Kinetics and Phenotype of Endogenous Pulmonary Cd4 T Cell Response to Viral Primary Influenza: An Animal Study

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Abstract
Background: Pneumonia of both bacterial and viral origin, are a major cause of pediatric death globally, with IAV Influenza A virus being the most common culprit causing viral pneumonia. IAV affects approximately 20% pediatric population yearly.
Objectives: The present study was conducted to assess the specialized subsets of CD4 T cells with influenza A virus specificity in the lungs. Also, the present study aimed to establish that influenza A virus-specific CD4 T cell response is regulated via antigen-presenting cells of the pulmonary region.
Methods: Female C57Bl/6 and BALB/c mice of the age of 8-12 weeks were used for all assessments. Age- and weight-matched groups of female C57Bl/6 mice were lightly anesthetized by isoflurane inhalation and infected intranasally.
Results: Frequency of CD4 T cells that produces IFNγ following peptide-pulsed splenic stimulator DC incubation was increased significantly in comparison to CD4 T cells incubated using non-peptide-pulsed splenic stimulator DC. Hetero subtypes protection could be provided by Influenza A virus-specific CD8 and CD4 cells having specificity for highly conserved epitopes existing within internal IAV protein.
Conclusion: The present study concludes that GAS and IAV are critical pathogens invading humans and leading to significant disease and mortality globally. For the development of targeted vaccinations and treatment, the understanding of immune response following the invasion of these pathogens is vital.
Keywords: CD4 cell, influenza a virus, isoflurane, mice, T cells.
Tob Regul Sci.™ 2022; 8(1): 206-211
DOI: doi.org/10.18001/TRS.8.1.19

INTRODUCTION

The most common entry points for pathogens to invade the body are the mucosal surfaces. This particularly holds for lung epithelium, which is largely exposed to the air and is constantly contacted by numerous disease-causing pathogens.[1] While millennia of natural selection have honed a sophisticated repertoire of defenses, owing to the gaseous exchange being the primary function of the lung parenchyma, there are various challenges encountered by the lung.[2]

Defense against viral, bacterial, and fungal pathogens is largely governed by the T cells in the lungs. The optimal response of the T cells is necessary for assessing the defense mechanism as low levels result in delayed clearance of infective pathogens, whereas, the excessive response might result in

considerable host tissue damage in an attempt to rapidly clear the tissue pathogens.[3] The response by T cells differs for different pathogens. Activation of the T cells is primarily dependent on the Dendritic cells which are the main Antigen Presenting Cell (APC), following primary activation, these cells also decide the course of response to the antigen via production of the cytokines. Another lung-specific response is innate immune system cell presentation with the production of chemokines and pro-inflammatory cytokines.[4] To allow adequate gaseous exchange, the preservation of lung architecture with complete elimination of pathogen is vital. T-cells are largely responsible for initiating and regulating the defense against viral, bacterial, and fungal pathogens.[4]

The optimal response of the T cells is necessary for assessing the defense mechanism as low levels result in delayed clearance of infective pathogens, whereas, the excessive response might result in considerable host tissue damage in an attempt to rapidly clear the tissue pathogens. The properties and rapidity of T cell response are different on different pathogens.[5]

Pathogen-associated molecular patterns (PAMPs) are present in the inhaled pathogens reaching the lung. These PAMPs are recognized by the Dendritic cells and respiratory epithelial cells and pattern recognition receptors (PRRs) are expressed by these cells. PRRs include Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (12), NOD-like receptors (NLRs), and Toll-like receptors (TLRs). Either by the activation of the dendritic cells via PAMP or by the production of cytokines by epithelial mature cells followed by their entry to draining lymph node of the lung. All signals needed to activate naive T cells are expressed than at the draining lymph node.[6]

The antigen-presenting cells (APC) like dendritic cells play a vital role in initiating the T cells and govern the course of the action through cytokine and chemokine production. Two discrete subsets of helper T cells were first identified in 1986 and were termed type 1 and type 2 T helper cells.[5,6]

MATERIALS AND METHODS

From the National Cancer Institute female mice, C57Bl/6 and BALB/c mice were purchased. The age of the mice was 8-12 weeks used for the study. Mice were randomly selected for the groups of experiments.

Influenza A virus infection of mice

The female mice, C57Bl/6 after weight and age matching, were anesthetized lightly using isoflurane inhalation. Followed by this, mice were intranasally infected using mouse-adapted A/Puerto Rico/8/1934 (H1N1) (PR8) following the indicated dose and in 50µL of Iscove's media. The virus was grown and cultured at 37 ºC for two days in 10 days old allantoic fluid of embryonated hen eggs, which was stored at -80 ºC before its use.

After 48 hours of intranasal infection via virus, the mice were anesthetized and were given 75µL of clodronate liposomes via the intranasal route.

Cells Preparation

For cell preparation, lungs were harvested in 10ml of Iscove's s DMEM. After harvesting, to get a single cell suspension, lungs were mashed using wire mesh followed by filtration using nylon mesh. Mincing and digestion of lungs were done in Iscove's media having 0.02mg/mL DNAse (Sigma for 15 minutes at 37 ºC before mashing) and 1mg/mL collagenase (Sigma), in cases where it was indicated. The quantification of lymphocytes was done with a hemocytometer and trypan blue exclusion

Flow Cytometry

Obtained monoclonal antibodies were then utilized for the studies as follows: anti-CD8α (53-6.7) and anti-CD4 (GK1.5 and RM4-5). Cells were suspended with indicated antibodies at 1x106 cells/100µL FACS buffer (sterile PBS, 2% heat-inactivated fetal calf serum, 0.02% Sodium Azide) followed by ice incubation for 30 minutes to attain the surface staining. This was followed by fixing using BD FACS Lysing Solution (BD Biosciences, San Diego, CA). For transcription factors and intracellular staining, the surface staining was done following the same procedure followed by permeabilization, staining, and fixation following manufacturer's instruments on e-Bioscience Foxp3 intracellular staining kit (eBioscience, San Diego CA).

RESULTS

The present study was conducted to assess the specialized subsets of CD4 T cells with influenza A virus specificity in the lungs. Also, the present study aimed to establish that influenza A virus-specific CD4 T cell response is regulated via antigen-presenting cells of the pulmonary region. The study was conducted on female C57Bl/6 and BALB/c mice of the age of 8-12 weeks were used for all assessments. Age- and weight-matched groups of female C57Bl/6 mice were lightly anesthetized by isoflurane inhalation and infected intranasally.

S. No	The strain of the mice and specificity	Sequence	MHC	Tested	Result
1.	**C57Bl/6**				
a)	NP316-330	Irpnenpahksqlvw	I-A^b	Yes	-
b)	NP311-325	Qvyslirpnenpahk	I-A^b	Yes	++++
c)	NP276-290	Lpacvygpavasgyd	I-A^b	Yes	-
d)	NP201-215	Indrnfwrgengrkt	I-A^b	Yes	-
e)	NP136-150	Mmiwhsnlndatyqr	I-A^b	Yes	++
f)	NP218 – 229	Ayermcnilkgk	I-A^d	Yes	+++
g)	NA79 – 86	Irgwaiys	I-E^d	Yes	-
h)	NA69 – 89	Iltgnsslcpirgwaiyskdn	I-E^d	Yes	+++
2.	**BALB/c**				
a)	HA317-329	Vtglrnipsiqsr	I-E^d	Yes	-
b)	HA306-318	Pkyvrsaklrmvt	I-E^d	Yes	+
c)	HA195-209	Nayvsvvtsnynrrf	I-A^d	Yes	-
d)	HA 186 – 200	Stnqeqtslyvqasg	I-E^d	No	n/a
e)	HA 178 – 195	Yiwgihhpstnqeqtsly	I-E^d	No	n/a
f)	HA159-170	Klknsyvnkkgk	I-E^d	Yes	+
g)	HA141-153	Hntngvtaacshe	I-A^d	Yes	+++
h)	HA110-120	Sferfeifpke	I-E^d	Yes	-
i)	HA81 – 97	Netwdlfverskafsnc	I-A^d	No	n/a
j)	HA72 – 92	Hrildgidctlidallgdphc	I-A^d	No	n/a
k)	HA71-91	Lgnphcdvfqnetwdlfvers	I-A^d	No	n/a
l)	HA48 – 67	Tgkicnnphridgidctli	I-A^d	No	n/a

Table 1: Molecule binding of MHC II molecule for CD4 T cell epitome

The present study also assessed the MHC class II molecule binding for the identified CD4 T cell epitomes in mouse-adapted A/Puerto Rico/8/1934 strain, the results are depicted in Table 1. It was seen that there was a significant rise in CD4 T cell frequency that produce IFNγ after incubating with peptide-pulsed splenic stimulator DC in comparison to CD4 T cells where incubation was done using non-peptide-pulsed splenic stimulator DC.

The study results also showed that there was depletion in pulmonary ACPs with increased mortality following a sublethal dose of the Influenza A virus. Following infection of female C57BI/6 intranasally, with 0.05LD50 or 0.1LD50 inoculums of PR8. Also, pulmonary depletion of half pulmonary ACP was seen following intranasal clodronate liposomes administration. IAV infected mice with pulmonary APC depletion and IAV infected mice were monitored for survival daily.

DISCUSSION

Assessment of human pulmonary immune response to influenza A virus infection is scarce owing to the limitation of obtaining a sample from the lung.[7] A detailed understanding of lung immune response in mice can help in understanding human lung concerning its immune response and cells as seen in blood or lavage fluid of bronchioalveolar region, as these perform a vital function in the lung tissues.[8] First, the extent of plasticity of IAV-specific CD4 T cells in the lungs during infection. In other studies conducted on other models assessing infection, it was seen that during the priming stage of activation of CD4 T cells, specific signals are needed. However, the lineage commitment of this cell requires additional signals to reinforce or change such lineage.[9]

During the IAV infection course, changes are seen in transcription factors determining lineage changes within individual CD4 T cells is an area that needs to be focused on. It is speculated that during Influenza A virus infection, rather than a complete shift (from T-bethiFoxp3- to T-bet-Foxp3hi), upregulation/amplification of alternative lineage determining transcription factor (T-bet+/intF oxp3+/int) could be seen. During the murine model of M. tuberculosis, Foxp3+T-bet+ cells arise, which are efficacious at Th1-mediated inflammation site trafficking and suppression of cytokine production and proliferation by Th1 cells.[10]

An anti-viral and pro-inflammatory environment is vital during IAV infection during the presence of the virus in the lungs. However, later repair and inflammation resolution becomes vital.[11] During this shift to resolution from pro-inflammation, other subsets perform a necessary function. These include IAV-specific Foxp3+T-bet+ cells. This specific CD4 T cell response is finely-tunes and has several advantages and is prominent keeping in consideration that in the lung inflammation and excess of cellular infiltration can compromise the effective gaseous exchange.[12]

Supporting the fact that CD4 T cells express lineage that decides transcription factors in IAV infection, it is shown by a study that at day 10 following IAV infection, approximately 60% of NP311+ CD4 T cells are Bcl-6+, and 80% NP311+ cells are T-bet+. However, this seems impossible considering mathematics except when few NP311+ cells express both transcription factors.[13]

The results of the present study were also in agreement with other studies where authors reported that reduction in IL-2 helps in developing Tfh during Influenza A virus infection when Bcl-6+ Tbet+ cells appear. Also, Bcl-6+T-bet+ cells can be seen concerning infection by Influenza A, owing to isotype bias of the antibody response and Th polarization of the response.[14]

Using microscopy and flow cytometry, assessing secondary lymphoid organs concerning the germinal center formation and kinetics of IAV-specific T-bet+Bcl-6+ cells can lead to further clarification in such cases. Specific cell function after IAV infection can be indicated by quantitively analyzing the IAV-

specific CD4 T lung cells proportion, data about localization of IAV-specific T-bet+Bcl-6+ cells relative to germinal centers, and LN and spleen expressing T-bet and Bcl-6 over time.[15]

CONCLUSION

Within its limitations, the present study concludes that GAS and IAV are alarming human pathogens causing severe disease and mortality worldwide. Developing appropriate vaccination and intervention is directed towards the adequate understanding of the immune system specifically concerning these pathogens. During infection of IAV, there is an accumulation of CD4 T cells specific to IAV which reaches a maximum at 10 days postoperatively. On the 10th day postoperatively, CD 4 T cells in the majority were T-bet+, and these produce IL-17, IL-13, IL-2 in less concentration compared to IFN γ produced in high concentration. In both mice strains, BALB/c as well as C57Bl/6, IAV-specific Foxp3+ or Bcl-6+ cells are seen inconsiderable amount, whereas, RORγt+ cells are seen in small number, and GATA-3+ cells are not seen.

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