While nTregs are produced in the thymus as a functionally mature population, iTregs differentiate from naive T cells in the periphery acquiring both Foxp3 expression and suppressive capacity.

In order to harness the therapeutic potential of Tregs, it is necessary to have reliable markers so one can identify or isolate them. Thus, several markers have been proposed to distinguish Treg populations under different physiological conditions. The constitutive expression of Foxp3, the interleukin (IL)-2-receptor alpha-chain (CD25), the cytotoxic T-cell-associated antigen-4 (CTLA-4) or the glucocorticoid-induced tumor necrosis factor receptor family-related gene/protein represent the classical phenotype of most Treg cells, and the expression of these molecules is stabilized and amplified by Foxp3.[10] Moreover, nTregs display high expression of programmed

Immune Regulation and Tolerance Research Group, Programa Disciplinario de Inmunología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile
Correspondence: Professor K Pino-Lagos, Immune Regulation and Tolerance Research Group, Programa Disciplinario de Inmunologia, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Av. Independencia 1027, 3er Piso, Pabellón H, Santiago 8380453, Chile.
E-mail: kpino@med.uchile.cl or karina.p.lagos@gmail.com
Received 30 May 2014; revised 24 August 2014; accepted 24 August 2014

cell death-1, CD73, the transcription factors Helios and Eos[11–13] and Neuropilin-1 (Nrp1), the focus of this work.

Nrp1 is a multifunctional co-receptor first described to be involved in migration and axonal guidance in central nervous system[14,15] and later shown to be an essential component of the immunological synapse in humans, given its expression on dendritic cells and T cells, and the partial inhibition of cell proliferation in allogeneic co-cultures in the presence of anti-Nrp1 antibodies.[16] In the last decade, Nrp1 has been repeatedly proposed as a Treg cell marker since it was found to be preferentially expressed on $CD4^+$ $CD25^+$ Tregs with unchanged expression levels upon activation, in contrast to $CD4^+$ T-conventional cells, which loose Nrp1 expression under this condition.[17] In addition, Nrp1 expression on Treg cells correlate with Foxp3 expression and suppressive capacity.[17] One of the contributions of this molecule might consist of prolonging the interaction between dendritic cells and Treg cells during antigen presentation by homotypic interactions.[18] Recently, it has been proposed that, under certain physiological conditions, Nrp1 can be used to distinguish between nTregs (expressing high levels, or $Nrp1^{high}$) from iTregs ($Nrp1^{low}$),[13,19] and it has been demonstrated that molecules such as transforming growth factor-β and IL-6 induce and inhibit Nrp1 expression, respectively.[19]

As mentioned above, $Foxp3^+$ Treg cells are known to suppress inflammatory responses.[20] However, under certain physiological conditions Treg cells can change (or be 'reprogrammed') toward a pro-inflammatory phenotype.[21] It is unclear to what extend that Treg cells reprogramming occurs under physiological circumstances.[21,22] Recently, it has been described that loss of Eos, a co-repressor expressed on $Foxp3^+$ Tregs, has a critical role in mediating the IL-6-dependent transition of certain subset of $Foxp3^+$ Tregs from suppressor cells to 'helper-like' Treg cells without a loss on Foxp3 expression.[23] In addition, one study observed decreased mRNA expression levels of Eos on peripheral blood $CD4^+$ $CD25^{high}$ $CD127^{low/-}$ Treg cells from stem cell transplantation patients affected with acute or chronic graft-versus-host disease.[24] These results correlate with those of a previous report, suggesting reduced immune suppressive function of Tregs in graft-versus-host disease patients.[25] Conversely, Eos expression is upregulated on *in vitro* T-cell receptor-activated $CD4^+$ T cells as well.[26] Altogether, these observations suggest an unclear role of Eos in $CD4^+$ T-cell biology.

Given that little is known about Nrp1 expression on Treg cells in the context of the immune response to transplant, specifically regarding to the identity and phenotypic stability of Treg cells, our aim was to study the role of Tregs during graft rejection using Nrp1 as a surface marker in a murine model of skin transplantation.

RESULTS

Among T cells, Nrp1 is mainly expressed on Tregs

Initially the expression of Nrp1 was detected on neurons, but later its presence on dendritic cells, B cells and T cells was also proved.[27] In order to study the dynamic of Nrp1 expression on T cells, we first analyzed the expression of Nrp1 on $CD4^+$ and $CD8^+$ T cells from the spleen, peripheral lymph nodes and thymus that were removed from intact wild-type (WT) C57Bl/6 mice. As shown in Figure 1a, Nrp1 expression is mostly found in approximately 15–20% of $CD4^+$ T cells from the spleen and peripheral lymph nodes. In thymus, both $CD4^+$ and $CD8^+$ T cells expressed Nrp1 at similar levels (~10%). When we gated on $CD4^+$ $CD25^{high}$ T cells, ~90% of them were $Nrp1^+$, and ⩾80% express Foxp3; Figure 1b (left and middle columns) and Supplementary Figure 1. We consider $CD4^+$ $CD25^{high}$ T cells as Tregs, and to confirm this we performed the same staining and analysis strategy on cells from C57Bl/6-$Foxp3^{GFP}$ mice. As depicted in the right column, $CD4^+$ $CD25^{high}$ T cells correspond to Tregs as ⩾80% of them are $Foxp3^{GFP+}$ and, similar to WT cells, all Tregs are $Nrp1^+$.

Nrp1 expression is downregulated on Tregs during skin graft rejection

During an inflammatory response, iTreg cells can become $Nrp1^+$ in the inflamed zone, while splenic iTregs remains $Nrp1^{low}$.[19] It is also described that conventional effector T cells can express Nrp1 but not Foxp3, indicating an effector-activated memory status.[13,19] Based on

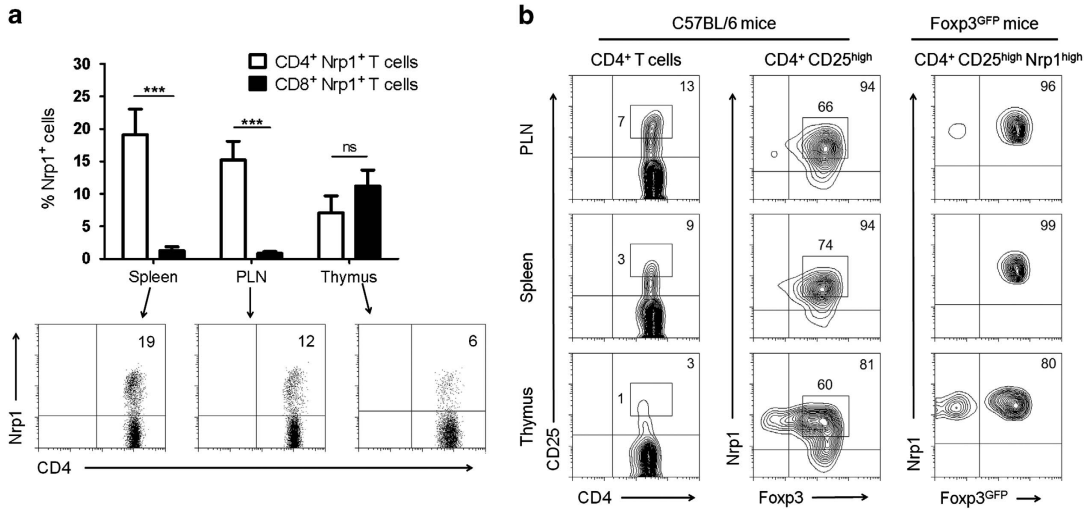

Figure 1 Nrp1 is expressed on $CD4^+$ T cells, mainly Tregs, among different immune organs. To analyze the expression of Nrp1 on $CD4^+$ and $CD8^+$ T cell compartment, cell suspensions were prepared from the thymus, spleen and peripheral lymph nodes (PLN), previously removed from WT C57Bl/6 mice, stained with specific antibodies to recognize Nrp1 on $CD4^+$ and $CD8^+$ T cells, and analyzed by flow cytometry. (**a**) Graph with the frequency of Nrp1 expression on $CD4^+$ and $CD8^+$ T cells from the mentioned tissues are depicted, with a representative dot plot for the $CD4^+$ T cell compartment. (**b**) Using the same procedure than in **a**, cells were stained for CD4, CD25, Foxp3 and Nrp1 to identify and associate Nrp1 expression on $Foxp3^+$ regulatory T cells (Tregs) from either WT C57Bl/6 mice (left and middle column) or C57Bl/6-$Foxp3^{GFP}$ reporter mice (right column). Bars correspond to s.d., and the statistical significance was assessed by analysis of variance, $***P=0.001$, $n=6$ independent experiments.

this, we wanted to address if the inflammatory milieu driven by the recognition and rejection of allo-antigens affect the frequencies of Tregs and the expression of Nrp1 in an *in vivo* model of skin graft transplantation. C57Bl/6 recipients received either syngeneic (C57Bl/6) or allogeneic (C57Bl/6 × Balb/c, F1) skin grafts as described before,[28] and at day 10, skin graft-draining lymph nodes were removed to analyze the expression of Nrp1. As shown in Figure 2a, the frequency of total activated $CD4^+$ $Nrp1^+$ T cells is increased during allograft rejection, but if we restricted the population to Tregs ($CD25^{high}$), the expression level of Nrp1 is significantly reduced (~50% versus ~70% in syngeneic condition). As the function of Tregs is to suppress an immune response enabling allograft acceptance, one could presume that during rejection Tregs are not suppressive due to a loss on their stability. To test this, we studied Eos on $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells, a molecule associated with Treg cell functional stability.[23] Interestingly, and according to the changes observed for Nrp1, the expression of Eos is downregulated during inflammation, where ~50% of Tregs express Eos in the allogeneic condition, compared with ~60% in syngeneic and ~80% in non-grafted mice (Figure 3), suggesting that under inflammation triggered by an allogeneic immune response Tregs may loose functional stability leading to the rejection of the graft.

$Nrp1^+$ Tregs promote skin survival and are necessary for a greater Nrp1 and Eos expression by effector $CD4^+$ T cells

It has been reported that the transfer of $CD4^+$ $Nrp1^+$ T cells into heart-transplanted mice allows the acceptance of the graft.[29] Based on this information, we wanted to understand the mechanism by which this process takes place. For this, we designed an *in vivo* approach in which $CD4^+$ $CD25^{high}$ $Nrp1^+$ Tregs (>90% expressing $Foxp3^+$ as confirmed by intracellular staining, as shown in Supplementary Figure 1) sort purified from WT C57Bl/6 mice and effector $CD4^+$ $CD25^-$ Nrp1-$Foxp3^{GFP-}$ T cells isolated from congenic (CD45.1 or $Ly5.2^+$) C57BL/6 $Foxp3^-$ GFP reporter mice are adoptively transferred into $RAG^{-/-}$ recipient animals (see scheme in Figure 4a). One day post cell transfer, all recipient mice received an allogeneic graft (F1 skin) and transplant survival was monitored over time. As shown in Figure 4b, the cotransfer of $Nrp1^+$ Tregs with effector $CD4^+$ $CD25^-$ Nrp1-$Foxp3^{GFP-}$ T cells promoted skin graft acceptance in ~60% of mice, as compared with the rejection control ($CD4^+$ $CD25^-$ Nrp1-$Foxp3^{GFP-}$ T cells only), in which all mice rejected their grafts. Next, we wanted to analyze the phenotype of the transferred cells. By gating on $CD4^+$ $Ly5.2^-$ T cells, which corresponds to the transferred $Ly5.1^+$ Tregs, we found that the expression of Nrp1 was reduced by ~10% of Tregs in both syngeneic and allogeneic inflammatory milieu, as compared with freshly sorted Tregs (⩾ 98% $Nrp1^+$; Figure 4c, top contour plots and graph). When we analyzed the phenotype of the effector $Ly5.2^+$ $CD4^+$ T cells, we observed that ⩾50% of them gained Nrp1 expression. Interestingly, in the allogeneic condition, the upregulation of Nrp1 expression by the effector $Ly5.2^+$ $CD4^+$ T cells was higher when cotransferred with $Nrp1^+$ Tregs than when effector $Ly5.2^+$ $CD4^+$ T cells are alone (Figure 4c, bottom contour plots and graph). Furthermore, the effector $Ly5.2^+$ $CD4^+$ T cells did not upregulate Foxp3 expression suggesting iTregs (Th3-type) are not generated (Supplementary Figure 2), but ~50% of the newly Nrp1-expressing effector $Ly5.2^+$ $CD4^+$ T cells upregulated Eos expression

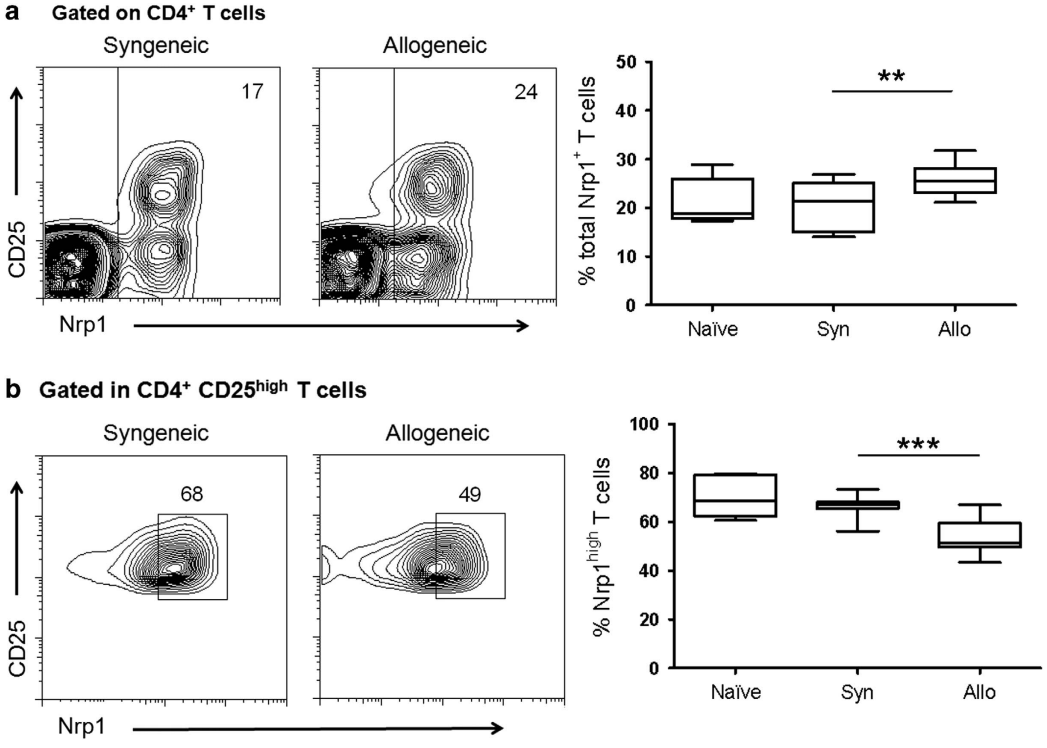

Figure 2 Tregs downregulate Nrp1 expression during allograft rejection. C57BL/6 mice were transplanted with either syngeneic (C57BL/6) or allogeneic (F1, C57BL/6 × Balb/c) skin grafts. Ten days after surgeries, skin graft-draining lymph nodes were removed and cell suspensions were prepared to study Nrp1 expression on the $CD4^+$ T-cell compartment. (**a**) The contour plot shows the expression of CD25 and Nrp1 on total $CD4^+$ T cells, and the frequencies (%) of $CD4^+$ $Nrp1^+$ T cells are depicted in the graph (right). (**b**) The contour plot shows the expression of CD25 and Nrp1 on Tregs. The frequencies (%) of Tregs expressing high levels of Nrp1 are depicted in the graph (right). Bars correspond to s.d., and the statistical significance was assessed by unpaired Student's *t*-test (Mann–Whitney *U*-test), $^{**}P = 0.01$, $^{***}P = 0.001$, n = at least two independent experiments.

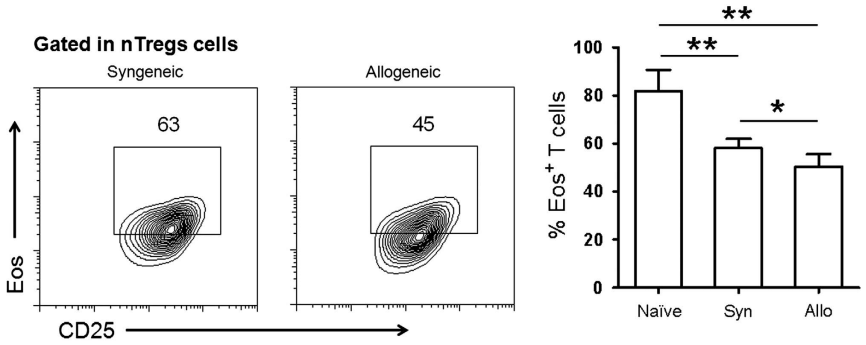

Figure 3 Tregs loose Eos expression during allograft rejection. C57BL/6 mice were transplanted with either syngeneic (C57BL/6) or F1 (C57BL/6 × Balb/c) skin grafts. Ten days after surgeries, skin graft-draining lymph nodes were removed and cell suspensions were prepared to study CD4, CD25, Foxp3 and Eos expression by flow cytometry. The contour plot shows the expression of Eos and CD25 on CD4$^+$ CD25^{high} Foxp3$^+$ Tregs, and the frequencies (%) of these cells are depicted in the graph (right). Bars correspond to s.d., and the statistical significance was assessed by unpaired Student's *t*-test (Mann–Whitney *U*-test), $*P=0.05$, $**P=0.01$, $n=$ two independent experiments.

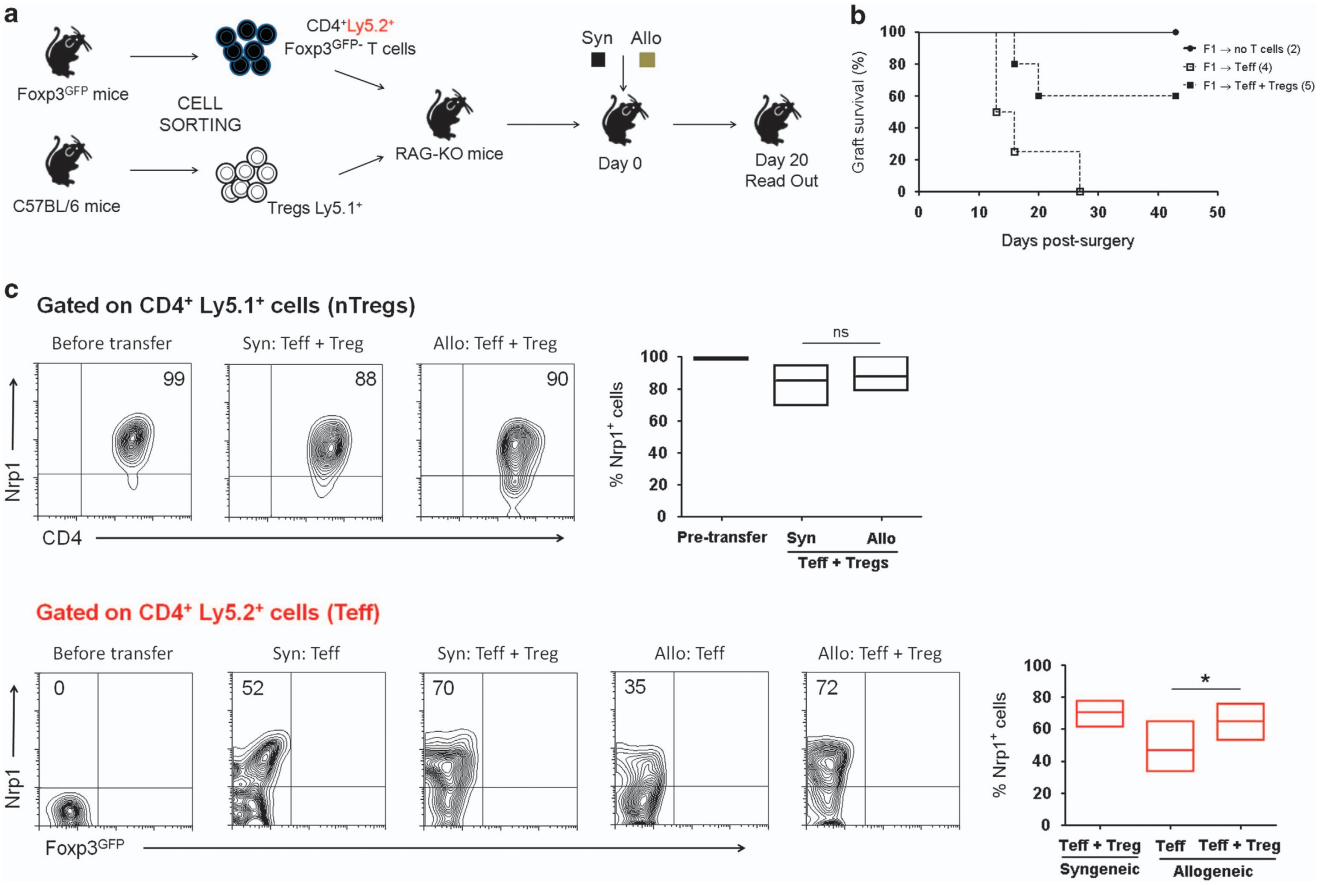

Figure 4 Nrp1$^+$ Tregs promote skin survival and are necessary for a greater Nrp1 expression by effector CD4$^+$ T cells. (**a**) Ly5.1$^+$ Treg and Ly5.2$^+$ CD4$^+$ Foxp3^{GFP-} T cells were sorted from C57BL/6 and Foxp3^{GFP} reporter mice, respectively, and transferred into RAG$^{-/-}$ mice (day 1). The next day, mice were transplanted with either syngeneic or allogeneic skin grafts (day 0) and graft survival was monitored two times per week. At day 20, skin graft-draining lymph nodes were removed and cell suspensions were prepared to study Foxp3, Nrp1 and Eos expression on both Ly5.2$^+$ or Ly5.1$^+$ T cells by flow cytometry. (**b**) Graph displaying the skin graft survival from mice receiving no T cells (filled circle), CD4$^+$ T-effector cells only (open square and dashed line) and CD4$^+$ T-effector cells plus Tregs (filled square). (**c**) The contour plots (top) show the expression of Nrp1 on Tregs together with the frequencies (%) of these cells (depicted in the graph) in freshly sorted Tregs cells, syngeneic and allogeneic conditions. On the bottom, contour plots depict the expression of Nrp1 and Foxp3^{GFP} on CD4$^+$ T cells ('Eff') under the conditions mentioned above, in addition to the frequencies (%) of these cells (graph on the right). For **c**, boxes correspond to minimum and maximum values, line is the mean, and the statistical significance was assessed by unpaired Student's *t*-test (Mann–Whitney *U*-test), $*P=0.05$, NS, nonsignificant; $n=$ three independent experiments.

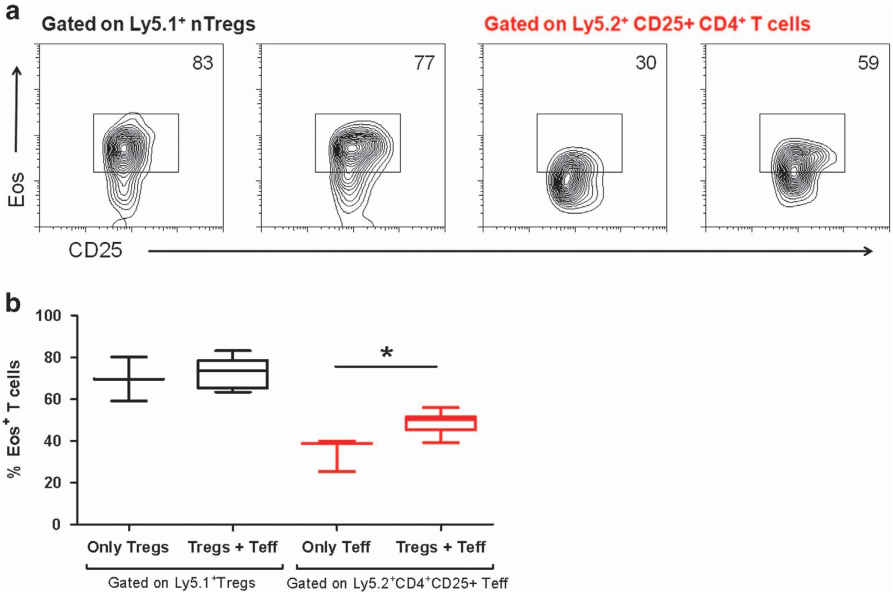

Figure 5 CD4+ T-effector cells gain Eos expression when cotransferred with Nrp1+ Tregs. Cell transfer and skin graft surgeries were performed as in Figure 4. (**a**) The contour plots show the expression of Eos and CD25 on Ly5.1+ Tregs when transferred alone or with effector CD4+ T cells ('Eff'), in the allogeneic condition. Similarly, same analysis is depicted when gating on Ly5.2+ CD4+ CD25high T cells instead (two plots on right). (**b**) Graph showing the frequencies of Eos+ cells on Ly5.1+ Tregs or Ly5.2+ CD4+ T-effector cells, when transferred alone or combined, in allogeneic condition. Bars correspond to minimun and maximun values, and the statistical significance was assessed by unpaired Student's *t*-test (Mann–Whitney *U*-test), $*P=0.05$. $n=$two independent experiments. KO, knockout.

when cotransferred with Nrp1+ Tregs, versus ~20% of Ly5.2+ CD4+ T cells alone (Figures 5a and b), confirming the results obtained above when WT animals are used as transplant recipients.

DISCUSSION

Evidence from both murine and clinical studies suggests that Nrp1 expression in CD4+ T cells may have an important role in the context of allograft rejection.[27] It has been reported that CD4+ Nrp1+ T cells transferred to ectopic heart-allograft recipient mice extend survival time of the graft by inhibiting the production of pro-inflammatory cytokines, enriching Foxp3+ Treg population and inducing anergy on effector T cells.[29] In the present work, we studied the dynamics on Nrp1 expression on CD4+ T-cell populations during allograft rejection. Our observations indicate that Nrp1 is mainly expressed on peripheral CD4+ T cells, and its expression is tightly associated with CD4+ CD25high Foxp3+ T cells, supporting previous data in which Nrp1 is proposed as a marker for nTregs.[17] Importantly, during *in vivo* transplant experiments we observed that Tregs downregulate Nrp1 expression only when an effect on the immune response is mounted against the allograft (that is, allogeneic versus syngeneic). As the presence of Nrp1 is associated with the suppressive capabilities of Tregs to perform their regulatory or inhibitory function,[17] one could presume that the downregulation of Nrp1 expression on Tregs during an allogeneic response is one of the mechanism by which rejection takes place. In the same experimental settings, the loss of Nrp1 on Tregs was associated with reduced expression of Eos, a molecular marker for Tregs stability.[23] Currently, it is accepted that T cells, including Tregs, can be reprogrammed under suppressive or inflammatory conditions, losing their initial profile and function.[30,31] In the particular case of Tregs, it has been reported that they can loose their Foxp3 expression on lymphopenic conditions or in autoimmune settings such as diabetes and experimental autoimmune encephalomyelitis (EAE), or that Tregs can acquire effector Th-cell characteristics while maintaining Foxp3 expression.[32] In line with this, it has been proposed that there is a heterogeneity in Foxp3+ cells, in which not all Foxp3+ cells are bona fide Tregs.[33] Recently, it has been proposed that the stable expression of Eos define a subpopulation of Tregs that remains suppressive upon inflammatory conditions, in comparison with Tregs lacking Eos expression that loose their suppressive capacity, without affecting Foxp3 expression.[23] Therefore, the loss of Eos together with Nrp1 could contribute to a defective Treg functionality leading to the rejection of the allograft. As mentioned above, Nrp1+ CD4+ T cells permit allograft tolerance,[29] and we recapitulate this observation in a murine skin transplantation model. Our data support the regulatory properties of the Nrp1+ CD4+ T cells, as the transfer of CD4+ CD25high Nrp1+ T cells into RAG−/− skin-grafted mice can be correlated with allograft acceptance and with changes in the inflammatory milieu surrounding the allograft, as the amounts of several inflammatory cytokines, such as IFN-γ, IL-17 and IL-6 are reduced in the skin grafts of Nrp1+ Tregs-treated animals (data not shown).

Most interesting is the fact that the cotransfer of Nrp1+ Tregs with effector Nrp1− CD4+ T cells permits the gain of Nrp1 and Eos expression by the effector counterpart, suggesting that Tregs may allow a fraction of the former to become suppressive, mediating the acceptance of the graft observed in the *in vivo* experiments. With the attempt to gain more insights on the mechanisms behind the results discussed here, preliminary work using an anti-Nrp1 blockade antibody showed, unexpectedly, that the blockade of Nrp1 did not abrogate the gaining of Nrp1 and Eos expression on effector T cells nor alter the acceptance of the allograft. Conversely, we observed a higher number of CD4+ effector T cells in skin graft-draining lymph nodes and augmented levels of inflammatory cytokines in the allograft (which can be a consequence of targeting Nrp1+ Tregs), and an upregulated expression of Nrp1 and Eos on CD4 T cells (which has been reported for recently activated T cells). Besides these arguments,

we believe this approach is not the optimal setting, considering that the amount of antibody injected may not be sufficient to block Nrp1 (in membrane and free). The use of Nrp1 knockout or conditional knockout mice would help to clarify these preliminary observations. Taking together, these data does not discredit the results shown in this article, but it implies that further studies are necessary for a better understanding of the mechanisms under Nrp1 and Eos function on both effector $CD4^+$ T cells and Tregs.

In the clinical setting, Nrp1 expression on T cells has been proposed as a putative predictor of transplant rejection, as a lower frequency of infiltrating $Nrp1^+$ lymphocytes was observed in kidney graft biopsies from patients under acute rejection, compared with biopsies from accepted transplants.[34] The authors suggested that the reduction of Tregs ($Nrp1^+$ cells) in transplants could be linked with the development of the rejection process.

Taken together, our data describe an unknown dynamic for Nrp1 expression on $CD4^+$ T cells during allograft rejection, and a new possible mechanism by which effector $CD4^+$ T cells could mediate allograft acceptance where the acquisition of the Nrp1 and Eos molecules may have a relevant role.

METHODS

Mice

Six- to eight-week-old WT C57Bl/6 ($CD45.2^+$ or $Ly5.1^+$), BALB/c, $RAG^{-/-}$ and Foxp3-eGFP reporter mice (C57Bl/6 background and $CD45.1^+$ or $Ly5.2^+$) were used in this study. The Foxp3-GFP reporter mice were kindly provided by J Rodrigo Mora (Harvard Medical School, Cambridge, MA, USA). BALB/c × C57BL/6 (or $F1^{dxb}$) mice (skin allograft donors) were obtained by crossing BALB/c mice ($H2^d$) with WT C57BL/6 mice ($H2^b$). Mice were maintained in accordance with the Bioethical Committee guidelines from the Facultad de Medicina, Universidad de Chile, Santiago, Chile.

Media and reagents

RPMI-1640 (Gibco BRL, Grand Island, NY, USA) was supplemented with 10 mM HEPES, 100 IU ml^{-1} penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (Gibco BRL) and 50 μM 2β-mercaptoethanol.

Flow cytometry and cell sorting

Flow cytometry analyses were performed using anti-mouse CD4 (cloneRM4-5), CD8 (clone 53-6.7), CD25 (clone PC61.5), Foxp3 (clone FJK-16S), Eos (clone ESB7C2), CD45.1 (clone A20; all from eBioscience, San Diego, CA, USA) and anti-Nrp1 (R&D Systems, Minneapolis, MN, USA), all conjugated with fluorescein isothiocyanate, phycoerythrin (PE), PerCP, PerCP-Cy5.5 or APC. Flourescence-activated cell sorting data acquisition was performed with flourescence-activated cell sorting Calibur (Beckton Dickinson, Franklin Lakes, NJ, USA), using CellQuest software (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (Tree Star, Canton, OH, USA).

For cell sorting experiments, $CD4^+$ T cells were enriched using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (StemCell, Vancouver, BC, Canada) according to the manufacturer's recommendations. Then, $CD4^+$ T cells were labeled with anti-CD4, anti-CD25, anti-CD45.1 and anti-Nrp1 antibodies. $CD4^+$ $CD25^{high}$ $Nrp1^+$ T cells (or Tregs) and $CD4^+$ Nrp1-$Foxp3^{GFP-}$ T cells (or effectors), from both WT C57Bl/6 and Foxp3-GFP reporter mice, respectively, were separated using a BD FACSAria III equipment (Franklin Lakes, NJ, USA), with purity ⩾96%.

Skin transplantation

Skin grafting was performed as described previously.[28] Briefly, tail skin (~1 cm^2) from C57BL/6 (syngeneic) or F1 (allogeneic) donors was transplanted onto the dorsal area of C57BL/6 WT or $RAG^{-/-}$ recipients. Survival of skin allografts was evaluated two times per week and grafts were considered rejected when 80% of the original graft disappeared or become necrotic. When indicated, skin grafts were collected, cut in small pieces and incubated in complete RPMI (at 1 mg ml^{-1} of tissue). After 2 h, supernatant were collected and stored at − 80 °C for cytokine quantification (enzyme-linked immunosorbent assay).

Adoptive transfer experiment

$RAG^{-/-}$ mice received 1.5×10^5 $Ly5.2^+$ $CD4^+$ Nrp1-$Foxp3^{GFP-}$ T cells and/or 5×10^4 $CD4^+$ $CD25^{high}$ $Nrp1^+$ T cells via tail-vein injection, 1 day prior to skin transplantation. At day 20 post-surgery, mice were killed and draining lymph nodes were removed to prepare cell suspensions for further flow cytometry analysis.

Statistical analysis

Data were analyzed using an unpaired Student's *t*-test or a Mann–Whitney *U*-test (two-tailed). Survival rate was analyzed by the Kaplan–Meier method, and comparisons were made by log-rank analysis. In all cases, $P<0.05$ was considered with statistical significance. For data analysis, GraphPad (Prism, La Jolla, CA, USA) software was used.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are very grateful to Dr Randolph J Noelle (Dartmouth College, USA) for helping with the acquisition of certain reagents, to Dr Arturo Ferreira (Universidad de Chile), Dr Mario Rosemblatt and Dr Rodrigo Pacheco (Fundación Ciencia & Vida, Chile) for facilitating the performance of some experiments (facility/equipment), to Chrysothemis Brown (King's College, London, UK) for reading and editing the manuscript and to Ruth Mora (Universidad de Chile) for technical assistance. This work was supported by CONICYT (Grant 791100001) and FONDECYT (Grant 11121309).

1 Cahalan MD. Imaging transplant rejection: a new view. *Nat Med* 2011; **17**: 662–663.
2 Celli S, Albert ML, Bousso P. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. *Nat Med* 2011; **17**: 744–749.
3 Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 2012; **30**: 531–564.
4 Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**: 775–787.
5 Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses?. *Int Immunol* 2009; **21**: 1105–1111.
6 Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev* 2011; **241**: 260–268.
7 Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity* 2013; **38**: 414–423.
8 Lin X, Chen M, Liu Y, Guo Z, He X, Brand D *et al.* Advances in distinguishing natural from induced Foxp3(+) regulatory T cells. *Int J Clin Exp Pathol* 2013; **6**: 116–123.
9 Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 2009; **30**: 626–635.
10 Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 2007; **445**: 771–775.
11 Getnet D, Grosso JF, Goldberg MV, Harris TJ, Yen HR, Bruno TC *et al.* A role for the transcription factor Helios in human CD4(+)CD25(+) regulatory T cells. *Mol Immunol* 2010; **47**: 1595–1600.
12 Himmel ME, MacDonald KG, Garcia RV, Steiner TS, Levings MK. Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *J Immunol* 2013; **190**: 2001–2008.
13 Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S *et al.* Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets *in vivo*. *J Exp Med* 2012; **209**: 1713–1722.
14 Fujisawa H. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. *J Neurobiol* 2004; **59**: 24–33.
15 Fujisawa H, Kitsukawa T, Kawakami A, Takagi S, Shimizu M, Hirata T. Roles of a neuronal cell-surface molecule, neuropilin, in nerve fiber fasciculation and guidance. *Cell Tissue Res* 1997; **290**: 465–470.
16 Tordjman R, Lepelletier Y, Lemarchandel V, Cambot M, Gaulard P, Hermine O *et al.* A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat Immunol* 2002; **3**: 477–482.
17 Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K *et al.* Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol* 2004; **34**: 623–630.

18 Sarris M, Andersen KG, Randow F, Mayr L, Betz AG. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* 2008; **28**: 402–413.
19 Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN *et al.* Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* 2012; **209**: 1723–1742.
20 Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 2009; **30**: 636–645.
21 da Silva Martins M, Piccirillo CA. Functional stability of Foxp3+ regulatory T cells. *Trends Mol Med* 2012; **18**: 454–462.
22 Sakaguchi S, Vignali DA, Rudensky AY, Niec RE, Waldmann H. The plasticity and stability of regulatory T cells. *Nat Rev Immunol* 2013; **13**: 461–467.
23 Sharma MD, Huang L, Choi JH, Lee EJ, Wilson JM, Lemos H *et al.* An inherently bifunctional subset of Foxp3+ T helper cells is controlled by the transcription factor eos. *Immunity* 2013; **38**: 998–1012.
24 Ukena SN, Geffers R, Buchholz S, Stadler M, Franzke A. Biomarkers for acute and chronic graft-versus-host disease in regulatory T cells. *Transpl Immunol* 2012; **27**: 179–183.
25 Ukena SN, Velaga S, Geffers R, Grosse J, Baron U, Buchholz S *et al.* Human regulatory T cells in allogeneic stem cell transplantation. *Blood* 2011; **118**: e82–e92.
26 Rieder S, Shevach E. The role of Eos in T cell function (P1059). *J Immunol* 2013; **190** (Meeting Abstracts 1) 121.1.
27 Campos-Mora M, Morales RA, Gajardo T, Catalan D, Pino-Lagos K. Neuropilin-1 in Transplantation Tolerance. *Front Immunol* 2013; **4**: 405.
28 Quezada SA, Bennett K, Blazar BR, Rudensky AY, Sakaguchi S, Noelle RJ. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J Immunol* 2005; **175**: 771–779.
29 Yuan Q, Hong S, Shi B, Kers J, Li Z, Pei X *et al.* CD4(+)CD25(–)Nrp1(+) T cells synergize with rapamycin to prevent murine cardiac allorejection in immunocompetent recipients. *PLoS ONE* 2013; **8**: e61151.
30 Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. *Immunology* 2013; **138**: 105–115.
31 Zhu J, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev* 2010; **238**: 247–262.
32 Hori S. Lineage stability and phenotypic plasticity of Foxp3+ regulatory T cells. *Immunol Rev* 2014; **259**: 159–172.
33 Hori S. Developmental plasticity of Foxp3+ regulatory T cells. *Curr Opin Immunol* 2010; **22**: 575–582.
34 Zhou H, Zhang L, Tong L, Cai M, Guo H, Yang C *et al.* Expression of neuropilin-1 in kidney graft biopsies: what is the significance? *Transplant Proc* 2007; **39**: 81–83.

The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)