RAPID COMMUNICATION

# Anti-C4d chimeric antigen receptor regulatory T cells suppressed allograft rejection in ABO-incompatible heart transplantation

ABO blood group-incompatible (ABOi) transplantation has been developed to overcome the serious problem of donor organ shortage. However, antibody-mediated rejection (ABMR) remains as the main limitation to successful ABOi transplantation. Introduction of desensitization treatment improved the outcomes of ABOi transplantation by suppressing ABMR; however, this strong, nonspecific immunosuppression also increases infectious complications. Recently, chimeric antigen receptor regulatory T cells (CAR Tregs) were developed to improve the antigen specificity, viability, and suppressive activity of Tregs. C4d deposition is a marker of ABMR and is also found in most ABOi allograft tissues. Based on these findings, we developed anti-C4d CAR Tregs to suppress ABMR in ABOi allografts. Anti-C4d CAR Tregs prepared by retroviral transduction of CAR into CD62L$^+$CD4$^+$CD25$^+$ Tregs, expressed forkhead box P3 (Foxp3), CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), latency-associated peptide (LAP), and glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) to similar extents as non-transduced Tregs. Anti-C4d CAR Tregs were activated by specific binding to C4d and suppressed *in vitro* T cell proliferation as well as non-transduced Tregs. Furthermore, adoptive transfer of anti-C4d CAR Tregs significantly prolonged mouse ABOi heart allograft survival ($P < 0.05$).

Regulatory T cells (Tregs) can contribute to donor-specific transplantation tolerance while having much fewer adverse effects than nonspecific immunosuppression. Infusion of Tregs has been shown to suppress allograft rejection. However, the number of antigen-specific Tregs is very low and Tregs often lose their viability and activity after infusion. Recently, chimeric antigen receptor (CAR) T cells showed strong anti-tumor effects by specifically targeting tumor antigens. In parallel, CAR Tregs can augment the antigen-specificity, viability as well as activity of conventional Tregs.[1–3] A CAR consists of a single chain variable fragment (scFv) of an antibody in the extracellular domain to provide antigen-specificity to Tregs; and they also possess costimulatory molecules in the intracellular domains to improve the viability and activity of Tregs.

Complement activation is often involved in ABMR and the deposition of complement component 4d (C4d) as a byproduct of antibody-mediated complement activation is included in the diagnostic criteria of ABMR.[4] Interestingly, C4d deposition is observed in 80—90% of ABOi transplantation cases as a result of either ABMR or accommodation, where antibody binding and activation of the proximal complement cascade occur without further tissue injury.[5] Based on high rate of C4d deposition in ABOi allografts, we hypothesized that anti-C4d CAR Tregs could infiltrate to the C4d$^+$ ABOi allograft and effectively suppress allograft rejection. In this study, we developed anti-C4d CAR Tregs and assessed their immunosuppressive effects on ABMR in a mouse ABOi heart transplantation model.

Through the phage enzyme immunoassay, several reactive clones to mouse C4d were identified as candidate clones. Next, we chose two anti-C4d scFv clones (SC-8, BF-2) which showed good binding affinity for C4d and the BF-2 clone was finally chosen for further study based on its binding activity and expression level (Fig. S1A). We successfully generated retroviral vectors containing anti-C4d CAR by cloning anti-C4d scFv into different regions of CD8, CD28, and CD3ζ in a second-generation CAR structure (Fig. 1A). A control CAR vector containing palivizumab scFv was also constructed (Fig. 1A). Detailed methods were described in the supplementary materials.
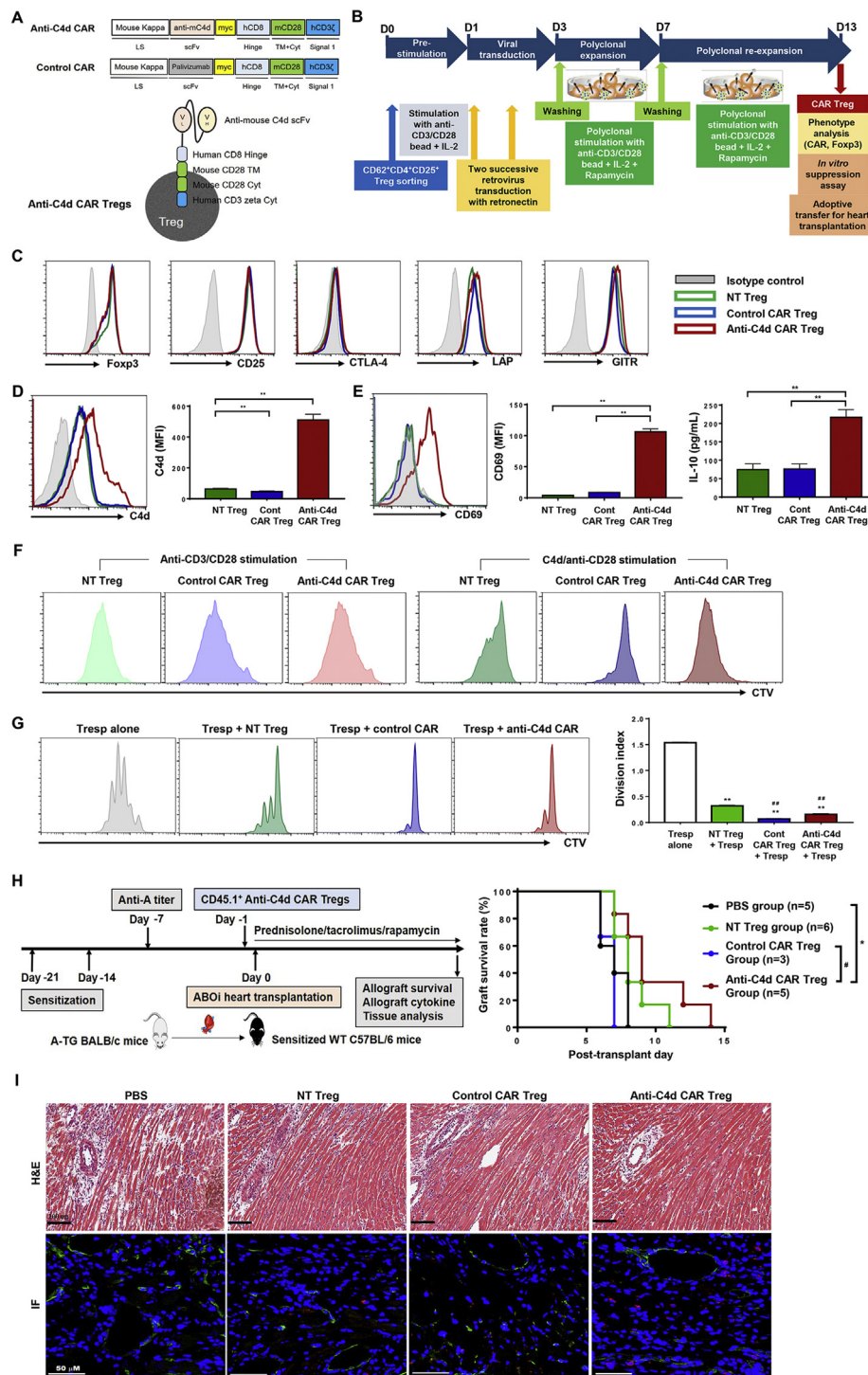
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**Figure 1**    (**A**) Structures of anti-C4d CAR, control CAR and anti-C4d CAR Tregs. CAR, chimeric antigen receptor; C4d, complement component 4d; Cyt, cytoplasmic domain; LS, leader sequence; mC4d, mouse complement component 4d; Myc, myc-tag; scFv, single chain variable fragment; TM, transmembraneous domain; Tregs, regulatory T cells; $V_H$, variable region of heavy chain; $V_L$, variable region of light chain. (**B**) Scheme of generation of anti-C4d CAR Tregs. Sorted $CD62L^+CD4^+CD25^+$ Tregs were transduced with retrovirus containing either anti-C4d CAR or control CAR and then stimulated by anti-CD3/CD28 beads in the presence of IL-2 and rapamycin. IL-2, interleukin-2; Foxp3, forkhead box P3. (**C**) Expression of Foxp3, CD25, CTLA-4, LAP, and GITR in anti-C4d CAR Tregs compared to that in control CAR Tregs and NT Tregs. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; Foxp3, forkhead box P3; GITR, glucocorticoid-induced tumor necrosis factor receptor-related protein; LAP, latency-associated peptide; NT, non-transduced. (**D**) Specific binding of anti-C4d CAR Tregs to C4d. **$P < 0.01$ compared to anti-C4d CAR Tregs group (Student's $t$ test). Cont, control; MFI, mean fluorescence intensity. (**E**) CD69 expression and secretion of IL-10 by activation of anti-C4d CAR Tregs in response to binding to C4d on Raji cells. **$P < 0.01$ compared to anti-C4d CAR Tregs group (Student's $t$ test). IL-10,

Figure 1B shows the basic protocol of CAR Treg generation and its detailed methods are described in the supplementary materials. Both anti-C4d and control CAR Tregs expressed CAR expression (Myc+) and showed good viability with preserved Foxp3 expression, whereas NT Tregs did not express Myc (Fig. S1B). Both CAR Tregs expressed Foxp3, CD25, CTLA-4, LAP, and GITR to a similar extent as NT Tregs (Fig. 1C), suggesting that the immunosuppressive function-associated molecules in Tregs were well-preserved in the anti-C4d CAR Tregs.

Soluble C4d-human Fc successfully bound to anti-C4d CAR Tregs, whereas it did not bind to control CAR Tregs or NT Tregs (Fig. 1D). Anti-C4d CAR Tregs upregulated CD69 expression and secreted much more IL-10 in response to C4d binding, than both control CAR Tregs and NT Tregs ($P < 0.01$, Fig. 1E). Next, C4d stimulation induced vigorous proliferation of anti-C4d CAR Tregs to similar extent as anti-CD3/CD28 stimulation (Fig. 1F). However, C4d stimulation induced proliferation of NT Tregs and control CAR Tregs to much lesser extent than anti-CD3/CD28 stimulation. These data show that anti-C4d CAR Tregs can specifically bind to C4d and are activated by their binding to C4d.

All three groups of Tregs suppressed in vitro T cell proliferation, although both CAR Tregs had slightly stronger suppressive effects than NT Tregs ($P < 0.05$, Fig. 1G). These results indicate that anti-C4d CAR Tregs are functionally active Tregs and exhibit the immunosuppressive activity of Tregs.

Finally, we assessed the in vivo immunosuppressive activity of anti-C4d CAR Tregs against ABMR in ABOi heart transplantation. Sensitized recipients developed high titers of anti-A IgM and IgG before transplantation (Fig. 1H, Fig. S1C). Hearts from human blood group A antigen-transgenic (A-TG) BALB/c mice were transplanted into the sensitized wild-type C57BL/6J mice. Tregs were transferred into recipient mice with administration of prednisolone, tacrolimus, and rapamycin. Anti-C4d CAR Tregs significantly prolonged the ABOi heart allograft survival rate compared to the phosphate buffered saline (PBS) control and control CAR Tregs ($P < 0.05$, Fig. 1H). When the expression of proinflammatory cytokines in heart allografts on day 7 was compared, the anti-C4d CAR Treg group showed lower IFN-$\gamma$ and TNF-$\alpha$ expression than the PBS control group ($P < 0.01$; $<0.05$); and NT Treg group ($P < 0.05$; $<0.01$, Fig. 1D). Histologic examination showed perivascular inflammation and C4d deposition, indicating that ABMR indeed occurred in ABOi heart transplantation (Fig. 1I). Vascular inflammation and tissue injury of heart allograft on day 7 seemed to be attenuated in the anti-C4d CAR Treg group (Fig. 1I). Infiltration of CD45.1+ anti-C4d CAR Tregs around C4d+ endothelial cells on day 7 was markedly observed in immunofluorescence images (Fig. 1I).

To date, anti-HLA-A2 CAR Tregs are the only CAR Tregs applied in the transplantation field and have shown good immunosuppressive effects on allograft rejection.[1-3] However, anti-HLA-A2 CAR Tregs targeting donor-specific HLA, cannot cover all donor–recipient pairs. In contrast, the anti-C4d CAR Tregs target C4d, a well-known ABMR-associated molecule and can be used to treat most ABMR regardless of the HLA combinations of the donors and recipients. One potential limitation of anti-C4d CAR Tregs is their low ability to suppress C4d-negative ABMR. On the other hand, anti-C4d CAR Tregs may prevent ABMR in ABOi transplantation by infiltrating C4d+ ABOi allografts, as C4d deposition occurs in most ABOi allografts with or without ABMR via the mechanisms of accommodation unique to ABOi transplantation.[5] Consistent with this hypothesis, we demonstrated that anti-C4d CAR Tregs significantly prolonged ABOi allograft survival.

This study could contribute to CAR Treg therapy and controlling allograft rejection in the transplantation field by proposing a quite new type of CAR Tregs that target C4d for both ABMR and ABOi transplantation. Further studies are needed to generate anti-human C4d CAR Tregs and assess their immunosuppressive effects against allograft rejection for future clinical application. In conclusion, anti-C4d CAR Tregs improve ABOi heart allograft survival by suppressing ABMR and are promising for application in human transplantation.

### Author contributions

Hyori Kim, Junho Chung, and Jaeseok Yang: study design, data curation, data analysis, and writing.

Sun-Kyung Lee, and Jerome Han: performance of experiments, data analysis, and writing, Honglin Piao, Nara Shin, and Joon Young Jang, Ji-Jing Yan: performance of experiments, data analysis.

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### Conflict of interests

The author declare no competing interests.

interleukin-10. (**F**) Proliferation of anti-C4d CAR Tregs in response to polyclonal (anti-CD3/CD28) or antigen-driven (C4d/anti-CD28) stimulation. CTV, Cell Trace Violet. (**G**) In vitro immunosuppressive activity of anti-C4d CAR Tregs against T cell proliferation. T cell proliferation was calculated as the division index. $**P < 0.01$ compared to effector T cells alone, $^{\#\#}P < 0.01$ compared to NT Tregs group (Student's t test). N = 3 per each group. Each value in the bar charts indicates the mean and standard error of the mean. Tresp, responder T cells. (**H**) Heart allograft survival rates in ABOi heart transplantation. Sensitized C57BL6/J mice underwent A-TG BALB/c heart transplantation one day after adoptive transfer of anti-C4d CAR Tregs, control CAR Tregs or NT Tregs. $*P < 0.01$ compared to PBS group; $^{\#}P < 0.01$ compared to control CAR Treg group (Log rank test). ABOi, ABO-incompatible; A-TG, human blood group A antigen-transgenic; PBS, phosphate buffered saline; WT, wild-type. (**I**) H&E staining imaging (magnification × 200) to show ABOi allograft injury and merged views in IF imaging (magnification × 400) to show infiltration of CD45.1+ anti-C4d CAR Tregs into C4d+ ABOi heart allograft tissues (C4d, green; CD45.1, red; DAPI, blue) on day 7 after heart transplantation. DAPI, 4,6-diamidino-2-phenylindole; H&E, Hematoxylin and eosin; IF, Immunofluorescence staining.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.07.001.

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