Immunomodulation Induced by a Novel Immunosuppressant, *NK026680* Plus Donor-Specific Transfusion Permits a Long-Term Cardiac Allograft Survival in Mice

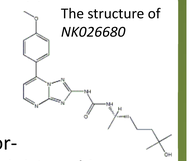
Shin Emoto[1], Ryoichi Goto[1], Susumu Shibasaki[1], Akihisa Nagatsu[1], Hitoshi Ono[1], Rumi Igarashi[1], Takeshi Aoyagi[1],
Moto Fukai[2], Tsuyoshi Shimamura[3], Kan Saiga[4], Akinobu Taketomi[1], Satoru Todo[5], Kenichiro Yamashita[2]

[1]Department of Gastroenterological Surgeryl, Hokkaido University [2]Department of Transplant Surgery, Hokkaido University [3]Division of Organ Transplantation, Hokkaido University Hospital [4]Nippon Kayaku Co. (Tokyo) [5]St. Mary's Hospital

Background

- *NK026680* (*NK)* is a novel triazolopyrimidine-derivative. We have previously reported that *NK* suppresses T cell activation and proliferation through suppression of p38 MAPK cascades, and prolongs allograft survival (Shibasaki S, et al. Transplant Immunol 2012; 26: 42-49.).
- Donor-specific transfusion (DST) has immunomodulatory effects in a donor-specific manner, especially when administered in combination with certain immunosuppressants or costimulatory blockers.

The structure of *NK026680*



Aim

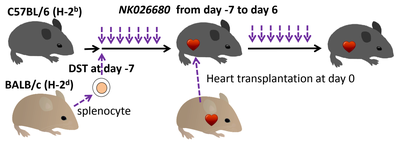
To investigate whether *NK* in combination with DST has immunomodulatory effects.

Materials and Methods

1. mice C57BL/6 (H-2^b) and BALB/c (H-2^d) male mice were purchased from Nihon SLC (Tokyo, Japan). Mice were used at age over 8 weeks old.

2. Reagents *NK026680* was provided by Nippon Kayaku Co. (Tokyo Japan). PE-Cy7-CD3ε (clone 17A2), FITC-CD4 (RM4-4), APC-H7-CD4 (GZK-1.5), V500-CD8a (53-6.7), FITC-CD44 (IM7), PE-CD62L (MEL-14), FITC-IFN-γ were purchased from BD Pharmingen (San Diego, CA, USA),and APC-Foxp3 (FJK-16s) from eBioscience (San Diego, CA, USA). Collagenase IV (Sigma Aldrich, St. Louis, MO, USA) was used at a concentration of 2 μg/ml.

3. Transplantation and Treatments C57BL/6 mice were used as recipients and BALB/c as donors. Vascularized heart transplantation was performed intraabdominally as previously described (Corry RJ, et al. Transplantation, 1973; 16: 343.). Rejection was accessed by the daily palpation of the graft through the abdominal wall. *NK026680* was suspended in 0.5 % carboxymethylcellulose (Kanto Chemical CO., INC. Tokyo, JAPAN) and orally administered at 40 mg/kg/day from 7 days before transplantation (day -7) to day 6. BALB/c splenocytes (20 x 10^6) were suspended by phosphate buffer serine (200μl) and injected intravenously at day -7 as DST.



4. Proliferation assay To investigate cell proliferation against donor antigens, mixed lymphocyte reaction (MLR) was performed. Splenocytes were retrieved from C57BL/6 and BLAB/c mice. After red blood cells were lysed by ACK lysing buffer (Lonza, Walkersville, MD USA), splenocytes were suspended with culture medium made from RPMI1640 (Sigma Ardrich), 10% heat-inactivated fetal bovine serum, penicillin (100 U/L), streptomycin (100 U/L), and 2-mercaptoethanol (50 μg/ml). C57BL/6 splenocytes were used as responders and 30Gy-irradiated BALB/c splenocytes as stimulators. Responder cells were stained by 5μM 5,6-carboxyfluorescein diacetate saccinimidyl ester (CFSE). C57BL/6 splenocytes ($5.0x10^5$) and BALB/c splenocytes ($5.0x10^5$) were mixed in 96 well round bottom plates (BD Falcon, San Diego CA, USA), incubated at 37℃, 5%-CO_2 and harvested 3 days after plating. Cell proliferation was detected with CFSE dilution.

5. In vivo donor-antigen stimulation BALB/c splenocytes ($20x10^6$) were intravenously injected into recipient mice for *in vivo* donor-antigen stimulation at day 0 (Sawaitzki B, et al. J Exp Med 2005; 201(12): 1925-35.) and splenocytes were obtained 24 hr later.

6. Interferon-γ (IFN-γ) ELISPOT assay Cells were suspended with culture medium described above. C57BL/6 splenocytes ($5.0x10^5$) were cocultured with irradiated BALB/c splenocytes ($5.0x10^5$) in MultiScreen 96-well Plates (Millipore Corporation, Billerica, MA) precoated with mouse IFN-γ ELISPOT Capture antibody (BD Bioscience). 24 hr after plating, IFN-γ spots were detected by a detection antibody (BD Bioscience) and visualized using streptavidin-HRP (BD Bioscience), followed by adding the substrate solution (BD Bioscience). Spots were counted using KS ELISPOT (Carl Zeiss, Germany).

7. Retrieval of cells in the graft Cells in the graft were retrieved by incubating RPMI medium containing 2 μg/ml Collagenase IV for 0.5 - 1.0 hr at 37℃, 5%-CO_2.

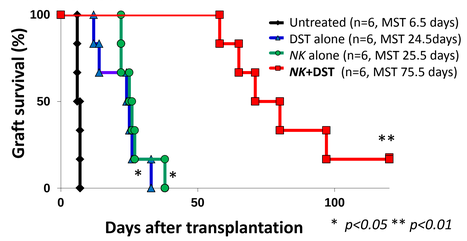
8. Flow Cytometric Analyses (FCM) An intracellular transcription factor Foxp3 was stained according to the manufacturer's protocol (eBioscience). For intracellular IFN-γ staining, cells were pulsed with Phorbol Myristate Acetate (40 ng/ml) and Ionomycin (1.0 μg/ml) for 6 hr, and incubated with Monencin (2 μg/ml) for 2 hr. Cells were permeabilized with the Cytofix/Cytoperm buffer (BD Bioscience).

9. Statistical analyses *NK* in combination with DST treated group (*NK*+DST) was compared with an untreated group, DST and *NK* monotherapy groups. Statistical evaluation was performed with StatMate (ATMS, Co,. Ltd, Tokyo JAPAN). One-Way ANOVA with the Tukey-Kramer post hoc test was applied for multiple comparisons. Allograft survival was analyzed using a Kaplan-Meier method and data compared by applying a log-rank test.

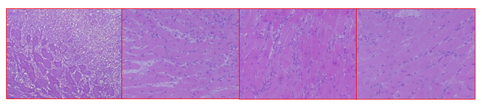
Results

Result #1. NK+DST markedly prolongs cardiac allograft survival and preserves graft structure.

A. Graft survival



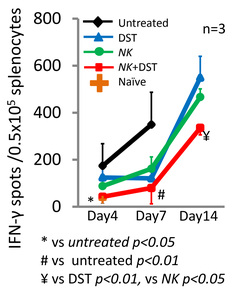
B. Histology of the cardiac grafts on day 7



hematoxylin-eosin, original magnification x200

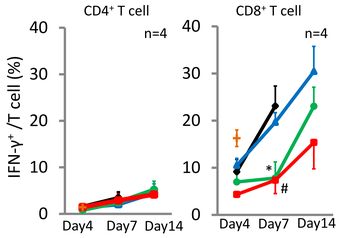
Result #2. NK+DST suppresses post-transplant activation and IFN-γ production of CD8⁺ T cells.

A. Alloreactive IFN-γ producing cells in the spleen (ELISPOT)



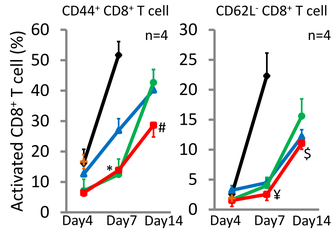
* vs *untreated p<0.05*
\# vs untreated *p<0.01*
¥ vs DST *p<0.01,* vs *NK p<0.05*

B. Percentage of IFN-γ producing T cells in the spleen (FCM)



* vs untreated, DST *p<0.001*
\# vs untreated, DST *p<0.001*

C. Percentage of activated CD8⁺ T cells in the spleen (FCM)

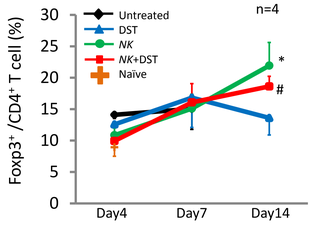


* vs untreated *p<0.001*
\# vs DST *p<0.05*, vs *NK p<0.01*
¥ vs untreated *p<0.001*
$ vs *NK p<0.01*

To investigate the post-transplant systemic effector function, we at first focused on the IFN-γ production against donor-antigens in the spleen by ELISPOT. IFN-γ production was suppressed in *NK*+DST group compared with in DST or *NK* groups even after administration of *NK* was stopped (A). The major population producing IFN-γ was $CD8^+$ T cells (B). Activation of $CD8^+$ T cells was also suppressed in *NK*+DST group (C).

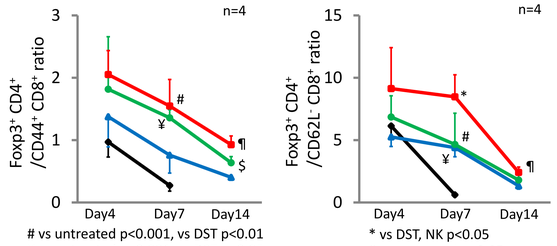
Result #3. NK+DST accelerated increase of Foxp3⁺ CD4⁺ T cell in the spleen, shifting the balance toward regulatory environment.

A. Percentage of Foxp3⁺ CD4⁺ T cells (FCM)



* vs DST *p<0.001*
\# vs DST *p<0.01*

B. Foxp3⁺ CD4⁺ T cell/ activated CD8⁺ T cell ratio



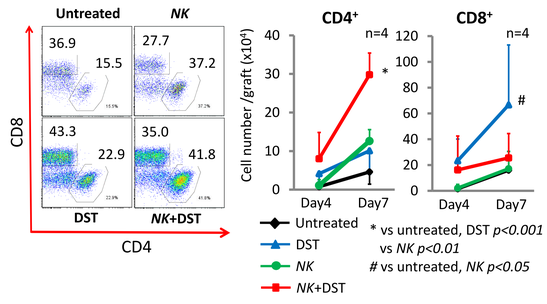
\# vs untreated p<0.001, vs DST p<0.01
¥ vs untreated p<0.001, vs DST p<0.05
¶ vs DST p<0.001, vs NK p<0.01
$ vs DST p<0.05

* vs DST, NK p<0.05
\# vs untreated p<0.05
¥ vs untreated p<0.05
¶ vs DST p<0.05

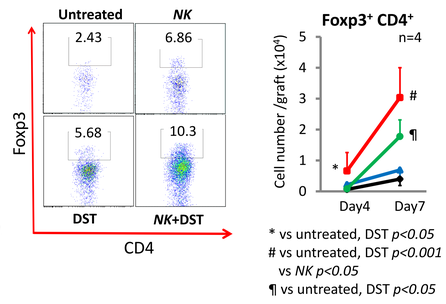
To investigate systemic regulatory function, we next focused on the population of $Foxp3^+$ $CD4^+$ T cell in the spleen. Although there was no difference between all groups at day 7, $Foxp3^+$ $CD4^+$ T cells expanded in *NK* and *NK*+DST groups at day 14 (A). The ratio of $Foxp3^+$ $CD4^+$ T cells to activated $CD8^+$ T cells was highest in *NK*+DST group among the all groups at day 4, 7, and 14 (B).

Result #4. Foxp3⁺ CD4⁺ T cells increase in the graft by the NK+DST treatment.

A. Percentage of graft infiltrating T cells



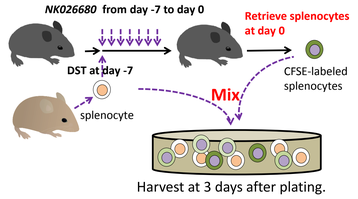
B. Percentage of graft infiltrating Foxp3⁺ CD4⁺ T cells



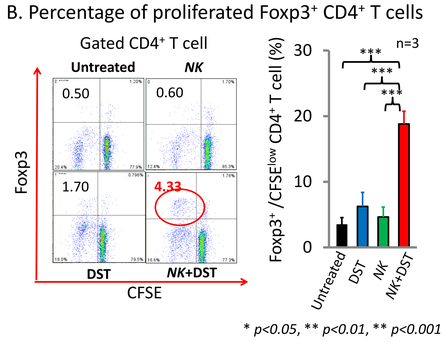
DST accelerated $CD8^+$ T cell infiltration into the graft, whereas *NK* promoted infiltration of $CD4^+$ but not $CD8^+$ T cells in the graft (A). A marked accumulation of $Foxp3^+$ $CD4^+$ T cells was observed in *NK*+DST group (B).

Result #5. NK+DST promotes Foxp3⁺ CD4⁺ T cell expansion after donor-antigen stimulation in vitro.

A. MLR Protocol



B. Percentage of proliferated Foxp3⁺ CD4⁺ T cells

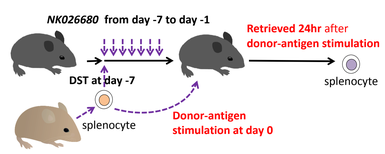


* p<0.05, ** p<0.01, ** p<0.001

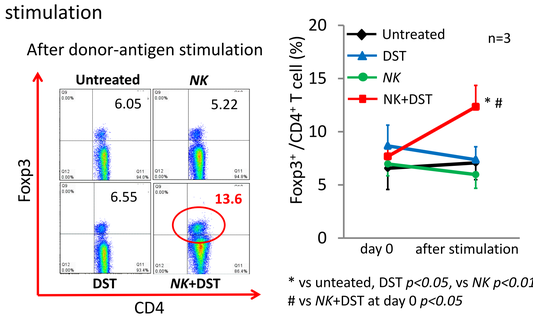
Experimental protocol is shown in (A). In *NK*+DST group, $Foxp3^+$ $CD4^+$ T cells expanded after stimulation with donor-antigens at the time of transplantation (B).

Result #6. NK+DST promotes Foxp3⁺ CD4⁺ T cell expansion after donor-antigen stimulation in vivo.

A. Protocol of *in vivo* donor-antigen stimulation



B. Percentage of $Foxp3^+$ $CD4^+$ T cells after donor-antigens stimulation



* vs unteated, DST *p<0.05*, vs *NK p<0.01*
\# vs *NK*+DST at day 0 *p<0.05*

The experiment of *in vivo* donor-antigen stimulation (A) resulted in the significant increase of $Foxp3^+$ $CD4^+$ T cells in the spleen 24 hr after stimulation in *NK*+DST group (B), consistent with Result #5.

Conclusion

NK026680 together with DST exerts immunomodulatory effects which promotes $Foxp3^+$ $CD4^+$ T cell expansion and shifts the balance to regulatory environment after transplantation, thereby markedly prolongs cardiac allograft survival in mice.