Report format

1. ***Title of the research project funded by the Mizutani Foundation***
   Regulation of Natural Killer cell function by O-GlcNAc modification.

2. ***Name of the principal investigator and collaborators.***
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3. ***Institution.***
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4. ***Abstract of objectives, methods, and results.***

O-GlcNAc modification (O-GlcNAcylation) is a reversible post-translational modification which involves the addition of N-acetylglucosamine to serine/threonine residues of cytosolic and nuclear proteins.[1, 2] An elevated cellular level of O-GlcNAc is best described under chronic hyperglycemic conditions. Although the number of proteins found to be O-GlcNAcylated in immune cells is growing, knowledge on the functional significance of O-GlcNAcylation in controlling immune cell function remains rudimentary. We are interested in studying the role of O-GlcNAcylation in innate immune system, specifically its role in Natural Killer (NK) cells. We found that global inhibition of O-GlcNAcylation increases NK cell cytotoxicity against cancer cells. We identified the pool of O-GlcNAcylated proteins in NK cells by mass spectrometry and found that perforin and granzyme-B are O-GlcNAcylated in NK cells, which are the key effector molecules for NK cell mediated cytolysis. We also observed elevated perforin/granzyme release after O-GlcNAc inhibition, which might account partially for the observed increase in cytotoxicity. This study reveals a novel role of O-GlcNAcylation in NK cells and contributes to improve the NK cell mediated killing of cancer cells and to develop a better therapeutic approach for NK cell adoptive therapy.

We chose Acute myeloid leukemia (AML) as the cancer model. AML is the most common acute leukemia in adults. Unfortunately, the current AML therapeutics have poor efficacy and high toxicities. Only 20% of patients over age 56 survive two years.[3] New therapeutic approaches are urgently needed. One potential strategy is immunotherapy with Natural Killer (NK) cells. NK cells are cytotoxic lymphocytes of the innate immune system.[4] The advance of NK cell immunotherapy has been hampered in large part by a lack of suitable expansion systems that generate large amounts of functional, highly active cytotoxic NK cells in doses suitable for administration in humans. We used a novel NK cell expansion system using artificial antigen presenting cells (aAPCs) transfected with membrane-bound IL-21 and other NK cell stimulating molecules.[5] Peripheral blood NK cells grown in this system can rapidly expand in 3 weeks up to 47,000-fold and exhibit higher cytotoxic activity.[5] These expanded NK cells are capable of decreasing tumor burden in xenogeneic models of neuroblastoma and myeloma and are now being used in phase 1 clinical trials for multiple malignancies. Though

this NK cell expansion system has promise, these cells still exhibit suboptimal NK cell killing of target cancer cells. We have found that targeting O-GlcNAc modification dramatically increases the ability of NK cells to kill cancer cells. Thus the combination of aAPC expansion and stimulation by O-GlcNAc inhibitors might result in clinically relevant doses of highly cytotoxic NK cells with better therapeutic potential. Based on this, we propose to further characterize these O-GlcNAc inhibited NK cells as well as develop a platform to manufacture these NK cells for clinical use.

5. ***Progress after the granted period***

To confirm the O-GlcNAcylation in perforin and granzyme proteins, we overexpressed perforin and granzyme (V5 Tagged) in NK cells by viral transductions and immunoprecipitated perforin and granzyme from NK cells. We performed western blotting using anti O-GlcNAc antibody to confirm perforins and Granzymes are indeed O-GlcNAcylated in NK cells.

Next, we investigated a pathologically relevant condition that elevates cellular O-GlcNAcylation with potential to alter NK cell function. Hyperglycemic conditions are known to elevate OGlcNAcylation. Hence, we isolated NK cells from blood of diabetes patients as a relevant model to study the pathological role of O-GlcNAcylation in NK cell function. NK cells from diabetic patients are known to exhibit significant functional defects.[6] However, specific causes for this dysfunction are less clear. Type 1 diabetic NK cells reported to exhibit decreased NKG2Ddependent cytotoxicity and interferon-γ secretion,[7] while type 2 diabetic patients are highly susceptible to infection and have an increased incidence of some tumors, possibly due to NK cell dysfunction.

Interestingly, we found that NK cells from diabetic patients, who are known to have elevated O-GlcNAcylation levels, exhibited a reduced ability to kill target AML cells as compared to normal donor NK cells. This supports our in vitro results using PUGNAc (PUGNAc inhibits O-GlcNAcase, a beta-exo-N-acetylhexosaminidase which cleaves beta-O-linked-N-acetylglucosamine residues from glycoproteins) to elevate O-GlcNAcylation, and suggests that pathological hyperglycemia and associated increase in O-GlcNAcylation might be a mechanism that compromises NK cell function. To further confirm that the impaired NK cell function in diabetic patients is indeed due to increase in O-GlcNAcylation, we treated the diabetic NK cells with DON (6-Diazo-5-oxo-L-norleucine, a glutamine analogue, is used also as an OGT inhibitor) and studied their cytotoxic function. As expected, DON treated diabetic NK cells showed enhanced efficacy in killing AML cells. These results suggest that high O-GlcNAc modification of proteins may directly contribute to NK cell function.

6. ***Future perspective.***

We will identify the O-GlcNAcylation sites in perforin and granzyme and will mutate these sites in NK cells to further explore biochemical and functional consequence of those mutations. We will also analyze the endogenous O-GlcNAc transferase (OGT, the enzyme which adds O-GlcNAc to proteins) binding to overexpressed perforin/granzyme. Next, we will examine signal-induced binding of OGT to perforin/granzyme and analyze different deletion mutants of perforin/granzyme

and OGT to identify the binding region. We will also study perforin/granzyme -OGA interaction in a similar manner as described above. These studies will identify the discrete binding domains in OGT, OGA and perforin/granzyme and we will employ these short peptides to modulate perforin/granzyme O-GlcNAcylation in vivo. We will develop a specific monoclonal antibody to detect mutant O-GlcNAcylated perforin/granzyme. This could be a valuable tool to explore the biochemistry of O-GlcNAcylation-dependent perforin/granzyme signaling in NK cells. These molecules could prove valuable tools to inhibit perforin/granzyme O-GlcNAcylation in vivo and thereby glycosylation dependent perforin/granzyme functions like the enhanced secretion and tumor apoptosis.

7. Publications (a maximum of 10 references) should be given using the following style.

1) Smith AB, Jones CD. Title, *J. Biol. Chem.* (168): 1758-1764, 1976

References

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2. Hart GW, Slawson C, Ramirez-Correa G, Lagerlof O. Cross talk between O-GlcNAcylation and phosphorylation: Roles in signaling, transcription, and chronic disease... *Ann. Rev. Biochem*. (80):825–858, 2011
3. Estey E, Dohner H. Acute myeloid leukaemia. *Lancet*. (368):1894–1907, 2006.
4. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells*. Nat Immunol*. (9):503–510, 2008
5. Denman CJ1, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, Singh H, Hurton L, Maiti SN, Huls MH, Champlin RE, Cooper LJ, Lee DA. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One.* (7) 2012
6. Qin H, Lee IF, Panagiotopoulos C, Wang X, Chu AD, Utz PJ, Priatel JJ, Tan R. Natural killer cells from children with type 1 diabetes have defects in NKG2D-dependent function and signaling. *Diabetes.* 60(3):857-66, 2011
7. Berrou J, Fougeray S, Venot M, Chardiny V, Gautier JF, Dulphy N, Toubert A, Peraldi MN. Natural killer cell function, an important target for infection and tumor protection, is impaired in type 2 diabetes. *PLoS One*. 8(4):2013