Therapeutic Anti-Amyloid β Antibodies Cause Neuronal Disturbances

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Abstract

Recent published clinical trial safety data showed that 41% of Alzheimer patients experienced amyloid-related imaging abnormalities (ARIA), marks of microhemorrhages and oedema in the brain, following administration of Biogen's *Aduhelm/* aducanumab (amino acids 3-7 of the Aβ peptide). Similarly, Janssen/Pfizer's Bapineuzumab (amino acids 1-5 of the Aβ peptide) and Roche's Gantenerumab (amino acids 2–11/18–27 of the Aβ peptide) also displayed ARIA in clinical trials, including microhemorrhage and focal areas of inflammation or vasogenic oedema respectively. The molecular mechanisms underlying ARIA caused by therapeutic anti-Aβ antibodies remain largely unknown, however, recent reports demonstrated that therapeutic anti-prion antibodies activate both neuronal apoptotic and allergenic proteomes following cross-linking cellular prion protein. Here, we report that treatment of human induced pluripotent stem cells-derived neurons (HSCN) from a non-demented donor, co-cultured with human primary microglia with anti-$A\beta_{1-6}$, or anti-$A\beta_{17-23}$ antibodies activate a significant number of both apoptotic and allergenic-related proteins as assessed by mass spectrometry. Interestingly, a large proportion of the identified proteins included cytokines such as IL-4, IL-12, and IL-13 suggesting a type-1 hypersensitivity response. Following flow cytometry analysis, several proinflammatory cytokines were significantly elevated following anti-$A\beta_{1-6}$, or anti-$A\beta_{17-23}$ antibody treatment. These results justify further and more robust investigation of the molecular mechanisms of ARIA during immunotherapy study trials of AD.

Keywords: Alzheimer's disease; anti-Aβ antibody; neuronal type 1 hypersensitivity; human primary microglia; iPSC-derived neurons

Main

Alzheimer's disease (AD) is the most common form of dementia, characterized by severe, permanent, and progressive memory loss accompanied by cognitive and behavioural changes (Graham et al., 2017; Huang and Mucke, 2012; Scheltens et al., 2016). Amyloid beta (Aβ) is considered as a prominent target for the development of AD modifying therapeutics because of its putative causal contribution to the pathogenesis of AD (Murphy and LeVine 3rd, 2010). Several active and passive immunization approaches have been developed for the prevention and treatment of AD, but most approaches have failed in clinical trials due to their lack of efficacy (Avgerinos et al., 2021). To date, several humanized monoclonal anti-Aβ antibodies have been developed for the treatment of AD. The effect of some of these antibodies on adverse event risks in phase III randomised clinical trials in AD, including bapineuzumab (Salloway et al., 2014), solanezumab (Doody et al., 2014), gantenerumab (Ostrowitzki et al., 2017), crenezumab (Cummings et al., 2018a, 2018b), and aducanumab (Sevigny et al., 2016), led to amyloid-related imaging abnormalities (ARIA) with MR signal alterations representing either vasogenic oedema (ARIA-E) and microhemorrhages/hemosiderosis (ARIA-H) respectively (Avgerinos et al., 2021). The molecular mechanisms underlying immunotherapy related ARIA in AD and other potential neurotoxic effects caused by anti-Aβ antibody-based therapies remain largely unknown. We describe the deleterious effects caused by anti-Aβ antibodies, similar to those used in phase III randomised clinical trials in AD described above, following their application directly on human induced pluripotent stem cell-derived neurons (HSCN) and on human primary microglia cells (HPM) co-cultured with HSCN. We report here that direct anti-Aβ antibody treatment (DAT) led to activation of a large number of apoptotic- and allergenic-related genes as assessed by DAVID (Huang et al., 2009a, 2009b) and AllerGAtlas (Liu et al., 2018b) database respectively. In addition, co-culture of anti-Aβ antibody-treated HPM with HSCN (direct microglia treatment, DMT) and simultaneous treatment of HPM with HSCN (direct microglia antibody treatment, DMAT) with anti-Aβ antibodies also led to activation of a significant number of apoptotic- and allergenic-related genes. Of note, some of the activated allergenic-related genes were part of the same interactome as the high affinity IgE receptor (FcεRI), IgG FcγR1 as well as FcγR2 and FcγR3. Flow cytometry analysis displayed higher levels of GM-CSF, IL-6, IL-9, IL-12, IL-17A, IFN-α, and TNF-α following treatment with anti-Aβ antibodies. This study demonstrates for the first time that anti-Aβ antibody treatment leads to a neuronal hypersensitivity-like reaction and apoptosis.

Together, our data support the inclusion of a comprehensive toxicity assessment of therapeutic antibodies before entering clinical studies for AD.

Anti-Aβ Antibodies Cause Neuronal Hypersensitivity-like and Apoptosis Reaction

Direct antibody treatment of human induced pluripotent stem cell-derived neuron: Direct antibody treatment (DAT) of human induced pluripotent stem cells-derived neurons (HSCN) with anti-Aβ1-6 and anti-Aβ17-23 polyclonal antibodies (pAbs) (**Figure 1**) led to the identification of 1182 and 1255 proteins (p <0.05) respectively after liquid-chromatography mass spectrometry (LC-MS) analysis when compared with untreated cells. Differentially expressed proteins were considered using at least 2 identified unique peptides and a confidence score ≥ 40. The stringent parameters used here led to the identification of 389 and 422 proteins following treatment with anti-Aβ1-6 and anti-Aβ17-23 pAbs respectively **(Table S1)**. Of note, the parameters used to identify proteins associated with antibody treatment are unusually high and would allow elimination of 'false-negatives' post LC-MS analysis. Initially, the 389 and 422 proteins were then assessed for allergenicity using AllergGAtlas database (Liu et al., 2018b) and 20 and 22 allergy related proteins following anti-Aβ1-6 (termed as $_{Allg}DAT_{Aβ1-6}$) and anti-Aβ17-23 (termed as $_{Allg}DAT_{Aβ17-23}$) pAb treatment respectively. These treatments led to activation of key allergenic proteins such as PSAT1, PTPRN, RHOA, MYLK, HSPB1, CTSD, PIK3C2A, PARK7, PRDX1, GSTP1, LDHA, DOCK2, DOCK8, PPIA, PHB, PGK1, HSP90B1, and VIM **(Table S2)**. Among the 20 allergenic-related $_{Allg}DAT_{Aβ1-6}$, 6 were upregulated and 14 were downregulated when compared to untreated control. Further, 8 were upregulated and 14 were downregulated amongst the 22 allergenic-related $_{Allg}DAT_{Aβ17-23}$ when compared to untreated control **(Table S2).** Interestingly, over 80% of the allergy related protein (18 proteins) were common between $_{Allg}DAT_{Aβ1-6}$ and $_{Allg}DAT_{Aβ17-23}$ **(Figure 2A)** suggesting a shared molecular pathway. Notably, 7 out of the 18 common proteins have been reported to a key role in pro-inflammatory responses, including RHOA (Segain et al., 2003), MYLK (Xiong et al., 2017), PGK1 (Chang et al.), PRDX1 (Chu et al., 2016), DOCK2 (Chen et al., 2018), GSTP1 (Wetering et al., 2020), and PPIA (Nigro et al., 2011). On the other hand, other proteins common to both treatments were shown to be associated with the pathogenesis of allergy and such proteins included PPIA (Stemmy et al., 2011), GSTP (van de Wetering et al., 2021), MYLK (Wang et al., 2014b), PHB (Yurugi and Rajalingam, 2013), and RHOA (Zhang et al., 2020). Importantly, our analysis demonstrated activation of 4

common allergy-related proteins previously been shown to be associated with allergy and included LDHA, PRDX1, PGK1, and VIM (Adhikari et al., 2021a). However, reactome pathway associated with allergenic response such as neutrophil degranulation (Kämpe et al., 2011) and autophagy (Liu et al., 2016b) were associated with $_{Allg}DAT_{A\beta 1\text{-}6,}$ but not with $_{Allg}DAT_{A\beta 17\text{-}23}$ in overrepresentation test by PANTHER classification system v14.0 (Mi et al., 2019) **(Table S3).**

During phase III clinical trials, administration of monoclonal anti-Aβ antibodies to AD patients resulted in oedema/vasogenic oedema, and microhemorrhages, defined as ARIA (Avgerinos et al., 2021). The molecular mechanisms underlying ARIA remain unknown. We verified whether the allergy-related proteins are associated with endothelial dysfunction, oedema or microhemorrhages and uncovered 8 vascular related proteins (RHOA, MYLK, PIK3C2A, PARK7, PRDX1, LDHA, DOCK2, DOCK8, VIM) common to $_{Allg}DAT_{A\beta 1\text{-}6}$ and $_{Allg}DAT_{A\beta 17\text{-}23}$. For instance, RHOA and MYLK were previously shown to be associated with endothelial dysfunction (Shen et al., 2010; Yao et al., 2010) and oedema (Carles et al., 2010; Shen et al., 2010).

In addition, Metascape gene enrichment analysis (Zhou et al., 2019) of the 18 common allergy related proteins demonstrated the involvement of RHOA in the regulation of NIK/NF-kappaB signalling, neutrophil degranulation, and hemostasis, conforming its role in endothelial dysfunction, inflammatory processes, and allergic response (Lacy, 2006; Pleines et al., 2012; Yao et al., 2010; Zhang et al., 2020) (**Figure 2B**;**Figures S1A-D;Table S4**). Moreover, RHOA was previously identified as a therapeutic target for AD (Aguilar et al., 2017) and found to be associated with prion pathogenesis (Kim et al., 2017). Finally, ModEnrichr gene enrichment analysis (Kuleshov et al., 2019) highlighted the involvement of the regulation of protein kinase activity (Mazrouei et al., 2019), neutrophil degranulation (Kämpe et al., 2011), and regulation of apoptotic process (Su et al., 2018) in endothelial dysfunction, inflammatory processes, immune response, and allergy (**Figures S2A-B**). The molecular complex detection (MCODE) algorithm in Metascape (Bader and Hogue, 2003) was used to identify the densely connected proteins and RHOA was shown to interact directly with PARK7, VIM, and DOCK8 (all of which are associated with vascular function), implying that these proteins are associated with endothelial dysfunction, inflammation, and allergy (**Figure 2C; Figures S1E-F)**.

We have previously shown that treatment of neuronal cell lines with anti-PrP monoclonal antibodies leads to a hypersensitivity-like reaction (Adhikari et al., 2021a). We wanted to investigate whether the 18 common allergy related proteins were involved in a type 1 and/or 2

hypersensitivity reaction. Here, we show that RHOA interacts directly with both FcγR1 and FcγR2 and PTPRN interacts directly with FcγR1, FcγR3, and FcεR1A, indicating the involvement of $_{Allg}DAT_{Aβ1-6}$ and $_{Allg}DAT_{Aβ17-23}$ proteins in provoking a hypersensitivity reaction **(Figure S2C)**.

We then assessed the 389 and 422 proteins for apoptotic activity using DAVID bioinformatics resources (Huang et al., 2009b), and 40 and 46 apoptosis-related proteins were identified following anti-Aβ1-6 (termed as $_{Apop}DAT_{Aβ1-6}$) and anti-Aβ17-23 (termed as $_{Apop}DAT_{Aβ17-23}$) respectively, including DOCK8, LDHA, PARK7, PHB, GAPDH, PDIA3, ANXA6, PRDX2, GSTP1, HSPB1 were identified **(Table S5).** Among the 40 apoptosis-related $_{Apop}DAT_{Aβ1-6}$ proteins, 12 were upregulated and 28 were downregulated, however, 17 proteins were upregulated and 29 were downregulated amongst the 46 apoptotic-related $_{Apop}DAT_{Aβ17-23}$ when compared to untreated control **(Table S5).** Over 78% (34 proteins) apoptosis related proteins were common to treatments with $_{Apop}DAT_{Aβ1-6}$ and $_{Apop}DAT_{Aβ17-23}$ **(Figure 2D; Table S6).** Among 34 proteins, 12 proteins (SOD1, DOCK8, HSPB1, SCRIB, LDHA, PARK7, PRDX2, NPM1, DDX5, CFL1, HSPA1A, HNRNPK) were related to vascular functions. For instance, NPM1 is associated with vascular inflammation and endothelial dysfunction (Rao et al., 2021) while PRDX2 was shown to be associated with both endothelial dysfunction (El Eter et al., 2014) and protective effect on vascular endothelial cells (Kang et al., 2011). Moreover, reactome pathway associated with apoptosis such as chaperone mediated autophagy in the formation of apoptosis (Wu et al., 2019) was found to be associated with $_{Apop}DAT_{Aβ17-23}$, while interleukin-12 signalling in the execution of apoptosis (Fan et al., 2002), neutrophil degranulation (Iba et al., 2013), and signaling by Rho GTPases (Aznar and Lacal, 2001) were found to be associated with both $_{Allg}DAT_{Aβ1-6}$ and $_{Apop}DAT_{Aβ17-23}$ in the overrepresentation test by PANTHER (**Table S7**).

Metascape gene ontology analysis confirmed the involvement of the identified apoptosis related proteins with the endothelial dysfunction, cell death, regulation of apoptosis, cytokine production, and interleukin signaling such as VEGFA-VEGFR2 signalling pathway (CFL1, PRDX2, LDHA etc.)(Shaik-Dasthagirisaheb et al., 2013), cellular response to stress (SOD1, PRDX2 etc.)(Boyce and Yuan, 2006), regulation of extrinsic apoptotic signalling pathway (PRDX2, PARK7 etc.)(Fulda and Debatin, 2006), regulation of protein kinase activity (Lavin et al., 1996), and interleukin-12 signalling (CFL1, SOD1 etc.)(Fan et al., 2002) (**Figure 2E; Figures S3A-D; Table S8**). ModEnrichr gene enrichment analysis also confirmed the association of endothelial dysfunction, apoptosis and cell death, and interleukin signaling for the identified $_{Apop}DAT_{Aβ1-6}$ and $_{Apop}DAT_{Aβ17-}$

$_{23}$ proteins (**Figures S2D-E**). Herein, NPM1was found to interact directly with ACTC1, PALG4, RPS6, and RPS3; however, PRDX2 directly interacts with PDIA3 and AARS1 during MCODE algorithm analysis (**Figure 2F**). STRING analysis showed that GAPDH protein interacts directly with FcγR1, FcγR2, FcγR3, and FcεR1A (**Figure S2F**). In addition, DOCK8 was found to be directly interacting with FcγR2, FcγR3, and FcεR1A. Herein, endothelial dysfunction related proteins NPM1 and PRDX2 were also shown to be directly interacting with GAPDH as well as with PARK7, PHB, YWHAE, HSPB1, and HSPA5. Therefore, the data revealed that $_{Apop}DAT_{Aβ1-6}$ and $_{Apop}DAT_{Aβ17-23}$ proteins, especially GAPDH, have strong interactions with FcγRs, indicating their involvement in hypersensitivity response.

Co-culture of anti-Aβ antibody-treated human primary microglia cells with human induced pluripotent stem cells-derived neuron: Co-culture (DMT) of anti-Aβ1-6- ($DMT_{Aβ1-6}$) or anti-Aβ17-23 antibody-treated ($DMT_{Aβ17-23}$) human primary microglia cells (HPM) with human induced pluripotent stem cells-derived neuron (HSCN) (**Figure 1**) led to the identification of 1124 and 1061 proteins (p <0.05) respectively after LC-MS analysis when compared with untreated cells. After consideration of at least 2 unique peptides and a confidence score of ≥ 40, 340 and 315 proteins were identified respectively **(Table S9).**

The allergenicity assessment of the 340 and 315 proteins showed that 18 and 16 proteins are associated with allergy following anti-Aβ1-6 (termed as $_{Allg}DMT_{Aβ1-6}$) and anti-Aβ17-23 (termed as $_{Allg}DMT_{Aβ17-23}$) treatment respectively, including TYK2, PCNA, CTSD, ANXA1, MYLK, PPARD, PIK3C2A, PRDX1, PGK1, ALB, EEF1A1, HSP90B1, ACTB, PPIA, VIM, and PHB **(Table S10).** Among the 18 allergenic-related $_{Allg}DMT_{Aβ1-6}$, 9 were upregulated and 9 were downregulated. 8 were upregulated and were downregulated amongst the 16 allergenic-related $_{Allg}DMT_{Aβ17-23,}$ when compared to untreated control **(Table S10).** Over 82% of the allergy related protein (n=14; TYK2, PCNA, CTSD, ANXA1, MYLK, DSG1, PPARD, PIK3C2A, PRDX1, PGK1, ALB, EEF1A1, HSP90B1, ACTB) were common to $_{Allg}DMT_{Aβ1-6}$ and $_{Allg}DMT_{Aβ17-23}$ **(Table S6; Figure 3A);** notably, 8 out 14 (MYLK, PGK1, PRDX1, TYK2, PCNA, ANXA1, PPARD, ALB) are involved in pro-inflammatory responses (Chu et al., 2016; Kullmann et al., 1996; Magzal et al., 2017; Moraes et al., 2018; Poelzl et al., 2021; Wang et al., 2015a; Youssef and Badr, 2004; Zhao et al., 2016) and 7 out of 14 (MYLK, PPARD, PRDX1, PGK1, ALB, ACTB, EEF1A1) have previously been shown to be involved in the pathogenesis of allergy (Adhikari et al., 2021a). Reactome pathway associated with allergenic responses such as IL-4 and IL-13

signalling (Gour and Wills-Karp, 2015) was found for $_{Allg}DAT_{A\beta17\text{-}23,}$ but not for $_{Allg}DAT_{A\beta1\text{-}6}$ in overrepresentation test by PANTHER classification system (**Table S11**). 9 out of 14 proteins were vascular related (MYLK, TYK2, PCNA, PPARD, PIK3C2A, PRDX1, PGK1, ALB, EEF1A1) where PPARD, MYLK, and ALB have an established association with endothelial dysfunction (Cheang et al., 2017; Kadono et al., 2010; Shen et al., 2010). In addition, gene enrichment analysis through Metascape for the identified common 14 allergy-related genes confirmed their involvement in allergenic responses, inflammation, and endothelial dysfunction through the IL-4 and IL-13 signalling (Gour and Wills-Karp, 2015), neutrophil degranulation (Kämpe et al., 2011; Lacy, 2006), signaling by Rho GTPases (Yao et al., 2010; Zeng et al., 2022), cellular responses to stress (Silva et al., 2012), and response to hypoxia (Janaszak-Jasiecka et al., 2021) **(Figure 3B; Figures S4A-B; Table S12).** Herein, HSP90B1 was found to be involved with IL-4 and IL-13 signaling, cellular responses to stress, and response to hypoxia. Finally, ModEnrichr gene enrichment analysis of the $_{Allg}DMT_{A\beta1\text{-}6}$ and $_{Allg}DMT_{A\beta17\text{-}23}$ related proteins confirmed their involvement in allergy and inflammatory processes through the regulation of apoptotic process (Akdis et al., 2004; Su et al., 2018), cytokine mediated signaling pathway (Athari, 2019), and platelet aggregation (Kasperska-Zajac and Rogala, 2007; Palma-Carlos et al., 1991) (**Figures S5A-B**). The MCODE algorithm showed that HSP90B1 is directly interacting with PIK3C2A and PCNA which are associated with vascular function (**Figure 3C**). The protein-protein interaction analysis of the $_{Allg}DMT_{A\beta1\text{-}6}$ and $_{Allg}DMT_{A\beta17\text{-}23}$ related proteins through STRING showed that ALB interacts directly with FcγR1, FcγR2, FcγR3, and the cytokine IL-4 and IL-13. On the other hand, ACTB interacts directly with FcγR2, FcγR3, and the cytokine IL-4 and IL-13. In addition, HSP90B1 directly interacts with both ACTB and ALB (**Figure 3D**).

The 340 and 315 proteins were then assessed for apoptosis using DAVID bioinformatics resources, and 31 and 27 apoptosis-related proteins were identified following $_{Apop}DMT_{A\beta1\text{-}6}$ and $_{Apop}DMT_{A\beta17\text{-}23}$ respectively, including ANXA1, PPARD, HSP90B1, HNRNPK, ALB, PRDX2 (**Table S13**). Among the 31 apoptosis-related $_{Apop}DMT_{A\beta1\text{-}6}$, 16 were upregulated and 15 downregulated; and amongst the 27 apoptotic-related $_{Apop}DMT_{A\beta17\text{-}23,}$ 15 were upregulated and 12 downregulated when compared to untreated control **(Table S13**).

Nearly 80% (23 proteins) of the apoptosis related proteins were common to treatment with $_{Apop}DMT_{A\beta1\text{-}6}$ and $_{Apop}DMT_{A\beta17\text{-}23}$ (**Figure 3E; Table S6**). Among 23 proteins, 7 proteins (ANXA1, PPARD, NPM1, PRDX2, CFL1, HNRNPK, ALB) were found to be vascular related where

PPARD, PRDX2, and ALB were found to be associated with vascular dysfunction (Cheang et al., 2017; El Eter et al., 2014; Kadono et al., 2010). Moreover, reactome pathway associated with apoptosis such as cellular responses to stress (Boyce and Yuan, 2006), IL-12 signaling in the execution of apoptosis (Fan et al., 2002), chaperone mediated autophagy in the formation of apoptosis (Wu et al., 2019), and signaling by Rho GTPases (Aznar and Lacal, 2001) were found to be associated with both $_{Allg}DMT_{A\beta1\text{-}6}$ and $_{Apop}DMT_{A\beta17\text{-}23}$ in the overrepresentation test by PANTHER (**Table S14**).

Metascape gene enrichment analysis showed the involvement of the identified 23 common apoptosis related proteins of $_{Apop}DMT_{A\beta1\text{-}6}$ and $_{Apop}DMT_{A\beta17\text{-}23}$ with apoptosis, cell death, interleukin signaling, and inflammation by exhibiting the gene ontology such as regulation of extrinsic apoptotic signaling pathway and cell death (HNRNPK, P4HB, ANXA1, EEF1A2, PHB1 etc.)(Fulda and Debatin, 2006), interleukin-12 signalling (CFL1, HSPA9, P4HB, TCP1)(Fan et al., 2002), signaling by Rho GTPases (CFL1, HSP90AB1, ITSN1 etc.)(Aznar and Lacal, 2001), platelet degranulation (ALB, CFL1, HSPA5)(Hottz et al., 2014), cellular response to stress (ALB, HSPA5, HSPA9, HSP90AB1, P4HB, PRDX2 etc.)(Boyce and Yuan, 2006), VEGFA-VEGFR2 signalling pathway (ALB, ANXA1, CFL1, GAPDH, P4HB, PRDX2 etc.)(Shaik-Dasthagirisaheb et al., 2013) (**Figure 3F; Figures S4C-D; Table S15**). These proteins were also found to be densely connected in the MCODE algorithm analysis (**Figure 3G**). Finally, ModEnrichr gene enrichment analysis also confirmed the association of these identified $_{Apop}DMT_{A\beta1\text{-}6}$ and $_{Apop}DMT_{A\beta17\text{-}23}$ proteins with apoptosis (**Figures S5C-D**). The protein-protein interaction analysis of the $_{Apop}DMT_{A\beta1\text{-}6}$ and $_{Apop}DMT_{A\beta17\text{-}23}$ related proteins through STRING showed that ALB directly interacts with FcγR1, FcγR2, and FcγR3. In addition, RPS6, HSP90BA1, HSP90B1, P4HB, CFL1, PRDX2, ANXA1, ACTC1, NEFL, ANXA6, HSPA5 directly interact with ALB, indicating that ALB plays a central role in these interactions (**Figure 3H**).

Direct antibody treatment of human primary microglia cells and human induced pluripotent stem cells-derived neuron co-culture: Direct anti-Aβ1-6- ($DMAT_{A\beta1\text{-}6}$) or anti-Aβ17-23 ($DMAT_{A\beta17\text{-}23}$) treatment of human primary microglia cells (HPM) and human induced pluripotent stem cells (HSCN) co-culture (DMAT) (**Figure 1**) resulted in 861 and 740 differentially expressed proteins respectively, after LC-MS analysis. After consideration of at least 2 unique peptides and a confidence score of ≥ 40, 271 and 232 proteins were identified following treatment with anti-Aβ1-6 and anti-Aβ17-23 antibodies respectively (**Table S16**).

The allergenicity assessment of the 271 and 232 proteins showed that 16 and 5 proteins are associated with allergy following anti-Aβ1-6 (termed as $_{Allg}DMAT_{Aβ1-6}$) and anti-Aβ17-23 (termed as $_{Allg}DMAT_{Aβ17-23}$) treatment respectively, including TYK2, ANXA1, HSP90B1, PPIA, ITGB4, PGK1, TBC1D4, CTSD, VIM, EEF1A1, TF, PRDX1, HSPD1, MYLK, PCNA, ACTB protein for $DMAT_{Aβ1-6}$ and MYLK, PCNA, ACTB, DSG1, PPARD protein for $DMAT_{Aβ17-23}$ (**Table S17**). Among the 16 allergenic-related $_{Allg}DMAT_{Aβ1-6}$, 6 were upregulated and 10 downregulated; and amongst the 5 allergenic-related $_{Allg}DMAT_{Aβ17-23,}$ 2 were upregulated and 3 downregulated when compared to untreated control (**Table S17**).

About 28% (3 proteins) of the allergy related proteins (MYLK, PCNA, ACTB) were common to $_{Allg}DMAT_{Aβ1-6}$ and $_{Allg}DMAT_{Aβ17-23}$ (**Table S6; Figure S6A**); MYLK and PCNA are involved in pro-inflammatory responses (Kullmann et al., 1996; Wang et al., 2015a). However, 5 allergy related proteins (TYK2, ANXA1, PPIA, PGK1, PRDX1) (Chu et al., 2016; Moraes et al., 2018; Nigro et al., 2011; Poelzl et al., 2021; Zhao et al., 2016) specific for $_{Allg}DMAT_{Aβ1-6}$ and PPARD specific for $_{Allg}DMAT_{Aβ17-23}$ (Youssef and Badr, 2004) are associated with pro-inflammatory responses.

Moreover, 9 proteins (MYLK, ACTB, ANXA1, ITGB4, PPARD, PRDX1, PGK1, EEF1A1, PPIA) related to both $_{Allg}DMAT_{Aβ1-6}$ and $_{Allg}DMAT_{Aβ17-23}$ were previously shown to be involved in the pathogenesis of allergy (Adhikari et al., 2021a; Liu et al., 2018a; Ng et al., 2011). Reactome pathway associated with allergenic response such as IL-4 and IL-13 signaling (Gour and Wills-Karp, 2015) was found for $_{Allg}DMAT_{Aβ1-6,}$ but not for $_{Allg}DMAT_{Aβ17-23}$ in overrepresentation test by PANTHER classification system (**Table S18**).

Finally, 7 proteins specific for $_{Allg}DMAT_{Aβ1-6}$ (PCNA, TYK2, VIM, PPARD, PRDX1, PGK1, EEF1A1, TF) were vascular related proteins; and 1 protein specific for $_{Allg}DMAT_{Aβ17-23}$ (PPARD) was vascular related and associated with endothelial dysfunction (Cheang et al., 2017).

In addition, gene enrichment analysis through Metascape for the identified 16 allergy-related proteins of $_{Allg}DMAT_{Aβ1-6}$ confirmed their involvement in allergenic responses, inflammation, and endothelial dysfunction through IL-4 and IL-13 signalling (ANXA1, HSP90B1, TYK2, VIM)(Gour and Wills-Karp, 2015), neutrophil degranulation (CTSD, DSG1, EEF1A1, PPIA)(Kämpe et al., 2011; Lacy, 2006), signaling by Rho GTPases (ACTB, DSG1, MYLK)(Yao et al., 2010; Zeng et al., 2022), and cellular responses to stress (HSPA5, HSP90AB1, P4HB, RPS3A, RPS27A, PRDX2, HSP90B1, HYOU1, CCAR2) (Silva et al., 2012) (**Figures S6B-D;**

Table S19). Finally, gene enrichment analysis of the $_{Allg}DMAT_{Aβ1-6}$ related proteins using ModEnrichr confirmed their involvement in allergy and inflammatory processes through the regulation of apoptotic process (Akdis et al., 2004; Su et al., 2018), cytokine mediated signaling pathway (Athari, 2019), and platelet aggregation (Kasperska-Zajac and Rogala, 2007; Palma-Carlos et al., 1991) (**Figures S6E-F**).

The protein-protein interaction analysis of the $_{Allg}DMAT_{Aβ1-6}$ related proteins through STRING showed that ACTB directly interacts with FcγR2, FcγR3, and the cytokine IL-4 and IL-13. On the other hand, the pro-inflammatory proteins TYK2 and ANXA1 directly interact with ACTB **(Figure S6G**). Thus, the data indicate the presence of allergenic response for the $_{Allg}DMAT_{Aβ1-6}$ proteins.

The 271 and 232 proteins were then assessed for apoptosis using DAVID bioinformatics resources, and 29 and 19 apoptosis-related proteins following $_{Apop}DMAT_{Aβ1-6}$ and $_{Apop}DMAT_{Aβ17-23}$ respectively were found (**Table S20**). Among the 29 apoptosis-related $_{Apop}DMAT_{Aβ1-6}$, 13 were upregulated and 16 downregulated; and amongst the 19 apoptotic-related $_{Apop}DMAT_{Aβ17-23,}$ 9 were upregulated and downregulated when compared to untreated control (**Table S20**).

About 58% (14 proteins) apoptosis related proteins were common to treatment with $_{Apop}DMAT_{Aβ1-6}$ and $_{Apop}DMAT_{Aβ17-23}$ (**Figure S7A; Table S6**). Among 29 proteins of $_{Apop}DMAT_{Aβ1-6}$, 5 proteins (NPM1, PRDX2, CFL1, NF1, HNRNPK) were vascular related. On the other hand, 5 proteins (NPM1, PRDX2, CFL1, PPARD, DDX5) were vascular related among the 19 proteins of $_{Apop}DMAT_{Aβ17-23}$. PPARD and PRDX2 were found to be associated with vascular dysfunction (Cheang et al., 2017; El Eter et al., 2014).

Reactome pathway associated with apoptosis such as IL-12 signaling in the execution of apoptosis (Fan et al., 2002) was found to be associated with $_{Apop}DMAT_{Aβ17-23,}$ but not with $_{Allg}DMAT_{Aβ1-6}$ in the overrepresentation test by PANTHER **(Table S21**). Metascape gene enrichment analysis showed the involvement of the identified 29 proteins of $_{Apop}DMAT_{Aβ1-6}$ and 19 proteins of $_{Apop}DMAT_{Aβ17-23}$ with neuronal apoptosis and IL-12 signaling respectively, suggesting a neuroinflammatory process and neuronal cell death (Fan et al., 2002; Fulda and Debatin, 2006) (**Figures S7B-D; Table S22**). Cellular response to stress (Boyce and Yuan, 2006) and VEGFA-VEGFR2 signaling pathway (Shaik-Dasthagirisaheb et al., 2013) highlighted an association of $_{Apop}DMAT_{Aβ1-6}$ and $_{Apop}DMAT_{Aβ17-23}$ proteins with vascular related function (**Figures S7B-D**). The ModEnrichr gene enrichment analysis also confirmed the neuroinflammatory process and neuronal cell death via apoptosis and IL-12 signaling (**Figures S7E-F**).

The protein-protein interaction analysis of the $_{Apop}DMAT_{A\beta 1\text{-}6}$ and $_{Apop}DMAT_{A\beta 17\text{-}23}$ related proteins through STRING showed that GAPDH directly interacts with FcγR2, and FcγR3. In addition, NES, INS, ANXA6, RPS27A, HSP90AB1, PRDX2, CFL1, ACTC1, TCP1, NPM1 directly interact with GAPDH indicating that GAPDH plays a central role in these interactions (**Figure S7G**).

Treatment with anti-Aβ antibodies Induces Caspase 3-dependent Apoptosis

It is believed that caspase-3 is a vital executor of apoptosis since it is either largely or completely responsible for the proteolytic cleavage of numerous important proteins (Adhikari et al., 2021b; Porter and Jänicke, 1999). Apoptosis may be initiated via different pathways, and *in vivo* studies in AD indicated that caspase 3-dependent pathway is activated (Banwait et al., 2008; Louneva et al., 2008). Pro-caspase-3 protein (32 kDa) is cleaved into 20, 19, or 17 kDa subunits (Fernandes-Alnemri et al., 1996), therefore, we analyzed these active subunits by Western blotting (**Figure 4**). Major and minor bands corresponding to the 17 and 19 kDa subunits, respectively, were detected in HSCN following $DAT_{A\beta 17\text{-}23}$, $DMAT_{A\beta 1\text{-}6}$ or $DMAT_{A\beta 17\text{-}23}$ (**Figure 4**), whereas these subunits were undetectable in $DAT_{A\beta 1\text{-}6}$, $DMT_{A\beta 1\text{-}6}$ or $DMT_{A\beta 17\text{-}23}$ (**Figure 4**).

Treatment with anti-Aβ antibodies Induces production of proinflammatory cytokines

Cytokines are a group of heterogeneous multifunctional proteins that generally act locally. Cytokines can be classified as either pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α, control inflammation through priming cells and tissues in their immediate surrounding (Boshtam et al., 2017). However, the primary role of anti-inflammatory cytokines, such as IL-4, IL-6, IL-10, IL-11, IL-13, and TGF-β, is to inhibit the excess inflammatory response of pro-inflammatory cytokines (Opal and DePalo, 2000).

Cytokine profile of human induced pluripotent stem cells following treatment with anti-Aβ antibodies: In this study, we investigated the effect of anti-Aβ antibody treatment on the cytokine profile. The proinflammatory cytokines TNF-α, IL-6, GM-CSF were significantly increased following $DAT_{A\beta 1\text{-}6}$ compared to the untreated control **(Figure 5A; Table 1)**. Similarly, TNF-α and GM-CSF were also elevated following $DAT_{A\beta 17\text{-}23}$ **(Figure 5B; Table 1)**. Of note, TNF-α directly interacts with RHOA, MYLK, PRDX1, CTSD, PPIA, HSPB1, HSP90B1 proteins identified following $DAT_{A\beta 1\text{-}6}$ and $DAT_{A\beta 17\text{-}23}$.

Interestingly, $DMT_{A\beta 1\text{-}6}$ led to a significant decrease of the proinflammatory IFN-α and IL-17A cytokines, while the pleiotropic cytokines IL-9 and IL-6 displayed significant increase when compared to untreated control (**Figure 5C; Table 1**). As noted above, IL-6 plays a central role in the PPARD-TYK2-ALB-ACTB-ANXA1-HSP90B1 interactome identified following $DMT_{A\beta 1\text{-}6}$ (**Figure 5C**). Whereas both TNF-α and IL17A were decreased following $DMT_{A\beta 17\text{-}23}$, however, IFN-α displayed significant increase, contrasting with levels of IFN-α observed with $DMT_{A\beta 1\text{-}6}$ treatment (**Figure 5D; Table 1**). TNF-α also plays a central role in the PPARD-TYK2-ALB-ACTB-PRDX1-PPIA-MYLK-ANXA1-HSP90B1-CTSD interactome identified following $DMTA_{\beta 17\text{-}23}$ (**Figure 5D**). Interestingly, both $DMAT_{A\beta 1\text{-}6}$ and $DMAT_{A\beta 17\text{-}23}$ led to significant decrease of both pleiotropic cytokines IL-9 and IL-6, contrasting levels observed with treatment with $DMT_{A\beta 1\text{-}6}$ (**Figure 5E; Table 1**). However, significant increase of IFN-α was associated with both $DMAT_{A\beta 1\text{-}6}$ and $DMAT_{A\beta 17\text{-}23}$ whereas $DMAT_{A\beta 17\text{-}23}$ also led to significant increase of IL-12 and TNF-α (**Figure 5F; Table 1**). IL-6 directly interacts with ACTB, TYK2, ANXA1, and HSP90B1 identified following $DMAT_{A\beta 1\text{-}6}$ (**Figure 5E**), while TNF-α was part of the ACTB-PPARD-MYLK interactome following $DMAT_{A\beta 17\text{-}23}$ treatment (**Figure 5F**).

Discussion

In addition to a reported case of mortality, a staggering 41% of AD patients displayed amyloid-related imaging abnormalities (ARIA), showing the signs of microhaemorrhages and oedema in the brain following administration of the U.S. Food and Drug Administration (FDA)-approved Biogen's Aduhelm/ aducanumab that targets Aβ3-7 linear epitope (Salloway et al., 2022). The ARIA adverse effects are not unique to Aduhelm as Gantenerumab, that targets the Aβ2–11/18–27 conformational epitopes, also displayed ARIA in clinical trials (Ostrowitzki et al., 2012). Further, a higher dose of the bapineuzumab "3D6" antibody, raised against Aβ1–5 peptide was also linked with increased microhemorrhage incidence (Black et al., 2010; DeMattos et al., 2012; Salloway et al., 2009; Schroeter et al., 2008; Sperling et al., 2012). These immunotherapy-associated ARIA side effects were largely recognised during clinical trials and depended solely on radiographic evidence, and to our knowledge no studies were undertaken to evaluate the molecular mechanisms underlying ARIA and other possible toxic effects caused by anti-Aβ antibodies.

A 80 year old study by Crowe and co-workers linked allergy with cerebral oedema, also called cerebral allergic oedema (Crowe, 1942). Another study by Giardino and co-workers demonstrated

the presence of cerebral oedema in a 52-year-old patient affected with hereditary angioedema (Giardino et al., 2014). Additionally, Tan and colleagues demonstrated the first incidence of vasogenic cerebral oedema induced by a cell-mediated hypersensitivity reaction to a nickel-containing aneurysm clip (Tan et al., 2014). These previous findings highlighted the involvement of allergy in causing oedema, vasogenic oedema (VE), or cerebral oedema. In a more recent study, we showed that anti-PrP antibody treatment of mouse neuronal cells causes neuronal type-2 like hypersensitivity response (Adhikari et al., 2021a). Similarly, and considering the widespread and unexplained ARIA linked to treatment with anti-Aβ antibodies, we reasoned that some of the anti-Aβ antibodies used in clinical trials might also cause a hypersensitivity response in the brain. In this study, we used two commercial polyclonal antibodies produced against Aβ1-6 or Aβ17-23 peptide. These polyclonal antibodies share a large portion of their epitopes with Aduhelm (Aβ3-7) and Gantenerumab (Aβ2–11/18–27) respectively, with notable differences such as polyclonality versus monoclonality and amino acid sequence differences. Here, we identified several allergy-related proteins in DAT, DMT and DMAT. For instance, MYLK, PRDX1, and PGK1 activated by DAT, DMT, or DMAT are associated with proinflammatory responses. MYLK has been identified as potential therapeutic target for the treatment of inflammatory bowel disease, respiratory diseases, and cancer (Xiong et al., 2017). Zhou and co-workers demonstrated the role of non-muscle MYLK in asthma susceptibility, severity, and exacerbation (Zhou et al., 2015). PRDX1 is a multifunctional macrophage redox protein that is thought to play a critical role in regulating oxidative stress, inflammation, and apoptosis (Liu and Zhang, 2019; Yang et al., 2020). PRDX1 contributes to the development of cerebral ischemia-reperfusion damage by inducing TLR4-regulated inflammation and apoptosis in the brain (Liu and Zhang, 2019). In addition, PRDX1 was identified as an inflammation marker for colorectal cancer and its depletion is associated with increased reactive oxygen species (ROS) and cytokine IL-6, TNF-α expression *in vitro* (Chu et al., 2016). PGK1 is an enzyme that is required for the aerobic glycolysis pathway to function properly. The lack of PGK1 protein has been linked to myopathy, parkinsonism, and neurological dysfunction (Sotiriou et al., 2010). Additionally, PGK1 is overexpressed in blood of rheumatoid arthritis where IL-1β and IFN-γ were co-expressed, suggesting the involvement of PGK1 in pro-inflammatory process (Zhao et al., 2016).

Several studies have demonstrated the involvement of endothelial dysfunction in the aetiology of AD (Dede et al., 2007; Kelleher and Soiza, 2013). Borroni and colleagues established that

endothelial dysfunction occurs early in the course of AD (Borroni et al., 2002). Another study by Zuliani and co-workers also demonstrated the involvement of endothelial dysfunction in late onset AD and vascular dementia patients (Zuliani et al., 2008). Endothelial cells can secrete a wide range of cytokines and chemokines when stimulated, consequently, these cells participate in the immune response and rapidly release inflammatory mediators, including IL-6 and TNF-α (Shoda et al., 2016). Increased endothelial permeability owing to acute or chronic inflammation can cause tissue oedema (Mai et al., 2013). Additionally, the endothelium layer closely regulates fluid exchange, and its malfunction leads to uncontrolled fluid extravasation and oedema (Shoda et al., 2016). Furthermore, the endothelium can express growth factors such as GM-CSF, VEGF, and FGF in response to stimuli (Mai et al., 2013). In this current study, several proteins identified following anti-Aβ antibody treatment such as RHOA (DAT), MYLK (DAT and DMT), NPM1 (DAT), PPARD (DMT and DMAT), ALB (DMT), PRDX2 (DMT and DMAT) are associated with endothelial dysfunction.

The RHO family is known to play an important role in maintaining endothelial barrier integrity (Zhang et al., 2016). Furthermore, RHOA activation is linked to increased endothelial permeability, and the RHOA/ROCK pathway plays a key role in the pathophysiology of vascular endothelial dysfunction (Yao et al., 2010; Zhang et al., 2016). Inhibition of the RHOA/ROCK pathway has been proven to prevent endothelial dysfunction in a number of clinical situations (Yao et al., 2010). The MYLK protein is activated by inflammatory mediators, which results in increased vascular permeability and malfunction of the vascular barrier, as demonstrated in several *in vitro* and *in vivo* investigations (Barabutis et al., 2016; Shen et al., 2010). Nucleophosmin (NPM) is a multifunctional phosphoprotein that works as a pro-inflammatory molecule (Di Carlo et al., 2021). NPM stimulates atherosclerosis via activating the NF-κB signalling pathway, which results in vascular inflammation and endothelial dysfunction (Rao et al., 2021). Pfister and D'Mello demonstrated that whereas NPM1 is extensively expressed in neurones and is needed for neuronal survival, its overexpression results in neuronal death (Pfister and D'Mello, 2016). PPARD is a proinflammatory protein that regulates macrophages in inflammatory conditions, and ablation of PPARD increased the availability of inflammatory suppressors (Lee et al., 2003). Marx and colleagues have revealed that PPARD has a proinflammatory effect and that the BCL-6 protein effectively suppresses inflammation in the absence of PPARB/PPARD (Marx et al., 2004). Yin and colleagues, on the other hand, have demonstrated the preventive effect of PPARD against

endothelium injury (Yin et al., 2010). El Eter and colleagues demonstrated a substantial correlation between PRDXs (PRDX 1, 2, 4, 6) and endothelial dysfunction, as well as the severity of peripheral atherosclerotic disease in diabetic individuals (El Eter et al., 2014). In the absence of PRDX2, redox-dependent signalling is activated, resulting in an increase in TNF-α production. Kadono and colleagues demonstrated that abnormally high serum albumin levels are associated with vascular dysfunction (Kadono et al., 2010). On the other hand, albumin oxidant or antioxidant imbalance action causes endothelial cells to undergo early vascular alterations (Puiu et al., 2019). Although apoptotic mechanism has been well studied in relation to AD, its role in neuronal death remains unclear (Behl, 2000; Paquet et al., 2018). In AD, apoptotic cell death can be caused by an excess of pro-apoptotic or anti-apoptotic proteins (Friedlander, 2003; Zhang et al., 2012). Su and co-workers demonstrated that apoptosis is one of the leading causes of neuronal cell death in AD (Su et al., 1994). After addition of Aβ peptide in micromolar concentrations to cultured primary neurons, Loo *et al*. demonstrated that apoptosis might play an important role in AD associated neuronal loss (Loo et al., 1993). Several *in vitro* studies have demonstrated the Aβ induced neuronal apoptosis (Blasko et al., 2000; Calissano et al., 2009; Han et al., 2017; Kadowaki et al., 2005; Morishima et al., 2001). Paquet and co-workers revealed that immunization with AD vaccine AN1792, a pre-aggregated $A\beta_{1\text{-}42}$ with QS-21 adjuvants increases neuronal death (Paquet et al., 2018). Endothelial dysfunction is distinguished by increased endothelial cell death, decreased endothelial-dependent relaxation, and abnormal oxidative stress (Su et al., 2018; Wang et al., 2014a). Gao and colleagues demonstrated that apoptosis is involved in BBB disruption, neurological impairments, and brain oedema (Gao et al., 2017). Therefore, proteins associated with apoptosis might play a significant role in the formation of vasogenic oedema and cerebral microhemorrhages identified as ARIA-E and ARIA-H in radiographic analysis following anti-Aβ antibody treatment. In this study, we identified many apoptosis-related proteins following anti-Aβ antibody treatment. Both pro- and anti-apoptotic proteins were identified following DAT, DMT and DMAT. Importantly, apoptosis-related proteins such as NPM1, PPARD, PRDX2, and ALB are known to be associated with endothelial dysfunction, confirming their putative role in the formation of anti-Aβ antibody mediated vasogenic oedema and cerebral microhemorrhages.

Along with mast cells and basophils, helper T cells secreting IL-4, IL-5, and IL-13 are regarded to be the most critical players in chronic allergic inflammation (Colgan and Hankel, 2010). T helper type 2 cells generate IL-4 and IL-13 once the high-affinity receptor for immunoglobulin E (IgE)

is cross-linked. Furthermore, IL-6, IL-10, IL-12, IL-17, IL-18, and IFN-γ were also found to be linked to allergic asthma (Wong et al., 2001a) and allergies (Brombacher, 2000; Van Dyken and Locksley, 2013; Karo-Atar et al., 2018; Kelly-Welch et al., 2003). Of importance, activated microglia-derived IL-4 and IL-13 play a key role in neurotoxicity via regulating oxidative stress (Jeong et al., 2019; Nam et al., 2012). Our current study identified IL-4 and IL-13 signalling association with allergy-related proteins following DMT and DMAT indicating the role of these identified proteins with inflammation and hypersensitivity responses. IL-12 can also act as a pro-inflammatory cytokine due to its activation and regulatory roles on several cytotoxic immune cells including natural killer cells, macrophages, and T cells (Balasubbramanian et al., 2019). As a pro-inflammatory cytokine, IL-12 can act as a bridge between innate and antigen-specific adaptive immunity (Trinchieri, 1995, 1998). IL-12 was shown to be associated with allergen-induced airway inflammation (Meyts et al., 2006), and in allergic asthma patients (Wong et al., 2001b). In this study, the identified apoptosis related proteins following DAT, DMT, and DMAT were associated with IL-12 signalling indicating the contribution of the identified apoptosis related proteins in allergy, apoptosis, and endothelial dysfunction. Furthermore, we show that caspase 3-dependent pathway is activated following $DAT_{Aβ17-23,}$ $DMAT_{Aβ1-6}$ or $DMAT_{Aβ17-23}$ but not in $DAT_{Aβ1-6}$, $DMT_{Aβ1-6}$ or $DMT_{Aβ17-23}$. This dichotomy could be due to the involvement of apoptosis-related proteins with anti-caspase activity. TRAP1, HSP90B1, HNRNPK, HYOU1, NPM1 identified following $DAT_{Aβ1-6}$, $DMT_{Aβ1-6}$ or $DMT_{Aβ17-23}$ possess anti-caspase. For instance, a study by Gesualdi *et al.* has shown that higher expression of TRAP1 interferes with caspase 3 activation (Montesano Gesualdi et al., 2007). Furthermore, Xiao and co-workers demonstrated that overexpression of HNRNPK suppresses TRAIL-induced caspase-3 and caspase-7 activity independently of p53 status (Xiao et al., 2013).

FcϵRI is composed of FcϵRIA, one FcϵRIB (Ra et al., 2012) and two FcϵRIG (Gomez, 2019; Kinet, 1999; Turner and Kinet, 1999). FcϵRIA directly binds IgE with high affinity during a classic IgE-mediated allergic immune response (Gomez, 2019; Kraft et al., 2004; Lin et al., 1996; Ra et al., 2012). It was previously established that anaphylaxis could be induced following secretion of platelet-activating factor by basophils, macrophages, and neutrophils after FcγR stimulation by the IgG/FcγR pathway (Jönsson et al., 2011; Strait et al., 2002; Tsujimura et al., 2008). Furthermore, Falanga and colleagues showed that IgG allergen cross-linking of mast cells, basophils, and macrophages caused Fyn- and Lyn-regulated mediator release via IgG/FcγR2b and

IgG/FcγR3 pathways (Falanga et al., 2012). Of importance, Lyn was shown to play a key role in neurotoxicity triggered by Aβ and tau hyperphosphorylation by phosphorylating FcγRIIb2 (Gwon et al., 2019).

In this study, FcεR1A was shown to interact directly with RHOA, DOCK8, and GAPDH identified following DAT indicating that treatment with anti-Aβ antibodies led to a neuronal type 1 hypersensitivity response. Furthermore, ALB was shown to interact directly with FcγR1, FcγR2, FcγR3, IL-4, and IL-13 identified following DMT. Moreover, ACTB was shown to be part of the FcγR2-FcγR3-IL-4-IL-13 interactome identified following DMT and DMAT indicating its involvement with type 2 hypersensitivity response.

Passively administered anti-Aβ antibodies were previously shown to prime microglial cells to clear amyloid plaques through Fc receptor-mediated phagocytosis (Bard et al., 2000). However, microglial dysfunction associated with AD pathogenesis (Swanson et al., 2020) could affect the efficacy of antibody mediated Aβ plaques clearance and exacerbate the neuro-proinflammatory state recognised in AD. The receptor expressed on myeloid cells 2 (*TREM2*) was shown to increase the risk for AD (Colonna and Wang, 2016; Cuyvers et al., 2014; Guerreiro et al., 2013; Jonsson et al., 2013; Ulrich and Holtzman, 2016; Villegas-Llerena et al., 2016). Kleinberger *et al* have shown that TREM2 mutations or TREM2 knockout (KO) affect the capacity of microglia to phagocytose Aβ (Gernot et al., 2014). Moreover, TREM2 deficiency affected the efficacy of anti-Aβ antibody-mediated therapy (Xiang et al., 2016). Interestingly, the authors showed that therapeutic anti-Aβ antibody-mediated clearance of amyloid plaques was conditional on the presence of TREM2 (Xiang et al., 2016). Importantly, the authors demonstrated that TREM2 KO cells displayed significant increase of FcγRs, including cell surface FcγRI, FcγRIIB, and FcγRIII. In our current study we show that TREM2 directly interacts with HSPD1 that might influence TREM2 and FcγR-mediated phagocytosis. HSPD1 is a TREM2 agonist and was shown to stimulate the TREM2-dependent phagocytosis process in microglial *in vitro* (Stefano et al., 2009). Further, stimulation of TREM2 through HSPD1 increased microglia phenotypic conversion and reduced apoptotic neurones (Zhai et al., 2017). Our study showed that activation of HSPD1 was observed following DMT and DMAT but not with DAT, indicating the stimulation of TREM2 via HSPD1 agonist and activation of both TREM2-dependent phagocytosis process. This result highlighted a direct interaction of TREM2 with allergy- and apoptosis-related protein identified following DMT and

DMAT that might contribute to the activation/inhibition of TREM2 and FcγR-mediated phagocytosis.

The role of cytokines in AD pathogenesis remains unresolved, however, the presence of proinflammatory cytokines, suggest their active participation is at least exacerbating the severity of the disease. Abnormal Aβ clearance could be linked to alteration of microglia towards a pro-inflammatory state, associated with increased levels of pro-inflammatory cytokines. In fact, overexpression of microglial TREM2 inhibits the production of proinflammatory cytokines (TNF-*α*, IL-6, and IL-1*β*) following LPS stimulation (Zhang et al., 2017). The pro-inflammatory cytokine TNF-α was suggested to be detrimental in AD and anti- TNF-α therapy reversed deposition of Aβ, behavioural impairments and neuroinflammation in an AD mouse model (Detrait et al., 2014; Gabbita et al., 2015; Russo et al., 2012; Tweedie et al., 2012). Furthermore, IL-1β overexpression led to Aβ plaque reduction *in vivo* (Cherry et al., 2015; Ghosh et al., 2013), while IL-1R deficiency displayed lower level of microglia recruited to amyloid plaques (Halle et al., 2008). However, overexpression of the anti-inflammatory cytokine IL-10 intensified Aβ deposits as well as cognitive impairment (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015; Kiyota et al., 2012), but chronic astrocytic overexpression of TGF-β led to increased clearance of Aβ plaque (Wyss-Coray et al., 2001) and improved cognitive behaviour *in vivo* (Chen et al., 2015). Similarly, overexpression of IFN-γ resulted in increased clearance of Aβ deposits (Chakrabarty et al., 2010). Also, inhibiting IL-12 and IL-23 in an AD mouse model reverses Aβ plaque formation and cognitive impairment (Vom Berg et al., 2012). The cytokine biologics used in Aβ plaque clearance for the treatment of AD established a common mechanism where the involvement of microglia was common.

The pro-inflammatory cytokine TNF-α is expressed in both neurons and microglia (Gahring et al., 1996; Jekabsone et al., 2006; Renno et al., 1995). It was previously shown that TNF-α is expressed cortical neurons (Gahring et al., 1996) and can cause neuronal loss following microglial activation (Neniskyte et al., 2014). Floden and colleagues have shown that activation of microglia with fibrillar Aβ1-42 causes neuronal death via stimulation of TNF-α and NMDA receptors in a synergistic manner (Floden et al., 2005). In this current study, TNF-α increased in $DAT_{Aβ1-6,}$ $DAT_{Aβ17-23,}$ and $DMAT_{Aβ17-23}$ but decreased in $DMT_{Aβ17-23}$ confirming its secretion by both neurons and microglia. This confirms that the activation of neurons only or simultaneous activation of both neurons and microglia following treatment with anti-Aβ antibody leads to increased TNF-

α expression while activation of microglia only following anti-Aβ antibody treatment before co-culture with neurons leads to decreased TNF-α expression. Of interest, in a previous study, we did not find significant expression of TNF-α when we co-cultured the anti-PrP antibody-treated microglia with mouse primary neurons (Adhikari et al., 2021a). TNF-α is considered as a key mediator of the atopic allergy inflammation (Ying et al., 1991), human lung allergic reactions (Casale et al., 1996), and allergic rhinitis (Iwasaki et al., 2003; Mo et al., 2011) highlighting its association with hypersensitivity responses. TNF-α is regarded as a detrimental factor for AD progression (Detrait et al., 2014; Zheng et al., 2016) and it has been shown that prolonged TNF-α expression in neural cells of 3xTg AD mice resulted in a significant proportion of neuronal death (Janelsins et al., 2008). The pro-inflammatory cytokine IL-6 is also expressed in both neurons and microglia (Erta et al., 2012; Sallmann et al., 2000). Activated microglia were reported to have a deleterious effect on the surrounding brain tissue by inducing the production of IL-6 and TNF-α (Wang et al., 2015b). A study by Sébire and co-workers revealed that IL-6 and TNF-α are generated predominantly or exclusively by microglial cells in primary cultures of human embryonic CNS cells (Sébire et al., 1993). On the other hand, Ye and colleagues demonstrated that microglia cultivated from aged mice generate much more IL-6 than microglia cultured from neonates or adults (Ye and Johnson, 1999). IL-6 generation is inhibited by IL-10 production in microglia by lowering the activity of NF-κB in both murine microglia cell line N13 and primary microglia cultures (Heyen et al., 2000). In this study, IL-6 increased following $DAT_{Aβ1-6}$ and $DMT_{Aβ1-6}$ but decreased following $DMAT_{Aβ1-6}$ and $DMAT_{Aβ17-23}$ confirming that IL-6 expression might be inhibited by simultaneous activation of both neurons and microglia following treatment with anti-Aβ antibody. Neveu and colleagues revealed that allergic asthmatic individuals exhibit increased levels of the IL-6, but not TNF-α (Neveu et al., 2010). IL-6 is involved in the pathophysiology of experimental allergic bronchial asthma (Doganci et al., 2005; Rincon and Irvin, 2012). Of interest, Lyra e Silva and co-workers showed that IL-6 signaling can links between cognitive decline and metabolic changes in AD (Lyra e Silva et al., 2021). IL-9 is expressed in neurons and microglia (Donninelli et al., 2020; Fontaine et al., 2008), however, IL-9 and IL-9-R are mostly expressed in neocortical neurons *in vitro* (Fontaine et al., 2008). Further, Ding *et al*. showed that IL-9 has no effect on microglia proliferation (Ding et al., 2015). IL-9 is generated by Th2 and Th9 cells in response to allergen exposure and is thought to play a role in allergy (Angkasekwinai and Dong, 2021). In this study, IL-9 increased in $DMT_{Aβ1-6}$ but decreased in both

$DMAT_{Aβ1-6}$ and $DMAT_{Aβ17-23}$ confirming the antagonistic effect of both neuron and microglia in the reduced IL-9 expression. Wharton and colleagues revealed that changes in IL-9 levels were associated with AD in African Americans (Wharton et al., 2019). *In vitro* treatment of neural stem cells with GM-CSF resulted in increased neuronal differentiation (Krüger et al., 2007). However, GM-CSF treatment of microglia contributes to the signal transduction cascade (Liva et al., 1999). In this current study, GM-CSF increased in both $DAT_{Aβ1-6}$ and $DAT_{Aβ17-23}$ indicating the activation of neurons and release of GM-CSF following direct anti-Aβ antibody treatment. Numerous studies have demonstrated the involvement of GM-CSF in the regulation of Th2 immunity and allergic airway inflammation (Asquith et al., 2008), and pathology in chronic inflammation (Dougan et al., 2019) indicating the potential role of GM-CSF in hypersensitivity response. Potter *et al*. found that treating transgenic AD mice with GM-CSF for a short period of time resulted in an increase in activated microglia, reduction of amyloid burden, and restored spatial memory to normal levels (Potter et al., 2021). Another study found that GM-CSF is involved in the decrease of brain amyloidosis and the elevation of plasma Aβ (Kiyota et al., 2018).

The pro-inflammatory cytokine IFN-α is produced by both neuron and microglia (Li et al., 2018; Liu et al., 2016a; Tilg and Peschel, 1996). IFN-α directly contributes to the inhibition of neural stem cell proliferation, which results in a reduction in the generation of new neurons (Zheng et al., 2014). In a previous study, IFN-α was shown to contribute to cell apoptosis and human hippocampal neurogenesis via STAT1-mediated production of IL-6 (Borsini et al., 2018). We show in our current study that IFN-α increased in $DMT_{Aβ17-23,}$ $DMAT_{Aβ1-6,}$ and $DMAT_{Aβ17-23}$ while decreased in $DMT_{Aβ1-6}$ indicating that IFN-α expression depends mostly on the microglial activation following anti-Aβ antibody treatment. A study by Chalise and co-workers showed that IFN-α inhibit the increase of IL-6, IL-10, IL-12, IL-17, IFN-γ, and TNF production (Chalise et al., 2013). Similarly, we show that IFN-α displayed inhibitory effect on IL-6, IL-17, and TNF-α production associated with DMT and DMAT. IFN-α/β has been shown to adversely influence Th2 function and numerous components of the allergy response in humans (Gonzales-van Horn and Farrar, 2015) including asthma and inflammation (Kikkawa et al., 2012; Rich et al., 2020) indicating the involvement of IFN-α in hypersensitivity response. The proinflammatory cytokine IL-17A is expressed in both microglia and neurons (Kawanokuchi et al., 2008; Moynes et al., 2014; Das Sarma et al., 2009). IL-17A affects neurons directly, but it can also affect neuronal function through communicating with immune cells (Moynes et al., 2014). IL-17A stimulates

cortical microglia, resulting in an abnormally high rate of phagocytosis of neural progenitor cells (Sasaki et al., 2020). Sun and colleagues found that recombinant IL-17A promotes microglial activation and enhances pro-inflammatory cytokine production *in vitro* (Sun et al., 2015). In this study, IL-17A decreased in both $DMT_{A\beta1\text{-}6}$ and $DMT_{A\beta17\text{-}23}$ confirming activation of microglia following anti-Aβ1-6 and anti-Aβ17-23 antibody treatment. IL-17 stimulates the production of a variety of cytokines and chemokines, including IL-1, IL-6, IL-8, and IL-21 (Iwakura et al., 2011). IL-17A activation has been shown to affect the production of IL-1, IL-6, and TNF-α (Kuwabara et al., 2017). IL-17A decrease following anti-Aβ antibody treatment appears to promote the production of IL-6 and IL-9 in our study. Additionally, IL-17A play an important role in asthma and allergic rhinitis (Gu et al., 2017) indicating the involvement in hypersensitivity response. Individuals with AD have been found to have higher levels of IL-17A in both plasma and CSF (Chen et al., 2014, 2020) and IL-17 causes cognitive and synaptic impairments in early stages of AD (Brigas et al., 2021).

In this study, we identified a large number of allergy and apoptosis-related proteins, involved in pro-inflammatory responses and endothelial dysfunction. This study demonstrates and for the first time the association between anti-Aβ antibodies and apoptosis, hypersensitivity-like responses and endothelial dysfunction possibly associated with ARIA. The recent death of a patient after treatment with Aducanumab strongly argues and with a degree of urgency for a more comprehensive molecular characterisation of therapeutic antibodies for AD and other fields before proceeding to clinical trials.

Methods

Antibodies for Cell Treatment

We used two antibodies for the treatment of human induced pluripotent stem cells-derived neuron (HSCN) or human primary microglia (HPM) and the antibodies are rabbit polyclonal anti-human *β*-amyloid (1-6) antibody (Anaspec, USA) and rabbit polyclonal anti-human *β*-amyloid (17-23) antibody (Anaspec, USA).

Culture of Human Primary Microglia

Primary human microglia culture was isolated from human foetal brain tissue of 17- to 20-week-old foetuses collected after therapeutic termination with written informed consent. This study has been approved by the Human Research Ethics Committee of Western Sydney University (H14026) and Macquarie University (#5201200411). Microglia was prepared using a protocol adapted from

previously described methods (Guillemin et al., 2005) with slight modifications. Briefly, one gram of brain tissue was weighted and washed with phosphate-buffered saline (PBS) containing 1% antibiotic. Next, the tissue was dissociated mechanically by pipetting with a serological pipette. The dissociated tissues were transferred to uncoated tissue culture grade plates and maintained in DMEM supplemented with 5% FBS and 2% antibiotics. The cells were kept at 37^0C degree incubator with 5% humidified CO_2 atmosphere for 1 week. The culture was washed with PBS to remove unattached tissue fragments followed by addition of fresh medium.

Culture of Human Induced Pluripotent Stem Cell-derived neuron (HSCN) Cells

All human iPSC experiments were conducted in accordance with the requirements of the University of Wollongong Human Ethics Research Committee (HE 13/299). The iPSCs used in this study were derived from dermal fibroblasts of a 75 years old female non-demented donor from the same family bearing the wild-type PSEN 1 A246 genotype, and have been previously described (Muñoz et al., 2018). All iPSC cultures were maintained on Matrigel (Corning, USA) coated 60 mm tissue culture dishes (Greiner Bio-One, Austria) in mTeSR1 medium (Stemcell technologies, Canada) in a humidified incubator at 37°C and 5% O2. The mTeSR1 medium was changed daily and colonies were passaged every 5-7 days with Dulbecco's phosphate-buffered saline (DPBS) and EDTA.

Lentiviral particles were produced using the doxycycline-inducible lentiviral vector PLV-TetO-hNGN2-eGFP-PURO (NGN2; #79823, Addgene) and the reverse tetracycline trans-activator vector FUW-M2rtTA (TTA; #20342, Addgene) by polyethyleneimine (Sigma-Aldrich, Australia) and Opti-MEM (Life Technologies) transfection of HEK293T cells and were packaged using plasmids vSVG (#8454, Addgene), RSV (#12253, Addgene) and pMDL (#12251, Addgene), following established methods (Hulme et al., 2020).

For neuronal differentiation, the iPSCs were seeded at a density of 2.5 – 5.0 x 10^4 in each well of a 24 well plate in mTeSR1 medium supplemented with 10μM Y-27632 (Focus Bioscience, Australia). For lentiviral transduction, the mTeSR-1 medium was aspirated and replaced with fresh mTeSR1 medium supplemented with Y-27632 and containing the NGN2 and TTA lentiviruses (1μL/mL) and plates were incubated overnight. For lentiviral induction, 1μg/mL doxycycline (Sigma-Aldrich, Australia) was added to neural induction media [Neurobasal medium (Thermo, Australia), 1x N-2 supplement (Thermo, Australia), 1x B-27 with vitamin A supplement, 1x Insulin-transferrin-Selenium-A (Thermo, Australia), 1x GlutaMAX (Life Technologies, USA)]

supplemented with 10μM SB431524 (Stem Cell Technologies, Canada) and 0.1μM LDN193189 (Stem Cell Technologies, Canada). The medium was replaced with neural induction medium, and the plates were incubated overnight. The medium was replaced with fresh neural induction medium containing doxycycline, SB431524, LDN193189 and 0.5μg/mL puromycin (Sigma-Aldrich, Australia). The cells were incubated with daily media changes until day 4. On day 4, 25% neuronal medium [BrainPhys Neuronal Medium (Stemcell Technologies, Canada), 1x N-2 supplement, 1x B-27 with vitamin A supplement] was combined with 75% neural induction medium and supplemented with doxycycline and 10 ng/mL BDNF (Miltenyi Biotech, Germany) and incubated overnight. On day 5, 50% neuronal medium was combined with 50% neural induction medium and supplemented with doxycycline and BDNF. On day 9, the medium was aspirated and 100% neuronal medium, supplemented with BDNF, was added. The plates were incubated for 48 h, with half media changes every 48 h until day 22.

Treatment of iPSC and Primary Microglia Cells with Anti-Aβ Antibodies

We used HSCN and HPM cells for the treatment of different anti-Aβ antibodies to investigate the effects of anti-Aβ antibodies. The HSCN cells were used to assess hypersensitivity following direct application of anti-Aβ antibodies (DAT). The HPM cells, initially treated with anti-Aβ antibodies, were used to assess their effects on HSCN following direct co-culture (DMT). In addition, we performed another treatment approach, which we called DMAT, to assess the direct simultaneous effect of anti-Aβ antibodies to the HSCN-HPM co -cultures.

Direct Antibody Treatment (DAT): The protocol for direct antibody treatment (DAT) was adapted from the previously described methods with slight modifications (Adhikari et al., 2021a). Briefly, HSCN ($3x10^4$ cells/well) were plated and grown in 24 well cell culture plate following the above-mentioned protocol. The cells were then treated with 1μg of anti-Aβ1-6 or anti-Aβ17-23 antibody for 3 days. The cells were then removed from the plates and centrifuged at 800 rpm for 5 minutes. The cells were lysed with NP-40 lysis buffer (150mM NaCl, 1.0% Nonidet P-40 and Triton X-100, 50 mM Tris-Cl, adjust P^H to 7.4) with addition of AEBSF protease inhibitor (Sigma, USA) and stored at −80°C until further use. The supernatants were snap frozen in liquid nitrogen and stored at −80°C until further use for cytokine analysis.

Direct Microglia Treatment (DMT): The protocol for direct microglia treatment (DMT) was adapted from the previously described methods with slight modifications (Adhikari et al., 2021a). HPM were grown in 24 well cell culture plate with culture medium and incubated at 37^0C in 5%

CO_2. Cells were then treated with 1µg of anti-Aβ1-6 or anti-Aβ17-23 antibodies as above daily for 3 days. The antibody treated HPM were centrifuged at 800 rpm for 5 minutes before co-culturing with confluent HSC for 3 days. Finally, HSCN/antibody-treated HPM co-cultures were centrifuged at 800 rpm for 5 minutes and the pellet was lysed with NP-40 lysis buffer with addition of AEBSF protease inhibitor then stored at −80°C until further use. The supernatants were snap frozen in liquid nitrogen and stored at −80°C until further use for cytokine analysis.

Direct Antibody Treatment of Neurons and Microglia Co-Cultures (DMAT): To assess the direct and simultaneous effects of anti-Aβ antibodies on neurons/microglia co-cultures, we initially grew HSCN and HPM separately in 24 well cell culture plate. We then added HPM to HSCN to establish the HPM/HSCN co-cultures. Prior to co-culturing HPM with HSC, the cells were treated with trypsin-EDTA solution for 10 min at 37^0C. HPM were then scrapped gently with cell scraper and washed 1x only with serum free medium. The cells were then resuspended and added to the HSCN and kept at 37^0C in the incubator for 48 hours before the stating the anti-Aβ antibody treatment. The HSCN/ HPM co-cultures were then treated with 1µg of anti-Aβ1-6 or anti-Aβ17-23 antibodies as above daily for 3 days. Finally, the anti-Aβ treated HSC/HPM co-cultures were centrifuged at 800 rpm for 5 minutes and the pellet was lysed with NP-40 lysis buffer with addition of AEBSF protease inhibitor then stored at −80°C until further use. The supernatants were snap frozen in liquid nitrogen and stored at −80°C until further use for cytokine analysis.

In-Solution Trypsin Digestion for Liquid Chromatography-Mass Spectrometry

The protocol for in-solution trypsin digestion for liquid chromatography-mass spectrometry (LC-MS) sample preparation was adapted from the previously described methods with slight modification (Adhikari et al., 2021a). The cell lysates prepared above were used for LC-MS sample preparation. For in-solution trypsin digestion, 50µL of protein sample (220 µg/mL) was concentrated using Rotational Vacuum Concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). 3µL DTT (Roche Diagnostics Deutschland GmbH, Germany) (200mM DTT in Tris buffer, pH 7.8) was then added and the mixture was vortexed before addition of 15µL of 6M Urea into the sample then incubated for 1h at room temperature. 3µL iodoacetamide (Sigma-Aldrich, Australia) alkylating reagent (200mM iodoacetamide in tris buffer, pH 7.8) was then added, the sample mixture vortexed then followed by incubation for 1h at room temperature. The mixture was topped up with 112µL of distilled water before adding 5µL of trypsin (Promega Corporation, USA) solution and incubated overnight at 37^0C. Finally, the reaction was stopped,

and the pH of the solution adjusted to <6 with concentrated acetic acid. After trypsin digestion, the solution was purified using Solid Phase Extraction (SPE - Oasis HLB 1 cc Vac Cartridge, 30 mg) vacuum manifold (Waters Milford, Massachusetts, USA) then reconstituted in 15µL 0.1% formic acid, vortexed and kept for 30 minutes at 25^0C. The solution was then vortexed and sonicated for 3 minutes then centrifuged at 14,000 rpm for 10 minutes before transferring into labelled glass vials.

Liquid Chromatography-Mass Spectrometry Analysis

The protocol for sample running in the LC-MS system was adapted from the previously described methods (Adhikari et al., 2021a). The samples prepared above were carefully placed in a Waters Total Recovery chromatography sample vials for analysis. System specific cleaning protocol was run before loading the sample to avoid contamination in the system. LC-MS was performed using a Waters nanoAcquity UPLC equipped with a Waters nanoEase M/Z Peptide BEH C18 Column, 130Å, 1.7µm x 75µm x 100mm, thermostatted to 40^0C (Waters Corporation, USA). Briefly, solvent A consisted of ultrapure water (Milli-Q) plus 0.1% formic acid and solvent B consisted of LC-MS grade acetonitrile (Burdick and Jackson) plus 0.1% formic acid. Samples were injected onto a trapping column (Waters nanoEase M/Z Symmetry C18 Trap Column, 100A, 5µm, 180µm x 20mm) at 5µL/min at 99% Solvent A for 3 min before being eluted on the Analytical Column with a flowrate of 0.30µL/min. An initial solvent composition of 1% B was ramped to 85% B over 50 minutes. Injections of 1µL were made from sample solutions stored at 4^0C.

Mass spectrometry was performed using a Waters SYNAPT G2-Si (HDMS) spectrometer fitted with a nano electrospray ionization source and operating in positive ion mode. Mass accuracy was maintained by infusing at 0.5µL/min a lock spray solution of 1pg/µL leucine encephalin in 50% aqueous acetonitrile, plus 0.1% formic acid, calibrated against a sodium iodide solution. The capillary voltage was maintained at 3 kV, cone voltage at 30 V, source offset at 30 V, ion block temperature 80^0C, gas (N2) flows: purge gas 20L/hr., cone gas 20L/hr. MassLynx Mass Spectrometry Software (Waters Corporation, USA) was used to process the data. Each sample was run for three times in the LC-MS system and finally the collected data were run against the mouse proteome using Uniprot database and analysed using Progenesis QI software (Waters Corporation, USA).

Identification of the Apoptosis Related Genes

The apoptotic genes were identified using the DAVID (database for annotation, visualization and integrated discovery) v6.8 functional annotation bioinformatics microarray analysis tool (https://david.ncifcrf.gov/) (Huang et al., 2009a, 2009b) and UniProt database (https://www.uniprot.org/) (Consortium, 2021). DAVID bioinformatics resources are integrated biological knowledgebase and analytic tools that can be used for extracting biological meaning from massive gene/protein lists in a systematic manner (Huang et al., 2009b). The UniProt database is a large collection of comprehensive, high-quality, and freely available protein sequences and enriched with molecular function, biological process, and subcellular localization information (Consortium, 2021).

Identification of the Allergy Related Genes

To identify allergy-related genes in the final LC-MS dataset following DAT, DMT, and DMAT, the AllerGAtlas 1.0 (http://biokb.ncpsb.org/AlleRGatlas/) database was used. This database contains 1195 well-annotated human allergy-related genes that were identified through text-mining and manual curation (Liu et al., 2018b). The AllerGAtlas database's goals were to investigate the pathophysiology and epidemiology of individual cases, new diagnostic and prognostic biomarkers, individual therapy responses, and personalized medicine (Liu et al., 2018b).

Gene Enrichment and Protein-Protein Interaction Analysis

Initially, the statistical overrepresentation test for the identified allergenic and apoptosis related genes was performed using the PANTHER database (http://www.pantherdb.org/)(Mi et al., 2021; Thomas et al., 2003). The “reactome pathway” was selected as a parameter for statistical overrepresentation test; *Homo sapiens* was selected as “reference gene list” and “whole-genome lists”; “Fisher's Exact” as test type; and “Calculate False Discovery Rate” was selected as correction methods. The Gene enrichment and protein-protein interaction analysis of the identified allergenic and apoptotic genes were performed using the Metascape server (https://metascape.org/gp/index.html#/main/step1). Metascape is a web-based portal aimed to give experimental biologists with a comprehensive gene list annotation and analysis resource (Zhou et al., 2019). Metascape integrates functional gene enrichment, gene annotation search, protein-protein interaction analysis, and membership search to use over 40 separate knowledgebases into one integrated platform. It also allows for comparison studies of datasets from numerous independent and orthogonal experiments (Zhou et al., 2019). In Metascape, for enrichment

analysis, the minimum overlap, *p* value cut-off, and minimum enrichment was set as 3, 0.01, and 1.5, respectively. The gene enrichment was also performed using the modEnrichr server, a suite of gene list enrichment analysis tools (https://maayanlab.cloud/modEnrichr/) (Chen et al., 2013; Kuleshov et al., 2019). The protein-protein interaction was also predicted using the STRING v11.5 (https://string-db.org/)(Snel et al., 2000) applying the parameter confidence score and the interaction network was edited using the Cytoscape v3.9.0. Protein-protein interaction enrichment analysis was performed through the Metascape server. The Metascape server carried out the interactions using mainly STRING and BioGrid servers and considers only physical interactions (physical score > 0.132). It considers the network that contains the subset of proteins and have physical interactions with at least one other member in the gene list. If the network contains between 3 and 500 proteins, the Molecular Complex Detection (MCODE) algorithm10 is applied to the gene lists to identify densely connected network components.

Western Blot Analysis

In order to run and prepare samples for Western blotting, the previously reported procedures were used with minor modifications (Adhikari et al., 2021a). Briefly, cell lysates derived from DAT, DMT, DMAT was mixed with an equal volume of Laemmli buffer (Bio-Rad, CA, USA). The solution was vortexed then heated for 5 min to 95^0C. The solution was left to cool down before loading the sample into 12% SDS-PAGE gel (Bio-Rad, CA, USA) and run at 200 Volt for 5 min then 1h 30 min at 100V in running buffer (Bio-Rad, CA, USA). Following transfer at 18V for 2h 30 min in transfer buffer (Bio-Rad, CA, USA), the membranes were blocked using 5% skimmed milk for 1h. The blots were rinsed with TBST then primary antibody rabbit cleaved caspase-3 (1:1000) (Cell Signaling Technology, USA) or mouse β-actin (1:5000) (ThermoFisher, USA) was added for overnight incubation before washing with 0.1% TBST buffer. The secondary antibody anti-rabbit IgG (1:20000) (Sigma-Aldrich, USA) was then added for 1hour at room temperature. The blot was washed using 0.1% TBST then visualized using the Clarity Western ECL Substrate (Bio-Rad, CA, USA) in iBright™ CL1000 Imaging System (Thermo Fisher Scientific).

Cytokine Analysis

The MACSPlex human 12 cytokine kit was used to evaluate the levels of 12 different cytokines in the cell culture supernatants (Miltenyi Biotec, Germany). All processes were carried out in accordance with the manufacturer's instructions, and washing step was accomplished using a centrifuge. MACSQuant Analyzer was used to acquire flow cytometric data (Miltenyi Biotec,

Germany). MACSQuant® Express Mode was used to analyse the data (Miltenyi Biotec, Germany).

Statistical Analyses

Statistical analyses were assessed using GraphPad Prism 9 with unpaired t-test. The results were considered significant at $p<0.05$.

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