

Original Research Article

Desert hedgehog is a negative regulator of CD44-CD25⁺ double negative T lymphocytes developmental stage in thymic differentiation

Stephen M. Kariuki*

Department of Medical Laboratory Sciences, Mount Kenya University, Eldoret, Kenya

Received: 08 December 2017

Accepted: 09 January 2018

***Correspondence:**

Dr. Stephen M. Kariuki,

E-mail: skariuki@mku.ac.ke

Copyright: © the author(s), publisher and licensee Medip Academy. This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABSTRACT

Background: The process of thymic proliferation and differentiation in mammals is indispensable. The role of hedgehog family of proteins has been extensively studied in the recent past years. Specifically, scientists have carried out substantial amount of work on Sonic hedgehog (Shh) and Indian hedgehog (Ihh) and published on their roles on either T-cell development or peripheral T-cell activation. However, the role of Desert hedgehog (Dhh) the third member of the hedgehog family of proteins, in T-cell development has not been clearly understood. In this work, we aimed to investigate the role of Desert hedgehog in thymic differentiation, particularly in the double negative T cell developmental stages.

Methods: Thymuses of three Dhh^{-/-} mice, three Dhh^{+/-} and three Dhh^{+/+} were obtained by killing the mice using A prepared suspension of cells was analyzed by a three-color flow Cytometry and the acquired data analyzed using flow jo, a tree star software for flow cytometry data analysis. To establish the statistical significance of the findings, data was subjected to student t-test and significance reported at critical p-value of 0.05.

Results: The total number of thymic cells was found to be higher in Dhh KO mice relative to the WT control. Analysis of thymic subsets using flow cytometry showed that double negative CD4-CD8⁻ thymocytes were found to be relatively higher in Dhh^{-/-} mice compared to Dhh^{+/-} control. In particular the percentage representation of CD44-CD25⁺ DN3 thymocytes were significantly higher (p=0.03) in Dhh KO mice relative to the WT controls.

Conclusions: The findings of this study suggest that Dhh signal could be a negative regulator during early thymic differentiation. The data shown here is representative of three separate experiments. To get much clearer and replicative findings, these experiments can be repeated with a much larger sample size.

Keywords: CD44-CD25⁺ DN3 thymocytes, Desert hedgehog, Double negative thymocytes, Thymic differentiation

INTRODUCTION

The hedgehog family proteins (Desert hedgehog, Sonic hedgehog and Indian hedgehog) act as morphogens during vertebrate embryogenesis and organogenesis. Morphogens are signaling protein molecules secreted from a localized point that form a concentration gradient capable of determining the fate of developing cells located within this gradient in a concentration-dependent

fashion.^{1,2} In the recent past years, researchers have reported that hedgehog family of proteins are involved in thymocyte development.^{3,4} Sonic hedgehog (Shh) and Indian hedgehog (Ihh) have both been reportedly said to be regulators of T-cell development within the thymus.^{2,5-7} Shh is said to arrest thymocyte differentiation at the CD25⁺DN stages, derailing procession to DP CD4⁺CD8⁺ thymocytes.^{3,8} It is well documented in immunology literature that T lymphocytes are produced in the bone

marrow but then move to the thymus where they mature till they are released to the peripheral blood circulation as naïve CD4⁺ or CD8⁺ cells. In the thymus, T cells develop through the following stages, beginning from double negative stages (Named so because the developing cells have neither CD4 nor CD8 surface markers): DN1 (CD44⁺CD25⁻), DN2 (CD44⁻CD25⁺), DN3 (CD44⁻CD25⁺), DN4 (CD44⁻CD25⁻), DP (CD4⁺CD8⁺), SP (CD4⁺CD3⁺ OR CD8⁺CD3⁺).

The role of desert hedgehog (Dhh) in thymic development has not been clearly established yet. Away from thymic differentiation however, Dhh has been found to take part in regulating the morphogenesis of the testis.^{9,10} Elsewhere, researchers report that Dhh stimulates theca cells proliferation and steroidogenesis, and over expression of Dhh gives a range of embryonic and adult skin manifestations, demonstrating that it can regulate epidermal homeostasis, including tumor formation-in this case acting as a functional homologue of Sonic hedgehog Shh.¹¹⁻¹³

The hedgehog signaling pathway is said to play a pivotal role in development of a variety of human tumors such as breast cancer, gastric cancer, colorectal cancer, pancreatic cancer, prostate cancer, brain tumors and basal cell carcinoma when inappropriately activated.^{14,15} All of the above studies on the role of Hh family proteins were carried out on a murine model. In the current study, we aim to establish the exact role of Dhh in thymic differentiation using murine thymuses.

METHODS

Flow cytometry

In order to analyze the thymic phenotypes of different sub-populations and see whether there is any comparative abnormality (in homozygous Dhh^{-/-} and wild type Dhh^{+/+} mice) fresh thymocytes were stained with fluorescent-labeled mAbs anti: CD4-PE, CD8-TRI, CD3-FITC, CD5-FITC, CD44-PE and CD25-FITC (e-bioscience). Cells from smashed murine thymuses were prepared in suspension at 10⁶/ml in Phosphate buffered saline/5% fetal calf serum/0.01% sodium azide and for each stain 10⁵ cells were stained with the relevant antibodies for 30 minutes on ice. Following this incubation period, cells were washed using the above medium and centrifuged for 5mins at 1300rpm. They were then analyzed on a FAC Scan (BD Biosciences) at the Barts and the London school of Medicine and Dentistry. Live cells were gated by FSC/SSC profiles for analysis. The data shown (Appendix 1) represent more than 3 experiments.

Data analysis

After all experiments, data was analyzed using flowjo (Treestar USA) where dot plots were generated in batch and printed for further analysis. All data was simplified

by setting the WT control to one and expressing all other data relative to the wild type.

This data is presented here in graphs using Microsoft excel. Statistical test was carried out using paired Student's t test for every set of results and significance of the results was reported as anything equal or less than P=0.05.

RESULTS

Double negative T lymphocyte progenitors

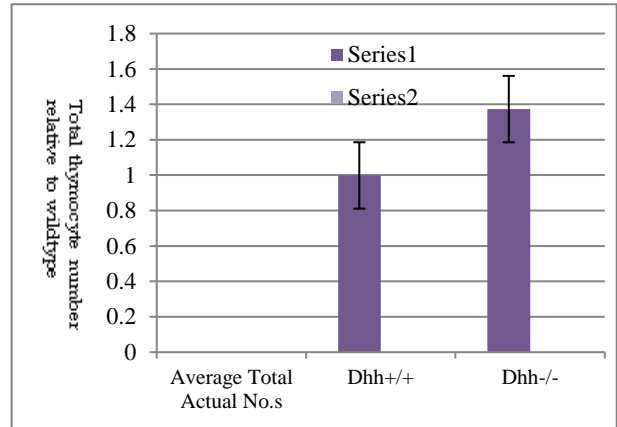


Figure 1: Dhh^{-/-} mouse thymic size relative to WT control.

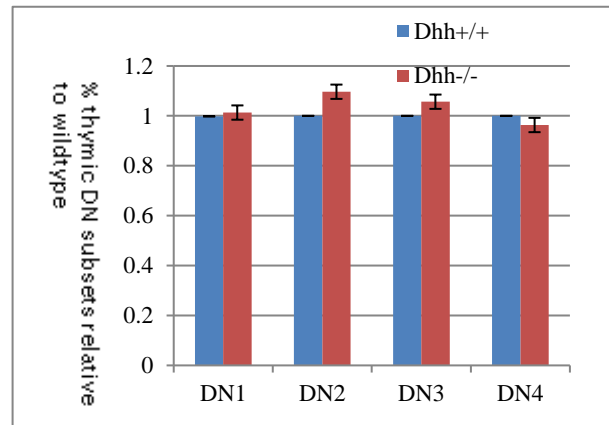


Figure 2: Graphic representation of DN percentages from gated cd4/8/3-tri cells.

Using a three-color flow Cytometry, all early thymic progenitors (CD4-CD8-DN thymocytes) were analyzed. The percentage representation of DN 2 (CD44⁺CD25⁺) and DN 3 (CD44⁻CD25⁺) thymic subsets showed markedly high in numbers in Dhh KO relative to WT control.

In absolute terms, the actual number of all DN thymic subsets in Dhh KO mice calculated by adjusting numbers according to thymus size, was found to be increased relative to their wildtype counterparts.

However, only the increase in DN3 (CD44⁺CD25⁺) thymic subset of Dhh KO mice was found to be statistically significant (P=0.03) relative to the wild type control. The results are presented in dot plots (Figure 4 and 5) and in bar graphs for both percentages (Figure 2) and actual numbers (Figure 3).

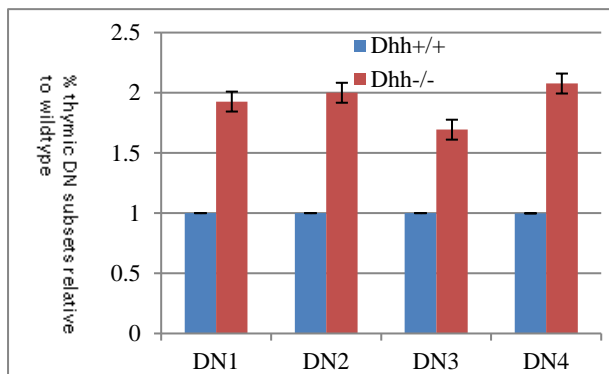


Figure 3: Actual numbers representation of DN subsets taking into account thymic size of Dhh KO mice relative to WT.

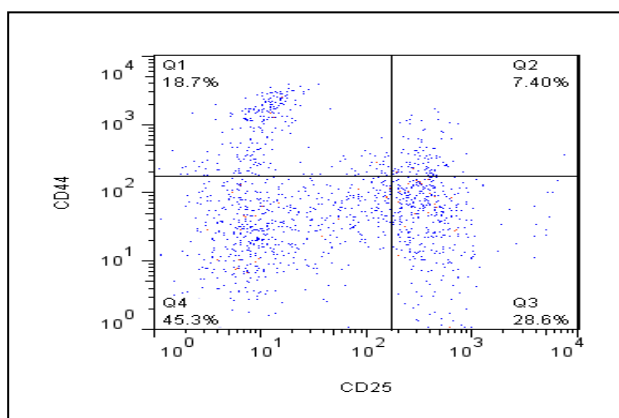


Figure 4: WT control showing percentage representation of DN1-Q1, DN2-Q2, DN3-Q3 and DN4-Q4.

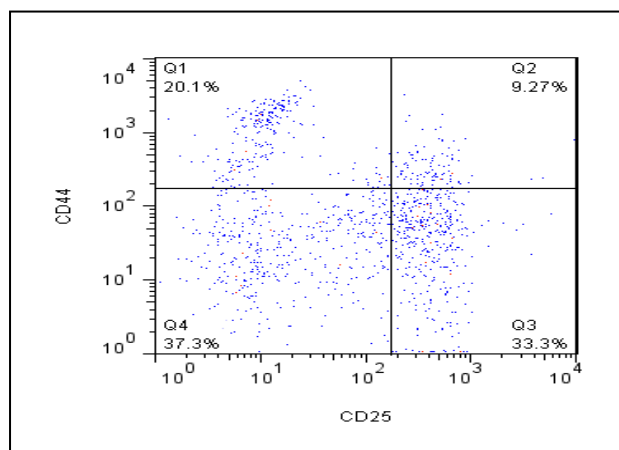


Figure 5: Dhh KO showing percentage representation of DN1-Q1, DN2-Q2, DN3-Q3 and DN4-Q4.

DISCUSSION

In the current study, thymic cells were analyzed in a three-color flow Cytometry where a general upward trend was noted in all early thymic progenitors in Dhh KO compared to the WT control in terms of numbers (from CD44⁺CD25⁻DN1 to CD44⁺CD25⁺DN4). While the actual percentage representation of CD44⁺c-kit⁺CD25⁻CD3⁻CD4⁻CD8⁻DN1 thymocytes was not significantly altered between the wild type and Dhh knockout mice, the actual percentage of DN2 and DN3 was significantly increased in Dhh KO mice relative to their wild type counterparts. Further, as you would expect when analyzing relative percentages, the percentage representation of DN4 (CD44⁺CD25⁻) subset was reduced in Dhh KO mice. According to research by Moore and Zlotnik, DN1 (CD44⁺CD25⁻) thymocytes have not committed to T cell lineage yet, whereas DN2 and DN3 thymic subsets have started to rearrange their TCR β -chains and are now committed to the T cell lineage.¹⁶ There was a marked increase in all CD25⁺ thymocytes (DN2 and DN3) (Figure 2 and 3) in Dhh KO where the biggest difference between Dhh KO and the WT control was found on CD44⁺CD25⁺DN3 cell population (P=0.03), considered statistically significant at P<0.05. This is consistent with past research where CD44⁺CD25⁺DN2 and CD44⁺CD25⁺DN3 were found to have the highest level of expression of smo, an important molecule in hedgehog signal transduction.³ Thus, a Dhh signal could be a negative regulator of thymic differentiation as early as the DN1 stage in development and then as TCR-chain genes are rearranged and expressed, these differences may become amplified subsequent to signalling through the TCR β -chain gene in the context of the pre-TCR complex. We believe the significant increase in CD44⁺CD25⁺DN3 thymocytes in Dhh KO mice relative to WT control can be attributed to this process of pre-TCR signaling as proliferation of this subpopulation is a well-documented downstream event following signaling through the pre-TCR. However, though there was still an upward trend in terms of numbers of the next CD44⁺CD25⁺DN4 thymic population (Figure 3), the difference between Dhh KO and WT control was not statistically significant. Furthermore, although an increase was observed in the actual number of DN4 subpopulation there was no increase in the actual percentage of DN4 which would reflect an increase in numbers overall following greater proliferation of the DN3 but not an increase in thymic differentiation from DN3 to DN4 itself.

Another reason why the difference between CD44⁺CD25⁺DN4 thymocytes in Dhh KO compared to the WT control was not significant, could be the low distribution of smo on this subpopulation of thymic cells as explained by Outram et al meaning that Dhh signaling at this stage usually does not have a big impact and so its absence on the KO mice makes little difference on thymic differentiation as suggested above.³ The observed difference however, might become statistically significant

with analysis of a larger sample animals so as to narrow the window of experimental errors.

Later stages in thymic development were analyzed using cell surface expression of CD4 and CD8. Live cells were gated from SSC-FSC profiles using flowjo (Treestar) and DPs (CD4⁺CD8⁺), CD4⁺CD8⁻ and CD4⁺CD8⁺SP thymocytes analyzed. The actual numbers of DP CD4⁺CD8⁺ thymocytes from Dhh KO mice were substantially higher than those isolated from the WT control. However, this difference was not up to statistical significance (calculated P = 0.79). Thymocytes at the DP stage have already committed to the T cell lineage according to Moore and Zlotnik, and have their $\alpha\beta$ -TCR genes rearranged and expressed on the cell surface in the context of the mature $\alpha\beta$ -TCR.¹⁶ These cells are ready to undergo positive and negative selection.¹⁷ It is only thymocytes that have a mature TCR on their cell surface that can undergo positive selection and differentiate to become a mature CD4 or CD8 single positive thymocyte.¹⁸ Research that was conducted by Outram et al³ show that the percentage distribution of Ptc and smo, the two important receptor molecules in Hh signaling pathway, on DP CD4⁺CD8⁺ thymocytes is lower than on all of the preceding stages of thymic development. Thus, the non-statistically significant difference between Dhh KO relative to the WT control counterpart at this stage of development could be attributable to this.

CONCLUSION

Desert hedgehog could be a negative regulator of development of thymocytes in mammals. This protein plays the biggest role of negative regulation at the double negative (CD4⁻CD8⁻) T cell developmental stages in the thymus, particularly at the DN3 (CD44⁻CD25⁺) stage. It is therefore highly probable that Dhh, Shh and Ihh could have overlapping roles in thymic proliferation and differentiation because the latter two proteins have also been found to give a negative signal during T-cell development.^{1,3} It is hypothesized that differences in the pattern of expression of the various hedgehog proteins will determine where and when in the process of thymic development these proteins play their specific roles.

Recommendations

To even more accurately establish the role of Dhh signaling, there would need for further investigation with a larger sample size and more number of experiments to get more statistically significant results.

ACKNOWLEDGEMENTS

Authors would like to thank Dr. S. V. Outram for her help, direction and counsel all through this work, my dad for his moral help, Gary Warnes at Barts and the London School of Medicine and Dentistry for his help with flow cytometry and Mr. Kevin Clough for some assistance in the UEL laboratory.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: Not required

REFERENCES

1. Varas A, Hager-Theodorides AL, Sacedón R, Vicente A, Zapata AG, Crompton T. The role of morphogens in T-cell development. *Trends in immunology*. 2003;24(4):197-206.
2. Furmanski AL, Saldana JI, Ono M, Sahni H, Paschalidis N, D'Acquisto F, et al. Tissue-derived hedgehog proteins modulate Th differentiation and disease. *J Immunol*. 2013;190(6):2641-9.
3. Outram S, Varas A, Pepicelli C, Crompton T. Hedgehog signalling regulates Differentiation from double negative to double positive thymocytes. *J Immunol*. 2000;13(4):584.
4. Miranda LH, Brundage K, Schafer R, Tou J, Barnett J. Prenatal cadmium exposure dysregulates Sonic hedgehog and Wnt/ β -catenin signaling in the thymus resulting in altered thymocyte development. *J Tox Appl Pharm*. 2010;242(2):136-45.
5. Shah D, Hager-Theodorides A, OS, Ross S, Varas A, Crompton T. Reduced thymocyte development in sonic hedgehog knockout embryos. *J Immunol*. 2004;142(4):2296-306.
6. Outram SV, Hager-Theodorides AL, Shah DK, Rowbotham NJ, Drakopoulou E, Ross SE, et al. Indian hedgehog (Ihh) both promotes and restricts thymocyte differentiation. *Blood*. 2009;113(10):2217-28.
7. Barbarulo A, Lau CI, Mengrelis K, Ross S, Solanki A, Saldaña JI, Crompton T. Hedgehog signalling in the embryonic mouse thymus. *J Develop Biol*. 2016;4(3):22.
8. Sacedón R, Varas A, Frías MCG. Hedgehog proteins. 2003;22(1):17-26.
9. Bitgood M, Shen L, McMahon A. Sertoli cell signalling by Desert hedgehog regulates the male germline. *J Curr Bio*. 1996;6(3):298-304.
10. Lijun C, Itaranta P, Zang S, Vainio S. Sprouty2 is involved in male Sex Organogenesis by controlling Fibroblast Growth Factor 9-Induced Mesonephretic Cell Migration to the Developing Testis. *J Endocrin*. 2006;147:83777-88.
11. Spicer LJ, Sudo S, Aad PY, Wang LS, Chun SY, Ben-Shlomo I, et al. The hedgehog-patched signaling pathway and function in the mammalian ovary: a novel role for hedgehog proteins in stimulating proliferation and steroidogenesis of theca cells. *Reproduction*. 2009;138(2):329-39.
12. Wijgerde M, Ooms M, Hoogerbrugge J, Grootegoed J. Hedgehog signaling in mouse ovary: Indian hedgehog and Desert hedgehog from granulosa cells induce target gene expression in theca cells. *J Endocr*. 2005;146(8):3558-66.
13. Christelle A, Narang M, Ellis J, Wicking C, Kaur P, Wainwright B. An in vivo comparative study of Sonic, Desert and Indian hedgehog reveals that

- hedgehog pathway activity regulates epidermal stem cell homeostasis. *Develop.* 2004;131:5009-19.
14. Katoh Y, Katoh M. Hedgehog signaling pathway and gastric cancer. *Cancer Biol Ther.* 2005;4(10):1050-4.
 15. Lehmann H, Felderer L, Frew IJ, Matsushita S, Levesque MP, Dummer R. Hedgehog Pathway Inhibitors Promote Adaptive Immune Responses in Basal Cell Carcinoma. 2015:1-10.
 16. Moore T, Zlonik A. T-cell lineage Commitment and Cytokine Responses of Thymic Progenitors. *Blood.* 1995;86(5):1885-60.
 17. Murphy K, Travers P, Walter P. *Janeway's Immunobiology.* 7th ed. U.S.A: Garland Science: Taylor & Francis Group;2008.
 18. Brandle D, Muller C, Rulicke T, Hengartner H, Pircher H. Engagement of the T-cell receptor during positive selection in the thymus-regulates RAG-1 expression. *Proc Natl Acad Sci.* 1992;89:9529-33.

Cite this article as: Kariuki SM. Desert hedgehog is a negative regulator of CD44⁻CD25⁺ double negative T lymphocytes developmental stage in thymic differentiation. *Int J Res Med Sci* 2018;6:734-8.